Tomas G. Villa · Miguel Vinas *Editors*

New Weapons to Control Bacterial Growth

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Introductory Chapter

Tomás G. Villa and Miguel Viñas

Resistance to antimicrobial agents has been known since the beginning of the antibiotic era. In fact, Alexander Fleming and Selman Waksman were already aware of the ability of bacteria to become resistant to antibiotics. In addition, it is likely that this antibiotic resistance has been enhanced and stimulated by the extensive use of antimicrobials in human and veterinary medicine, as well as by the somewhat illicit use of these compounds in intensive livestock feeding. In anyway, the emergence of antibiotic-resistant bacteria currently constitutes one of the major problems facing medicine, in such a way that, in some cases, we are almost out of drugs to combat infection. Indeed, respiratory tract infections caused by multiresistant *Pseudomonas aeruginosa,* as well as infections caused by methicillin-resistant *Staphylococcus aureus,* are notoriously hard to control. In addition, some microbes are naturally resistant to most antimicrobials. This is the case for mycobacteria, as these organisms have a very hydrophobic lipid layer on their external surface that prevents hydrophilic drugs from entering the cell. In summary, the prevalent scenario is defined by a diminishing effectiveness of conventional antimicrobials, an increasing virulence of pathogenic and opportunistic microbes and the need to explore alternatives. Both the WHO 2014 Antimicrobial Resistance Global Report and the 2014 US Presidential Report emphasized the need to address this antimicrobial resistance (AMR) problem; and AMR was also the subject of the special actions initiated in the USA and Europe (IMI).

An interesting scenario includes the use of bacteriocins, described in this book, as potent biological antibacterial polypeptides. These bacterial proteinaceous toxins

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inhibit the growth of closely related bacterial strains (Farkas-Himsley 1980). Gratia discovered bacteriocins in 1925, while searching for antibacterial compounds. He named his first discovery as "colicin" because of its ability to kill some *Escherichia coli* strains. These early discoveries were complemented by later studies, such as the use of bacteriophages as bacterial lytic agents (Shwartzman 1925). Hence, in 1950, when Joan F. Gardner (Sir William Dunn School of Pathology, Oxford) published the article entitled "*Some antibiotics formed by Bacterium coli*," she clearly stated: "*the recent work of Fredericq and Gratia* (Fredericq and Gratia 1950) *and their co-workers has resulted in the recognition of more than 17 distinct types of colicins*." From that point, it only took a short step to using these antibacterial compounds as antibiotics. In fact, in early years, colicin-producing bacteria were indeed treated as "antibiotic-producing bacteria" (Chabbert 1950). Even currently, bacteriocins show a great potential both in medicine and in food preservation, as reviewed here.

Bacterial RNases, such as those reported here (RNase R, P, Y, E, PNP, PH, I, II, III, barnase, binase, RNase D, H, L and T) have the potential to dramatically control the cellular RNAs levels, thus affecting bacterial growth and displaying either bactericidal or bacteriostatic roles on the target bacteria. In this sense, RNase inhibitors could regulate and control these enzymes. Following this line of thought, by the middle of last century, researchers were searching for acidic or anionic polymers (Fellig and Wiley 1959) capable of inhibiting RNases, which could be used as antiviral agents. This resulted in the discovery of a series of RNase inhibitors, such as the 2′ cytidilic acid, glutamic acid and aromatic amino acid copolymers (Sela 1962), iodoacetate (Lamden et al. 1962), and bentonite (Jacoli 1968). Additionally, Irie (1969) found that *Aspergillus saitoi* RNase was inhibited by nucleosides, and 2 years before Smeaton and Elliott (1967) reported that *Bacillus subtilis,* towards the end of its growth phase, produced a novel polypeptide (12.5 kDa) that inhibited RNases. This inhibitor forms a complex with the enzyme, and the inhibition is not reversed by urea, salt, heating, or sulphydryl reagents (in contrast to the mammalian system), and interestingly it does not affect pancreatic ribonuclease or ribonuclease T1. This research opened the possibility of using polypeptides as RNase inhibitors and, therefore, involved in the control of the cell cycle. In addition, some of these inhibitors can display anti-angiogenic properties and may fight tumor development, hence playing a role in tumor reduction (Polakowski et al. 1993). The studies on RNase inhibitors have had additional projections, such as their role in the treatment of HIV; indeed the reported RNase H inhibition by azidothymidylate (Tan et al. 1991) may at least partially account, for the sensitivity of HIV to AZT. Furthermore, novel RNase inhibitors, such as the pyridopyrimidinone HIV-1 RNase H inhibitors, are also useful in HIV treatment (Velthuisen et al. 2014).

Species belonging to *Clostridium* genus are part of the Firmicutes, a group of Gram-positive bacteria that are rod-shaped, produce spores and are obligate anaerobes (Bruggemann and Gottschalk 2009). The genus contains *ca.* 100 species and, while many of them are useful in different economic/industrial processes, others display extreme pathogenic abilities. The species include *Clostridium botulinum*, a bacterium that produces the most potent known neurotoxin in nature

and that causes many deaths by ingestion of contaminated foodstuffs, not only in adults, but also in infants due to the consumption of spore-bearing honey (Tanzi and Gabay 2002). Nevertheless, as it is the case with other poisons, this neurotoxin is used in medical/esthetical applications, under the name of botox, for correcting the visible signs of aging in the form of wrinkles (Carruthers and Carruthers 1992). The ability of *C. botulinum* to transfer neurotoxin genes to other *Clostridium* species, in particular species such as *Clostridium butyricum* used in the food industry, must be carefully considered. Another species belonging to this genus is *Clostridium tetani*, producing a very potent neurotoxin (second only to the botulinic toxin in potency) named tetanospasmin or spasmogenic toxin because of the violent spams it produces (as reported by M'Arthur in 1816), but also capable of producing other clinical syndromes, such as generalized tetanus, partial tetanus and tetanus of the wounds. *Clostridium perfringens* (formerly known as *Clostridium welchii*, or *Bacillus welchii*) is the third most common cause of food poisoning in the USA and UK, it produces gas gangrene or myonecrosis with tissue necrosis (due to an α-toxin, that contributes to the disruption of the cellular plasma membrane), bacteremia, and emphysematous cholecystitis. Bowlby and Rowland (1914) first reported this bacterium as the leading cause of gas gangrene while studying wounded soldiers in the trenchs of the Allied and German armies during World War I. Finally, another species that merits to be included here is *Clostridium difficile,* which produces pseudomembranous colitis (normally appearing after strong oral antibiotherapy), although it is also involved in gas gangrene and enterotoxemia.

Since the early years of antimicrobial research, much attention has been directed to compounds with the ability to stop bacterial DNA synthesis. This is the case for Berrah and Konetzka's (1962) report, which centers on the reversible inhibition of bacterial DNA synthesis by phenethyl alcohol; or the report by Gangadharam et al. (1963) that deals with the selective inhibition caused by isoniazid on nucleic acid synthesis in *Mycobacterium tuberculosis*. Quinolones, on the other hand, mainly target bacterial topoisomerase II as well as mammalian topoisomerase II. They can be used either as antibacterials or in the treatment of tumors, despite the fact that eukaryotic topoisomerases are at least 2 orders of magnitude less sensitive to quinolones than bacterial topoisomerases II (Hussy et al. 1986; Tabarrini et al. 1999; Kathiravan et al. 2013). Tabarrini et al. (1999) designed novel quinolone derivatives with a shift from antibacterial to antitumoral activity by synthesizing a series of modified tricyclic quinolones, in which the essential 3-carboxylic function is surrogated by phenolic OH and the classic C-6 fluorine atom is replaced by a NH_2 group. In addition, Rajabalian et al. (2007) found that introduction of O-benzylmoiety on oxime group of *N*-(2-oxyimino) piperazinyl quinolone series also changes the quinolone biological profile from antibacterial to an excellent cytotoxic activity. A mayor target for controlling viral diseases is the regulation of the viral DNA synthesizing enzymes, an area pioneered by Müller et al. (1975). They studied the effect of 9-beta-D-arabinofuranosyladenine (AraA) on the in vivo and in vitro synthesis of DNA, RNA, and protein in transformed cells. Evidently, the rational design of antitumor and/or antiviral agents must take advantage of the

biochemical differences between normal host cells and transformed cells, and this approach is investigated in the interesting classic publication by Müller (1977). The following years saw the rise of a number of antiviral/antitumor drugs with potential clinical use, such as azidothymidine triphosphate (an inhibitor of both human immunodeficiency virus type 1 reverse transcriptase, and DNA polymerase γ) (König et al. 1989) or abacavir (metabolized to the active compound carbovir triphosphate) that, being a planar molecule guanosine, acts on HIV-1 reverse transcriptase causing DNA synthesis chain termination (Ray et al. 2002).

Bacterial efflux pumps are proteins active as transporters that are located within the plasma membrane, which either utilize ATP as their energy source or are coupled to the electrochemical potential difference created by pumping either hydrogen or sodium ions from/to the outside of the cell, the latter is the case for symporters, uniporters, or antiporters. These efflux pumps are grouped into five superfamilies, namely (i) MFS (major facilitator superfamily), (ii) MATE (multi-antimicrobial extrusion protein family), (iii) RND (resistance-nodulation-cell division superfamily), (iv) ABC (ATP-binding cassette superfamily), and (v) SMR (small multidrug resistance superfamily). These efflux pumps play an important role in antibiotic resistance; hence Desnottes identified them, in 1999, as crucial targets for inhibition by novel molecules, as this would render those bacteria sensitive to antibiotics. Although no inhibitors have yet reached the market, there are several promising compounds, such as alkylaminoquinolines and related substances (Malléa et al. 2003) that could be used for reversing the ability of some bacteria to survive antibiotic treatment. These and other related topics are dealt with in this book.

Proteases or proteinases are perhaps the most ubiquitous group of enzymes found in animals, plants, bacteria, and archaea, as well as in viruses. They perform proteolysis by hydrolyzing peptide bonds, thus generating either small peptides or amino acids that bacteria can efficiently use through salvage mechanisms; but they can also generate new peptides with novel biological functions. The classic paper by Christensen and Macleod (1945), concerning the activation of serum protease by either chloroform or streptococcal fibrinolysin, merits to be cited here. This discovery has a potentially high application, with uses ranging from medicine (i.e., control of blood clotting) to industry (from better laundry detergents to improving bread), or simply as a basic biological research tool (Feijoo-Siota and Villa 2011). These enzymes have undergone many changes during evolution, giving rise to the 6 basic protease groups currently known, that include the serine proteases, threonine proteases, cysteine proteases, aspartic proteases, glutamic acid proteases, and metalloproteases; an updated classification of the protease superfamilies can be found in the MEROPS database (Rawlings et al. 2010). The use of inhibitors to control bacterial proteases has long been considered as an important means of controlling bacteria proliferation, as well as a way to control the bacterial proteasome to target the hydrolysis of unfolded or wrongly folded proteins. The protease inhibitors tested for this purpose include alpha 1-antitrypsin, alpha 1-antichymotrypsin and neuroserpin (Puente and López-Otín 2004). The protease

inhibitors research was spurred after these compounds were found useful in the fight against HIV (Oberg 1988).

The early studies on bacterial hormones involved these compounds in the development of competence and its regulation in either Gram-negative (i.e., *Haemophilus influenza*; Goodgal and Herriott 1961) or Gram-positive bacteria (*Streptococcus pneumoniae*; Pakula 1965 or *Bacillus subtilis*; Charpak and Dedonder 1965; Fontaine et al. 2014) as well as in the manifestation of the so-called *quorum sensing* in bacteria. This was later on followed by a multitude of publications (Fuqua et al. 1994) reporting *quorum sensing* in a variety of bacteria and its use as a potential target for novel antibacterial compounds (Williams 2002). In this regard, Rasmussen et al. (2005) reported several compounds with the ability to modulate the bacterial *quorum sensing*. Vattem et al. (2007) found that certain naturally occurring phytochemicals can inhibit the bacterial *quorum sensing* and related processes, and this opened up an exciting new strategy for antimicrobial chemotherapy, capable of overcoming, at least partially, the known phenomenon of antibiotic resistance. Unfortunately, the use of compounds that block the bacterial *quorum sensing* signals in hospitalized patients, treated with non-bactericidal antibiotics, was shown by Köhler et al. (2010) to select for virulence and cooperation in the bacteria, thus creating a new unexpected problem.

The study of bacterial transcriptomics represents a valid approach to identify novel antibacterial targets. This discipline studies the complete set of RNA transcripts produced by the genome, this is all RNA molecules present in a cell, including mRNAs, rRNAs, and tRNs as well as other non-coding RNAs. Among all these RNA molecules it seems inevitable that new antibacterial targets will be found, indeed a wide variety of targets are coming to light, some of which are described in this book. At the beginning, the use of transcriptomics was associated to cancer research, such as the search for tumor-associated antigens for immunotherapy (Vinals et al. 2001), for molecular typing of breast cancer (Bertucci et al. 2001), to define innovative cancer drug targets (Workman and Clarke 2001), for the functional study of food ingredients to fight colorectal cancer (Stierum et al. 2001), or for predictive toxicology (Storck et al. 2002); but already in 2002 Betts used transcriptomics as a tool for the identification of novel drug targets and for vaccine development in tuberculosis. Comparative transcriptomics have also been used (Yoder-Himes et al. 2012) to positively identify potential therapeutic targets against *Burkholderia.*

The term Achilles' heel has transcended the Greek mythology to signify a weakness in spite of overall strength, which can lead to downfall. Thetis held her son Achilles by his heel when submerging him into the magical waters of the River Styx, to give him invulnerability; unfortunately, Achiles' heel was not washed over by the water and this eventually caused Achilles' downfall. In this way, the term "Achilles' heel" is common in current expressions such as "*Achilles' heel of cancer*" (Vogelstein and Kinzler 2001; Yuneva 2008; Martinez-Outschoorn et al. 2011), "*the peritubular endothelium as the Achilles heel of the kidney*" (Rabelink et al. 2007) or "*dendritic cells in the host as Achilles' heel for mucosal pathogens*" (Niedergang et al. 2004). Microorganisms also have their Achilles' heel in lipid II,

involved in the synthesis of bacterial peptidoglycan. Lipid II is the target for several antimicrobials, including the glycopeptide vancomycin and the human beta-defensin 3. The synthesis of lipid II and the amount of this compound present in the bacterial cell wall must be precisely controlled, as any irregularity in its synthesis/accumulation can lead to bacterial cell wall fragility and result in microbial death (Danese et al. 1998).

The term microbiome was coined by Joshua Lederberg to denote the importance of the microbial populations that inhabit the human body, both in health and in disease (Lederberg and McCray 2001). The human microbiome can be defined as "an ecological community of commensal, symbiotic, and pathogenic microorganisms found in the human body." Obviously, the microbiome is a dynamic concept, as the microbial populations can change considerably, not only from a health to a disease stage, but also it evolves in response to environmental conditions and human life stages. From the moment of birth, the baby acquires a complex collection of microorganisms, some come directly from the mother and others from the environment, and many of them will remain in his/her body until death. The microbiome mainly includes nonpathogenic commensal microorganisms, following the concept of commensalism proposed by Pierre-Joseph van Beneden at the University of Louvain in the nineteenth century. The number of microbial species that constitute a putative "basic microbiome" is still under discussion, but we know that major changes can lead to diseases such as depression, anxiety, affection of the immune system, neonatal necrotizing enterocolitis, inflammatory bowel disease, vaginosis, or even cancer. This book deals with some of these matters, including cancer. It is generally accepted that the human microbiome contains *ca*. 100 trillion microbial cells, thus outnumbering the human cells by 10-fold (Savage 1977), and that it can affect many aspects of the human biochemistry and physiology. For example, in healthy individuals the microbiota provides a wide range of metabolic functions lacking in human beings (Gill et al. 2006). The United States National Institutes of Health launched in 2008 the "Human Microbiome Project," with the goal of identifying the microorganisms involved, both in human healthy and disease states. This initiative is also supported by other projects, such as the "Earth Microbiome Project" (it is estimated that just the oceans in our planet contain 1.3×10^{28} archaeal cells, 3.1×10^{28} bacterial cells, and 1×10^{30} virus particles; Curtis et al. 2002; Suttle 2007). The control of the human microbiome involved in carcinogenesis (including the case of *Helicobacter pylori*) is important and treated in the present book.

Healthy intestinal microbiota (including bacteria and their bacteriophages, fungi and protozoa) constitutes an important barrier to prevent microbial pathogens from invading the gut, and are also an effective means of controlling the passage of bacteria into the blood stream. It is estimated that 500–1000 species of bacteria live in the human gut (Savage 1977), and it is well established that animal life could not be possible without these microbial populations inhabiting their guts. The bacteria are essential not only to prevent the growth of pathogens, but also to generate vitamins, amino acids and all kinds of nutrients absorbed by the gut. An additional complication is the denominated "genetic cross-talk among microorganisms of the

gut" which can generate unwanted events, such as the transfer of genes encoding antibiotic resistance. This possibility was already suggested by Wells and James (1973), and finally demonstrated by Smith (1977). Small variations in the normal microbiota can cause transient syndromes or diseases in animals, but major alterations can result in death. For this reason, current studies investigate novel ways of replacing aberrant microbiota with normal flora, to cure some of these diseases. These and related subjects are scrutinized here.

Pathogenic microorganisms has constantly reduced the human life expectancy since the early times; according to Anderson and May (1991) Paleolithic people had an average life span of only 25 years, and infectious diseases were the main cause of premature death. Human longevity progressively increased with time, as hygiene improved in the different human societies. Introduction of antimicrobials and antibiotics, at the beginning of the twentieth century, greatly and rapidly increased human life expectancy to a current average of 80–90 years for developed countries, were vaccines and antibiotics are readily available. We are nevertheless aware that not all patients respond equally to antimicrobial and/or antibiotics treatment, in fact the response depends on many factors, but mutations on either the nuclear or the mitochondrial genomes are perhaps the main cause of such variation. This particular aspect is also examined here.

Resistance to antimicrobials is an important public health problem and the number of resistant strains increases constantly, to such extent that we now need to find new antimicrobials to continue being able to fight infectious diseases. Although antibiotic resistance in bacteria predates the use of antibiotics in human and veterinary medicines, 70 years of massive antibiotic use has resulted in the selection of resistant microorganisms. Fusté et al. (2012) demonstrated that if the antibiotics are removed, the resistant bacteria tend to revert to their original, nonresistant phenotypes. Such a radical measure cannot be implemented, but the problem could be ameliorated by returning to the parenteral way of antibiotic administration, instead of oral antibiotic treatment, as this would eliminate the horizontal antibiotic resistance transfer in the gut. As early as 1948, Duggar recognized the importance of a continual search for new antibiotics, and Malek et al. (1957) provided a variety of new methods to search for novel antibiotics. As a result of these early efforts a number of antibiotics were discovered, including the isoniazid-derived tuberculostatics (Zsolnai 1959a) and the benzimidazole derivatives containing phenolic hydroxyl groups (Zsolnai 1959b). This book includes an updated chapter that covers the problems of finding new antimicrobial compounds suitable for human treatment.

As the number of antibiotic-resistant bacteria increases, so does the urgency to find new antibiotics for use in medicine. One of the approaches that have gained recognition over the last few years is the use of novel synthetic peptides with antimicrobial capability and this is reviewed here. Glycosylated cyclic or polycyclic nonribosomal peptides are perphaps the archetype of these groups, and they include vancomycin, teicoplanin, telavancin, bleomycin, ramoplanin, and decaplanin. The first of these compounds was isolated in 1953 and approved by the FDA in 1958 by to treat penicillin resistant staphylococci. These polypeptides inhibit the synthesis

of the peptidoglycan layer in the bacterial cell wall by binding to the acyl-D-alanyl-D-alanine residue in peptidoglycan. A number of lipoglycopeptide derivatives are currently available, such as oritavancin and dalbavancin, and this approach is likely to continue producing new successful compounds to treat antibiotic-resistant bacterial strains. Another group of synthetic peptides, included in this book, displays a different mechanism of action, as they inhibit protein synthesis. The paragon of this group is vernamycin, an antibiotic described by Ennis (1965). Other applications of these compounds include the design of anti-caries peptides against *Streptococcus mutans* (Younson and Kelly 2004) and the coiled-coil peptides that can prevent the assembly and hence functionality of the three-secretion system in bacteria. The three-secretion system apparatus is responsible for the ability of bacteria to adhere to the gut mucosa; hence these peptides could provide an attractive tool to block *E. coli* enteropathogenic strains (Larzábal et al. 2010, 2013). These peptides as well as more recent developments, such as the thiopeptide antibiotics (Just-Baringo et al. 2014) are analyzed here.

The use of microorganisms for therapeutic purposes originated from Vuillemin's work in the nineteenth century (Vuillemin 1890). It was he who coined the term "antibiosis" to epitomize the tremendous fight constantly taking place in the microbial world in the interaction between predator and prey. In fact, the last three decades of the nineteenth century produced many publications on the subject of antibiosis. Pasteur and Joubert (1877) described the existence of antibiosis affecting *Bacillus anthracis* and what they called "normal bacteria." They described that animals simultaneously inoculated with the anthrax bacillus and a "normal bacteria," did not develop anthrax. Cantani (1885) further proved this theory by successfully treating a patient with a serious *Mycobacterium tuberculosis* infection by "insufflating" air containing a "normal" (nonpathogenic) bacterium. The discovery of bacteriophages (Twort and Mellanby 1912; Twort 1915; d'Herelle 1917, 1931; Gratia 1922) opened a novel way of fighting pathogenic bacteria, different from that proposed by Paul Erlich (see Fitzgerald 1911) under the concept of "Magic Bullet" (a compound that would get rid of parasites from the body following a single *dosa sterilisa magna*). The idea of employing bacteriophages against pathogenic bacteria, although apparently new in those early Twort-d'Herelle's years, had already been suggested by Hankin (1896). Hankin reported that the waters of the Ganges, in India, had a marked antibacterial activity against cholera bacilli, and he suggested that an unidentified substance was responsible for limiting the spreading of cholera epidemics. However, it was not until the twentieth century that the medical use of bacteriophages was developed into what it is currently considered the "enzybiotic way of fighting pathogenic bacteria." The term enzybiotic applies not only to the bacteriophages, but also to the lytic enzymes they produce during their lytic cycles. In a broader sense, the term also applies to the enzymes, namely 1,3-β-D-glucanases, 1,6-β-D-glucanases and chitinases, that lyse fungal cell walls (Villa and Veiga-Crespo 2010).

Polyalkylcyanoacrylate nanoparticles to deliver antineoplastic drugs into the blood stream were developed last century, towards the end of the 1970s (Couvreur et al. 1979a, b). Some years later, El-Samaligy and Rohdewald (1983) reported the

use of reconstituted collagen nanoparticles as a novel drug delivery system, and later on polyvalent polymeric drug carriers were first used by Vert (1986). Liposomes were soon found a good alternative system to deliver antibiotics to treat intracellular bacterial pathogens. Tom et al. (2004) used metal nanoparticles to carry active principles, such as antibiotics. Nath et al. (2008) used a modification of this gold nanoparticle method to design a high-throughput antimicrobial susceptibility assay, based on concanavalin-induced self-assembly of dextran-coated gold nanoparticles. Silver nanoparticles exhibit unique antibacterial properties that make them ideal candidates for medical applications and, if they are appropriately prepared, they can be used as antimicrobials (Kemp et al. 2009).

This book contains updated chapters written by scientists who are experts in the fields and it is our hope that the reader will find it useful.

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Antimicrobial Peptides Produced by Bacteria: The Bacteriocins

Beatriz Martínez, Ana Rodríguez and Evaristo Suárez

Abstract Bacteriocins are the subset of antimicrobial peptides (AMPs) produced by bacteria. They are small amphipathic peptides that interact with bacterial membranes leading to cell death. Most of the best known are produced by lactic acid bacteria used as food fermentation starters, because of their potential use as food preservatives. Bacteriocins are divided into two groups: lantibiotics that present posttranslational condensation rings and unmodified peptides. The first are subdivided into elongated versus globular lantibiotics, while four subgroups are recognized among unmodified bacteriocins. The genetic organization is in clusters that may reside into plasmids or transposons, formed by the structural gene, the export and immunity determinants, the quorum sensing governing production and any modification genes. Bacteriocins are active at extremely low concentrations (nM range) due to a dual mode of action: (a) binding to the membrane phospholipids and (b) specific recognition of surface components, both of which collaborate in pore formation. Development of resistance to bacteriocins is very infrequent due to the presence of two targets and is usually due to unspecific modifications of the cell envelope. Bacteriocins are used as food preservatives, either after total or partial purification or as extracts of producing bacteria. In situ production is also used, with the advantage of producing early lysis of the starter bacteria and ripening acceleration of the fermented product. They may also form part of hurdle technologies and be incorporated into packaging systems to allow extended liberation. Medical and veterinary applications are in their infancy but good results have been obtained against infection by Gram-positive bacteria and *Helicobacter pylori*.

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1 Introduction

Bacteriocins are the subset of antimicrobial peptides (AMPs) produced by bacteria. AMPs are synthesized by virtually all cellular organisms (the defensins generated by fungi, plants, insects, and vertebrates in general and the cathelicidins typical of mammals belong to this group). All of them have in common their size (usually less than 10 kDa.) and general structure, amphipathic molecules with a positive net charge (Nes et al. 1996; Perez et al. 2014). This promotes their interaction with plasma membranes, where they bind to the negatively charged phospholipids, sink into the fatty acid moiety and polymerize to form pores that abolish the membrane potential and allows leakage of cytoplasm solutes, leading to cell death (see below). AMPs from multicellular organisms tend to have a wide spectrum of action that comprises bacteria, fungi, protozoa and enveloped viruses, thus being a substantial component of their innate immunity. However, bacteriocins are usually only active against bacteria related to the producer (for example, those produced by *Escherichia coli* are specific for Enterobacteriaceae) although some, usually produced by Gram positive bacteria, may affect a wide range of other Gram-positive organisms and even inhibit spore germination. This selective toxicity may facilitate bacteriocin use for treatment of infectious diseases, especially those produced by antibiotic multiresistant bacteria (Smith and Hillman 2008; Hassan et al. 2012; Cotter et al. 2013).

Out of the 177 sequenced bacteriocins included in the specialized BACTIBASE web page (http://bactibase.pfba-lab-tun.org/main.php), 156 are produced by Gram-positive organisms, 18 by Gram-negative bacteria, and 3 by halophile archaea (i.e., they are not bacteriocins but illustrate on the universality of AMPs production). Out of the 156 Gram-positive bacteriocins, 113 are synthesized by lactic acid bacteria (LAB), a heterogeneous group of low G + C anaerobic organisms that generate lactic acid as the main end product of sugar fermentation. This may not reflect a higher capacity of synthesizing bacteriocins but, rather, a more profound search for them because LAB constitute the main source of fermented food starters, where in situ produced bacteriocins are believed to contribute to extend the shelf life of food products. Thus, research on bacteriocin biology has been mostly focused on LAB bacteriocins.

2 Bacteriocin Classification

Bacteriocins are divided into two major groups; class I is composed of small molecules (less than 5 kDa.) that present posttranslational modifications, while those of class II are made up of peptides with unmodified amino acids (Perez et al. 2014). The unusual residues present in class I bacteriocins arise from dehydration of serine and threonine to give 2,3-didehydroalanine and 2,3 didehydrobutyrine respectively. Frequently, thioether linkages with the sulfhydryl groups of neighboring cysteines

are formed. The residues arising from this condensation are called lanthionine and 3-methyl-lanthionine respectively, this being the reason why they are collectively termed as lantibiotics (Asaduzzaman and Sonomoto 2009; Bierbaum and Sahl 2009). Bacteriocins of classes I and II can be divided into different subgroups depending on the secondary structure of the molecule, the number of peptides that form the active antimicrobial, and even their spectrum of susceptible bacteria.

Lantibiotics constitute about one-third of all bacteriocins and are classified into two main types, A and B. The first comprises peptides that have an elongated secondary structure in at least part of the molecule. Those of type B are globular, thus presenting a compacted arrangement. The type A lantibiotics are further subdivided into subtype A(I), made out of molecules with a more or less lineal structure, while subtype A(II) members have an elongated end, the rest being globular (Asaduzzaman and Sonomoto 2009; Bierbaum and Sahl 2009). Nisin A and subtilin are examples of subtype A(I), lacticin 481 and plantaricin C belong to subtype A(II) and mersacidin is a typical type B lantibiotic. This classification may need some modification, first because subtype A(II) and type B lantibiotics present just one gene that codifies for the dehydration and cyclization of the precursor peptide while the subtype A(I) has two separate genes. In addition, there are lantibiotics whose final structure does not conform to any of those defined by types A and B. Examples include sublancin and the two-component lantibiotics, made out by two peptides, both of which are needed for their biological activity (lacticin 3147).

Class II bacteriocins are traditionally subdivided into four subclasses: pediocin-like (class IIa), two component (class IIb), circular (class IIc), and miscellaneous (class IId). Class IIa is composed of a series of peptides initially grouped by their ability to inhibit *Listeria monocytogenes*. Since all of them present the YGNGV/L conserved peptide toward their NH_2-end, it was postulated that this was responsible for their antimicrobial activity. However, it is now clear that other amino acids, forming a "cationic patch" in the NH_2-terminal moiety, are also needed for the initial electrostatic interaction with the membrane phospholipids. The less conserved amphiphilic C-terminal part recognizes the susceptible bacteria and penetrates the hydrophobic membrane core, thanks to a hinge that is placed between both bacteriocin regions. Pediocin PA1/AcH, produced by *Pediococcus acidilactici*, is the model bacteriocin of this group (Ennahar et al. 2000; Drider et al. 2006).

Class IIb bacteriocins are composed by two different peptides, associated in equimolecular proportion. Each peptide may (thermophilin 13) or may not (lactococcin G and lactacin F) have antimicrobial activity, but they show a synergistic effect when acting together and, sometimes, their spectrum of susceptible bacteria changes with respect to that of the individual peptides (Nissen-Meyer et al. 2009; Perez et al. 2014).

Class IIc comprises the so-called circular bacteriocins. They are quite large (between 58 and 70 amino acids) and, although being synthesized as lineal peptides, become circularized by covalent binding between the first and last residues. This conformation makes them very resistant to heat (some retain their activity after

treatment at 121 °C for 15 min), pH variation, and proteolytic digestion. Two subgroups have been recognized based on their physicochemical characteristics; the first comprises cationic peptides with isoelectric points close to 10, its model bacteriocin being AS-48, while the second has isoelectric points close to neutrality or clearly in the acid range (gassericin A and butyrivibriocin A respectively) (Sánchez-Hidalgo et al. 2011; Gabrielsen et al. 2014).

Finally, class IId is made out by all bacteriocins that cannot be included in any of the first three classes. It is a miscellaneous group that includes: (i) lineal peptides that do not present the YGNGV/L motif of pediocins, such as lactococcin A, (ii) bacteriocins that do not have a dedicated export system but use the general secretory mechanism of the cell; enterocin P and lactococcin 972 are well-known representatives of this group (Cintas et al. 1997; Martínez et al. 1999) (enterocin P presents the YGNGV/L motif of pediocins, so it may be classified as a class IIa bacteriocin as well) and (iii) leaderless bacteriocins, such as lacticin Q, which, significantly, has a formylated methionine as its first residue (Fujita et al. 2007).

3 Genetics and Regulation of Bacteriocin Synthesis

The genes involved in bacteriocin production are usually clustered and, frequently, form part of mobile genetic elements such as conjugative transposons (nisin) and plasmids (lactococcin 972) (Lubelski et al. 2008; Martínez et al. 1999). This facilitates their spread and explains, for example, the ability of *P. pentosaceus* and *Lactobacillus plantarum* strains to synthesize pediocin PA1/AcH (Miller et al. 2005). The clusters may also be located into the chromosome (subtilin) (Asaduzzaman and Sonomoto 2009).

In the case of lantibiotics, two genetic organizations are found. In both cases, the structural gene (*lanA*) codes for a prebacteriocin with no antimicrobial activity due to the presence of a NH_2-extension of between 23 and 59 amino acids. This leader peptide acts as a scaffold for the posttranslational modification enzymes and serves as an export signal for the mature lantibiotic (Fig. 1).

The differences between type A(I) and type A(II)/class B lantibiotic synthesis clusters start with the genes that encode the posttranslational modification enzymes. In the first case, separate genes encode the Ser and Thr dehydratase (*lanB*) and the cyclase (*lanC*) that catalyzes their condensation with Cys to give the lanthionine residues, while, in the second, the product of a single determinant (*lanM*) fulfills both functions. LanM presents some sequence identity with LanC but no relation is perceived with LanB. In some cases, further modifications may be introduced by enzymes encoded by *lanD* genes; for instance, the C-terminal cysteine of mersacidin and mutacin 1140 (a type A(I) bacteriocin) is decarboxylated and converted into S-amino vinyl-D-cysteine (Asaduzzaman and Sonomoto 2009).

Once the posttranslational modification is completed, the lantibiotic is exported and the leader peptide eliminated. For the type A(I) molecules, secretion is mediated by the ABC transporter LanT and processing is performed by the extracellular

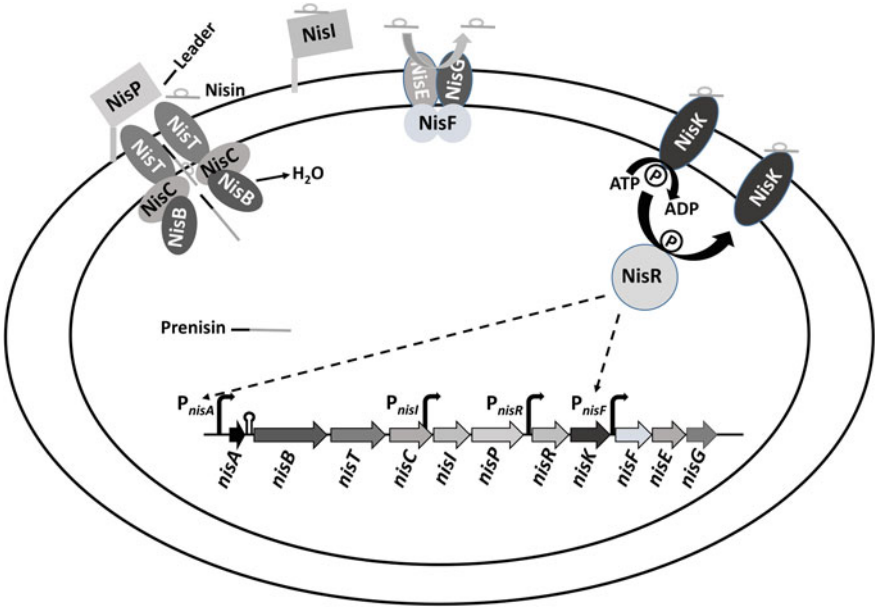

Fig. 1 Scheme of the lantibiotic nisin biosynthesis. The P_{nisI} and P_{nisR} promoters are constitutive and produce NisI that protects the cell and NisR-K that senses the presence of external nisin. Upon nisin-driven successive phosphorylation of NisK-NisR, this last protein induces expression of P_{nisA} and P_{nisF} and synthesis of prenisin occurs. This is recognized by the dehydratase NisB, folded by NisC, exported through NisT and the leader removed by NisP, to produce the active bacteriocin. NisI and the group NisE-F-G interact with mature nisin to avoid contact with the lipid II and the plasma membrane

protease LanP. LanT appears to recognize the leader peptide and, within it, the oligopeptide FNLD, located between positions −20 and −15 [this oligopeptide is a docking motive for LanB and LanC as well; Khusainov et al. (2011)] and a proline in position −2. Consequently, prepeptides and partially modified bacteriocins are secreted as well. In contrast, LanP only eliminates the leader peptide from totally modified molecules, this being the activation step of the bacteriocin. In the case of nisin, it appears that all intracellular biosynthesis steps are performed in multimeric complexes, bound to the internal layer of the membrane and formed by NisB, NisC and NisT dimers (Fig. 1) (Lubelski et al. 2008; Khusainov et al. 2011). Type A(II) and class B lantibiotics present a single gene (*lanT*) whose product fulfills both secretion and processing of the peptide, thanks to an extra NH_2-terminal peptidase domain that recognizes a double GG/GA motif close to the cleavage site (Altena et al. 2000; Asaduzzaman and Sonomoto 2009).

Bacteriocin activation occurs outside but in the vicinity of the plasma membrane. It becomes thus necessary to build a system that protects the producing cell. In the case of lantibiotics, this is fulfilled by LanI (for immunity) a lipoprotein located on the external layer of the membrane that binds the antimicrobial peptide and prevents

interaction with its targets. Additionally, a complex formed by the proteins LanEFG has the capacity to expel the lantibiotic from the membrane surface. Both systems appear to work synergistically, each one producing only 5–20 % of the full immunity level provided by both (Ra et al. 1999).

Production of bacteriocins in general and of lantibiotics in particular, is subjected to quorum sensing. The best characterized system is that of nisin (Fig. 1). In its biosynthetic cluster there are two contiguous genes, *nisR-nisK*, that form a constitutive operon and encode a response regulator and a histidine kinase, respectively. NisK protrudes though the membrane and recognizes extracellular nisin but not its precursors. Binding results in activation of the kinase activity, autophosphorilation of a His residue and subsequent transfer of the phosphate to NisR. NisR-P is a transcriptional activator that recognizes operator sequences located in the promoters of the main nisin operon, which governs expression of the *nisA-B-T-C-I-P* genes, and of *nisF-E-G*. Immediately behind *nisA* there is a transcription termination signal that is not absolute, thus allowing synthesis of high and moderate amounts of the prepeptide and the other proteins respectively. The rational of this is clear; nisin will be exported and diluted into the medium while the other proteins will remain associated to the cell. The increase of nisin concentration is paralleled with enhanced production of NisI and biosynthesis of the NisEFG proteins (Lubelski et al. 2008).

The genetic organizations for production of class IIa, IIb, and some IId bacteriocins are very similar. Two possibilities exist: a single operon that comprises all relevant genes or three clustered operons, one of which may have divergent transcription with respect to the other two (Ennahar et al. 2000). In the first case, the structural gene (or genes for class IIb) is/are followed by the immunity determinant, the ABC transporter that also eliminates the leader peptide (usually at a GG/GA motif) and a gene that encodes an accessory protein that might be involved in the immunity/export of the bacteriocin. This is the case for pediocin PA-1/AcH (class IIa) and lactococcin G (class IIb). In the case of the three-operon bacteriocins, the first includes the structural and immunity genes; the second, the ABC transporter/protease and the accessory protein determinants and the third carries the genes involved in regulation of bacteriocin production. This last operon is composed of three genes, the first of which encodes a pheromone peptide that structurally resembles a bacteriocin (small, cationic, amphipathic, heat-resistant, synthesized as a prepeptide that shares the processing signals) but without significant antimicrobial activity. The other two genes encode a membrane-associated histidine kinase and a response regulator, similar to those described for nisin (see above). Examples of this genetic organization are provided by sakacin P (class IIa) and plantaricin E/F (class IIb) (Brurberg et al. 1997; Nissen-Meyer et al. 2010).

The genetic organization of circular bacteriocin (class IIc) biosynthesis is not well understood. Between 5 and 7 genes appear to be needed, although some others, clustered with them, might play a role as well (Maqueda et al. 2008; Gabrielsen et al. 2014). Most proteins are hydrophobic, thus suggesting that bacteriocin synthesis is membrane associated. The structural gene encodes a prepeptide with a leader that may be as short as two amino acid residues. A second, cationic and

hydrophobic, peptide is involved in immunity. The system also includes an integral membrane protein that presents a DUF 95-like transport domain, which might be associated to an ATPase also present in the cluster and to several other proteins, all of which might participate in bacteriocin export. Complementarily, most clusters have in their vicinity an accessory operon encoding a putative ABC transport complex that might enhance bacteriocin production and/or immunity (Diaz et al. 2003).

4 Mode of Action

Bacteriocins are highly potent bactericidal agents. As mentioned before, some of them have a narrow spectrum of activity targeting only close related species but many, particularly those produced by lactic acid bacteria, are active against a wider panel of Gram positive bacteria. Remarkably, antibiotic and bacteriocin resistance are independent and, consequently, no cross-resistance has been recorded so far. Gram-negative bacteria are intrinsically resistant to peptide bacteriocins produced by Gram-positive organisms due to the protective role of their outer membrane that prevents reaching of their target, i.e., the plasma membrane. Consistently with this, Gram-negative bacteria may become susceptible to wide spectrum bacteriocins such as nisin, when combined with EDTA or organic acids that disrupt the outer membrane.

Pioneering studies on the mode of action of bacteriocins have been carried out with nisin, a lantibiotic produced by *Lactococcus lactis*, and with class IIa bacteriocins. Interaction with the plasma membrane and the formation of pores is a common theme among most LAB bacteriocins. However, in-depth structure–function studies have revealed that bacteriocins are not just mere pore formers but they may also interact with receptors or docking molecules, abrogating essential cell pathways which account for their potency. Moreover, non-membrane permeabilizing bacteriocins have also been described. A schematic representation of the mode of action of bacteriocins is depicted in Fig. 2. Unveiling the mode of action of bacteriocins is important to anticipate possible mechanisms of resistance as well as for a rational exploitation of their antimicrobial activity.

4.1 Pore Formation

The ability of many bacteriocins to interact with the plasma membrane resides on their physicochemical and structural properties. Pore-forming bacteriocins are unstructured in water but adopt an amphipilic α-helical structure in membrane mimicking environments that enables pore formation when present at high concentrations, i.e., in the micromolar range.

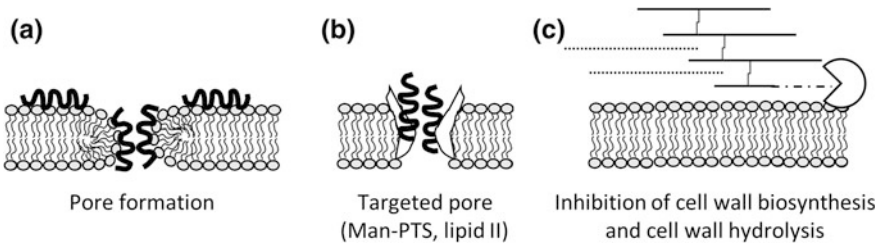

Fig. 2 Bacteriocin mode of action. **a** Upon interaction with the negatively charged cell envelope, pore-forming positively charged bacteriocins insert into the plasma membrane when present at high concentration (μM range). **b** Lipid II or the membrane components of mannose-PTS may act as receptors or docking molecules to promote pore formation. **c** Binding to the cell wall precursor lipid II inhibits the synthesis of peptidoglycan, which may be combined with pore formation (nisin and other lantibiotics) or not (Lcn972, mersacidin). Hydrolysis of the peptidoglycan is accomplished by activation of endogenous autolysins

Based on their cationic character, pore formation begins with electrostatic interactions between the peptide and the negatively charged bacterial surface. Using model membranes, nisin has been shown, for example, to initially interact with the anionic lipids by electrostatic forces, followed by peptide aggregation. Translocation of the C-terminus of nisin across the membrane phospholipids is favored by the negative membrane potential and a wedge-like transient pore is formed (Fig. 2) (Driessen et al. 1995). In class IIa pediocin-like bacteriocins, their conserved positively charged N-terminus interacts with the negative cell envelope constituents and folds into a three-stranded antiparallel ß-sheet connected by a hinge region to an α-helix and a C-terminal tail in a hairpin structure. Pore formation is achieved by insertion of this hydrophobic C-terminal domain into the cytoplasmic membrane, likely forming a barrel stave like arrangement (Drider et al. 2006; Nissen-Meyer et al. 2009). Two-peptide non-modified bacteriocins (class IIb) also adopt a helical structure in a hydrophobic environment, with the peculiarity that further structuring occurs upon interaction with their complementary peptides that finally results in insertion into the membrane. The two peptides are thought to interact through helix–helix interactions involving the GXXG motifs present in both peptides (Nissen-Meyer et al. 2009). Bacteriocins of class IIc, such as the circular enterocin AS-48, have a compact globular structure with 5 α-helixes whose conformation also changes in hydrophobic environments, facilitating membrane disruption (Sánchez-Barrena et al. 2003).

Alteration of the membrane permeability barrier leads to a total or partial dissipation of the proton motive force, ultimately causing energy exhaustion and cell death. The specificity of the pores varies, with some bacteriocins producing leakage of just monovalent cations and others that mediate the efflux of ions, amino acids, and ATP. Other factors, such as membrane lipid composition and a certain membrane potential threshold, may also be required to determine the fate of the pore (Moll et al. 1999).

4.2 *Docking Molecules or Receptors Involved in Bacteriocin Activity*

When compared to the eukaryotic antimicrobial peptides, one striking feature of pore-forming bacteriocins is their strong in vivo potency (nanomolar vs. micromolar range) and their narrower spectrum of activity. This is due to the formation of target-mediated pores, i.e., disruption of membrane permeability occurs upon high affinity interaction of the peptides with docking molecules or receptors present in the cell envelope (Fig. 2).

The cell wall precursor lipid II is a prominent docking molecule linked to the mode of action of several LAB lantibiotics including nisin, plantaricin C, lacticin 3147, nukacin ISK-1 and lacticin 481, among others (Breukink and de Kruijff 2006). Lipid II is an essential precursor of cell wall biosynthesis. Thereby, binding of lipid II by pore-forming lantibiotics results in the combination of two modes of action in the same molecule: inhibition of cell wall biosynthesis and pore formation (Wiedemann et al. 2001). In the case of nisin, it has been shown that lipid II stabilizes the pore and is part of the pore itself in a lipid II:nisin 4:8 stoichiometry (Hasper et al. 2004). Interaction of nisin with lipid II also implies that the cell wall precursor is delocalized from the sites where it is needed, interfering with normal cell growth and division (Hasper et al. 2006). It is worth mentioning the example of the lantibiotic lacticin 3147: in this case, one of its composing peptides, LtnA1, binds lipid II and the complex recruits LtnA2, promoting insertion into the membrane and generation of the pore (Wiedemann et al. 2006b). Pore formation upon lipid II binding seems to occur in a species-specific fashion in the case of more globular lantibiotics such as plantaricin C, highlighting the complex interactions of bacteriocins and their targets (Wiedemann et al. 2006a).

Lcn972 is so far the only non-lantibiotic bacteriocin known to interact with lipid II (Martínez et al. 2008a). Contrary to others, Lcn972 does not form pores on susceptible cells. Instead, it inhibits cell wall biosynthesis at the division septum. As a consequence, cells elongate and, finally, die. Lcn972 has a very narrow spectrum of activity, targeting lactococci exclusively, suggesting that it might require other receptor(s) that determine its specificity. Inhibition of septum formation during cell division has also been suggested for garvicin A (Maldonado-Barragán et al. 2013).

Several class II bacteriocins use mannose phosphotransferase transport systems (man-PTS) as receptors (Fig. 2). Lactococcin A, lactococcin B and several class IIa bacteriocins are membrane-disrupting peptides that form complexes with the man-PTS components IIC and IID, the two membrane-associated proteins of this sugar transporter (Diep et al. 2007). Further experiments demonstrated that class IIa bacteriocins that share a similar spectrum of activity specifically interact with a 40-residues N-terminal external loop of the IIC component, whereas lactococcin A requires further interactions with the man-PTS, explaining its spectrum of activity restricted to lactococci (Kjos et al. 2011). It is speculated that the conserved N-terminal β-sheet domain of these bacteriocins is involved in the initial recognition of the man-PTS receptor that further enables the C-terminal domain to insert into the

membrane to promote helix–helix interactions between the bacteriocin and the membrane proteins IIC/IID. It is not clear, however, how the integrity of the membrane is disrupted. Pores might be formed exclusively by bacteriocin molecules that assemble upon interaction with the man-PTS or, alternatively, the presence of the bacteriocin might force the transporter to remain in a permanent open-conformation (Kjos et al. 2011). Interestingly, the cognate immunity protein is part of the man-PTS-bacteriocin complex in the producer strains, suggesting that it may provide immunity by blocking efflux of intracellular solutes through the man-PTS.

Other putative bacteriocin receptors that have been recently proposed are the maltose ABC transporter for the circular peptide garvicin ML, a membrane metallopeptidase for the leaderless bacteriocin LsbB, and UppP, a membrane protein involved in cell wall biosynthesis, for the class IIb lactococcin G.

A large deletion encompassing several starch and maltose metabolic genes was identified in several independent *L. lactis* garvicin ML resistant mutants. Complementation with *malEFG* genes coding for the maltose ABC transporter restored sensitivity in *L. lactis* in a concentration-dependent fashion to wild-type levels, supporting its role in bacteriocin activity (Gabrielsen et al. 2012). The *L. lactis* Zn-dependent metalopeptidase YvjB was identified as the putative LsbB receptor using a cosmid library of a susceptible *L. lactis* strain. A resistant derivative transformed with this library was screened for susceptibility to LsbB and *yvjB* was identified as the only gene involved in restoration of the sensitive phenotype (Uzelac et al. 2013). Moreover, several independent resistant mutants were shown to carry mutations in the same gene and its heterologous expression in otherwise naturally resistant bacteria rendered them susceptible to LsbB. In this case, the last C-terminal 8 amino acids appears to be responsible of the interaction of LsbB and its putative receptor, as only peptides bearing these residues were able to block the inhibitory activity of LsbB (Ovchinnikov et al. 2014). Similarly, spontaneous *L. lactis* resistant mutants to lactococcin G mapped in *uppP*, which encodes a dispensable undecaprenyl pyrophosphate phosphatase involved in the synthesis of cell wall precursors. When this gene was expressed in the intrinsically resistant *Streptococcus pneumoniae*, it became susceptible to the bacteriocin (Kjos et al. 2014).

Physical interaction between these membrane proteins and the bacteriocins has not been experimentally proven yet. As described, their role as receptors is based on genetic and phenotypic evidences. Namely, knocking out the corresponding coding gene makes sensitive cells resistant, while its expression, even in heterologous hosts, is required for a sensitivity phenotype.

4.3 Other Modes of Action and Additional Killing Activities

The lantibiotics nisin, subtilin and bovicin HC5 inhibit spore outgrowth. The formation of lipid II targeted pores by nisin halts the establishment of the membrane potential, and consequently, inhibits metabolism in germinating spores which cannot develop further (Gut et al. 2011).

Besides binding to lipid II, nisin has been shown to bind to lipid-bound precursors of the wall teichoic acid (WTA) biosynthesis pathway and promote pore formation in artificial liposomes (Müller et al. 2012). Whether this binding blocks WTA biosynthesis and contributes to nisin killing in vivo is unknown so far.

Pore-forming bacteriocins may have other effects on target cells that contribute to killing (Fig. 2). Nisin and other lantibiotics have been shown to separate endogenous autolysins from their natural inhibitors, the anionic cell envelope polymers, by a cation exchange-like process, thus inducing cell lysis (Bierbaum and Sahl 2009). Accumulation of highly reactive hydroxyl radicals also contribute to the antimicrobial activity of the pore-forming lacticin Q. As described for some antibiotics, lacticin Q increased the levels of hydroxyl radicals and cell viability of lacticin Q-treated cells could be restored by radical scavengers (Li et al. 2013).

4.4 Mechanisms of Resistance

Bacteriocin resistance can be achieved by nonspecific and specific mechanisms. The first mostly alter the properties of the cell envelope to reduce, or even prevent, the interaction of these antimicrobials with the plasma membrane. Bacteriocin-specific mechanisms protect the cells against unique or a group of related bacteriocins; for example, as a consequence of the loss of a common receptor (Table 1).

One of the general strategies adopted by sensitive bacteria to reduce their susceptibility toward bacteriocins is to increase the number of D-alanine esters into the teichoic acids to reduce the net negative charge of the cell envelope. In this way the initial electrostatic interactions are weakened and the positively charged bacteriocins bind less efficiently to the cells (Peschel et al. 1999). The formation of stable pores can be further prevented by changes in the composition of membrane phospholipids that may alter its fluidity (Kaur et al. 2011). Production of exopolysaccharides, reduced surface hydrophobicity, and a thicker cell wall due to the increased activity of penicillin-binding proteins also contribute to prevent the

Table 1 Resistance mechanisms to bacteriocins

Bacteriocin	Mechanism	Consequence
Pore-forming	Changes on the cell envelope: Less negative surface Thicker cell wall Membrane fluidity	Impaired membrane insertion
Class IIa, lactococcin A, lactococcin B	Downregulation of man-PTS genes	Inhibition of pore formation
Nisin	Proteolytic degradation of nisin	Inactivation
Lacticin 3147/nisin	Active transport by ABC transporters or immunity-like proteins (immune mimicry)	Reduced local concentration

interaction of bacteriocins with the cytoplasmic membrane. All these changes on the cell envelope are often orchestrated by two-component systems that detect the stress caused by the bacteriocin and coordinate the expression of the genes involved in the phenotypes described above (Cotter et al. 2002; Martínez et al. 2007).

Multidrug resistance transporters and/or ABC transporters have been linked to resistance to nisin and other antimicrobials in several species (Majchrzykiewicz et al. 2010; Collins et al. 2010). These transporters provide protection by actively removing bacteriocin molecules out of the membrane, reducing their local concentration at the cell surface. In fact, immunity to several lantibiotics is often provided by such pumping devices in concurrence with small immunity proteins. Interestingly, bacteria carrying functional homologues of the ABC transporters involved in immunity have been detected in non-bacteriocin producers. This so-called immunity mimicry mechanism might be regarded as a specific bacteriocin resistance mechanism as it provides cross-resistance to bacteriocins that share similar immunity systems. Homologues to LtnFE, the ABC transporter involved in immunity to lacticin 3147, have been identified in non-lacticin 3147 producers (*Enterococcus, Bacillus*) and provide protection against this bacteriocin (Draper et al. 2009). These LtnFE homologues constitute a reservoir of bacteriocin resistance genes which may spread within lacticin 3147-sensitive populations.

Among bacteriocin-specific mechanisms, high resistance levels (over 1000 times) to all class IIa and some class IId bacteriocins have been linked to down-regulation of the man-PTS genes. Inactivating mutations have been mapped in *rpoN* that specifies the sigma factor σ^{54} and in the gene coding for ManR, a σ^{54}-dependent transcriptional regulator required for activation of the man-PTS genes (Kjos et al. 2011). A specific nisin-degrading enzyme, generally known as Nisin Resistance Protein (Nsr), has been detected in non-nisin producing *L. lactis*, *Bacillus* and several streptococcal species. *L. lactis* Nsr has been recently characterized as a lipoprotein with protease activity that specifically removes the last C-terminal amino acids of nisin (Sun et al. 2009).

Finally, several comparative genomic, transcriptomic, and phenomic studies of resistant strains have uncovered a possible role of global regulators on resistance to bacteriocins. This is the case, for example, of a putative regulator of an extracytoplasmic function sigma factor, which protected lactococcal cells against the cell wall-active bacteriocin Lcn972 (Roces et al. 2012a).

It is worth mentioning that resistance to bacteriocins usually implies energy costs that compromise the fitness of resistant mutants. In fact, despite of the wide use of nisin as a food preservative, resistance is not an issue for concern so far. Nevertheless, development of resistance to bacteriocins should be closely monitored as well as their impact in the physiology of the target bacteria. For example, development of bacteriocin resistance in *S. aureus* and *L. monocytogenes* inhibited infection of these food pathogens by specific bacteriophages, which are proposed as alternative preservatives in food (Martínez et al. 2008b; Tessema et al. 2011). In this scenario, the use of combined food preservation technologies (bacteriocins + phages) might become jeopardized.

5 Applications of Bacteriocins

Immediately after its discovery, nisin was applied to inhibit late blowing in cheeses caused by the gas formed by germinating *Clostridium tyrobutyricum* spores. The success of this approach along with the consumer's demands for foods without chemically synthesized preservatives encouraged the search for other bacteriocins to be applied as food preservatives. Furthermore, the current knowledge on bacteriocin biology is also paving the way for innovative strategies exploiting bacteriocins in other fields, from health to biotechnological processes (Fig. 3).

5.1 Preservation of Food Products

Bacteriocins produced by LAB have several attributes that make them suitable as food biopreservatives: ability to inhibit Gram-positive pathogenic and spoilage bacteria (*L. monocytogenes*, *S. aureus*, *Bacillus cereus*, *Clostridium botulinum*, *C. tyrobutyricum*, etc.); susceptibility to digestive proteases; stability in a wide range of temperature and pH values; no alteration of the organoleptic properties of food as they are taste-, odor-, and colorless; no toxicity to eukaryotic cells and simplicity to scale-up production.

Bacteriocins may be added to food as pure preparations with the advantage of knowing exactly the doses being added, which gives a complete control of their action. However, this increases the production costs and requires approval by

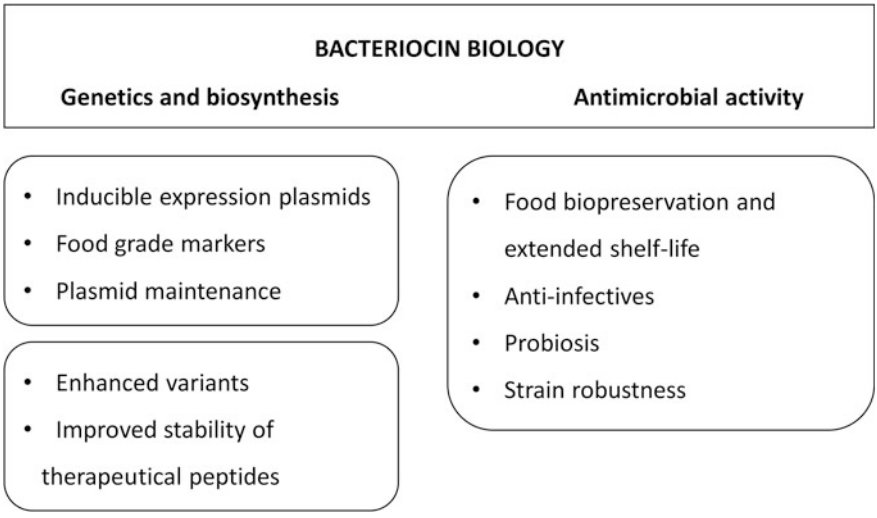

Fig. 3 Applications of bacteriocins based on their genetics and biosynthetic machinery as well as their antimicrobial activity

regulatory authorities. So far, nisin is the only bacteriocin approved worldwide as a food additive and is identified in the European Union as E234. A semi-purified preparation of Nisin A (Nisaplin® and Chrisin) is manufactured by Dupont Nutrition Biosciences ApS and Christian Hansen A/S, respectively. As a food additive, nisin shows high efficacy in protecting processed cheeses by controlling the outgrowth of spores of *C. botulinum* and *C. tyrobutyricum* (5–15 mg/kg), the development of *L. monocytogenes* in Riccotta cheese (2.5–5 mg/kg), also being used in stirred yogurt (0.5–1.25 mg/kg) to avoid overacidification through the shelf life (Delves-Broughton 2007). Nisin is less effective in meat products due to its adsorption to fat and enzymatic inactivation by gluthatione-S-transferases present in fresh beef muscle (Rose et al. 2002). However, nisin is effective in inhibiting the growth of lactic acid bacteria in vacuum-packed cooked ham stored for 60 days (Kalschne et al. 2014). Nisin and pediocins are also able to reduce *L. monocytogenes* in fresh-cut lettuce when used as a substitute of chemical disinfection in the washing solution (Allende et al. 2007).

In some countries, such as the US and Canada, it is allowed the use of fermentates as food ingredients, which may contain bacteriocins along with organic acids. They are produced by the fermentation of food grade substrates by bacteriocin-producing bacteria. Some already commercialized are: ALTA™ 2431 (pediocin PA-1/AcH, Quest International); MICROGARD™ (with a Propionibacterium bacteriocin; Dupont Nutrition Biosciences ApS); and Bactoferm F-LC (a mix of a pediocin and a sakacin; manufactured by Christian Hansen A/S).

Bacteriocins can also be applied in food by in situ production. This requires bacteriocinogenic strains (protective cultures) well adapted to the particular food matrix (e.g., milk) where they grow and produce bacteriocin under the particular processing, ripening, and storage conditions (Gálvez et al. 2007). This system is cost-effective and is not affected by legal regulations. Nisin-producing *L. lactis* inhibit *L. monocytogenes* in Camembert cheese (Maisnier-Patin et al. 1992), *S. aureus* in Afuega'l Pitu cheese (Rilla et al. 2004), *Listeria innocua* in semi-hard cheese made with raw milk (Rodríguez et al. 2000) and *Cl. tyrobutyricum*, a late blowing agent, in semi-hard Vidiago cheese (Rilla et al. 2003). Protection against late blowing was also provided by the bacteriocin producer *Lactobacillus gasseri* K7 (Bogovic Matijasic et al. 2007). Spraying lacticin 3147 producers on the surface of smear and cottage cheeses inhibited *L. monocytogenes* development (O'Sullivan et al. 2006; McAuliffe et al. 1999). These bacteriocinogenic starters are also able to control cheese quality by suppressing growth of nonstarter lactic acid bacteria (NSLAB) populations during ripening. In this way, the risk of flavor defects in low-fat Cheddar cheese was reduced (Fenelon et al. 1999). Likewise, bacteriocin-producing cultures accelerate cheese ripening by increasing the lysis of the starter strains with the consequent release of intracellular enzymes to the cheese matrix. This is the case of the strain *L. lactis* DPC 3286 (lactococcins A, B, M producer) in Cheddar cheese (Morgan et al. 2002) or *L. lactis* INIA 415 (lacticin 481 and nisin Z producer) in Hispánico cheese (Ávila et al. 2005).

Bacteriocinogenic enterococci have been used as adjunct cultures in both cheeses and dairy fermented beverages. *Enterococcus faecalis* AS 48-32, the AS-48

producer, clearly inhibited *Bacillus cereus* growth in nonfat hard cheese throughout ripening (Muñoz et al. 2004). The enterocin A producer *E. faecium* MMRA, as protective adjunct culture, halted the growth of *L. monocytogenes* but not its viability during cold storage of Rayeb (Rehaiem et al. 2012).

Bacteriocins can be used in combination with other methods of preservation (heat, chelating agents, carbon dioxide, other biocides, etc.) as part of hurdle technology to enhance their efficacy. There are some examples compiled in the literature, most of which aimed to broaden the inhibition spectrum, to reduce the concentration needed of each preservative or to control the proliferation of sublethally injured cells (Gálvez et al. 2007). Pediocin PA-1/AcH combined with a CO_2 atmosphere showed a synergistic effect against foodborne pathogens (Nilsson et al. 2000), while nisin activity against *C. sporogenes* was enhanced by nitrites (Rayman et al. 1981). Inactivation of this microorganism in milk was also enhanced by combining nisin and high pressure (Arqués et al. 2005). In addition, nisin was combined with pulse electric fields to inactivate *S. aureus* in milk (Sobrino-López and Martín-Belloso 2006), *L. innocua* in whey (Gallo et al. 2007) and *L. monocytogenes* in skim milk (Calderón-Miranda et al. 1999). Enhanced bactericidal activity against *L. monocytogenes* was shown by enterocin AS-48 in combination with chemical preservatives, essential oils and natural bioactive compounds (Cobo-Molinos et al. 2009).

The incorporation of bacteriocins in packaging systems allows slow release on the food products, thereby extending their shelf life. The antimicrobial efficacy may even be higher than that resulting from the addition of bacteriocins to the food matrix. In this regard, low-density polyethylene (LDPE) films coated with nisin inhibited *Micrococcus luteus* in raw milk (Mauriello et al. 2005), while enterocin 416K1 showed antilisterial activity on cheeses during cold storage (Issepi et al. 2008). Soy-based films impregnated with nisin and lauric acid reduced up to 6 log units the *L. monocytogenes* concentration on turkey bologna after 21 days at 4 °C (Dawson et al. 2002), while the immobilization of nisin on polyamide and cellulose lowered *S. aureus* concentration in Cheddar cheese (Scannell et al. 2000a) and in cheese slices under cold conditions for 3 months (Scannell et al. 2000b). Going further in the antimicrobial active packaging concept, nisin has also been incorporated (1–2 %) into polypropylene/montmorillonite nanocomposites to make packaging food material and inhibited *L. monocytogenes*, *S. aureus* and *C. perfringens* when tested on skimmed milk agar plates, thereby having a great potential to reduce post-process growth of food pathogens (Meira et al. 2014).

5.2 Medical and Veterinary Applications

The potency of bacteriocins in vitro, their low toxicity, and no cross-resistance with antibiotics supports their use as antimicrobials in clinical settings. Narrow spectrum bacteriocins can be used to fight against targeted pathogens without disturbing

commensal populations. By contrast, broad-spectrum bacteriocins might be effective against unidentified infections produced by Gram-positive bacteria.

Lantibiotics such as nisin and lacticin 3147 exhibit in vitro activity against *S. pneumoniae*, methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant enterococci (VRE), and *Clostridium difficile* (Piper et al. 2009). The combination of nisin with ramoplanin, an antibiotic that inhibits peptidoglycan biosynthesis, exerts a synergistic effect on MRSA and VRE (Brumfitt et al. 2002). Topic formulations containing bacteriocins active against *Propionibacterium acnes* have also been tested for the treatment of acne (Kang et al. 2009). Lacticin 3147 was effective in vivo to treat a systemic infection by *S. aureus* Xen 29 in a murine model by subcutaneal treatment (Piper et al. 2012).

Nisin and lacticin 3147 have also been tested against clinically significant Mycobacteria (*Mycobacterium tuberculosis*, *M. avium*, *M. kansasii*). Lacticin appeared to be more effective than nisin (MIC_{90} 7.5 vs. 60 mg/L) (Carroll et al. 2010).

Nisin appears to be an efficient alternative to antibiotics for treatment of mastitis treatment when applied as a topic solution (6 μg/mL) according to the results obtained in a clinical trial with the participation of eight women with clinical signs of staphylococcal mastitis (Fernández et al. 2008). In addition, an intramammary infusion containing nisin (MasOut®) is being developed as an alternative treatment for mastitis in lactating cows. The combination of lacticin 3147 with a commercial teat seal resulted in increasing protection against the mastitis-causing pathogen *Streptococcus dysgalactiae* (Ryan et al. 1998).

Another medical application of bacteriocins under exploration is their use against the etiological agent of gastroduodenal ulcer *Helicobacter pylori*, based on its in vitro susceptibility to nisin, pediocin O2, leucocin K, lacticins A164, BH5, JW3, and NK24 (Kim et al. 2003). Previously, a combination of nisin with glycerol monolaurate has been patented by Applied Microbiology Inc, as a method for treatment *H. pylori* infection and eradication of its colonization (Blackburn et al. 1997).

Currently, a few healthcare products containing bacteriocins are commercially available, including an edible toothpaste in Japan (www.neonisin.com) and a topical cream for treatment of skin infections (Biosynexus Inc, MA, USA). Nevertheless, some chemical properties of bacteriocins, in particular of lantibiotics, such as their low solubility, low activity at high pH, rapid degradation by intestinal enzymes and interaction with blood components should be improved to promote their use in health care.

5.3 Role of Bacteriocins in Probiosis

Bacteriocin production by probiotic bacterial strains may be regarded as an important trait in strain selection due to the putative advantage in the competitive dynamics established between bacteriocin producers and closely related species

within very complex microbial communities as those of the gastrointestinal tract. The mechanisms behind the probiotic effect of bacteriocin production in vivo are still obscure. The contribution of bacteriocins to a positive effect on host health might be attributed to: (i) their action as colonizing peptides that allow probiotics competition with the indigenous microbiota; (ii) the elimination of pathogens by virtue of their antimicrobial activity; (iii) their role as signaling peptides that recruit other bacteria and/or the immune system to eliminate infectious microorganisms (Dobson et al. 2012).

Up to 56 species of *Lactobacillus* have been found as part of the intestinal microbiota. Most of them produced bacteriocins in vitro, and some also in vivo (Gillor et al. 2005), with activity against both Gram positive and Gram negative pathogens. *L. johnsonii* LA1 (Gotteland and Cruchet 2003) and *L. acidophilus* LB (Coconnier et al. 1998) inhibited *H. pylori* bound to intestinal epithelial cells. Another interesting study demonstrated that *L. salivarius* UC118 produces in vivo the potent bacteriocin Abp118 that protected mice from *L. monocytogenes* infection (Corr et al. 2007). Similarly, *L. casei* L26 LAFTI inhibited enterohemorrhagic *E. coli* and *L. monocytogenes* in mice (Su et al. 2007). Moreover, it has been reported that bacteriocin production by probiotic lactobacilli inhibited pathogens (e.g., *Gardnerella vaginalis*) in the human vagina (Aroutcheva et al. 2001).

Probiotic supplements containing bacteriocins administered to broiler chickens resulted in an increased rate survival when they were challenged with *Salmonella pullorum* (Audisio et al. 2000). Bacteriocin production is also one of the factors associated to the antibacterial effect of probiotics used as biocontrol agents in aquaculture (Verschuere et al. 2000). They are used as food supplements to improve the performance of aquatic microorganisms, being effective as growth promoters and pathogen inhibitors.

5.4 Expanding the Biotechnological Potential of Bacteriocins

The primary metabolite nature of bacteriocins along with a rather simple biosynthetic pathway facilitates the design of novel molecules through gene-based peptide engineering and, consequently, may expand their use as antimicrobials (Perez et al. 2014). The spectrum of inhibition, stability, interaction with food matrices or specific activity of bacteriocins could be modified by genetic engineering. For instance, a single amino acid change in nisin Z (the methionine at position 21 was replaced by lysine) resulted in a fivefold increase of its solubility at pH 8, which was accompanied by inhibition of Gram-negative bacteria (Yuan et al. 2004). Similarly, a nisin A derivative (glycine at position 29 was replaced by serine) had an extended inhibitory spectrum toward foodborne pathogens such as *Cronobacter sakazakii*, *E. coli* and *Salmonella enterica* serovar Tiphymurium (Field et al. 2012).

It has been also possible to create bacterial strains that produce several bacteriocins by conjugation of multiple bacteriocinogenic plasmids. In this way, the performance of commercial dairy starters can be enhanced as exemplified by transferring the lacticin 3147-encoding conjugative plasmid pMRC01 to Cheddar cheese starter strains (Coakley et al. 1997).

Genes involved in bacteriocin biosynthesis have been used to develop tools for genetic engineering, including regulated gene expression in Gram-positive bacteria. An important property of the nisin-inducible promoters is that the degree of activation depends on the intensity of the stimulus, i.e., the concentration of the extracellular nisin. This has been used for designing the NICE system, made out by cells that express *nisR-K* and vectors with multicloning sites behind the *nisA* promoter, where protein determinants can be inserted. Production of these proteins will be a function of the concentration of nisin added to the cell culture (Mierau and Kleerebezem 2005). The immunity determinants have also been exploited as food-grade markers in cloning vectors that make use of bacteriocins as selecting agents and, more recently, the introduction of full bacteriocin operons into plasmids has demonstrated to prevent plasmid segregation under non-selecting conditions (Takala and Saris 2002; Campelo et al. 2014). The development of these plasmids helps to reduce the use of antibiotics in biotechnological processes with genetically modified bacteria. Other strategies encompassed the use of the nisin biosynthetic machinery to introduce thioether rings into foreign peptides to improve their stability under harsh conditions (Kluskens et al. 2005).

Finally, bacteriocins may be very useful tools to uncover the molecular mechanisms and the physiology behind the stress response in susceptible bacteria. For example, the use of the cell wall-active bacteriocin Lcn972 has been instrumental to define the core response of *L. lactis* to cell envelope stress and to identify biomarkers for strain robustness (Roces et al. 2012b).

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Control of Bacterial Growth Through RNA Degradation

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Abstract The amount of the different RNAs present in a bacterial cell must be perfectly coordinated in terms of rate of synthesis and degradation to allow balanced growth adjusted to the environmental conditions such as temperature, osmolarity, nutrients, pH, and the like. Any given compound causing imbalanced growth, causing acceleration of the degradation of mRNAs before they can be translated, or lack of RNA processing to properly form the tRNAs or even the ribosomes, may be at least in principle be looked upon as potential antibacterial compound.

1 Introduction

The chemical composition of a bacterial cell varies according to its growth rate and its physiological stage. In 1957, Holm, using continuous culture conditions, was able to identify the influence of the bacterial growth rate (μ) on the chemical composition of the cells. It was also noted, in batch cultures, that the transition from a logarithmic to a stationary growth phase produced many metabolic changes, and this also altered the cell composition (Ecker and Lockhart 1961).

Under certain nongrowing conditions, such as long-term starvation, bacteria catabolize their own ribosomal RNAs (rRNAs), although they may spare their ribosomal proteins (Julien et al. 1967). Hence, when bacterial cells recover from long starvation periods, they must resynthesize their rRNAs and reassemble their ribosomes (Jacobson and Gillespie 1968). RNA synthesis in *Escherichia coli* encompasses two distinct periods, separated by a 40–60 min catabolic interval.

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The RNA synthesized during the first period is mainly rRNA (70 %), whereas a variety of RNAs are produced during the latter period, as shown by its sedimentation properties.

Bacterial cells contain a high variety of RNases, and they display an array of functions, some of which will be addressed here. Messenger RNA (mRNA) degradation plays an important role in the bacterial cell cycle, as it is involved not only in the so-called "salvage pathways" but also in transcription control, in order to adapt to new growing or physicochemical conditions. By assessing the half-life of mRNAs of interest, it has been shown that ribonucleases control a variety of pathways involved not only in pathogenesis or virulence factors, but also in bacterial motility and cellular secretion. All of the above identify RNases as new potential targets for novel antimicrobials to control microbial pathogens. Apart from mRNAs, transfer RNAs (tRNAs) and rRNAs must be also taken into account, as they are essential players in the cellular translation machinery. They also represent putative novel antibacterial targets, as either their absence or malfunction would have a bacteriostatic or bactericidal effect on the bacterial pathogens.

Chemical compounds such as nitrofurans (Fig. 1) are known to have powerful antibacterial activity (Stillman et al. 1943), they include furazolidone, furylfuramide, nitrofurantoin, nitrofurazone, nifuratel, nifurquinazol, nifurtoinol, and nifuroxazide, with more than 3500 such compounds synthesized thus far. These chemicals have been banned from use in animals destined for human consumption, since nitrofuran-derived metabolites can remain in foodstuffs bound to proteins. Their activity includes inhibition of carbohydrate metabolism (by inhibiting the synthesis of acetyl-CoA from pyruvate), double-stranded DNA (dDNA) breakage, and interference with the normal role of ribosomes. These synthetic antibacterial drugs, when applied extensively, interfere with gene expression in a highly specific manner (Herrlich and Schweiger 1976). If applied at low concentrations, they do not affect transcription, but still inhibit translation of inducible genes. This discovery was, in fact, one of the first hints in molecular biology that suggested heterogeneity in the cellular mRNA population and, in turn, opened the possibility of selective action on the transcriptional/translational apparatus.

Several nitrofurans, however, have been found to be carcinogenic to mice, rats, hamsters, and dogs, and possibly humans (Morris et al. 1969; Cohen et al. 1970, 1973; Yahagi et al. 1974). They have also been reported to interfere with DNA synthesis in bacteriophages, as well as prophage induction (McCalla 1964), breakage of bacterial DNA (McCalla et al. 1971), and in general act as mutagenic

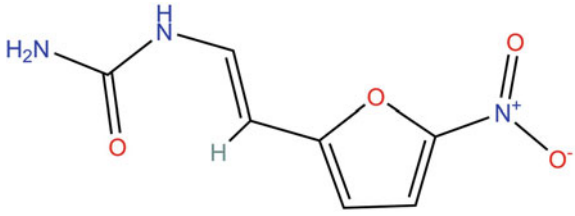

Fig. 1 Nitrofurazone molecule

agents (McCalla and Voutsinos, 1974). Mukherjee and Chatterjee (1992) concentrated on the binding of nitrofurans to bacterial DNA and found that there was a very strong interaction, in vitro, between nitrofurantoin and *Vibrio cholerae* DNA (association constant of 5.04×10^{-6} M), with an average of 0.015 binding sites per nucleotide. All the available data suggest that nitrofurans act by intercalation, resulting in an increase in the DNA melting temperature.

There are additional antibiotics that have the ability of controlling RNA synthesis, either by attaching to the DNA and blocking the formation of the transcriptional initiation complex, or by directly inhibiting the enzyme involved in transcription (DNA-dependent RNA polymerase). These antibiotics include actinomycins, rifamycins and streptovaricin, streptolydigin and tirandamycin, Microcin J25, sorangicins, myxopyronins, corallopyronin, ripostatins and fidaxomicin (for a comprehensive review, see Gacto et al. 2014). Actinomycins are mostly active against Gram-positive bacteria, but their therapeutic value is greatly reduced due to toxicity. They exert their action by binding to target DNA, thus inhibiting the formation of the transcription initiation complex, as well as to the DNA-dependent RNA polymerase. Rifamycins are active against both Gram-positive and Gram-negative bacteria and are produced by *Amycolatopsis mediterranei* (formerly known as *Streptomyces mediterranei* or *Nocardia mediterranei*). These antibiotics include streptovaricin and inhibit the DNA-dependent RNA polymerase by interfering with the enzyme's polymerizing activity, thus halting transcription when the RNA is only 2–3 nt in length (Campbell et al. 2001). This group of antimicrobial compounds is quite effective against intracellular nonreplicating tuberculosis bacteria (Shinnick 1996). Streptolydigin is produced by *Streptomyces lydicus* and displays the structural features of a typical tetramic acid compound (Gacto et al. 2014). Both streptolydigin and tirandamycin inhibit the initiation, elongation, and pyrophosphorolysis carried out by bacterial RNA polymerases. These antibiotics have been produced by chemical synthesis, accomplished by assembling commercially available precursor compounds (Pronin and Kozmin 2010). Microcin J25 is produced by certain strains of *E. coli* and shows antibacterial activity by inhibiting RNA polymerase. The proposed mechanism of action for microcin J25 is a two-step inhibition of transcription; it first binds onto the secondary channel on the RNA polymerase, and then obstructs this channel like "a cork in a bottle" (Gacto et al. 2014). Sorangicins are complex macrolides polyether antibiotics, isolated from the myxobacteria *Sorangium cellulosum*, that inhibit only the bacterial DNA-dependent RNA polymerase at the formation of the initiation complex (Irschik et al. 1985). Myxopyronins are α-pyrone antibiotics produced by the myxobacteria *Myxococcus fulvus* that inhibit bacterial RNA polymerases without affecting their eukaryotic counterparts (Tupin et al. 2009). They are active against a high number of both Gram-positive and Gram-negative bacteria, with the latter usually more resistant due to their possession of strong efflux pumps. Corallopyronin is also a α-pyrone antibiotic produced by the myxobacteria *Corallococcus coralloides*, this drug is similar to, although less potent than, myxopyronins (Gacto et al. 2014). Ripostatins are macrocyclic lactone produced by the myxobacteria *S. cellulosum* and their antibacterial properties result from

inhibition of the bacterial RNA polymerase, in a similar way to the action of myxopyronins but with a narrower spectrum and potency. Finally, fidaxomicin is a macrocyclic lactone produced by *Dactylosporangium aurantiacum*, *Actinoplanes deccanensis,* and *Micromonospora echinospora* (Gacto et al. 2014). It displays a potent antibacterial activity against most Gram-positive bacteria, including *Clostridium difficile,* as well as some Gram-negative, by interfering with their RNA synthesis.

We will now concentrate on the characteristics of a series of microbial RNases that play a role in RNA metabolism, in turn affecting bacterial growth, and hence are putative new targets for the development of novel antibacterial drugs.

2 Ribonuclease R

This RNase was described as a phosphodiesterase by Roth, as early as 1954, as it displayed this activity in rat liver and kidney, splitting the RNA diester phosphate links and giving rise to mixtures of mono and oligonucleotides soluble in acid or acid-alcohol. Roth also found that these RNases did not produce any inorganic phosphate.

However, it was McLellan and Vogel (1970) who provided evidence on the role of environmental conditions on the expression level of some *E. coli* genes. These authors were able to dissect translational from transcriptional processes when studying the synthesis of arginine in *E. coli* K12, using either ArgR+ or ArgR− strains. They found that while in the presence of excess arginine the argR+ strain produces relatively little translation, which is not the case for the argR− strain. This was "*due to an accelerated degradation of mRNA in the argR+ strain under repressive conditions.*"

Messenger RNAs are some of the least stable molecules in biology (Brenner et al. 1961) and this is mainly due to the existence of RNases. Furthermore, each mRNA has a particular rate of decay, in *E. coli* this varies from as little as 30 s to as long as 8 min, or even longer if the incubation temperature drops below 37 °C. Srivastava et al., in 1992, coined the term RNase R (R meaning residual). These authors found that crude extracts from the *E. coli* RNase I-less mutant contained both RNase M (an enzyme with a Mw similar to that of RNase I, 23–28 kDa, and a preferred activity on pyrimidine–adenosine bonds) and RNase R (a 24 kDa enzyme with a 3′–5′ exoribonuclease activity and encoded by the *rnr* gene located at 95 min on the *E. coli* chromosome). RNase R was not an abnormal product of the mutant *rna* gene and, interestingly, it was active on any phosphodiester bond; although its activity was 100 times lower than that of RNase I. This finding suggested that RNase R was an additional enzyme involved in mRNA degradation and salvage pathways. RNases with greater specificity must have the primary function of processing specific RNA molecules at a limited number of unique sites. RNase R is not capable of degrading either complete RNA–RNA or DNA–RNA hybrids, or hybrids with a 4-nucleotide 3′-RNA overhang. Additionally, it cannot digest DNA

oligomers, suggesting that it does not bind DNA (Cheng and Deutscher 2002). In fact, Vincent and Deutscher (2006) found that tight binding and activity of RNase R required a minimum 7 nucleotides 3′ overhang, whereas 10 or more nucleotides were required for optimum binding and enzymatic activity. RNase R and polynucleotide phosphorylase (PNPase) mediate a quality control process that removes defective rRNAs, hence, when these RNases malfunction, rRNA fragments accumulate, and ribosomal maturation does not occur (Cheng and Deutscher 2003). RNase R is also important for the removal of mRNA fragments exhibiting secondary structures and, as such, directly involved in the mRNA decay pathway (Cheng and Deutscher 2005). In Gram-positive bacteria, such as *Bacillus subtilis,* four 3′–5′ exoribonucleases have been identified: PNPase, RNase R, RNase PH, and YhaM (Oussenko et al. 2005). PNPase is the major enzyme involved in mRNA turnover, although RNase R is also capable of degrading the mRNAs (Oussenko et al. 2005).

Hong et al. (2005) highlighted the importance of RNase R in the bacterial cell cycle and found that this enzyme, a highly conserved 3′–5′ exoribonuclease, is required for the selective degradation of single-stranded RNA (ssRNA) in stalked cells of *Caulobacter crescentus*. Therefore, it plays an important role in the control of cell cycle progression. Additionally, placing bacteria under stress can produce a dramatic increase in their RNase R activity. Accordingly, Chen and Deutscher (2005) found that when *E. coli* entered stationary phase or suffered from starvation, its response involved a considerable increase (up to 10-fold) in RNase R activity. This fact was later confirmed by Andrade et al. (2006), who demonstrated that RNase R synthesis is induced in the stationary phase of growth, and that this enzyme is involved in the posttranscriptional regulation of ompA mRNA, correlating with and increase in the amount of OmpA protein found in the bacterial outer membrane. This report represented the first evidence linking RNase R levels to the phase of bacterial growth, rather than to the growth rate, and suggesting that this enzyme is a modulator of gene expression in bacterial cells in stationary phase. RNase R and PNPase span an S1 domain that allows these enzymes to overcome certain constraining superstructures in the RNAs (including the genesis of multimeric forms) that may protect them from hydrolysis by other RNases (Amblar et al. 2007).

RNAse R appears to play a different role in phytopathogenic Gram-negative bacteria, such as *Pseudomonas syringe*, as compared to *E. coli*. Purusharth et al. described in 2007 that RNase R interacts with RNase E in the so-called "degradosome" (see below) in cold-adapted bacterial strains, preventing them from dying when subjected to low temperatures (presumably by optimizing the RNA metabolism and turnover rates). When null mutants of this enzyme were used in the same experiments, they accumulated large amounts of mRNA fragments, as well as untrimmed additional ribonucleotides at their 3′ ends, which resulted in defficient protein synthesis and eventually led to cell death. The role of RNase R as a stress-induced protein was also confirmed in Gram-positive bacteria, such as *Streptococcus pneumoniae* (Moreira et al. 2012), and has also been shown to play an important role in the establishment of virulence in several pathogenic bacteria.

Additionally, RNase R is involved in trans-translation processes, such as rescuing bacteria containing high numbers of stalled ribosomes.

Bacteria lacking an active form of RNase R may fail to gain "genetic competence," as described for the facultative intracellular pathogen *Legionella pneumophila* (Charpentier et al. 2008). As lack of genetic competence puts the bacteria in a bacteriostatic situation, we can infer that either an RNase imbalance or stimulation of its enymatic activity by drugs could lead to bacteria not growing, hence offering a new therapeutical target. Sulthana et al. (2011) isolated an Antarctic bacterium (*Pseudomonas syringae* Lz4W) that produces a heat-labile RNase R (largely inactivated at 37 °C), and requires Mg^{2+} and Mn^{2+} for activity. The enzyme shows maximum activity on poly(A) and poly(U) ribohomopolymers, although it is also capable of hydrolyzing the mRNA from *mal E/F genes*. This enzyme, apart from releasing 5′-nucleotide monophosphates with its processive exoribonuclease activity, also produces four-nucleotide end products (instead of the three-nucleotide compounds produced by *E. coli*). Three of those products, namely ATP, GTP and CTP, can inhibit RNase R. Recently, Jacob et al. (2013) reported a quality control mechanism in ribosomes, involving both RNase R and YbeY (an endoribonuclease), which degrades defective 70S ribosomes and could impair the normal bacterial translational machinery. Once more, these results suggest that RNase R is a good target for the development of novel antibacterial drugs. Ribosomes play an important role in cell homeostasis, and most of the RNase R molecules are located in ribosomes in growing cells. On the other hand, the free form of the enzyme is quite unstable, with a half-life of only 2 min, and deleterious to the cell (Liang and Deutscher 2013).

3 Ribonuclease P

Purification of RNase P (EC 3.1.26.5) was described in 1972 by Robertson et al. as a specialized form of RNase found in *E. coli* and involved in the processing of tyr tRNA (see also Bikoff and Gefter 1975). In fact, later on RNase P was shown to be an endoribonuclease that catalyzed the 5′ maturation of precursor tRNAs in all organisms, from bacteria to archaea/eukarya (the only exception so far is the archaeon *Nanoarchaeum equitans*; Randau et al. 2008). It consists of a small autocatalytic RNA (ribozyme, see below) that is assisted by a variety of proteins (for the RNase P in the eukaryotic nucleolus, these are hPop1, hPop5, Rpp20, Rpp25, Rpp30, Rpp38, and Rpp40, plus three additional proteins associated with at least a subset of RNase MRP particles, i.e., Rpp14, Rpp21 and Rpp29; Mattijssen et al. 2011) to perform its tRNA processing role. This is not the case in human mitochondria, where RNase P is exclusively composed of proteins (Holzmann et al. 2008; Walker and Engelke 2008). In 2011, Mattijssen et al., found that this enzyme can also cleave viperin mRNA (VIPERIN stands for Virus Inhibitory Protein, Endoplasmic Reticulum-associated, INterferon-inducible). Viperin can be induced in a variety of cell types by different cellular factors, such as type I, II and III

interferons, DNA and RNA viral proteins, poly (I: C), and polysaccharides, and it may also play a role in transcription mediated by RNA polymerases I and III (Reiner et al. 2006). RNase P is a good target for antimicrobials directed to both prokaryotic and eukaryotic microorganisms, since its malfunction would result in the depletion of the cellular aminoacil tRNAs, and that, in turn, would make the cells go into stringent response (Haseltine and Block 1973). RNase P is responsible for the processing of precursor RNA to generate the bacterial 4.5S RNA, and is also involved in the in vitro cleavage of a bacteriophage phi80-induced RNA which is 62 nucleotides long (Bothwell et al. 1976). Stark et al. (1978) found this enzyme to be rather unusual, since its activity on precursor tRNA substrates can be abolished by pretreatment with either ribonuclease A, proteases, or by thermal denaturation. RNase P spans a main RNA fragment (necessary for enzymatic activity) and a polypeptide, with a buoyant density in CsCl of 1.71 g/ml, which is typical of a protein–RNA complex. A year later, Kole and Altman (1979) described that this peculiar enzyme could be reconstituted in vitro from its inactive precursor RNA and polypeptide. Kole and Altman, in 1981, fully characterized the *E.* coli RNase P, they reported that the polypeptide (inactive by itself) had a molecular weight of 17,500 Da and became active when mixed with a RNA species referred to as M1 RNA. These authors also found that the rate at which RNase P cleaves any particular tRNA precursor molecule depends on the identity of that tRNA (for a review, see Gegenheimer and Apirion 1981). Interestingly, the different RNase P enzymes from Gram-positive bacteria, such as *B. subtilis,* were found to have a similar biochemical composition and mode of action, and they were closely related to their *E. coli* counterpart (Gardiner and Pace 1980). Our understanding on the mode of action of RNase P was greatly increased by the work of Guerrier-Takada et al. 1983. They found that the RNA moieties of the ribonucleases P purified from either *E. coli* or *B. subtilis* could cleave tRNA precursors, but only in the presence of either spermidine or other proteins. Thus, the RNA portion, not the protein, acted as the catalytic moiety, but the protein helped modulate substrate affinity and specificity and stabilized the catalytic RNA moiety (Subbarao et al. 1984). Lawrence and Altman (1986) used site-directed mutagenesis on the RNA portion to modulate its biological activity. The nucleotide sequence of these catalytic RNAs varies between Gram-positive and Gram-negative bacteria, although both share a conserved core region. The different enzymes possess or lack additional structural domains, according to the particular RNA processing requirements in different bacteria (James et al. 1988). There is no structural homology between bacterial and archaeal-eukaryotic RNase P proteins. This is probably due to the enzyme being originally composed of "RNA-alone" and also because the bacteria and Archaea lineages diverged very early in evolution and, hence, used completely different strategies in the recruitment of protein subunits during the transition from the "RNA-alone" to the "RNA-protein" composition of the enzyme (for a comprehensive review, see Hartmann and Hartmann 2003).

RNase P is not only involved in the processing/generation of tRNAs, but may also be involved in the physiology of some of the viruses that infect higher plants, such as in the turnip yellow mosaic virus RNA.

There are some intriguing facts concerning RNase P, such as the discovery by Gold et al. (1988) that in some autoimmune diseases, such as systemic lupus erythematosus and related rheumatic diseases, there are antibodies that selectively react with the RNase P isolated from HeLa cells. This discovery could represent not only a new target for antibodies, but also the development of novel antibacterial targets sensitive to these specific antibodies. The fact that RNase P contains immunological determinants that are well conserved, from bacteria to humans (Mamula et al. 1989), must be taken into consideration when designing future drugs directed against the RNase P of pathogenic bacteria. For example, the RNase P in HeLa cells is composed, as is the case for other RNase P enzymes, of a RNA subunit associated with at least nine protein subunits (Rpp14, Rpp20, Rpp21, Rpp29 (hPop4), Rpp30, Rpp38, Rpp40, hPop1, and hPop5), and each of these subunits represent putative targets for antibacterials (Guerrier-Takada et al. 2002). To date, only a few compounds have been found that can activate/inhibit RNase P, these include calcipotriol (Fig. 2) a molecule that has a bimodal action on the enzyme. This compound, initially targeted for osteoporosis treatment, is now successfully used in the formulation of ointmens for treating psoriasis. Calcipotriol can act both as an activator and an inhibitor of tRNA maturation, depending on the dose used. This indicates that calcipotriol may have a direct effect on tRNA biogenesis and, as such, has a good potential as a novel antibacterial (Papadimou et al. 2000a). In addition, calcipotriol and other antipsoriasis compounds, such as anthralin, may have additive effects (Papadimou et al. 2000b).

These studies on the biological effects of the enzyme are bringing to light new unexpected and extremely interesting facts. Trang et al. (2000) found an effective inhibition of human cytomegalovirus replication by a ribozyme directly derived from the catalytic RNA subunit of RNase P from *E. coli*, which strongly supports the putative use of this ribozyme as an antiviral. RNase P (and in particularly the ribozyme moiety) could fulfill the requirements of a good antiviral (high specificity with a minimum of side effects). As pointed out by Cobaleda and Sánchez-Garcia (2001), RNase P may represent a great improvement over antisense RNA. On the one hand, RNase P catalytic turnover greatly enhances the enzyme's efficiency in removing the

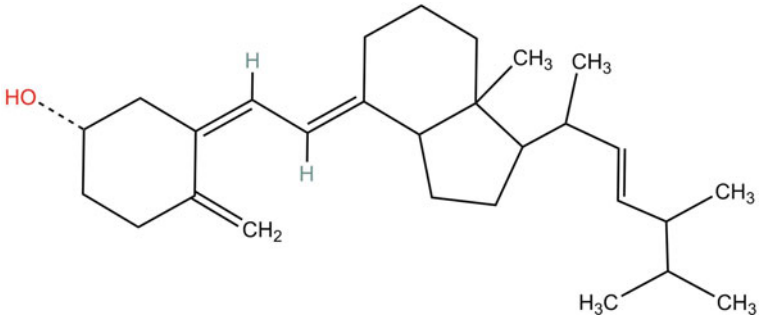

Fig. 2 Structure of calcipotriol

undesired mRNA, while on the other hand, as the enzyme recognition system is based on structure, this ribozyme works better than all others available to date.

This type of research was expanded by Dunn et al. (2001), who found that RNase P mediated the inhibition of not only cytomegalovirus protease expression, but also viral DNA encapsidation by oligonucleotide external guide sequences complementary to a target mRNA, thus recruiting intracellular RNase that specifically degrades the corresponding target RNA. Furthemore, Trang et al. (2002) demonstrated that engineering ribozymes from RNase P could result in increased biological action and a greater anti cytomegalovirus efficiency. Pursuing this idea of using modified RNases P as efficient antiviral drugs, Kim et al. (2004) designed a ribozyme from the catalytic RNA subunit of *E. coli* RNase P, to target the overlapping region of two human cytomegalovirus mRNAs encoding the protease and capsid assembly proteins. This resulted in a drastic reduction of viral proliferation, thus demonstrating the feasibility of using ribozymes either in gene therapy for antiviral applications, or simply as antivirals. For instance, Ikeda et al. (2006) reported the suppression of HIV-1 replication by a combination of endonucleolytic ribozymes (RNase P and tRNnase ZL). In fact, since the discovery of the first group I intron ribozyme in 1982, many new classes of ribozymes have been found, including hammerhead, hairpin, hepatitis delta, varkud satellite, groups I and II intron, as well as RNase P ribozymes and the spliceosome. As indicated above, ribozyme engineering opens an array of putate novel therapeutic uses for these enzymes, either as anticancer agents or in antiviral therapy (Bagheri and Kashani-Sabet 2004). In 2008, Bai et al. succeeded in inhibiting cytomegalovirus proliferation using a ribozyme derived from RNase P catalytic RNA. They transfected recombinant DNA expressing the ribozyme into cytomegalovirus-infected animals and found that the organs expressing the ribozyme (liver and spleen) had viral titers far lower than those of untreated animals. As a result, the survival rate of the ribozyme-treated infected animals was significantly higher than that of the untreated ones, hence demonstrating once more the utility of RNase P ribozymes in in vivo gene targeting applications.

Bacterial virulence genes can be also modulated, even inhibited, by external guide sequences of RNase P. Although there are a few publications exemplifying this, we feel that the work by Ko et al. (2008) specially merits inclusion here, as it presents a novel form of controlling *Yesinia pestis*, the microorganism that causes the "Black Death," with a maximum specificity and minimum of side effects.

From this and other studies, we are now aware that the strong antibacterial effect of certain antibiotics, such as streptomycin and other aminoglycosides, arises from their ability to interfere with RNAse P activity, thus preventing the bacterial cell from attaining fully processed mRNAs that can be translated (Hertweck et al. 2002). Kawamoto et al. (2008) further the research into the mechanism of inhibition of bacterial ribonuclease P by several aminoglycoside derivatives. For this purpose, they synthesized hexa-guanidinium and lysyl conjugates of neomycin B and found that the length of the side chain accounted for the inhibitory potency of the aminoglycoside-arginine conjugates (AACs), and that these conjugates are able to displace Mg^{2+} ions. The most interesting fact is that their conjugates can

discriminate between a bacterial and anarchaeal/eukaryal RNase P, which lays the foundations for the design of aminoglycoside conjugates with the ability of selectively inhibiting bacterial RNase P.

The work of Soler Bistué et al. (2009) also merits inclusion here. At a time when the rise in multiresistant bacteria harboring AAC(6′)-Ib has limited the effectiveness of amikacin and other aminoglycosides, these authors showed that recombinant plasmids coding for external guide sequences, short antisense oligoribonucleotides that elicit RNase P-mediated cleavage of a target mRNA, induce inhibition of expression of aac(6′)-Ib, and concomitantly result in a significant decrease in the levels of resistance to amikacin. More recently, Sala et al. (2012), using the same technology, were able to inhibit bacterial cell division by targeting their ftsZ ring, thus demonstrating once more that RNase P can represent an excellent target for the design of tailor-made novel antibacterial compounds.

Papadimou et al. (2003) are also worth of being cited here, for their studies on the control of RNase P by different compounds. They investigated the effect of synthetic retinoids (all-trans retinoic acid, acitretin) and arotinoids (Ro 13-7410, Ro 15-0778, Ro, 13-6298, and Ro 15-1570) on the activity of RNase P from epidermal keratinocytes (NHEK). They found that, all of the compounds listed above, behave as classic competitive inhibitors of RNase P, with Ro 13-7410 being the strongest inhibitor. Porphyrins and porphines also inhibit RNase P in vitro, and they do so more strongly than any other inhibitor so far described, which makes these unusual substrate-binding compounds very important in antibacterial reagent design (Hori et al. 2002, 2005). As chemical synthesis of these compounds is easily feasible (Rothemund 1935), we can predict that, in the future, hundreds of these derivatives will be synthesized and tested as inhibitors of RNase P and, hence, used as antimicrobials.

Because of the peculiar mode of action of RNase P, some of their RNA-derived ribozymes combine the dual attributes of antisense and ribozyme technologies, and this allows them to transcend some of their limitations. In fact, RNase-based therapy has been used to inhibit HIV replication and, to a certain extent, its pathogenesis (Hnatyszyn et al. 2001). An additional interesting fact is that the bacterial RNase P ribozyme can exert its action on a hairpin RNA with the classic CCA-3′ tag sequence, as well as on a cloverleaf pre-tRNA. On the other hand, ribozymes can only cleave the cloverleaf RNA at a magnesium concentration below 10 mM, but, at a magnesium concentration of 10 mM or higher, ribozymes can also cleave the hairpin RNA with a CCA-3′ tag sequence (Ando et al. 2003).

Since RNase P is essential for life in both prokaryotic and eukaryotic organisms, Willkomm et al. (2003) evaluated the possibility of manipulating RNase P activity as a way to control bacterial growth. These authors studied the possibility of inactivating the catalytic RNA subunit of bacterial RNase P by antisense-based strategies. For this purpose, they designed RNA hairpin oligonucleotides targeted at the tRNA 3′-CCA binding site and found substantial inactivation of RNase P, resulting in cessation of bacterial growth. Gruegelsiepe et al. (2006) also reported specific drug targeting of RNase P in live bacteria that resulted in bacterial growth inhibition. They were also the first to describe a duplex-forming oligomer that

invades a structured catalytic RNA and inactivates the RNA by different mechanisms, including perturbing the coordination of catalytically relevant Mg^{2+} ions. *Salmonella* has emerged as a good vector for oral delivery of RNase-derived ribozymes to control viral infections in a cytomegalovirus model (Bai et al. 2011). These authors provided evidence that oral delivery of M1GS RNA by *Salmonella*-based vectors effectively inhibited cytomegalovirus gene expression and replication in mice.

In summary, as outlined by Altman (2011), the gene coding for the RNA subunit of ribonuclease P is essential in all free-living organisms. It encodes an RNA subunit with enzymatic activity that appeared early on in evolution (probably already essential in an RNA world). This enzyme specifically cleaves substrate RNA molecules, at the junction in single-stranded molecules, and at the 5′ end in double-stranded molecules. The specificity of this enzyme can be targeted in the design of novel drugs for specific antibacterial or antiviral therapies.

4 Ribonuclease Y

Type Y RNases are endoribonucleases found in Gram-positive bacteria, such as *Bacillus subtilis*. They were first described by Shahbabian et al. (2009) as enzymes capable of in vitro cleaving the 5′ monophosphorylated mRNA, but unable to lyse nonterminated full-length mRNA. Initially, the enzyme was denominated YmdA, but is currently known as RNase Y. *Bacillus subtilis* possesses three essential enzymes, thought to be involved in mRNA decay (i.e., RNase Y, RNase J1, and RNase III), that can work sequentially. In this manner, degradation of a significant number of RNA transcripts dependents on first endonucleolytic cleavage by RNase Y, followed by degradation of the resulting RNA fragment by the 5′–3′ exoribonuclease RNase J1 (Lehnik-Habrink et al. 2011a, b; Durand et al. 2012a). In Gram-negative bacteria, such as *E. coli*, RNA degradation is mainly carried out by the ssRNA-specific endonuclease RNase E (see below), followed by hydrolysis by either 3′-5′ exoribonucleases or by RNA pyrophosphohydrolase RppH (Deana et al. 2008). In Gram-positive bacteria, there are two mechanisms involved in the turnover of large RNA molecules, such as mRNAs. The first mechanism involves an endoribonuclease (membrane-bound RNase Y, with a minor role for RNase III; DiMari and Bechhofer 1993), followed by several exonucleases, as is the case for Gram-negative bacteria, as indicated above. The second mechanism relies on RNase J1/J2, an RNase that degrades full-length primary transcripts once the 5′ triphosphate group has been converted to a 5′ monophosphate, by the *B. subtilis* ortholog of RNA pyrophosphohydrolase, BsRppH, or a related enzyme (see Durand et al. 2012b). In this case, 3′–5′ hydrolysis is less important, since 3′ polyadenylation prevents it from happening. RNases J1 and J2 affect the expression levels of hundreds of genes, through the degradation of specific structural mRNAs, and also control the expression of a polycistronic transcript by generating transcripts with different stabilities (Mäder et al. 2008). RNase J1 is also involved in rRNA

maturation and 5′ stability of mRNA (Mathy et al. 2007), as well as in the turnover of the *trp* leader sequence (Deikus et al. 2008).

Yao and Bechhofer (2010) shed more light on the role of RNase Y on mRNA decay. In particular, these authors studied the initiation of decay of *Bacillus subtilis* rpsO mRNA (a small monocistronic mRNA that encodes ribosomal protein S15). This is mediated by endoribonuclease RNase Y, and they found that the endonuclease cleavage occurred in the body of the mRNA, rather than the usual degradation from the 3′ end of the molecule. In order to assess the global impact of RNase Y, Lehnik-Habrink et al. (2011a, b) studied the transcriptomes in *B. subtilis* compared their expression levels of RNase Y. They found that RNase Y depletion resulted in increased mRNA half-lives, circa an hour, and that some 350 mRNAs were less abundant under these conditions. These results emphasize, once more, the role of RNase Y as the global acting endoribonuclease for Gram-positive bacteria. Bruscella et al. discovered, also in 2011, an interesting new role for RNase Y, as they demonstrated that this enzyme was involved in uncoupling the expression of translation factor IF3 from that of the ribosomal proteins L35 and L20. This means that bacteria with either a genetic background lacking, or producing low levels of, RNase Y will generate unprocessed and nonfunctional IF3 transcripts, which could lead to translation malfunction and, hence, hampered microbial growth. In summary, it appears that bulk RNA degradation in Gram-positive bacteria does not follow the same pathways as in Gram-negative organisms. According to Lehnik-Habrink et al. (2012), RNA degradation in Gram-positive bacteria is initiated by RNase Y, followed by sequential exoribonucleolysis of the generated fragments in both 3′-to-5′ and 5′-to-3′ directions. This, again, strongly suggests that controlling the cellular RNase enzymatic activity could provide a way of controlling bacterial growth. Other publications, like that by Laalami et al. (2013) reinforce this point. Indeed, Figaro et al. (2013) recently reported that *B. subtilis* null mutants, lacking RNases Y and J1, although still viable, have notably higher doubling times, aberrant morphologies, disordered peptidoglycan layers, defects in sporulation and, most importantly, phenotypes that are hypersensitive to a variety of antibiotics.

5 Ribonuclease E

Ribonuclease E (EC 3.1.26.12) is an enzyme that participates in the processing of bacterial ribosomic RNA (for instance, 9S to 5S rRNA) and in the chemical degradation of bulk cellular RNA. This enzyme, as is the case for RNase III and RNase P, is associated with the bacterial cytoplasmic membrane (Miczak et al. 1991). The term “degradosome” was coined (Bessarab et al. 1998) to represent the association of different size rRNAs with RNase E, an enzyme that cleaves A+ U-rich single-stranded regions of mature 16S, 23S and 5S rRNAs. In fact, RNase E oversees the protein interactions in the RNA degradosome (Vanzo et al. 1998). Liou et al. (2001) were able to physically visualize this degradosome, by, electron microscopy. They used immunogold labeling and freeze-fracture techniques to demonstrate that

degradosomes indeed exist in vivo in *E. coli*, and that they are associated to the cytoplasmic membrane via the N-terminal region of RNase E. The study of degradosomes may be of great important in the fight against intracellular microbial pathogens, such as *Mycobacterium tuberculosis,* since it may lead to the design of novel powerful drugs specifically directed to mycobacterial pathogens. Further to this, we must mention here the work by Kovacs et al. (2005), who were able to clone and dissect the degradosome of *M. tuberculosis* H37Rv, and found that the mycobacterial RNase E was associated with GroEL and two other proteins, products of the Mb1721 (inorganic polyphosphate/ATP-NAD kinase) and Mb0825c (acetyltransferase) genes, thus opening new potential therapeutic targets. The degradosome complex can even contain rather unusual enzymes, such as glycolytic enolase (Chandran and Luisi 2006), a protein that has been shown to be involved in the turnover of some RNA transcripts. As it is well known, the 48 kDa protein enolase is a universally conserved enzyme, from archaea to eubacteria and all eukaryotes, and catalyzes the interconversion of phosphoenolpyruvate and 2-phospho-D-glycerate. In bacteria such as in *E. coli*, roughly one-tenth of the total enolase is sequestered in the degradosome. Formation of this multienzyme complex requires energy, as is the case for RNA helicase (RhlB; it contains a DEAD-box motif that binds Mg-ATP), phosphoryltic exoribonuclease, polynucleotide phosphorylase (PNPase), and the endoribonuclease RNase E, with the latter responsible for the formation of a superstructure including the rest of the components.

The work of Taghbalout and Rothfield (2007) suggests that this superstructure is in fact a component of the *E. coli* cytoskeleton. This would represent a previously unrecognized role for the bacterial cytoskeleton, thus providing pseudo-compartmentalization spaces where the degradosome (RNA processing and RNA bulk degradation) could carry out its essential role. RNase E must, however, remain anchored to the innermost part of the cytoplasmic membrane for normal bacterial growth to occur (Khemici et al. 2008).

RNase E was first described, by Klee, in 1965, as a product of the degradation of pancreatic RNase A by porcine elastase. Apirion and Lassar (1978) identified a similar enzyme while working with a conditional lethal mutant of *E. coli.* This mutation affects the processing of ribosomal RNA at nonpermissive temperatures (thus originating an unusual 9S rRNA; Ghora and Apirion 1979). The authors concluded that the mutant strain contained a temperature-sensitive processing endoribonuclease, RNase E, and that the enzyme, at the permissive temperature, cut the growing rRNA chain somewhere between the 23 S and the 5 S rRNA cistrons. Ehretsmann et al. (1992) analyzed many RNase E sequences and suggested that the consensus sequence RAUUW (where R = A or G and W = A or U) was needed at the cleavage site. However, there is no universal acceptance of that consensus sequence, and some authors claim that the A+U content, rather than a particular nucleotide order, determines the specificity of RNase E cleavage (McDowall et al. 1994). RNase E is a70 k Da protein that was purified by Misra and Apirion in 1979, and characterized as a rRNA processing enzyme. RNase E is, however, originally transcribed and translated into a larger polypeptide of 180 kDa, which is later processed into both the native RNAse E and the RNase K, an enzyme specifically

involved in the cleavage of the OmpA mRNA (Mudd and Higgins 1993; Casaregola et al. 1994). RNase E does not contribute, and neither do RNase III and RNA P, to the RNA turnover in the bacterial cell (Apirion and Gitelman 1980). This enzyme copurifies with RNAse P, as shown by Carpousis et al. in 1994. They found that the purified RNase E also exhibited polynucleotide phosphorylase activity, as deduced from sedimentation and immunoprecipitation experiments. Thus, the discovery of such a specific association between RNAase E and PNPase raises the possibility that these two enzymes act in coordination in the processing and degradation of RNA, and therefore offers an interesting possibility for controlling bacterial populations. The RNase E encoding gene, *rne* (monogenic in *E. coli;* Ray and Apirion 1982; also known as *ams* or *hmp*), was cloned into λ Charon 25, producing a 71 kDa polypeptide (Ray and Apirion 1980). The role of RNase E was broaden by Ray and Apirion (1981), as the found that the enzyme is not only involved in the processing of rRNA, but also in the genesis of tRNAs; this means that inhibition of this enzyme could eventually lead to cessation of cell division (Goldblum and Apririon 1981).

Inactivation of this enzyme in *E. coli* can have unexpected outcomes, such as the one reported by Tomcsányi and Apirion (1986). These authors found that RNase E inactivation led to the accumulation of a short oligonucleotide (100 nt), identified as RNA I (the natural substrate for RNAse E), which inhibits plasmid DNA replication (in this case replication of the col E1 plasmid). Two years later, Mudd et al. (1988) described that RNase E is also involved in the metabolism of the bacteriophage T4 mRNAs, as they were clearly stabilized in an RNase-free background. This means that the enzyme is involved in the degradation of many T4 mRNAs (Mudd et al. 1990a). These authors latter found that the same was true for *E. coli* RNase E, and they were able to demonstrate the enzyme's general role in the decay of *E. coli* mRNAs (Mudd et al. 1990b). Bouvet and Belasco (1992) described that RNase E cleaves at a specific point at the 5′ end of the molecule, since it preferentially cleaves RNAs that have several unpaired nucleotides at their 5′ end, and this may explain how determinants near the molecule's 5′ end can control the rate of mRNA decay in bacteria.

As indicated above, RNase E is a key enzyme that controls mRNA degradation in bacteria. The question of how bacteria control the synthesis of this enzyme has been elusive for years, until finally Jain and Belasco (1995) showed that RNase E repressed its own synthesis by reducing its cellular concentration. They also found that the enzyme's autoregulation is accomplished, in turn, by controlling the longevity of the cellular mRNAs. In the complete absence of RNAase E, the unprocessed mRNAs can still be partially processed by an enzyme that Woo and Lin-Chao (1997) described as a "*previously unknown RNase E-independent mechanism for degradation of rne transcripts,*" that depended entirely on the culture conditions, (for a comprehensive review on the role of RNase E in the degradosome, see Carpousis 2007).

As pointed out above, *Bacillus subtilis* contains two paralogous endoribonucleases, termed RNases J1 and J2 (Even et al. 2005), with functional homologies to RNase E, but no sequence similarities. RNases J1 and J2 are sensitive to the

5′ phosphorylation state of their substrates, in a site-specific manner, and appear to be involved both in the regulatory processing of specific mRNAs and in the global mRNA degradation process.

In Gram-negative bacteria, such as *E. coli*, the balance of transcripts is affected by RNase E, which not only recycles RNA, but also processes RNA precursors. The 5′ terminus of the substrate, even when located away from the catalytic site, determines the enzymatic activity of RNase E. Callaghan et al. (2005) were able to crystalyze the catalytic domain of this enzyme, and explain why the enzyme can act as a RNA processing enzyme, in some instances, rather than as a RNA-lysing enzyme.

Zhao et al. (2006) studied the fine regulation of RNase E, and described that the protein RraA acts as an inhibitor of this essential enzyme. Hence, ectopic expression of RraA affects more than 700 transcripts in *E. coli* and, in addition, the stability of the RraA transcript is dependent on RNase E activity, which suggests a feedback mechanism controlling the regulation of RraA levels in *E. coli.* Additionally, RNase E can autoregulate its own synthesis, by directly binding to a stem loop in the rne 5′ untranslated region (Schuck et al. 2009).

Ribosomal proteins can also be involved in the regulation of RNase E activity. Singh et al. (2009) showed that L4 interacts with a site outside the catalytic domain of this enzyme and inhibits RNase E-specific cleavage in vitro, as well as stabilizing mRNAs targeted by RNase E in vivo.

RNAase E involvement in the overall cellular survival metabolism is becoming clearer every day. Manasherob et al. (2012) reported the unexpected role of RNase E in the regulation of the SOS response in *E. coli*, this regulation is dynamic and integrated in the canonical RecA/LexA-dependent signal transduction pathway.

6 Ribonuclease Z

RNase Z (EC 3.1.26.11; CAS: 98148-84-6) catalyzes the endonucleolytic cleavage of RNA, removing the extra 3′ nucleotides from the tRNA precursor and generating the tRNAs mature 3′ termini. This enzyme leaves a 3′-terminal hydroxyl group on the tRNA, and a 5′-phosphoryl group on the trailer molecule (Kunzmann et al. 1998). RNase Z was first purified by Kunzmann et al. (1998) from potato mitochondria and found to be responsible for editing the 5′ of tRNA (that event must occur before 3′ processing can take place). RNase Z activity is stable over a broad pH and temperature ranges, with peak activity at pH 8 and 30 °C. The optimal concentrations for $MgCl_2$ and KCl ions are 5 and 30 mM, respectively. Plant nuclei also contain a RNase Z but, as described by Mayer et al. (2000), the nucleic enzyme is different from its mitochondrial counterpart, in terms of its physicochemical properties and processing abilities. RNase Z has also been found in the archaeon *Haloferax volcani* (Schierling et al. 2002) and, although in general terms it is related to the eukaryotic enzyme, its biochemical properties do not resemble either

those of the nucleic or mitochondrial enzymes, but it shares some properties with both RNases.

It is well established that transfer RNAs originate as longer transcripts, which must be processed at the 5′ terminus by RNase P (see above), and at the 3′ terminus by either exo or endoribonucleases. The 3′-terminal reaction is catalyzed by RNAse Z, this enzyme produces a tRNA ready for the addition of the CCA motif (Li de la Sierra-Gallay et al. 2005). RNase Z has also been detected in Gram-positive bacteria, such as *B. subtilis*. In these bacteria, the protein is encoded by the yqjk ORF (Pellegrini et al. 2003), so decreased yqjK expression leads to in vivo accumulation of tRNAs that do not contain the CCA motif. Consequently, these authors renamed the gen as rnz. Although it was originally thought that many Gram-negative bacteria, such as *E. coli,* did not produce RNase Z, Perwez and Kushner (2006) were able to demonstrate the presence of this enzyme in the bacterium (encoded by the *elaC* gene). The RNase Z from *E. coli* is capable of exerting its action on other bacteria, such as *B. subtilis,* processing the tRNAs lacking a CCA determinant both in vivo and in vitro. Most importantly, the authors found that RNase Z played a significant role in mRNA decay. The enzyme was later shown (Dutta and Deutscher 2009) to possess both exonuclease and endonuclease activities.

In yeast mitochondria, functional tRNAs also require 5′ and 3′ editing, and these activities are carried out by RNase P and RNase Z, respectively. Using proteomics, Daoud et al. (2012) were able to demonstrate that these enzymes are part of a large, stable complex of 136 proteins, of which 7 are involved in RNA processing.

7 Polynucleotide Phosphorylase

Polynucleotide Phosphorylase (PNPase; EC 2.7.7.8) catalyzes either the reaction of RNA ($n + 1$) and orthophosphate to yield RNA(n) and a nucleoside diphosphate, or the reverse reaction. It is a bifunctional enzyme with a phosphorolytic 3′–5′ exoribonuclease activity and a 3′–5′ polymerase activity (Fig. 3). In bacteria, PNPase has been shown to be the key player in the regulation of small RNAs, that in turn control the expression of outer membrane proteins (Andrade and Arraiano 2008). The enzyme (encoded by the *pnp* gene in *E. coli*) is located at 69 min on the bacterial chromosome, adjacent to the *rpsO* gene (which encodes the ribosomal protein S15; Regnier et al. 1987). It both lyses the RNA molecules, starting from the 3′ end, and synthesizes long 3′ tails, including polyA tails, if the normal polyadenylylation enzyme is absent (Symmons et al. 2000). PNPase is a ubiquitous enzyme, found in all phyla, from bacteria to humans, involved in mRNA processing and degradation (Sarkar and Fisher 2006). Grunberg-Manago and et al., in 1955, were the firs to report its enzymatic activity, and the enzyme was first purified from *Azotobacter agilis*, also by Grunberg-Manago et al. (1956). A year later, PNPase activity was reported in the cell-free extracts of a variety of bacteria (Brummond et al. 1957). The enzyme was purified from *E. coli* by Kimhi and Littauer in 1968;

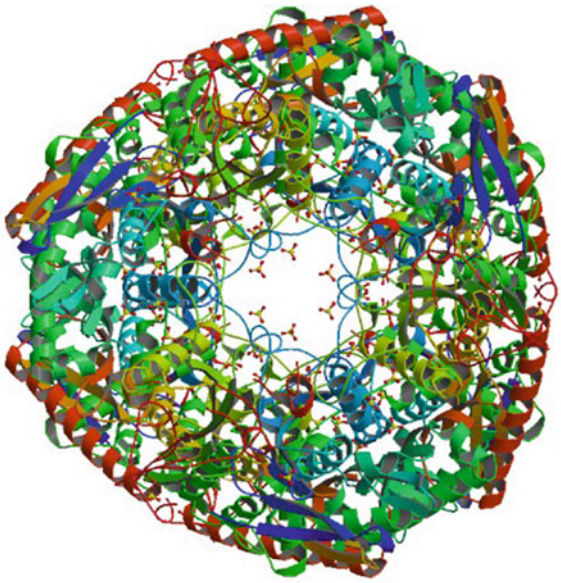

Fig. 3 Crystal structure of the enzyme PNPase (*Streptomyces antibioticus*), downloaded from the RCSB Protein Data Bank: http://www.rcsb.org/pdb/explore/images.do?structureId=1E3P. The structure is a trimer of multidomain subunits, and it is believed to have originated by duplication of a gene encoding an RNase PH-like enzyme (Symmons et al. 2002)

and, later on, purified from cucumbers by Khan and Fraenkel-Conrat (1983). These authors found that, although the biological activity of the two enzymes was similar, the structure of the molecule purified from cucumbers was totally different from that of the bacterial enzyme. PNPase, in addition to RNase I and RNase II, was reported by Kaplan and Apirion (1974) to be involved in the degradation of stable ribonucleic acid during carbon starvation in *E. coli* 249. The following year, these authors suggested that the endonucleolytic degradation of ribosomal RNA was the primary event in the degradation of ribosomes, under starvation conditions, and that the resulting RNA fragments were further degraded into nucleotides by RNase II and PNPase, while the ribosomal proteins remained attached to the cell membrane (Kaplan and Apirion 1975).

It was latter on found that this enzyme may be more complex than originally thought, at least in some instances. Craine and Klee (1976) reported the presence of a second enzymatic activity (deoxyadenylate kinase) tightly associated with highly purified preparations of polynucleotide phosphorylase in *Micrococcus luteus*, which might represent a new type of polynucleotide phosphorylase. In bacteria, such as *E. coli* or related species, the *rpsO* gene originates a variety of mRNA species, either due to the termination of transcription or because the primary transcripts are processed and their 3′ terminal stem loop removed, thus facilitating the action of 3′–5′ exonucleases, such as phosphorylase or RNase II (Hajnsdorf et al. 1994). In fact, the (polynucleotide phosphorylase) can play a part in mRNA decay in *E. coli*, in the absence of RNase II (Kinscherf and Apirion 1975). PNPase promotes 3′ polyadenylation in bacteria and affects the half-life of mRNAs (O'Hara et al. 1995). These authors found, by Northern analysis of the *trxA*, *ompA*, and *lpp* mRNA

transcripts, that when polyadenylylation was almost completely removed, the half-lives of the mRNAs increased. Crofton and Dennis (1983) cloned the PNPase gene from a large *Sal* I fragment of chromosomal DNA, and demonstrated an increase in PNPase polymerizating activity in recombinant cell extracts carrying the PNPase plasmid. In addition, these authors purified PNPase from *E. coli*, an 84 kDa polypeptide, and found that inactivation of the gene resulted in the loss of the protein. Genetic manipulation of the PNPase gene can produce rather unexpected results in *E. coli,* as described by McMurry and Levy in 1987. These authors found that A Tn5 insertional inactivation of the gene caused an increase in the bacteria becoming susceptibility to structurally and functionally diverse antibiotics, and that the hypersensitivity due to this insertion was only partially complemented by expression of a recombinant *pnp* gene.

Apart from processing cellular RNAs, PNPase can play additional roles, such as direct participation in the establishment of lysogeny and immunity in certain bacteriophages. Piazza et al. published an interesting paper in 1996, demonstrating that this enzyme is required for the establishment of bacteriophage P4 immunity. This bacteriophage possesses a unique immunity mechanism, in which the immunity factor is not a protein, but a short stable RNA (CI RNA). Hence, expression of the replication operon is prevented by premature transcription termination, rather than by repression of transcription initiation, and this role is played by the bacterium (*E. coli* in this case) PNPase.

PNPase regulation is important in healthy bacterial metabolism and may be essential when mesophilic bacteria are grown at low temperatures. If *E. coli's* growing temperature is changed from 37 to 15 °C, PNPase activity increases and, when this enzyme is not sufficient to compensate for the cellular demands, other RNases, such as RNase II and RNase PH join in this task (Awano et al. 2008). Similar results were described for the human pathogen *Campylobacter jejuni* (Haddad et al. 2009), where long-term survival of this bacterium at low temperatures is drastically dependent on polynucleotide phosphorylase activity. Additionally, when the culture conditions (i.e., depletion of the nitrogen source) put bacterial growth at risk, the alarmone, ppGpp (GDP; 3′-diphosphate) is synthesized in idling ribosomes by the *real A* gene, which regulates the expression of an array of genes. The alarmone also inhibits the synthesis of ribosomal and transfer RNAs, decreasing the levels of ribosomal and tRNA synthesis and, hence, protein synthesis (for a review, see Srivatsan and Wang 2008). In addition, recent studies with microarrays indicate that the expression of several hundred genes is affected by changes in ppGpp levels in *E. coli* (Durfee et al. 2008). In other organisms, such as *B. subtilis,* ppGpp is involved in sporulation, stress survival and virulence (Eymann et al. 2001). In the case of soil-dweling bacteria, such as *Streptomyces,* a stringent response also occurs in amino acid starvation conditions, with a concomitant increase in the amount of ppGpp, as was the case for *E. coli*. One of the main targets for ppGpp, in addition to RNA polymerase, turned out to be PNPase, but only for actinomycetes where it inhibited the enzyme while increasing the stability of bulk mRNA. The alarmone displayed a clearly negative regulatory effect in "rare" actinomycetes, belonging to genus *Nonomuraea* (Siculella et al. 2010), but

E. coli PNPase was fully resistant to such inhibition (Marcha and Jones 2010). Other cellular messengers, such as cyclic diguanylic acid (c-di-GMP), are involved in many bacterial pathways, such as biofilm formation and virulence. This molecule has also been found to directly affect the PNPase activity of *E. coli* and, therefore, mediate signal-dependent RNA processing (Tuckerman et al. 2011). Other metabolites originating from the bacterial central metabolism appear to also regulate PNPase activity (Nurmohamed et al. 2011). These metabolites include some produced in the Krebs cycle, such as citrate; a compound that affects the activity of *E. coli* PNPase, and, in turn, greatly affects cellular metabolism. In vitro, citrate directly binds to and modulates PNPase, either by inhibiting the enzyme (when metal-chelated citrate is used), or by activating it (when metal-free citrate is utilized). These data, taken together, point to the conclusion that RNA degradation pathways communicate directly with the central metabolism, rendering them a putative and very interesting key for controlling bacterial growth.

After the contribution by Chang et al., in 2008, it became evident that the enzymatic behavior of PNPase followed two modes of action. One model applies to Gram-positive bacteria (represented by *Streptomyces coelicolor* or *S. antibioticus*), while a second, rather different mechanism, is displayed by *E. coli* and related bacteria. Both models differ in their kcat, which is *circa* twofold higher for the *E. coli* enzyme than for the enzyme isolated from Streptomycetes. Additionally, the *S. coelicolor* PNPase is more effective than its *E. coli* counterpart in digesting substrates exhibiting 3′ stem-loop structures. In the case of *E. coli* and related bacteria, PNPase is autogenously regulated posttranscriptionally, thus controlling its own expression at the level of mRNA stability and translation; both the KH and S1 domains of PNPase are necessary for autoregulation and bacterial growth at low temperature (Matus-Ortega et al. 2007). The enzyme's primary transcript is efficiently processed at its 5′ terminus by RNase III (Portier et al. 1987), and the processed pnp mRNA is rapidly degraded in a PNPase-dependent manner (Carzaniga et al. 2009). ATP can also regulate PNPase (Del Favero et al. 2008); this nucleotide binds to the enzyme, and allosterically inhibits both its phosphorolytic and polymerization activities. As the authors point out, these findings clearly connect RNA turnover with the cell's energy levels, and highlight yet unforeseen metabolic roles for PNPase.

In eukaryotic microorganisms, such as the yeast *S. cerevisiae* and particularly in humans, PNPase may have additional roles, as suggested by Rainey et al., in 2006. PNPase exhibits a typical N-terminal-targeting sequence, which is cleaved by the mitochondrial matrix-processing peptidase at the inner membrane level. This localization in the mitochondrial intermembrane space (Wang et al. 2010a, b), suggests a unique role for this enzyme, clearly distinct from its highly conserved function in RNA processing in both eukaryotic organelles and bacteria. This creates a novel working framework for PNPase in human mitochondria, concerning organelle morphology and respiration (Chen et al. 2006), as well as reduction of oxidative RNA damage (Wu and Li 2008). PNPase is also involved in DNA repair, when damaged by UV light radiation, as shown by Rath et al. (2012). They found that, the absence of PNPase, makes *E. col*i cells more sensitive to UV, indicating

that PNPase plays a critical role in bacterial survival after UV radiation; therefore bacterial strains lacking *uvrA pnp, uvrB pnp,* and *uvrC pnp* exhibit UV hypersensitivity phenotypes.

PNPase appears to be also involved in the generation of spontaneous mutations in Gram-negative bacteria (Becket et al. 2012). These authors suggest that, the increase in rNDP pools generated by this enzyme is responsible for the spontaneous mutations observed in MMR-deficient bacteria. The PNP-derived pool also appears responsible for the mutations observed in bacteria with a *mutT* mutator background (but not in *mut Y*), as well as those obtained by treatment with 5-bromodeoxyuridine.

8 RNase PH

The enzyme RNase PH was first described in *E. coli* by Deutscher et al., in 1988, as an enzyme that accurately processed RNA precursors in vitro in an exonucleolytical manner. This reaction required inorganic phosphate (hence the name "RNase PH") and was clearly distinct from the activity of PNPase, an enzyme already known at the time, since RNase PH exhibited a molecular mass of *ca* 45–50 kDa and favored tRNA precursors as its substrates. The new enzyme also catalyzed a synthetic reaction characterized by the addition of nucleotides to the 3′ end of RNAs (Ost and Deutscher 1990). Ost and Deutscher, in 1991, showed that the RNase PH protein was encoded by an open reading frame (orfE) upstream of *pyrE*, located at 81.7 min on the *E. coli* chromosome. These authors suggested that the orfE be renamed *rph. In E. coli*, orfE and *pyrE* form a dicistronic operon (Jensen et al. 1992) but, in other bacterial species, such as *Pseudomonas aeruginosa*, the genes *pyEe* and *rph* are separated by the gene *crc* (catabolite repression control; MacGregor et al. 1996). RNase PH was soon demonstrated to be essential for both tRNA processing, and that bacterial viability may be compromised in RNase-deficient *E. coli* cells (Kelly et al. 1992). Additionally, RNase PH, together with PNPase, plays an essential role in ribosomal metabolism; in this function, the two enzymes cannot be replaced by any of the hydrolytic exoribonucleases present in the cell (Zhou and Deutscher 1997). In *Aquifex aeolicus*, RNase PH contains the typical alpha/beta conformation, which forms a hexameric ring structure as a trimer of dimers, resembling the structure displayed by the polynucleotide phosphorylase core domain homotrimer (Ishii et al. 2003).

An *rph* gene homologue has been identified in *B. subtilis*, and this gene has been shown to suppress cold-sensitive mutations in unrelated bacteria, such as *E. coli* (Craven et al. 1992). As in *E. coli*, the enzyme requires phosphate to display its exo-RNase activity and remove the 3′ nucleotides from precursor tRNAs. The gene is located at 251°, adjacent to the *gerM* gene, on the *B. subtilis* linkage map and plays a major role in the exonucleolytic maturation of CCA-containing tRNA precursors (Wen et al. 2005).

E. coli contains many RNases, but bacterial strains lacking exoribonucleases RNase II, D, BN, T, and PH are inviable, although introduction of either RNase

T > RNase PH > RNase D > RNase II > RNase BN, in decreasing order of efficiency, is enough to render the cells viable again. This indicates that these five genes share overlapping functions in vivo (Kelly and Deutscher 1992). However, all five genes are required for the appropriate 3′ processing of *E. coli* tRNA precursors in vivo (Reuven and Deutscher 1993).

Eukaryotes have the so-called exosome complex that is built around the backbone of a 9-subunit ring, similar to RNase PH and PNPase. Such a ring is devoid of any detectable catalytic activity, except possibly in plants (Chlebowski et al. 2011). In Archaea, the exosome is also a proteinic complex and, as in eukaryotic organisms, it plays a role in the 3′ processing and degradation of RNA. It is functionally similar to bacterial PNPase (including the requirement for divalent ions for catalysis) and to RNase PH, as it uses inorganic phosphate to progresively cleave RNA substrates, thus releasing nucleoside diphosphates (Lorentzen and Conti 2012).

Inhibition of RNase PH putatively represents another good target for novel drugs that would produce, if not cell death, at least a bacteriostatic state in the target bacteria.

9 RNase I

The enzyme RNase I (EC 2.7.7.17) in *E. coli* is currently referred to as RNase T2 (EC 3.1.27.1) RNase II, base-nonspecific ribonuclease, nonbase-specific RNase, RNase (nonbase specific), nonbase-specific ribonuclease, nonspecific RNase, RNase Ms, RNase MII, *E. coli* ribonuclease II, ribonucleate nucleotido-2′-transferase (cyclizing), acid ribonuclease, RNAase CL, *E. coli* ribonuclease I' ribonuclease PP2, ribonuclease N2, ribonuclease M, ribonuclease PP3, ribonucleate 3′-oligonucleotide hydrolase, ribonuclease U4, (Reddi and Mauser 1965; Uchida and Egami 1967; Garcia-Segura et al. 1986). RNase I catalyzes a two-stage endonucleolytic cleavage of nucleoside 3′-phosphates and 3′-phosphooligonucleotides, producing 2′, 3′-cyclic phosphate intermediates. The enzyme is involved in scavenging exogenous RNAs in the bacterial periplasmic space. The 16S ribosomal RNA plays a crucial role in the specific inhibition of RNase I, in particular the helix 41 of 16S ribosomal RNA. Accordingly, h41-mutant *E. coli* strains exhibit a lower survival rate, than wild-type strains, when in a stationary phase of growth. These phenotypic defects, in the mutants, are accompanied by significant RNA degradation, caused by RNase I (Kitahara and Miyazaki 2011).

10 RNase II

RNase II (EC 3.1.4.1) is encoded by the gene *rnb* in *E. coli*, and can act as a phosphodiesterase, 5′-exonuclease, 5′-phosphodiesterase, 5′-nucleotide phosphodiesterase, oligonucleate 5′-nucleotidohydrolase, 5′ nucleotide phosphodiesterase/alkaline

phosphodiesterase I, 5′-NPDase, 5′-PDase, 5′-PDE, 5′NPDE, alkaline phosphodiesterase, nucleotide pyrophosphatase/phosphodiesterase I, orthophosphoric diester phosphohydrolase, PDE I, phosphodiesterase, or exonuclease I. It is an enzyme (Khorana 1961) that sequentially removes 5′-nucleotides from the 3′-hydroxyl termini of 3′-hydroxy-terminated oligonucleotides. It can attack both ribonucleotides and deoxyribonucleotides and, contrary to that found for true RNase, displays low activity toward polynucleotides (i.e., **it must not be confused with one of the acceptions given to RNase I, see the previous section**). The in vivo role of RNase II is unclear. Kivity-Vogel and Elson (1968) and Lennette et al. (1971) reported that the in vivo rate of mRNA degradation was proportional to the level of RNase II present in the cell. On the other hand, Donovan and Kushner (1983) found no difference in the in vivo rate of mRNA degradation in either the absence of RNase II activity or when the enzymatic activity was increased 10-fold over the normal levels found in wild-type strains. What is clear, however, is that cells lacking both PNPase and RNase II are not viable (Donovan and Kushner 1986).

11 Ribonuclease III

The RNase III enzymes are, as described by Lamontagne et al. (2001), capable of binding and cleaving dsRNA. In bacteria, such as *E. coli*, rRNA operons are transcribed as 30S precursor molecules that must be extensively processed to generate the mature ribosomal 16S, 23S and 5S rRNA, and RNase III is the enzyme that plays this role. These universal enzymes are usually grouped into three categories, class 1, class 2, and class 3. The first type is mainly found in bacteria, bacteriophages, chlorella viruses (PBCV-1; Zhang et al. 2003), and, interestingly, in some fungi. The genes coding for RNase III are *rnc* (in *E. coli*), Pac1p (in *Schizosaccharomyces pombe*), and Rnt1p (in *Saccharomyces cerevisiae*). The prokaryotic ribonuclease III (EC 3.1.26.3) gene, originally sequenced by Nashimoto and Uchida (1985), Watson and Apirion (1985), and March et al. (1985), is an enzyme that digests double-stranded RNA and is beneficial for the normal growth of *E. coli*, becoming indispensable for growth at high temperatures (Apirion and Watson 1975; Apirion et al. 1976). It is involved in processing ribosomal RNA precursors as well as processing certain mRNAs.

The enzyme was first purified by Robertson et al. (1968), and later on by Srivastava and Srivastava (1996), from a recombinant *E. coli* strain harboring the *rnc* gene. Wu et al., in 2000, purified the human RNase III counterpart. It is a 160 kDa protein involved in preribosomal RNA processing and an endonuclease specific for double-stranded RNA. The enzyme, purified by DEAE- Sephadex and CM-Sephadex chromatography, showed high specificity for dsRNAs, whereas it did not digest ssRNAs, ss DNAs or dsDNAs. Wu et al. (2000) also found that the enzyme had an optimal pH range between pH 7.6 and pH 9.75, and required divalent cations, such as Mg^{++} and Mn^{++}, as well as monovalent cations, such as NH^{+}, K^{+}, and Na^{+} for its activity. RNase III was later on purified from *E. coli* by

Dunn (1976), by Sephadex G-100 chromatography, and displayed a molecular weight of 50 kDa; this suggested the purified enzyme had a dimeric conformation. Apart from dsRNA, RNase III can specifically cut certain single-stranded RNAs, such as the bacteriophage T7 early RNA, at primary or secondary sites, depending on the ionic strength. The enzyme recognizes duplex RNA structures and makes single-stranded incisions, favoring areas adjacent to UMP residues over AMP residues (Bishayee and Maitra 1976).

In fungi, RNase III processes precursors to small nuclear RNAs (snRNA) and small nucleolar RNAs (snoRNA).

Class 2 RNase III includes the Drosha family of enzymes, which play a role in miRNA maturation. Finally, Class 3 RNase III molecules include the Dicer family of enzymes, known to function in RNA interference (RNAi).

RNase III was first purified by Robertson and et al., in 1968, and was later on postulated to be the enzyme involved in rRNA processing (Duncan and Gorini 1975; Robertson and Dunn 1975; Ginsburg and Steitz 1975). RNase III is also capable of cleaving adenovirus mRNAs, as well as mammalian 28S and 18S ribosomal RNAs. As indicated by Westphal and Crouch (1975), the RNA fragmentation produced by this enzyme is not random but, in every case, a specific array of products is generated, thus abolishing the ability of adenovirus mRNA to direct cell-free synthesis of viral polypeptides. In fact, *E. coli* RNase III has been used to cleave double-stranded RNA (dsRNA) into endoribonuclease-prepared siRNA (esiRNA), to target multiple sites within a given mRNA, and eliciting the destruction of the target mRNA (Yang et al. 2002). This simple idea has high potential, as it opens the possibility of using this enzyme as a way to either control tumor cells or to prevent bacterial growth.

According to Kharrat et al. (1995), the double-stranded RNA-binding domain (dsRBD) of RNase III protein consists of two moieties, a N-terminal catalytic domain (ca. 150 oligo nucleotides long) and a C-terminal recognition module (ca. 70 amino acids long), which are homologous to other dsRBDs and displaying an α-β-β-β-α topology.

Bacterial motility is a rather complex mechanism that relies on a variety of genes, such as the *fla* (flagella formation) or those involved in the sensory system (i.e., *che*). Apirion and Watson, in 1978, mutated the gene responsible for RNase III in *E. coli* and found that this caused an interesting pleiotropic effect, since *rnc* mutants were unable to form flagella and, hence, did not exhibit motility, either under light microspe examination or when cultured in semisolid agar plates. Electron microscopy studies later revealed that the bacteria totally lacked flagella. These authors also found that some motility-mutation revertants still lacked RNase III, suggesting that other RNases (now known to be RNase P, see above) could replace RNase III in that role. Plautz and Apirion, in 1981, found that, in the absence of the three major RNA processing enzymes (RNase III, RNase E and RNase P), hardly any endonucleolytic RNA processing occurred in *E. coli*. Lack of only RNase III activity cannot seriously affect *E. coli* survival (although this enzyme is essential for *B. subtilis*, as discovered by Herskovitz and Bechhofer 2000 by silencing toxin genes harbored by lysogenic bacteriophagues; Durand et al.

2012b), but it may cause unexpected effects, such as that reported by Price et al. (1999). These authors showed that, in *S. coelicolor*, RNase III activity is necessary for antibiotic production. Additionally, Sello and Buttner (2008) found, also in *S. coelicolor*, that the enzyme was needed for proper sporulation.

The RNase III operon is autoregulated in *E. coli*, as demonstrated by Bardwell et al., in 1989, and also reported to be the case in *S. coelicolor* by Xu et al. in 2008. These authors found that *rnc* mutant strains overproduced both the operon's mRNA and its protein products, but a single cleavage in the mRNA 5′-noncoding region was enough to inactivate it. This autoregulation is, however, independent from the *rnc* translation rate (Matsunaga et al. 1997). All these findings will be of particular relevance in the future to aid in the design of novel drugs capable of halting the bacterial RNA metabolism, as an effective way of controlling their growth, and hence with considerable therapeutic potential.

In vivo, RNase III can be regulated by protein kinases, as demonstrated by Mayer and Schweiger in 1983. These authors found that *E. coli's* RNase III activity can be stimulated fourfold by infection with bacteriophage T7, and that the mechanism of activation is dependent on the phosphate transfer catalyzed by the viral protein kinase (with serine being the phosphorylated amino acid in the bacterial RNase III). In the lambda bacteriophage system (Wilson et al. 2002), RNase III cleaves the N nucleotide leader and prevents N-mediated translation repression of N gene expression. The N antitermination polypeptide is a heavy-duty protein on which lambda depends for successfull initiation of its lysogenic cycle in *E. coli*. It forms an antitermination transcription complex to overcome transcription terminators in the early operons (p(L) and p(R)). Plant viruses containing RNase III, such as that produced by the sweet potato chlorotic stunt virus (SPCSV), use the enzyme to abolish the plant's RNA interference, thus depleting their host from a potent antiviral (Cuellar et al. 2009). Similar roles have been reported for insect ascovirus-encoded RNase III (Hussain et al. 2010). *Neisseria meningitidis* is another example of the specific role played by RNase III, as it cleaves the terminal internal repeats (26/27 nt long) of the correia small insertion elements. The enzyme effectively processes dsRNAs containing 26/26 or 27/27 repeats, but it is unable to act on 26/27 termini (De Gregorio et al. 2003). The enzyme, when heterologously expressed in the yeast *S. cerevisiae,* induces abnormal cell morphology resulting in death (Pines et al. 1988). However, in other systems such as in vaccinia virus, the gene can entirely complement the paralogous counterpart (E3L) that encodes the protein playing the same role as bacterial *rnc* (Shors and Jacobs 1997).

12 Barnase and Binase

These RNases (EC 3.1.27.3) catalyze a two-stage endonucleolytic cleavage from nucleoside 3′-phosphates and 3′-phosphooligonucleotides to Gp, producing 2′,3′-cyclic phosphate intermediates. They are know by a variety of names, including: guanyloribonuclease, *Aspergillus oryzae* ribonuclease, RNase N1, RNase N2,

ribonuclease N3, ribonuclease U1, ribonuclease F1, ribonuclease Ch, ribonuclease PP1, ribonuclease SA, RNase F1, ribonuclease C2, RNase Sa, guanyl-specific RNase, RNase G, RNase T1, ribonuclease guaninenucleotido-2′-transferase (cyclizing), ribonuclease N3, and ribonuclease N1. These enzymes are related to RNase T1 (Takahashi 1961; Kasai et al. 1969), the best known member of this enzymatic class, a well-studied enzyme from *A. oryzae*. In fact, barnase and birnase represent the RNase T1 bacterial counterparts, in *B. amyloliquefaciens* and *B. intermedius,* respectively (for a recent review, see Ulyanova et al. 2011).

Both enzymes are cationic proteins exhibiting isoelectric points of 9.2 and 9.5, respectively. Interestingly, they are stable over a broad range of pH (from 3 to 9), although their optimum pH is close to 8.5. They hydrolyze RNAs afer a purine (guanine being the preferred nucleotide) and their end products contain 3′ mono or dinucleotides. Barnase (as well as binase, but with much lower efficiency*)* is inhibited in vitro by barstar (Hartley 1989), forming a tight and highly complementary complex that involve the positively charged residues (Lys27, Arg59, Arg83, and Arg87) of barnase and the negatively charged amino acids (Asp35, Asp39 and Glu76) of barstar (Wang et al. 2010a, b; Ulyanova et al. 2011).

Undoubtedly, both barnase and binase must be related to enzymes from nucleotide salvage pathways. First, because they are extracellular, second, because they originate mono or dinucleotides, and finally, because their genetic neighborhood includes, in order of proximity, proteins such as triphosphate pyrophosphate hydrolase, ATPase/kinase, gluconeogenesis factor, regulatory protein, phosphocarrier protein, and *N*-hydroxyarylamine *O*-acetyltransferase, which points toward such trophic function (Ulyanova et al. 2011). These two enzymes might even be important in the generation of bacterial strains capable of colonizing particular ecological niches, acting as toxins for the competing organisms. Even in the strains that produce these enzymes, such as in *B. subtilis,* they can have a role in induction of the so-called “bacterial cannibalism” (González-Pastor et al. 2003). Barnase and binase were originally believed to have potential uses in biotechnology, but finally they seem to have found their appropriate niche in medicine, in applications such as cancer therapy, to specifically attack malignant cells (as well as bacterial pathogens). Barnase has a specific inhibitor (barstar) that forms a tight complex with the enzyme, and this provides a good model for studying protein–protein noncovalent interactions (Guillet et al. 1993) that has been useful in the study of other RNase inhibitors.

13 RNase D

RNase D (E.C.3.1.13.5) belongs to one of the seven exoribonucleases families so far described in *E. coli* (it is similar to RNase III) and, as a 3′–5′ exoribonuclease, it is involved in the 3′ processing of various stable RNA molecules (Zuo et al. 2005), for example in the addition of the 3′ CCA sequence to prokaryotic tRNAs.

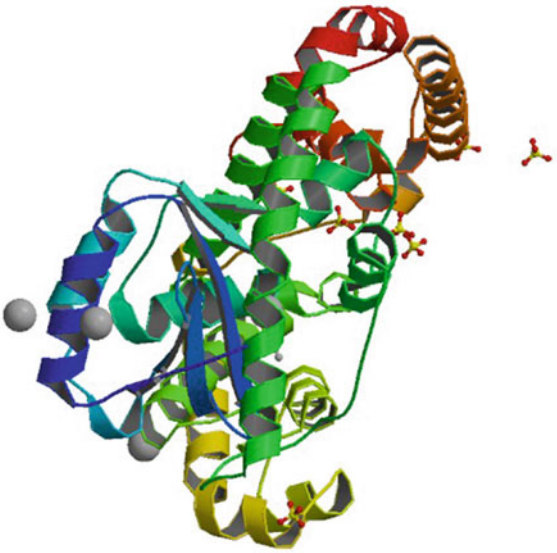

Fig. 4 Crystal structure of the ribonuclease RNase D as downloaded from the RCSB Protein Data Bank: http://www.rcsb.org/pdb/explore/images.do?structureId=1YT3

The enzyme (Fig. 4) was partially purified by Rech et al., in 1976 as a double-stranded RNA-specific ribonuclease (RNAse D) from Krebs II ascites cells. Two years later, Ghosh and Deutscher (1978) were able to identify a nuclease in *E. coli* (also RNase D) that acted on structurally altered transfer RNAs, hardly displayed any activity on intact tRNA, and was entirely inactive against synthetic polyribonucleotides (poly(A) and poly(U)) or double-stranded poly(A) and poly (U). This enzyme required Mg^{2+} for its activity, was stimulated by the monovalent cations K^+ and $NH4^+$, and its final product was 5′-mononucleotides. At the time, the enzyme did not correlate to any known *E. coli* ribonuclease enzymatic type (Ghosh and Deutscher 1978), and it was suggested to be involved in salvage pathways, thus recovering ribonucleotides from denatured tRNAs and other inactive RNA molecules. In 1980, Cudny and Deutscher were able to show an apparent involvement of ribonuclease D in the 3′ processing of tRNA precursors. RNase D removed nucleotides randomly and its activity slowed down when reaching the -C-C-A sequence, thereby allowing the tRNA to be aminoacylated and protected from further degradation. In 1988a, Zhang and Deutscher demonstrated that RNase D could act at the 3′ terminus of tRNA in vivo, thus supporting the conclusion that this enzyme participates in tRNA metabolism (Zhang and Deutscher 1988a).

Cudny et al. (1981a) finally purified the enzyme from *E. coli* and found that RNase D is composed of a single polypeptide with a molecular weight of 40 kDa, and an isoelectric point of circa 6.2. The enzyme is sensitive to inactivation by high temperatures, but can be protected by a variety of RNAs, including those RNAs that are not substrates for hydrolysis. Studies performed by Cudny et al. with purified protein (Cudny et al. 1981b) revealed that RNase D is an exonuclease that initiates hydrolysis at the 3′-terminus of the RNA and ramdomly removes

5′-mononucleotides. Zaniewski and Deutscher (1982) determined the position of the *rnd* locus at 39.5–40 min in *E. coli*'s chromosome, and defined it as the RNase D structural gene. The gene, however, is not essential for either cellular viability or for normal cell growth (Blouin et al. 1983); on the contrary, when the gene is expressed to high yields, RNase D shows deleterious effects on *E. coli* metabolism (Zhang and Deutscher 1988b).

14 RNase H

RNase H is a nonspecific endonuclease found in nearly all organisms, from *Archaea* to *bacteria* and *eukaryotes.* The enzyme cleaves the 3′-O-P bond of RNA in a DNA/RNA duplex (it does not degrade DNA or single RNA) to produce residues containing 3′-hydroxyl and 5′-phosphate residues. In DNA replication the enzyme removes the RNA primer, allowing completion of the newly synthesized DNA strand. The crystallographic structure of RNase H is shown in Fig. 5 and consists of a 5-stranded β-sheet surrounded by a distribution of α-helices (Schmitt et al. 2009). In retroviruses, reverse transcription is carried out by two different enzymatic activities, both accomplished by this enzyme. First, RNase H degrades the RNA strand in the RNA–DNA duplex/es that served as template for synthesis of

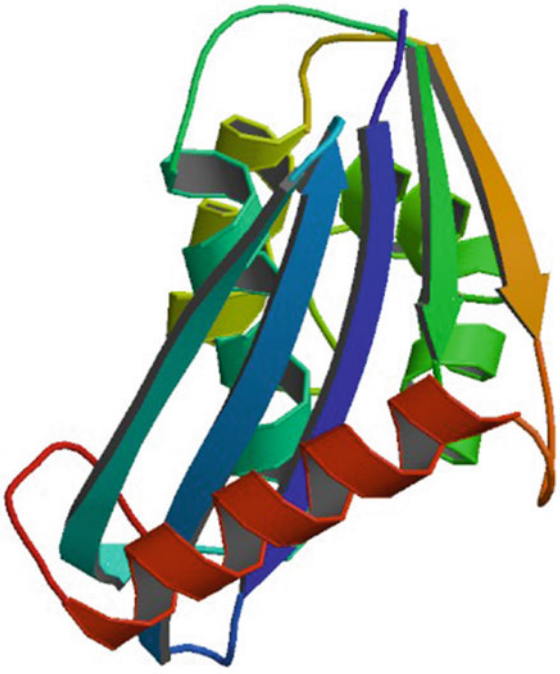

Fig. 5 Crystal structure of *E. coli* RNase HI, as determined by Goedken and Marqusee in 2001

the DNA minus-strand and, later on, removes the minus and plus strand primers, once the dsDNA synthesis is complete (for a comprehensive review, see Adamson and Freed 2010).

15 RNase L

RNase L (L, for latent), also known as ribonuclease 4 or 2′–5′A-dependent ribonuclease, is an interferon-induced ankyrin-repeat containing ribonuclease activity that is part of the body's innate immune defense which, upon activation, destroys all kinds of RNA, either cellular or viral, inside the cell (Samuel et al. 2006). This enzyme, in humans, is encoded by the gene RNASEL (Squire et al. 1994). It is becoming more and more apparent that RNase L does play an important role in innate immunity, and that it does so in different ways. For instance, it is involved in the synthesis of β interferon through the genesis of short ribonucleotides, as well as in protecting against viral-induced nerve demyelination (Chakrabarti et al. 2011). RNase L is normally present in the cell in low amounts, but interferon greatly increases its transcription (as well as that of an additional 300 genes) to accomplish the so-called "*antiviral state*." Double-stranded RNA stimulates 2′–5′-oligo(A) synthetase to produce 2′–5′ oligoadenylic acid, which in turn activates RNase L. The enzyme (EC 3.1.26.-, 3.1.27.-) exhibits an estimated molecular weight of 105 kDa, by SDS-PAGE, whereas the molecular weight estimated by ultracentrifugation is 85 kDa (Dougherty et al. 1980).

Strictly speaking, this RNase should not be included in this review, but as the expression in *E. coli* of interferon-inducible recombinant human RNase L causes bacterial RNA degradation, resulting in bacterial death (Pandey and Rath 2004), and as its role in the immunological response against nonviral pathogens is gaining momentum (Decker et al. 2005), we feel justified to mention RNase L in this chapter. On the one hand, nucleic acids from vertebrate pathogens, such as viruses or bacteria, as well as LPS from Gram-negative bacteria, activate a defense transduction pathway that converges on a transcription factor required for interferon production, which in turn is required for successful eradication of viral/bacterial infection (particularly intracellular bacteria). While, on the other hand, infection by Gram-positive bacteria, such as *Bacillus anthracis,* can also lead to interferon production (Kang et al. 2008), therefore making interferon a key player in antibacterial immunity. Indeed, RNase L-lacking mice mutants showed increased mortality rates when challenged with either *B. anthracis* or *E. coli*. This indicates that RNase L is required for optimal induction of proinflammatory cytokines to achieve a good antiviral/antibacterial state (Li et al. 2008) that includes production of endolysosomal protease, cathepsin-E, and endosome-associated activities directed toward destroying intracellular pathogens. In addition, RNase L deficiency exacerbates experimental colitis in mice (Long et al. 2013), and lack of this enzyme attenuates macrophage functions (Yi et al. 2013). Therefore, as Li et al. pointed out

in 2008: "*RNase*-L *represents a viable therapeutic target to augment host defense against diverse microbial pathogens.*"

16 RNase T

RNase T was first identified in *E. coli* (Deutscher 1990) and purified as a dimeric protein of 50 kDa, capable of in vitro removing the terminal AMP residue from uncharged tRNA, generating tRNA-C-C (Deutscher and Marlor 1985). The dimeric nature of RNase T is similar to other bacterial exoribonucleases and, in fact, the catalytic residues in this enzyme are organized in a very similar way to the catalytic domain of RNase D from *E. coli*, which is monomeric (Zuo et al. 2007). The corresponding gene (*rnt*) is localized at min 36 in the *E. coli* linkage map (Case et al. 1989). Huang and Deutscher suggested, in 1992, that RNase T is part of an operon, and its interruption prevents the bacterium from recovering from bad metabolic conditions (Padmanabha and Deutscher 1991). As demonstrated by Li and Deutscher in 1995, RNase T generates the mature 3′ terminus of the 5S RNA, although the completely processed 5S RNA molecule is not essential for cell survival. This enzyme is also involved in the complete maturation of bacterial 23S rRNA (Li et al. 1999), although, once again, lack of 23S rRNA processing by inhibition of RNase T has little effect on cellular growth. All these facts will have to be taken into account if/when designing drugs targeting RNase T, since such drugs are not expected to induce bactericidal action and, at best, would only exhibit bacteriostatic effects. The potent DNase activity displayed by RNase T (Viswanathan et al. 1998) may be involved in end trimming reactions during DNA recombination and/or DNA repair and, therefore, has the potential to be a target for novel drugs directed toward preventing horizontal transfer of antibiotic resistance in bacteria. Additionally, RNase T ameliorates the UV sensitivity exhibited by *E. coli* strains lacking Exo I, Exo VII and Rec J, the three classical single-strand DNA exonucleases (Viswanathan et al. 1999).

17 RNase Inhibitors

Even in the middle of last century (Gulick et al. 1958) researchers were trying to find acidic polymers, capable of inhibiting RNases, that could be used as antiviral agents. Nelson and Hummel, in 1961, described the use of 2′ cytidylic acid as an RNase inhibitor. The porcine ribonuclease inhibitor is a leucine-rich large polypeptide, used as a classic RNase inhibitor, with a molecular weight of *ca* 49 kDa and an isoelectric point of 4.7. It possesses a horseshoe shape, which forms extremely tight complexes with certain ribonucleases and is apparently involved in regulating the lifetime of RNA (Kobe and Deisenhofer 1993; Shapiro 2001).

Azidothymidine (AZT), a nucleoside analogue reverse transcriptase (RT) inhibitor, was the first drug approved for the treatment of HIV infection (Mitsuya et al. 1985). This molecule, as well as its derivatives the nucleoside/nucleotide RT inhibitors and the non-nucleoside RT inhibitors, blocks the retrotranscriptase from HIV by chain termination. The emergence of resistance against RNase inhibitors was originally believed to be limited to the first ~300 amino acids of retrotranscriptase, but Delviks-Frankenberry et al. demonstrated, in 2010, that mutations in the C-terminal domain of retrotranscriptase, specifically in the connection subdomain and RNase H domain, can also increase resistance to these antivirals.

For many years now, researchers have tried to develop antiretroviral drugs targeting RNase H, in addition to the acidic polymers. This task, however, has been elusive and, to the best of our knowledge, not a single compound has, thus far, been approved for clinical use (Jochmans 2008), although, as pointed out by Adamson and Freed (2010): "*RNase H remains an attractive drug target, as its activity is essential for HIV-1 replication.*" Di Santo (2011) recently reported that pyrrolyl and quinolinyl diketo acid derivatives act as dual inhibitors of the human immunodeficiency virus type 1 integrase and reverse transcriptase RNase H domains.

As indicated above, several compounds are currently used as inhibitors of RNase P, these include retinoids (Papadimou et al. 1998), calcipotriol (Papadimou et al. 2000a, b), certain antibiotics such as puromycin, amicetin and blasticidin S (Kalavrizioti et al. 2003), arotinoids (synthetic polyaromatic retinoid derivatives of vitamin A; Papadimou et al. 2000c), aminoglycoside-arginine conjugates (Eubank et al. 2002; Berchanski and Lapidot 2008), porphyrins and porphines (Hori et al. 2002), oligonucleotides causing RNA misfolding (Childs et al. 2003), vitamin D analogues (Tekos et al. 2004), antisense oligomers against the P15 loop region of RNase P (Gruegelsiepe et al. 2006), as well as spermine conjugates with acidic retinoids (Magoulas et al. 2009).

RNase E can be specifically inhibited by stem loops, as suggested by McDowall et al. (1995). Other inhibitors include RraA protein (Zhao et al. 2006; Yeom et al. 2008).

Early publications describe the use of a 6-mercaptopurine riboside 5′-diphosphate as an inhibitor of polynucleotide phosphorylase (Carbon 1962), and deoxyadenosine diphosphate has also been claimed to be a potential RNase inhibitor (Chou and Singer 1971); but more recently, Slomovic and Schuster (2008) proposed the use of RNase E in RNAi enzyme silencing.

In the case of RNase L, Bisbal et al. (1995) reported a 68 kDa polypeptide, in mice, that competes with the 2′–5′A binding ability and the nuclease activity of endogenous RNase L. According to Martinand et al. (1998), low levels of this inhibitor induce cellular resistance to certain viruses. Additionally, Ueno et al. discovered, in 2003, a specific substrate inhibitor against this RNase.

Despite all the advances made in recent years, concerning RNase inibitors with a potential use in therapy, much still remains to be done before the pharmacological needs for these novel drugs to fight bacterial or viral infections are satified.

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Control of *Clostridium botulinum* in Foods

Eric A. Johnson

Abstract The global burden of foodborne and waterborne diseases on human health and economics is enormous. Historically, humans have relied on preservation methods for foods such as drying, salting, smoking, acidification, and fermentation to ensure their microbiological safety. As our understanding of food preservation developed, a variety of chemical antimicrobials and processing techniques became available to control infections and toxin production by foodborne pathogens. A current global trend in food manufacturing and consumption is the preference by consumers for minimally processed, convenient, fresh-tasting, healthful, non-GMO, and organically produced foods. These requirements challenge the food industry in the production of safe food products and limit the use of chemical preservatives and antibiotics in foods and the food chain. There is considerable need for the development of efficacious natural antimicrobials and bactericidal systems for use in foods to prevent disease and food spoilage. A pathogen of major concern in global foodborne disease, and the central focus of this chapter, is the spore-former *Clostridium botulinum*. *C. botulinum* produces the most poisonous toxin known to humankind and the intramuscular lethal dose is estimated to be 0.1–0.5 ng per kg body weight, and the oral dose 0.2–1.2 μg per kg in adults, while infants are likely susceptible to a considerably lower lethal dose. Due to its high potency, botulinum neurotoxin (BoNT) is also considered a potential agent of bioterrorism. The goal of this chapter is to describe new developments in natural antimicrobials and antimicrobial systems to prevent growth and BoNT production in foods and in the human intestine and wounds by *C. botulinum*. These developments for food safety are important to enhance the quality of foods, to enable production of foods with extended shelf life and wide geographic distribution, and to address the challenge of antimicrobial resistance.

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1 Introduction

The global impact of foodborne and waterborne diseases on human health and worldwide economics is enormous (WHO 2015). Systematic estimates of the effect of foodborne disease on human morbidity, mortality, and economics is available for several developed countries due to established infrastructure for epidemiology and reporting and disease diagnosis. In contrast, data on the incidence and impact of foodborne is lacking for many developing countries but the burden is likely very large. The majority of diseases are caused by enteric bacteria, viruses, and mycotoxins. However, serious diseases of high health impact are caused by spore-forming bacteria including pathogenic clostridia, most notably *Clostridium botulinum* (Hatheway 1993; Johnson 2013).

Clostridium botulinum produces botulinum neurotoxin (BoNT), which is the most potent toxin known to humankind and is a serious cause of foodborne, intestinal, and wound botulism worldwide (Dolman 1964; Smith and Sugiyama 1981). Due to the extraordinary potency of BoNT, it is also considered to be a potential agent of bioterrorism (Arnon et al. 2001). Remarkably, BoNTs have also been developed as effective pharmaceuticals in the treatment of a myriad of neuromuscular diseases (Schantz and Johnson 1992). *C. botulinum* produces seven serotypes (A-G) of BoNTs, which are defined by neutralization of toxicity in mice by homologous antisera (Hatheway 1993). Botulism is caused solely by the action of BoNT on nerve-muscle tissue, and it usually affects motor neurons innervating skeletal muscle (Johnson and Montecucco 2008). Recent evidence indicates that BoNTs also affect sensory neurons controlling sensation and pain, and that BoNTs affect the CNS and alleviate diseases including migraine and tension headaches, and moods such as depression (Truong et al. 2013). Due to the severity of the disease botulism, the control of *C. botulinum* and toxin formation is of paramount concern of the global food industry, medicine, biosecurity, and governmental agencies.

The endospore of *C. botulinum* is the most heat-resistant biological form that can cause foodborne disease, with the notable exception of prions (Setlow and Johnson 2013). Several food regulations have been designed to control growth and BoNT production in foods (Hauschild 1989; Johnson 2013). As a primary example, thermal treatments (12D "botulinum cooks") are used in food processing to inactivate spores of foods packaged in hermetic containers such as cans and pouches (Setlow and Johnson 2013). Additionally, the prevention of BoNT formation in low-acid foods (pH > 4.6 and a_w > 0.85) was established to prevent growth of *C. botulinum* in food commodities (Glass and Johnson 2002). In practice, most low-acid foods commonly receive minimal processing such as nominal heat treatment, which can inactivate vegetative bacterial competitors present in foods but not the spores of *C. botulinum* and other pathogenic clostridia (Glass and Johnson 2002). Minimal processing often permits growth and BoNT formation by eliminating vegetative spoilage bacteria, since *C. botulinum* is not a good competitor against other microbiota in foods and in the gut (Jay 1997; Johnson 2013). The inability to successfully compete with the mature microbiome is evident in infant

botulism, in which the limited microbiota in the intestine of the infant can allow growth and toxin formation by *C. botulinum* (Arnon 2004).

Traditionally, the primary means for controlling *C. botulinum* in foods is to inactivate the spores by heat, or to prevent their growth, primarily by acidification, high salt levels, and by the use of chemical preservatives (Glass and Johnson 2002). With the growing trend of consumer preference for minimally processed foods and lack of chemical preservatives, there is a crucial need for new antimicrobials and antimicrobial systems for the control of *C. botulinum* and other spore-forming clostridial pathogens in foods and human infections. This chapter will focus on preservation of foods to control growth and toxin production by *C. botulinum*. Although *C. botulinum* is highlighted in this chapter, similar principles can be applied to control the growth of other spore-forming and vegetative pathogens in low-acid foods.

2 Description and Diagnosis of Botulism

Botulism is a rare disease, but is characterized by high morbidity, long hospital stays, the need for artificial respiration, and historically a high fatality rate (Johnson and Montecucco 2008). Foodborne botulism is a true intoxication caused by the consumption of foods contaminated with preformed botulinal neurotoxin, while infant and wound botulism are caused by opportunistic infections by *C. botulinum* spores and BoNT formation in the intestine or in the wound (Johnson 2013). Human botulism is caused predominantly by neurotoxins formed by Group I (proteolytic) *C. botulinum* types A and B and Group II (nonproteolytic) types B and E (Hatheway 1993; Johnson 2013). Serotypes C and F have rarely been associated with human illness, and types D and G have not been reported to cause human botulism (Hatheway and Johnson 1998). Symptoms generally onset 12–36 h after consumption of contaminated foods, but cases with onset times of 2 h to 10 days have also been reported which is related to the serotype and quantity of BoNT consumed (Cherington 1998; Johnson and Montecucco 2008). Patients often first exhibit gastrointestinal symptoms including nausea, vomiting, and diarrhea. Subsequent neurological symptoms develop including blurred and double vision, dry mouth, difficulty in swallowing, speaking and breathing, and peripheral muscle weakness. In severe cases, the patient is completely paralyzed, and these cases generally require assisted ventilation for several weeks or months (Cherington 1998; Johnson and Monteucco 2008). Death may occur due to respiratory failure and possibly cardiac arrest and concurrent microbial infections. Fatality due to botulism has significantly decreased during the latter part of the twentieth century as a result of prompt administration of antitoxin and good supportive care, particularly by intubation and artificial ventilation and intragastric feeding (CDC 1998; Maslanka et al. 2013). Mortality due to botulism occurred in about 60 % of the cases prior to 1949, while since 1980 the mortality rate has occurred in 5–10 % of the cases (Johnson 2013).

Clinical diagnosis of botulism is often difficult since the symptoms can mimic other neurological diseases and intoxications and many physicians do not have experience in diagnosing the disease. Botulism symptoms can resemble those exhibited by Guillain–Barré syndrome, myasthenia gravis, stroke, ingestion of chemical poisons, tick paralysis, and certain other neurological syndromes (Johnson and Montecucco 2008). The definitive diagnosis of a confirmed case botulism is by detection of BoNT in clinical samples (serum, stool, vomit) and in foods (Maslanka et al. 2013).

Currently, there are no antidotes for human botulism. Cases can be treated with equine antitoxin raised against the various serotypes of BoNT (Johnson and Montecucco 2008). However, antitoxin is not effective once BoNT enters the nerves and thus, the time window for administration of antitoxin is limited. A human-derived antitoxin (BabyBIG®) is available to reduce symptoms and hospital stays in infants and is more effective than in acute foodborne botulism since *C. botulinum* in the intestinal tract of infants continually produces BoNT over a period of weeks to months (Arnon et al. 2006). Since, there is no antidote for botulinum poisoning, intoxicated patients must relay on hospital care and in severe cases intubation and artificial respiration. Partly due to the limited number of ICUs with required respiratory equipment, BoNT is considered as a potential agent of bioterrorism (Arnon et al. 2001). The lack of an antidote for intoxicated humans emphasizes the need to develop new antimicrobials to prevent growth and toxin production in the food supply and in infected humans.

3 Control Measures to Inhibit Growth and Toxin Production by *C. botulinum*

The primary technologies for preventing the hazard *C. botulinum* and many other pathogens in foods are (a) preventing contamination of the raw food commodity; (b) inactivating pathogens including spores by physical treatments such as an extensive thermal treatment (e.g. 12D botulinum cook); (c) formulating botulinal-safe foods by using inhibitory values of pH, a_w, E_h, temperature control and; (d) use of efficacious antimicrobials. The various treatments can act in combination or ideally in synergy, and this forms the basis of "hurdle" technology for production of safe foods (Leistner 1995).

Clostridium botulinum occurs free-living in the environment worldwide and is commonly found in soils and aquatic sediments (Dodds 1993; Hauschild 1989, 1993), and is generally not associated with an animal host (as compared to obligatory association of enteric pathogens such as *Salmonella and E. coli* O157:H7 with animals). The levels of *C. botulinum* spores may increase with certain agricultural practices such as cultivating foods in soils, including most vegetables which are the most common vehicles of botulism in several countries. Furthermore, the expanding trend of using organic methods for growing foods by using manure as a fertilizer may lead to high levels of spores during cultivation and harvesting.

Growing practices should be carefully evaluated including the use of manure as fertilizer. The inactivation of *C. botulinum* spores by physical means, particularly thermal treatments and newer methods such as high pressure and UV and gamma irradiation, as well as other physical technologies, have been reviewed (Setlow and Johnson 2013) and may have utility for specific food commodities. The area that has been least studied but which holds considerable potential in the development of novel natural antimicrobials to prevent growth and toxin formation by *C. botulinum*.

4 Antimicrobials and Antimicrobial Systems to Prevent BoNT Production in Foods

Novel antimicrobials, particularly from natural sources such as plants, animals, and nonpathogenic microorganisms hold considerable potential in assuring food safety and food sustainability (Cowan 1999; Daeschel 1989; Gálvez et al. 2014; Hammes and Tichaczek 1994; Ludwig-Müller 2014; Sofos et al. 1998; Taylor 2015). Table 1 portrays a compilation of natural antimicrobials from plants, animals (including eggs and milk), and microorganisms that have desired antimicrobial properties in foods and in the human and animal intestine.

An ideal antimicrobial would have the following properties: (a) it rapidly and selectively affects the target organism(s); (b) it is microbicidal rather than bacteriostatic and kills the target organism; (c) it requires low concentrations and few hits to kill the target organism; (d) it rapidly disperses through the food matrix; (e) it has minimal partitioning into the lipid phase of the food which usually does not harbor microorganisms; (f) it is stable in foods; (g) its use does not lead to resistant mutants; (h) it has minimal effects on organoleptic properties, and (i) it is not toxic to humans at the level used. In addition, ideally it would positively interact or be synergistic with other preservation methods and antimicrobials (Gould and Jones 1989). Of the many antimicrobials used for control of botulinal toxin production as well as other foodborne pathogens, synthetic organic acids and their salts have been most extensively evaluated (Glass and Johnson 2002). When acidulants are added to foods, it should be kept in mind that it may require several hours to days for the acid to equilibrate within a food, during which time the food should be kept refrigerated to prevent *C. botulinum* growth and toxin production in nonacidified microenvironments of the food during acid dispersal. As expected, the efficacy of monocarboxylic acids and salts is related to both concentration used and their respective pK_a. Their antibotulinal effects are enhanced as the pH of the substrate decreases, shifting the equilibrium to the undissociated form. Organic acid salts with higher pK_a values, such as acetate and propionate, may be more effective in low-acid foods. Potassium sorbate and sorbic acid, traditionally added to foods as antimycotic agents, have been reported to inhibit *C. botulinum* growth (Glass and Johnson 2002). Sodium nitrite has also been used as an effective anti-botulinal agent in meats (Glass and Johnson 2002)

Table 1 Natural antimicrobials from plant, animal, and microbial sources

1. *Plants*
Phenolics and polyphenols
Quinones
Flavones, flavonoids, flavonols
Hops acids
Tannins
Terpenoids and essential oils
Thiosulfinates
Phytoalexins
Falcarindiol
6-Methoxymellien
Myristicin
2. *Animals and insects*
Lysozyme
Lactoperoxidase
Glucose oxidase
Lactoferrin
Ribosome-inactivating proteins
Killer toxins
Defensins
Royalisin
Cecropins
Chitosans
Monoacylglycerols
3. *Microorganisms*
Bacteriocins
Chelators
Lytic enzymes
Natamycin
Nonantibiotic antimicrobials
Inhibitory lactic acid bacteria
Probiotics
Phages
Compounds formed during indigenous fermentations

The antimicrobial properties of phosphate salts and polyphosphate compounds have been demonstrated in several food products (Ellinger 1972). Phosphates function as antimicrobials in their ability to enhance moisture sequestration, inhibit oxidation of food components, stabilize emulsions, and modify pH. Polyphosphates have also been reported to inhibit enzymes involved in cell division, and to physically interact with cells forming channels and promoting leakage and cell lysis (Kornberg 1999).

Bacteriocins, including nisin and pediocin, have been evaluated for the delay botulinal toxin production in temperature-abused low-acid foods (Delves-Broughton 1990; Hammes and Tichaczek 1994; Mazzotta et al. 1997; Reis et al. 2012). However, the antibotulinal effect of nisin may be limited by a number of factors. The efficacy of nisin was reduced as a function of high phospholipid or protein concentration, pH $\geq$ 6.0, and at higher storage temperatures. Development of resistance in botulinal spores and vegetative cells to bacteriocins should also be considered. Mazzato et al. (1997) showed the ability of *C. botulinum* to concurrently develop resistance to nisin and bacteriocins from other lactic acid bacteria upon repeated exposure to nisin. Nisin resistance can be reduced by combining antimicrobials and conditions such as reducing the pH to 5.5 and adding 3 % NaCl to the food substrate.

More recently, naturally occurring antimicrobials primarily from plants and lactic acid bacteria have attracted interest as secondary barriers against pathogenic bacteria (Cowan 1999; Daeschel 1989; Gálvez et al. 2014; Hammes and Tichaczek 1994; Ludwig-Müller 2014; Sofos et al. 1998; Taylor 2015). These include phenolic compounds, terpenoids and essential oils, flavonoids, coumarins, alkaloids, lipid compounds, lectins and polypeptides, and other poorly characterized antimicrobials (Table 1). Although, many of these compounds have been demonstrated to inhibit pathogenic bacteria, spoilage fungi, and human viruses in vitro, relatively few trials have been performed in foods and more research is warranted.

5 Role of Competitive and Spoilage Microorganisms in Ensuring Food Safety

Fermentation and the utility of competitive bacterial cultures have been used for centuries to ensure the safety of fermented foods including many indigenous fermented foods from many cultures (Johnson 1991). However, the role of fungi, yeasts, and bacteria in the historical safety of indigenous fermented foods is poorly understood but offers considerable potential for the discovery of new antagonistic cultures and likely the discovery of new antimicrobials. More recently, designed competitive microbial cultures have been added to certain fermented foods including sausages and cheeses to inhibit growth and toxin production by pathogens such as *Staphylococcus aureus* and *C. botulinum*. In general, lactic acid starter cultures enhance the safety of fermented foods by production of lactic acid and other antimicrobials such as bacteriocins, hydrogen peroxide, and diacetyl (Daeschel 1989; Gálvez et al. 2014; Glass and Johnson 2002).

Mesophilic lactic acid bacteria are also utilized as protective cultures for low-acid refrigerated foods. If the product is temperature abused, the lactic acid bacteria can grow leading to acidification of the product, and prevention of the growth of pathogens. Often the foods show softening and emit unsavory gases, and these changes in sensory attributes serve as a deterrent to consumption. Other innovative approaches may include incorporating competitive bacteria in edible

films or packaging. The presence of selected nonpathogenic, non-spoilage bacterial systems can inhibit growth of pathogenic and spoilage bacteria in the food products (Glass and Johnson 2002).

Food manufacturers continually strive to reduce microbial loads in incoming raw ingredients and in the manufacturing plant environment as a means to enhance food safety and improve shelf life. However, the continuous removal of the entire microbiota in some food processing plants could lead to colonization by pathogens or undesirable production organisms (Jay 1997). For example in many wineries and certain other fermented foods sanitation is limited to allow the survival of yeasts and lactic acid bacteria that are beneficial to the fermentation process. The intentional colonization of manufacturing plants with nonpathogenic forms of pathogens such as *Listeria innocua* or infectious phages could prevent the growth and survival of pathogenic *Listeria monocytogenes*.

6 Use of Predictive Modeling and Challenge Studies to Define Control Limits

When designing antimicrobial systems for foods, it is valuable to model the food system for growth of pathogens and to validate the efficacy of the systems using challenge studies with the target pathogen. Several predictive and processing models have been developed for food safety (Baranyi and Roberts 2000; Haas 2014; Maas 1993; McKellar and Lu 2004; Perez-Rodriguez 2013). Among the earliest applications of predictive food microbiology was the use of thermal death time models to determine commercial sterility for canned foods (Setlow and Johnson 2013). Predictive models are advancing to more precisely evaluate growth and toxin production of *C. botulinum* in food systems. With the advances in genomics, it is becoming feasible to identify genes and metabolic systems that are essential for pathogen growth and to target certain metabolic systems in several pathogens including *C. botulinum* (Ihekwaba et al. 2015; Peck 2009). In the future, these advances in genomics should enable the design of antimicrobial systems to specifically target the pathogen of major concern including *C. botulinum*.

7 Perspectives and Conclusions

The global impact of foodborne disease is of extremely high significance in affecting the morbidity and mortality of humans and animals, sustainability of the food supply, as well as the economic impact on society. Due to its formation of the most potent toxin known to humankind, *C. botulinum* has been used as a premier model organism for evaluation and enhancement of food safety. There exists considerable potential for developing novel antimicrobials from natural sources and

for the use of microbial systems to outcompete and prevent the growth of pathogens. Many existing and potential antimicrobials are natural in origin and are derived from plants, animals, and microorganisms. Other sources could be derived from indigenous fermentations that have been used worldwide in human societies for centuries. Predictive modeling and validation systems are available for the design and implementation of antimicrobial systems for enhancing the safety of foods and sustainability of the global food supply through prevention of spoilage. The advances in genomics will be extremely valuable in identifying target genes and proteins and for the precise design of antimicrobial systems.

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DNA-Synthesizing Enzymes as Antibacterial Targets

Carlos Barreiro and Ricardo V. Ullán

Abstract The antimicrobial resistance is an antibiotics inherent problem, which goes hand in hand with their discovery, evolution and clinical use. The quest for new valid targets and the development of new drugs is capital in these days, when the latter antibiotic barrier (vancomycin) has been teared down. Thus, the DNA replication machinery (replisome) gathers lot of characteristics and hopes to be a robust antibacterial target. Five enzymes of the replisome focus the research: (i) topoisomerase type II, (ii) DNA primase, (iii) DNA helicase, (iv) DNA polymerase and (v) DNA ligase. These enzymes, which present characteristics that differentiate the replisome of bacteria from that of virus, archaea or eukaryotes, have been extensively studied in the last decades as antibacterial targets with divergence in outcomes as stated in this chapter.

1 Introduction

World Health Organization (WHO) describes the antimicrobial resistance as 'the resistance of a microorganism to an antimicrobial drug that was originally effective for treatment of infections caused by it' (WHO 2015). In addition, they differentiate between: (i) antibiotic resistance to refer specifically to the resistance to antibiotics that occurs in common bacteria that cause infections and (ii) antimicrobial resistance, which is a broader term, including resistance to the drugs to treat infections caused by other microbes as well, such as parasites (e.g. malaria), viruses (e.g. HIV) and fungi (e.g. *Candida*) (WHO 2015).

As result of the use and more particularly due to the traditional misuse and overuse (abuse) of the antimicrobial drugs, the emergence of drug-resistant strains have been accelerated, which is a natural trend of the antibiotic–producer microorganisms. Hence, methicillin-resistant strains of *Staphylococcus aureus* (MRSA) and

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methicillin- and vancomycin-resistant *Enterococcus* spp. (VRE) in Gram-positive bacteria and *Acinetobacter* and *Pseudomonas* spp as Gram-negative bacteria, where some strains are multi- or even pan-drug resistant, are the most notorious and alarming examples (Robinson et al. 2012).

WHO recognizes that (i) the poor infection control practices, (ii) an inadequate sanitary conditions and (iii) the inappropriate food-handling have boosted the further spread of antimicrobial resistance (WHO 2015). The resistance of bacteria to antibiotics and similar drugs (antimicrobials) is considered a major public health threat by the Food and Drug Administration (FDA) and its counterparts around the world (FDA 2013). It is one of the most important challenges to the healthcare sector in the twenty-first century, as well as an economic burden (WHO 2012; Wright 2012). As a result, this lack of efficiency in the antibiotic treatments leads to: (i) longer illnesses, (ii) more complicated diseases, (iii) more doctor visits, (iv) the use of stronger and more expensive drugs and (v) more deaths caused by bacterial infections (FDA Consumer Health Information 2011).

Resistance mechanisms emerged over the past decades have become myriad, which have been widely disseminated worldwide through bacterial populations (Wright 2012). Basically, the exhibited bacterial mechanisms to protect themselves from antibiotics can be distributed in four groups: (i) antibiotic modification (e.g.: β-lactamases, which cleave the four membered β-lactam ring); (ii) prevention of the antibiotic entering the cell or pumping it out; (iii) alterations in the primary site of action which avoid the antibiotic activity due to the structural changes in the target molecule and (iv) alternative target production in parallel to the original sensitive target (Hawkey 1998; Lewis 2013).

The idea that antibiotic resistance in bacteria is a contemporary phenomenon, based on the recent resistance increase, is wrong. Metagenomic analyses of ancient DNA demonstrated the existence of highly diverse collections of genes encoding resistances in 30,000-year-old samples (D'Costa et al. 2011). Hence, if the bacteria are ready from the antibiotic survival for a long time, how this resistance emergence? It is mainly a question of balance between the potential development of tolerance or resistance to that compound in relation with the time when it is first employed and also the mechanism of resistance against that drug (Davies and Davies 2010). In example, one year after the penicillin adoption in clinic the first resistance was described whereas glycopeptide antibiotics (e.g.: vancomycin) have been an intriguing exception for almost 30 years from the clinical introduction (Santos-Beneit et al. 2014).

This microbial 'perfect storm' could mean a literal return to the pre-antibiotic era for many types of infections (Spellberg et al. 2008). But, how the health care system has arrived to this disturbing situation? A short review of the antibiotics evolution can give the key and probably part of the solution. Initially, a wrong idea on microbial genetics supported the problem; since, taking into account the low frequency of spontaneous mutations observed in nature, it was suggested that the development of resistance during therapy was unlikely (Méndez-Alvarez et al. 2000). Unfortunately, the antibiotics discoveries have been mitigated by the appearance of resistant microbes which threw to the ground that traditional genetic idea (Wright 2007; Livermore 2009) (Fig. 1).

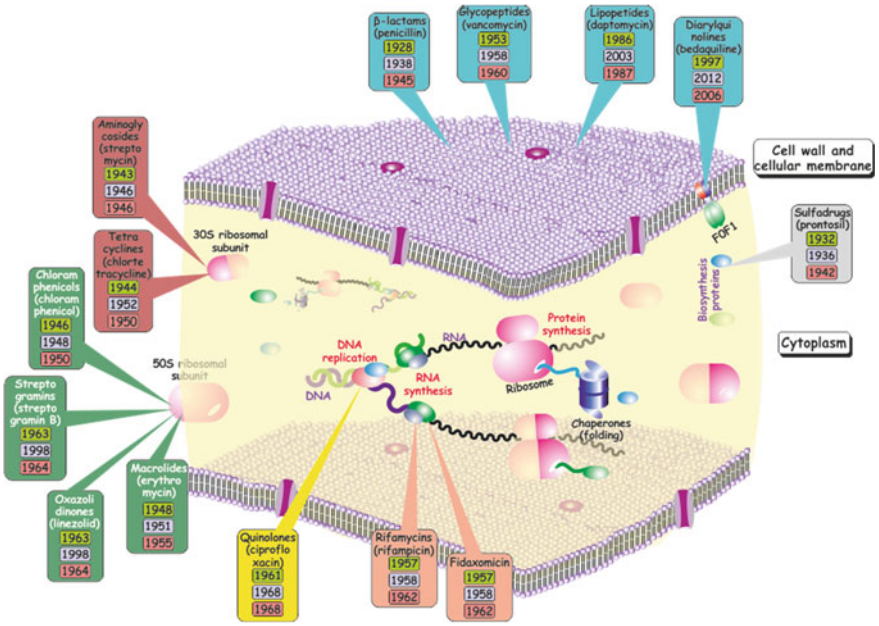

Fig. 1 Location of different antibiotic targets in the bacterial cell. *Boxes* show the antibiotic groups with their year of discovery (*green*), year of introduction (*light blue*) and year of first observed resistance (*red*). *Box colours* are in concordance with the drug target: biosynthetic proteins (*light grey*); membrane, cell wall or membrane proteins (*light blue*); 30S ribosomal unit (*brown*); 50S ribosomal unit (*dark green*); DNA synthesis (*yellow*) and RNA synthesis (*light red*). Based on Lewis (2013)

Nowadays, the political commitment is needed to impel regulations, which supported by clinical, farming and manufacturing efforts will help to decrease the antimicrobial resistance problem. Thus, antibiotics must be always prescribed appropriately when needed to ensure that the treatment is correctly followed, to avoid their used in agriculture or aquaculture and to abolish the substandard and counterfeit products (WHO 2012). In example, the decrease of antibiotics use as growth- and health- promoting compounds of livestock, fish and poultry farming for food production by means of their substitution by probiotics, prebiotics, enzymes or vaccines is a current milestone in the antibiotic resistance decrease (WHO 2012; Santos-Beneit et al. 2014). Anyway, the antibiotics are commonly used in the US in food animals today. Thus, the FDA estimates that 14.6 million kg of antibiotics were used in animals in 2012, which represents more than four times those sold for human use in 2011 (Teillant and Laxminarayan 2015).

Anyway, a paradoxical situation is right in front of our eyes. The appearance of resistance mechanisms contrast with the low number of new antibiotics brought to market and the pharmaceutical industry point of view about antibiotics as a poor investment, even though this, probably, is the Golden Age of understanding antibiotics mechanisms and resistance origin (Wright 2012). Thus, the antibiosis

rethinking or the turn back to the scientific theories of antibiotic resistance is a trending topic on public health care (Landecker 2015). Fortunately, at present, most infections can be still treated including the antibiotics in the drug arsenal. On one hand, modifications of the existing antibiotics or new ones are being carried out (although slower than recommendable). On the other hand, indiscriminate antibiotic use is being legally regulated (Jovetic et al. 2010).

2 Bacterial Replisome

The general understanding of bacterial cell death induced by antibiotics is focused on the essential cellular functions inhibited by the drug. Most current bactericidal antimicrobials inhibit DNA, RNA, cell wall or protein synthesis (Kohanski et al. 2010) (Fig. 1). The inhibition of the DNA synthesis is the core of the present chapter which is as an underexploited drug target (Robinson et al. 2012).

At this point, an overview of the bacterial replisome can help to clarify the proposed antibiotic targets. From a unique site (*ori*) to the terminus region (*ter*), the DNA replication in bacteria progresses creating two bidirectional replication forks. The replisome is a multi-component molecular machine which carries out replication of DNA (Table 1). Initially, it unwinds double-stranded DNA into two single strands to generate a new complementary sequence of DNA for each one. In example, *Bacillus subtilis* presents a network of 91 specific interactions linking 69 proteins (Noirot-Gros et al. 2002), which shows the complex structure of the system. The bacterial replisome composition and architecture seem highly stable along evolution, which underlines its efficiency responding to stress and avoiding problems during crucial steps of DNA synthesis. This fact is highlighted when it is taken into account that *Escherichia coli* and *B. subtilis* were separated by over two billion years of evolution (Battistuzzi et al. 2004; Bates 2008; Beattie and Reyes-Lamothe 2015).

Due to the opposing polarity of the DNA templates, the new DNA strands are synthesized differently. One (leading strand) is synthesized continuously, whereas the lagging strand is synthesized as a series of short Okazaki fragments. Basically, the process is carried out in three stages: (i) initiation at the origin of replication, (ii) DNA synthesis (elongation) at replication forks and (iii) termination (Schaeffer et al. 2005; Beattie and Reyes-Lamothe 2015).

2.1 Replication Initiation

The replisome structure of the Gram-negative bacterium *E. coli* is currently the best understood due to decades of genetic and biochemical research (Beattie and Reyes-Lamothe 2015). It can be used as a model, even though some exceptions in its machinery have been defined [e.g.: ε exonuclease function (Rock et al. 2015)]. Thus, this bacterium will be used as example of the replication steps. A 4.7-Mb circular

Table 1 Summary of the proteins involved in the DNA replication of the well-known model strains *E. coli* (Gram negative), *B. subtilis* (Gram positive), as well as the Actinobacteria strains *Corynebacterium glutamicum* and *Streptomyces coelicolor* (Based on genome annotations, homology analysis and references of: Johansson and Dixon 2013; Beattie and Reyes-Lamothe 2015). See extended version of Robinson et al. (2012)

Protein function	*E. coli*	*B. subtilis*	*C. glutamicum*	*S. coelicolor*
Origin activator	DnaA	DnaA	DnaA	DnaA
Helicase loader	DnaC	DnaI	–	–
Helicase	DnaB	DnaC	DnaB	DnaB
Primase	DnaG	DnaG	DnaG	DnaG
Replicase core	Pol III (αεθ)	PolC	DnaE	DnaE
Polymerase subunit	α (DnaE)	PolC	α (DnaE2, DnaE1);	α (DnaE, *SCO1739*)
Proofreading subunit	ε (DnaQ)	PolC	ε (*cg0302*, dnaQ2, *cg2321*)	ε (*SCO1827*)
Primer extension	–	DnaE/DnaQ	–	–
Leading/lagging-strand pol	Pol III (αεθ)	PolC	α (DnaE2, DnaE1); ε (*cg0302*, dnaQ2, *cg2321*)	α (DnaE, *SCO1739*); ε (*SCO1827*)
Clamp	β	β	DnaN	DnaN/*SCO1180*
Clamp loader	$\tau_3\delta\delta'\chi\psi$	$\tau_3\delta\delta'$	DnaZX, dnaX/*cg2576*	–
Accessory replicative polymerase	–	DnaE	DnaG	DnaG
Okazaki fragment processing	Pol I	Pol I	Pol A	Pol A
ssDNA binding protein	SSB	SSB	SSB	*SCO2683*/ *SCO3907*
Gyrase	GyrA/B	GyrA/B	GyrA/B	GyrA, *SCO5836*/GyrB, *SCO5822*

genome like *E. coli* presents a 260-bp sequence that constitutes a unique origin of replication (*oriC*). The bacteria present multiple copies of short DNA sequences recognized by replication initiator proteins. Thus, the DnaA protein recognizes five copies of a 9-bp DnaA-box in the replication origin to oligomerize into a helical filament destabilizing the neighbouring duplex unwinding element, which results in a bubble of SSB-coated ssDNA. This one is the substrate of the homohexameric helicase DnaB which plays a central role in the replisome by interaction with DnaA. It unwinds the parental DNA duplex, as well as, encircles single-stranded DNA (ssDNA) on the lagging strand. In contrast, when DnaB is complexed with six molecules of the loading partner DnaC, its activity is inhibited due to the closure of the DnaB central channel. The interaction with DnaA allows the activity of DnaB. At this stage, DnaB is able to interact with a specialist RNA polymerase (DnaG primase) that synthesizes short RNA primers on the ss-DNA regions. This is a key step to start the elongation step (Schaeffer et al. 2005; Beattie and Reyes-Lamothe 2015).

2.2 DNA Elongation Stage

The elongation stage is carried out by the DNA polymerase III (Pol III). On the one hand, the leading strand is continuously extended from *oriC* without enzyme dissociation. On the other hand, the lagging strand is discontinuously generated by means of 1-kb length Okazaki fragments which are processed by DNA polymerase I and joined by DNA ligase.

Pol III is a very efficient enzyme which operates with nearly perfect fidelity due to the collaboration of ten different holoenzyme subunits [the core (α, ε, θ), the β_2 sliding clamp, the clamp loader (δ′, γ, τ, δ, ψ, χ)] (Schaeffer et al. 2005). This multi-component molecular machine needs to be an absolutely stable assembly to accomplish replication of long chromosomes. In example, *E. coli* takes over 40 min of continuous replisome work with a synthesis rate of 1 kb per second (Beattie and Reyes-Lamothe 2015).

2.3 Termination of Replication

A monomeric site-specific DNA-binding protein, named Tus, is responsible for replication termination, which binds to a 23-bp Ter site opposite to the *oriC* region. Ten Ter sites conform a replication fork trap in *E. coli*, whereas in *B. subtilis* the replication terminator protein (RTP), which has a different structure, binds as a dimer of dimers (Schaeffer et al. 2004, 2005). The replication speed of the replisome machinery defines the efficiency of the Tus–*ter* termination system (Elshenawy et al. 2015).

In contrast, those bacteria such as *Streptomyces* genus that present multinucleoid cells with lineal chromosomes contain long-terminal inverted repeats and covalently bound terminal proteins which act as telomeres ending the replication (Yang et al. 2002; Ruban-Ośmiałowska et al. 2006; Bates 2008).

3 DNA-Synthesizing Enzymes as Targets

The large number of the replisome components work in a well-coordinated and defined pattern which is essential for cell survival as previously described. The activity distortion or inhibition of those enzymes affect the DNA replication that detriment the cell survival. Thus, several of the enzymatic components have a real potential as antibiotic targets whose identification raises hopes to lead novel antibacterials. However, a few of these novel targets have become the objective of an antibacterial drug candidate into clinical trials probably due to the identification approaches employed. In example, the protein–protein interaction instead of the enzymatic activity could be an alternative strategy to obtain new antibacterial compounds (Sanyal and Doig 2012; Marceau et al. 2013).

Nowadays, five replication associated proteins are the most promising drug targets: (i) topoisomerase type II, (ii) DNA primase, (iii) DNA helicase, (iv) DNA polymerase and (v) DNA ligase.

3.1 Topoisomerase Type II

Type II of topoisomerases are enzymes that modulate the eukaryotic and prokaryotic DNA topology in vital processes such as protein synthesis, DNA replication and repair, as well as, DNA decatenation. Two types of type II topoisomerases are present in bacteria: (i) DNA gyrase (GyrA, GyrB) which relieve torsional strain during replication and introduces negative supercoils in DNA at the expense of ATP hydrolysis; (ii) topo IV (ParC, ParE) that catalyzes the essential decatenation separating the two DNA molecules. Besides, both enzymes relax the supercoiled DNA (Sanyal and Doig 2012; Heide 2014).

Usually, topoisomerase inhibitors constrain both topo IV and DNA gyrase even though the primary target varies between the bacterial species. Sometimes, this variation is due to the lack of one of the enzymatic systems in the microorganism (Mdluli and Ma 2007). This fact has helped to delay the bacterial resistance development (Takei et al. 2001).

The gyrase inhibitor Novobiocin was discovered in the 1950s and introduced as Albamycin into human anti-infective therapy in 1964. Subsequently, more potent compounds as clorobiocin and coumermycin A1 were found and these were the three initial aminocoumarins, which act as competitive inhibitors of gyrase were also found. Before those three, just a few compounds have been discovered as natural aminocoumarins (rubradirin, simocyclinones or coumabiocins A-F) (Samuels and Garon 1993; Cheenpracha et al. 2010; Heide 2014). Nowadays, the knowledge of the biosynthetic gene clusters of the aminocoumarin antibiotics is allowing the use of combinatorial biosynthesis, mutasynthesis, structure-guided discovery and synthetic biology to exploit the generation of new aminocoumarins. Therefore, the expression of modified biosynthetic gene clusters, the addition of structural moieties to allow the compound uptake by the bacterium or the feeding with synthetic analogs to defective mutant strains are offering new varieties of synthetic aminocoumarins (Galm et al. 2004; Anderle et al. 2007; Heide 2009a, b, 2014; Alt et al. 2011; Tari et al. 2013; Trzoss et al. 2013) (Table 2).

The poor penetration across the Gram-negative outer membrane is a shortcoming of the aminocoumarins, as well as, their low solubility which means a pharmaceutical problem. However, the development of novel compounds such as novclobiocin 401 which is easily imported through the Gram-negative cell envelope opens new hopes. Besides, the combination of fluoroquinolones and aminocoumarins strongly reduce the resistance development against fluoroquinolones of *S. aureus*, which increase the antibiotic viability (Vickers et al. 2007; Heide 2014).

In many ways, aminocoumarins are considered as the 'Cinderellas' of the gyrase inhibitors, mostly in comparison with fluoroquinolones. Even though they bind

Table 2 Summary of compounds described as inhibitors of the DNA gyrase (Based on: Sharma et al. 2009; Collin et al. 2011)

Compounds	Comments	References
Quinolones	Antibacterials targeted to DNA gyrase	
Nalidixic acid Oxolinic acid Pipemidic acid	*First generation* Gram negative bacteria Extremely protein bound drugs Short half-life	Lesher et al. (1962), Shimizu et al. (1975), Gleckman et al. (1979)
Norfloxacin Enoxacin Ciprofloxacin Ofloxacin Lomefloxacin	*Second generation* Protein binding (50 %) Longer half-life than previous Better activity against Gram-negative bacteria.	Goldstein (1987), Campoli-Richards et al. (1988), Jaber et al. (1989), Smythe and Rybak (1989), Wadworth and Goa (1991)
Levofloxacin Gatifloxacin	*Third generation* Gram-negative bacteria Gram-positive bacteria	Perry et al. (2002), Croom and Goa (2003)
Moxifloxacin Gemifloxacin	*Fourth generation* Broad spectrum of Gram bacteria Anaerobes and atypical bacteria	Lowe and Lamb (2000), Keating and Scott (2004)
Aminocoumarins	Also known as coumarins contain a 3-amino-4,7-dihydroxycoumarin ring	
Novobiocin Clorobiocin Coumermycin A1	Antibiotics produced by different *Streptomyces* strains	Ward and Meyer (1957), Heide (2014)
Simocyclinones	Hybrid antibiotics containing both aminocoumarin and polyketide elements	
Simocyclinone D8	Modest antibacterial properties: Most potent against Gram-positive Ineffective against Gram-negative	Sissi et al. (2010)
Toxin-antitoxin		
CcdB	11.7-kDa protein involved in the maintenance of the copy number of the F plasmid that is produced as a part of a two-component toxin–antitoxin system	Miki et al. (1984)
ParE	The ParE toxin produced by the RK2 plasmid of *E. coli* inhibits DNA synthesis and to stabilize the gyrase–DNA cleavage complex	Jiang et al. (2002)
Protein inhibitor		
GyrI (SbmC or YeeB)	An 18-kDa *E. coli* protein, which inhibits DNA gyrase	Nakanishi et al. (1998)
MurI	Glutamate racemase (L-glutamate to D-glutamate) is involved in bacterial cell wall synthesis and inhibits the *E. coli* DNA gyrase	Ashiuchi et al. (2002)

(continued)

Table 2 (continued)

Compounds	Comments	References
Cyclothialidines	Cyclic peptides with antibacterial activity produced by streptomycetes, which works as competitive inhibitors of the gyrase ATPase reaction blocking the binding of ATP to subunit GyrB	Oblak et al. (2007)
Cinodine	Glycocinnamoylspermidine antibiotic class produced by *Nocardia* species	Greenstein et al. (1981)
Clerocidin	Diterpenoid cytotoxic and antibacterial agent isolated from the fungus *Oidiodendron truncatum*	Gatto et al. (2001)
Albicidin	Main component of the family of antibiotics and phytotoxins by the sugarcane scald pathogen *Xanthomonas albilineans*	Birch and Patil (1985)
Microcin B17	Enterobacteria carrying the pMccB17 plasmid produce the 3.1-kDa glycine-rich peptide named Microcin B17	Davagnino et al. (1986)

strongly to gyrase, they have not reached significant clinical success (Collin et al. 2011). An accidentally detected by-product of chloroquine synthesis (antimalarial) resulted in the nalidixic acid discovery. It was the base for the fluoroquinolones development (Lesher et al. 1962), since the clinically significant fluoroquinolone class compounds are synthetic fluorinated analogs of naldixic acid. They stabilize the cleaved DNA by covalently binding to the GyrA active site tyrosine which results in the DNA strand breakage and release, stoppage of DNA topology and incomplete DNA strands. This fact activates the SOS response. Thus, the inhibition of the SOS to avoid the remediation of the hydroxyl radical damage will help to potentiate the bactericidal drug effect of quinolones, which actively stimulate the oxidative damage that contribute to cell death (Yoshida et al. 1991; Kampranis and Maxwell 1998; Heddle and Maxwell 2002; Dwyer et al. 2007; Kohanski et al. 2007; Sharma et al. 2009; Sanyal and Doig 2012). Quinolones are by far the most successful antibacterial compounds targeted to DNA gyrase (Collin et al. 2011) (Table 2).

In Gram-positive bacteria, often topo IV is the primary target of the fluoroquinolones, whereas in Gram-negative bacteria, typically gyrase is the primary target. The fluoroquinolones have been a successful example of gyrase-targeted drugs, but the increase of bacterial resistance against these agents is boosting the screening of new compounds, as well as, new modes of inhibition of this enzyme (Collin et al. 2011). From the first generation of quinolones (nalidixic acid and oxolinic acid), which had relatively weak antimicrobial activity, the synthesis of the

fluoroquinolones over several generations has improved their efficiency. Thus, the second generation (norfloxacin, ciprofloxacin), third generation (levofloxacin, gatifloxacin) or the fourth generation (moxifloxacin, gemifloxacin) have led to a range of potent antibacterial compounds with great clinical and commercial success (Table 2) (King et al. 2000; Oliphant and Green 2002; Emmerson and Jones 2003; Collin et al. 2011).

In clinical isolates of *Mycobacterium tuberculosis,* the resistance to fluoroquinolones remains uncommon, but high resistance levels have been observed in laboratory-generated strains of *M. tuberculosis* and *Mycobacterium smegmatis* due to the amino acid substitutions in the putative fluoroquinolone-binding region of the GyrA (Mdluli and Ma 2007). These substitutions outside and inside of the fluoroquinolone-binding pocket have been described as the most common bacterial mechanism to gain resistance against fluoroquinolones (Lahiri et al. 2015).

Quinolones have some adverse effects (nausea, headache, dizziness and confusion) which rarely can originate prolongation of the corrected QT interval, phototoxicity, liver enzyme abnormalities, arthropathy, and cartilage and tendon abnormalities. Due to this fact, the new fluoroquinolones are rarely first-line agents and should be employed carefully (Oliphant and Green 2002).

3.2 DNA Primase (DnaG)

The description of DNA primase inhibitors is highly valuable for the elucidation of biochemical pathways, as well as, for the drug design to block bacterial diseases (Frick and Richardson 2001). These compounds must be really efficient against the DNA primase, since low levels of the protein are enough to keep the microbial growth (Kuron et al. 2014). DNA primase catalyzes the synthesis of short RNA fragments (4–15 nt) which act as primers of the DNA polymerase in the elongation process of the lagging strand. Most DNA primases can be divided in two groups: (i) bacterial and bacteriophage enzymes and (ii) heterodimeric eukaryotic primases. The prokaryotic enzymes contain three domains involved in: (i) DNA binding by a zinc ribbon motif, (ii) RNA polymerization, (iii) association with the DNA helicase (DnaB) (Frick and Richardson 2001; Sanyal and Doig 2012).

The traditional view of this class of proteins is evolving due to the diverse pathways in which these enzymes are employed. Thus, their reclassification in the primase—polymerases category within the wider group of polymerases has been recently recommended (Guilliam et al. 2015).

The nucleotide analogs have demonstrated their activity as DNA primase inhibitors (e.g.: 2′,3′-dideoxynucleoside 5′-triphosphates, 2′-deoxy-2′-azidocytidine) (Frick and Richardson 2001). Plant extracts (e.g.: *Polygonum cuspidatum*) containing phenolic saccharides have also showed inhibition activity against bacterial DNA primases (Hegde et al. 2004). Besides, fermentative products from fungi (*Penicillium verrucosum*) as Sch 642305 and its epimers present interesting inhibitory activities (Chu et al. 2003; Mehta and Shinde 2005; García-Fortanet et al. 2007).

Recently, the development of non-radioactive in vitro assays to identify DnaG inhibitors has been updated for pathogenic microorganisms as *Bacillus anthracis* and *M. tuberculosis*. Thus, coupling the activity of the primase DnaG to that of an inorganic pyrophosphatase allowed the development of a primase—pyrophosphatase assay. A 2500-molecules screening assay presented DNA intercalating and non-intercalating drugs such as tilorone, doxorubicin, suramin and ellagic acid, which works as primase inhibitors (Biswas et al. 2013a, b). In case of *M. tuberculosis*, which is probably the most-studied bacterial target for primase inhibitors, some clinically used anthracyclines and aloe-emodin have also demonstrated their activity against the mycobacterial primase (Gajadeera et al. 2015).

However, some of the described compounds inhibit the enzyme action by means of the direct binding to the DNA substrate. Thus, the main concern regarding these DNA intercalating compounds is the toxicity due to their non-selective activity (Sanyal and Doig 2012).

3.3 DNA Helicase

The separation and/or nucleic acid duplexes rearrangement fuelled by the adenosine triphosphate (ATP) hydrolysis is the aim of the helicases, which are ubiquitous motor proteins encoded by bacteria, viruses and human cells. DNA primase and helicase are functionally connected in the replisome. Thus, the helicase (DnaB) stimulates the primase (DnaG) activity in the in vitro assays (Sanyal and Doig 2012; Shadrick et al. 2013).

The bacterial helicase targets of the drugs include DnaB-like and RecBCD-like helicases which coordinate DNA replication and prepares DNA for homologous recombination and/or repair, respectively (Sanyal and Doig 2012; Shadrick et al. 2013).

The high-throughput screening of huge compound libraries have been carried out by means of the optimization of helicase inhibition assays as: (i) combination of fluorescent versus quencher moiety labelled oligonucleotides to analyze their helicase-mediated dissociation; (ii) radiometric assays of helicase activity utilizing as substrate radiolabelled 60-mer oligonucleotides; (iii) fluorescence polarization-based DNA unwinding assays; (iv) time-resolved fluorescence energy transfer (TRET); (v) bioassays based on the phage T4 *gene 2* mutants ability to grow in *E. coli* and (vi) microplate ethidium bromide displacement assays (Aiello et al. 2009; Sunchu et al. 2012; Bannister et al. 2013).

Those analytical methods permitted the screening of: (i) 78,588 compounds from the Microbiotix, Inc. collection against the *B. anthracis* helicase; (ii) 108,026 compounds from the National Screening Laboratory for the Regional Centers of Excellence in Biodefense and Emerging Infectious Disease against *S. aureus*; (iii) 230,000 commercially available compounds against *Pseudomonas aeruginosa* (iv) 446 small molecules from the Clinical Collection library of the National Institutes of Health (NIH) to screen inhibitors of the PriA helicases of *Neisseria*

gonorrhoeae and *E. coli* and (v) 326,100 small molecules from the NIH molecular libraries to detect inhibitors of the AddAB helicase of *Helicobacter pylori*. As a result, the research groups have described the discovery of (i) a good antibacterial inhibitor against the *P. aeruginosa* DnaB helicase (triaminotriazine) with cytotoxicity toward mammalian cells which acted as based for analogs with significant reduction in cytotoxicity; (ii) fifteen validated hits against the *B. anthracis* helicase; (iii) three active compounds against the *S. aureus* helicase; (iv) several benzobisthiazole derivative candidates against *B. anthracis* and *S. aureus* helicases; (v) one drug (CGS 15943) that induces conformational change in *N. gonorrhoeae* and *E. coli* PriA decreasing its catalytically proficient; (vi) a drug-like pipemidic acid thiourea (ML328) which inhibits the AddAB helicase of *Helicobacter pylori* and (vii) a ubiquitous natural product in plants (myricetin) which inhibits several enzyme classes (e.g. DNA polymerases, RNA polymerases, reverse transcriptases, telomerases, kinases and helicases), as inhibitor of the *E. coli* DnaB helicase. Among the detected compounds are coumarins, benzothiazoles, rhodanines, triazines, nitrofuran amides, pipemidic acid, thioureas *N*-phenylpyrroles, benzobisthiazole derivatives and some not easily classified compounds. Intriguingly, some helicase inhibitors presented a portion of the aminocoumarin scaffold without activity against the gyrase enzyme (McKay et al. 2006; Griep et al. 2007; Aiello et al. 2009; Bannister et al. 2013; Li et al. 2013; Shadrick et al. 2013).

3.4 DNA Polymerase

DNA generation by assembling nucleotides based on the chromosomal DNA sequence is the central role of the DNA polymerases. They are essential to DNA replication and their inhibition has been shown to be bactericidal (Sanyal and Doig 2012). The model bacteria *E. coli* presents five DNA polymerases: (i) Pol I (*polA*) involved in the Okazaki fragments processing and in the gap filling in the excision-repair processes; (ii) Pol II (*polB*) is an accurate DNA polymerase which role is not well defined, but it acts in DNA replication of the lagging strand, editing of the Pol III errors and in the restart of the replication after DNA damages; (iii) Pol III is the main replicative polymerase and (iv) Pol IV (*dinB*) and (v) Pol V (*umuDC*) are Y-family DNA polymerases, which present higher frequencies of base misincorporation in the absence of proofreading-exonuclease subunits or domains to correct the incorrect bases insertions (Hastings et al. 2010).

DNA polymerase III acts in a tripartite replicative machine which contains the Pol III, a processivity factor (β2) and a complex ATPase (DnaX). This polymerase differs between Gram-positive and Gram-negative bacteria in addition to the mammalian DNA polymerase. Besides, the bacterial DNA polymerase III diverges in E-type (*dnaE*) and C-type (*polC*), which co-exists in *B. subtilis*; whereas, another bacteria encode another Pol III (*ImuC*) as *P. aeruginosa* (McHenry 2011a, b; Sanyal and Doig 2012). These divergences among polymerases have strongly suggested the selection of the polymerases as therapeutic targets to decrease the

antibiotic resistance. Hence, the DNA pol IIIC has been validated as an antimicrobial target for drug development. However, the effectiveness of drugs, especially against pol IIIC of Gram-positive resistant bacteria, remains clinically undetermined (Ali and Taylor 2005; Berdis 2008).

On the one hand, some well-known drugs in previous described applications have been recently presented as DNA pol inhibitors. This is the case of non-steroidal anti-inflammatory drugs as carprofen, bromfenac or vedaprofen which act over the DNA polymerase III β subunit of *E. coli* (Yin et al. 2014). On the other hand, specific drugs have been screened. Thus, at the beginning of the 1970s the 6-(*p*-hydroxyphenylazo)-uracil/-isocytosine compounds, which acts by hydrogen bonding with cytosine and thymidine residues, were analyzed as inhibitor of the replication of Gram-positive bacteria (Neville and Brown 1972; Ali and Taylor 2005). From then to the present, several companies and research groups have applied for different patents of candidate compounds: (i) substituted phenyl azo and hydrazino pyrimidine derivatives (Imperial Chemical Industries); (ii) *N*-3 alkyl (anilino)uracils series (University of Massachusetts); (iii) uracil thioethers (Bayer AG); (iv) 4-substituted 2-amino-6-(anilino)pyrimidines, 6-substituted pyrazolo[3,4-*d*]pyrimidin-4-ones and *N*-3 alkyl and phenyl substituted uracils series (Merck) and (v) *N*-3 substituted uracil derivatives series which include an analog of the fluoroquinolone drug ciprofloxacin that suggest a 'dual activity' compound (Microbiotix) (Ali and Taylor 2005).

The *N*-3, C-6 substituted uracil derivatives showed antibacterial activity due to their activity against DNA polymerase IIIC (*polC*) which is consistent with their restricted antibacterial spectrum. Additionally, some compounds have been also described as competitive inhibitors of DNA polymerase IIIE from Gram negative or positive bacteria. Thus, these targets are valid candidates for the development of new drugs or derivates and the research on these compounds remains active, even though the industry tends to the abandonment of such antibacterial drug discovery at this time. However, the development of their pharmacokinetics or animal efficacy trials is uncertain up to date (Tarantino et al. 1999; Ali and Taylor 2005; Wright et al. 2005; Berdis 2008; Xu et al. 2011; Sanyal and Doig 2012).

3.5 DNA Ligase

The DNA polymerases synthesize DNA in the 5′ to 3′ direction, thus the DNA antiparallel strand is synthesized discontinuously as Okazaki fragments that are joined by DNA ligase to generate a complete strand. The precise joining of proper DNA ends in DNA replication, recombination and repair processes is the role of this specific class of enzymes. Humans and bacteria present a DNA ligase that plays the same functional role. However, the bacterial ligase (LigA class) uses NAD^+ as the cofactor, while the viruses, archaea and eukaryote ones (LigI class) depend on ATP to develop their function. The mammalian cells present three DNA ligases (I, III and IV) which play specific roles within the cell (Wilkinson et al. 2001; Weller and Doherty 2001; Sanyal and Doig 2012; Hale et al. 2015).

The understanding of the mechanisms and structures of these enzymes are well-known, which boosts the screening of compounds that selectively inhibit the bacterial DNA ligases without affecting the human DNA ligase role. That is the case of some inhibitors that bind the adenosine binding site of LigA (Wilkinson et al. 2001; Weller and Doherty 2001; Sanyal and Doig 2012; Hale et al. 2015). Thus, LigA is being the most frequently used candidate to look for bacterial DNA ligase inhibitors. However, some concerns in case of it use in antistaphylococcal chemotherapy have appeared due to the observed tolerance of staphylococcal cells (Podos et al. 2012). Anyway, different approaches have been used to face up the use of DNA ligases as drugs targets:

- The arylamino compounds, which are commonly used as anti-inflammatory and antimalarial compounds, as well as, the bisquinoline compounds have been observed in *Salmonella* as specific inhibitors of eubacterial DNA ligase (Ciarrocchi et al. 1999).
- The N^1, N^n-bis-(5-deoxy-a-D-xylofuranosylated) diamines have showed their specific inhibition of the *M. tuberculosis* LigA, but no effect was observed against the viral T4 ligase or the human DNA ligase I (Srivastava et al. 2005).
- The screening of small molecules capable of inhibiting the DNA ligase of *Streptococcus pneumoniae* presented the 2,4-diamino-7-dimethylamino-pyrimido[4,5-*d*]pyrimidine as a suitable candidate with a broad spectrum of antibacterial activity (Meier et al. 2008).
- Substituted adenosine analogs inhibit the NAD^+-dependent DNA ligase activities of *E. coli*, *Haemophilus influenzae*, *Mycoplasma pneumoniae*, *S. pneumoniae* and *S. aureus* without any effect against the ATP-dependent human DNA ligase 1 or the bacteriophage T4 ligase. Besides, their in vivo efficacy was validated in murine thigh and lung infection models (Mills et al. 2011; Stokes et al. 2011, 2012).
- The guided design, the adenosine binding pocket analysis and the synthesis of molecules to be used as inhibitor of NAD^+-dependent DNA ligase of Gram-positive bacteria have reached divergent results. Thus, aminoalkoxy-pyrimidine-carboxamides achieved moderate results as Gram-positive antibacterial, whereas the 2-amino-[1,8]-naphthyridine-3-carboxamides reached better results (Surivet et al. 2012; Wang et al. 2012; Murphy-Benenato et al. 2014).

In addition of the compounds, the methodological and knowledge advances are helping to the development of new drugs. In example, *Brugia malayi* is a lymphatic filarial parasite which contains an essential endobacteria (*Wolbachia*) for its development, viability and fertility. The bacterial DNA ligase of *Wolbachia* has been deeply analyzed to allow its use in tests for validating antifilarial drugs (Shrivastava et al. 2012). Besides, significant differences in the DNA repair systems have been recently described between mycobacteria and others microorganisms. This fact suggests the development of inhibitors able to distinguish between bacteria (Khanam and Ramachandran 2014).

4 Conclusions and Future Perspectives

From the very beginning of this chapter, a paradoxical situation can be observed. Since, one of the best known and phylogenetically conserved cellular systems as is the case of the DNA replication machinery, which offer several components that can act as species-specific drug targets, just offers a few inhibitors available for clinical purposes. On the one hand, Robinson et al. (2012) suggest that the inhibition of the bacterial replisomes is inherently difficult and even Nature has struggled to obtain inhibitors against them. On the other hand, Sanyal and Doig (2012) established part of the problem in the complex multicomponent mechanisms with large protein–protein interactions and extensive conformational changes by which the replisome function is difficult to study and exploit. This is a taxonomically stable system along the evolution and probably due to this the business is hardly difficult. In contrast, there are peculiarities that differentiate bacteria, virus and mammalian machineries that support the screening of these new molecules.

Where Nature does not arrive, the technological advances are trying it. Thus, the development of tests for specific inhibitors, analytical method for detection of binding domains, the knowledge on protein–protein interactions, the chemical synthesis of analogs and the structure-guided discovery or the synthetic biology are currently available for the developed of high-throughput screening programs. This fact introduces a new player in the game that could crystalize the hopes in the DNA replication machinery as a suitable target to tackle the worldwide question of the antibiotic resistances.

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Mechanisms of Drug Efflux and Strategies to Overcome Them as a Way to Control Microbial Growth

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1 Introduction

The discovery of antibacterials during the twentieth century has been one of the most important events in the history of medicine. The development, production, and use of these new drugs revolutionized clinical practice and industrial microbiology. Certainly, since the beginning of the antibiotic era many lives have been saved. Nevertheless, shortly after the first descriptions of the effects of antibiotics against pathogenic bacteria, the phenomenon of antimicrobial resistance began to be quoted by different researchers. At first this was not considered a significant clinical event. Nowadays, antibiotic resistance is an extremely important public health problem (Neu 1992). In fact, WHO, FAO, and OIE have signed documents jointly making recommendations on rational use of antibiotics to prevent the appearance of resistant strains of pathogenic microorganisms. Those recommendations include suppression of unnecessary treatments, dose adjustment to avoid wrong intervals or insufficient amounts and regulation of the use of antibiotics as growth promoters for veterinary use (Joint FAO/OIE/WHO expert workshops. Geneva 2003, Oslo 2004, Seoul 2006, Rome 2007).

But, how does a sensitive microorganism acquire resistance to an antimicrobial? Basically, by modification of the genetic endowment which may occur by chromosomal mutation or by gene transfer mechanisms. Resistant strains become predominant by antibiotic selective pressure which wipes out sensitive bacteria. Bacterial phenotypes expressing resistance to a wide range of structurally dissimilar

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chemotherapic drugs may lead to an even more serious problem: the multidrug resistance. There are certain human infections for which there is no effective treatment at the present time.

There is increasing evidence of the role of efflux pumps in bacterial resistance to antimicrobials. Although they do not confer high levels of resistance by themselves, their overexpression combined with other resistance mechanisms is of clinical importance.

The inhibition of efflux pumps is a promising strategy for restoring the activity of existing antibiotics and a useful diagnostic tool, since they allow detecting the presence of efflux in clinical isolates as a mechanism of resistance.

2 Resistance Mechanisms

Bacteria become resistant to antibiotic by developing basically three types of defense mechanisms against antimicrobials and other drugs:

1. Enzymatic inactivation: some bacteria produce hydrolytic enzymes able to hydrolyze the antimicrobial, such is the case of beta-lactamases, or enzymes which turn the molecule into an inactive derivative, such as the enzymes that phosphorylate or acetylate aminoglycosides (Livermore 1995; Mingeot-Leclercq et al. 1999).
2. Chemical alteration of antimicrobial targets: this prevents or hinders the action of the antimicrobial by modifying targets that are usually bacterial metabolic essential enzymes or by expressing alternative targets not likely to be inhibited by the molecule (Spratt 1994).
3. Bacterial modifications that prevent the antimicrobial reach its target: either lowering the natural membrane permeability by modifying porines' size or number, or actively secreting the intracellular antimicrobial by using proteic pumps that form efflux systems (Delcour 2009; Kumar and Schweizer 2005).

3 Efflux Pumps

Efflux pumps are membrane transporters generally involved in the extrusion of toxic compounds from the cell inside to the external environment. They are localized in the cytoplasmic membrane of all kinds of cells and function via an energy-dependent mechanism called active efflux. In antibiotic producers, for example, efflux systems confer autoimmunity, but in most systems they are involved in excretion of toxic compounds, intercellular communication, or maintaining homeostasis (Marchetti et al. 2011).

As mentioned above, all genomes sequenced so far contain different efflux pumps, this clearly indicating an ancient origin. In fact, many phylogenetic evidences show they may derive from a common ancestor, hence maintaining

sufficient similarity not only of structure but also of function throughout evolution (Van Bambeke et al. 2000).

Efflux pumps can be substrate specific or be able to transport a wide variety of chemically different compounds. In the latter case, they may be related to the well-known Multiple Drug Resistance (MDR) phenomenon. MDR pumps are a serious problem in antibiotherapy because their occurrence can decrease susceptibility to a wide range of chemically unrelated drugs (Weber and Piddock 2003). It is possible because the substrate identification is based on physicochemical properties (hydrophobicity, aromaticity, ability to bind hydrogen, ionizable character), and not in defined chemical properties as is the case of enzyme-substrate or ligand–receptor recognition (Van Bambeke et al. 2003).

As MDR systems are ubiquitous and can pump a large number of antibiotics, they are the objective of a promising research line involving the search for pump inhibitors, which would make MDR bacteria more sensitive to current drugs. As it will be seen later, it has been proved that the inhibition of such systems in different bacterial strains decreases minimal inhibitory concentrations of many drugs (Romanova et al. 2006; Okusu et al. 1996).

Recent studies suggest that antibiotics are only opportunistic substrates of these physiological transporters, with efflux pumps also playing a major role in extrusion of poorly diffusible endogenous molecules and protection of bacteria against exogenous, potentially harmful, diffusible substances (Piddock 2006). In this context, antibiotics have probably provided the necessary pressure that selects for efflux pump overexpression as a nonspecific mechanism of resistance (Van Bambeke et al. 2010).

4 Efflux Systems: Families and Organization

The organization of efflux pumps is based on the homology of their amino acid sequence and secondary structure. Efflux pumps are organized in five super families:

ABC (ATP-binding cassette)
MFS (major facilitator super family)
MATE (multidrug and toxic compound extrusion)
SMR (small multidrug resistance)
RND (resistance nodulation division)

All of them are active transporters, since they move substrates against their electrochemical gradient, thereby requiring an input of energy. Primary active transporters obtain the required energy from a change in the chemical state of one of the reactants. Members of the ABC super family are primary active transporters that use energy from ATP hydrolysis. The rest of super families transport molecules by secondary active transport, also known as co-transport or coupled transport, in which the energy is provided by the electrochemical potential difference created by pumping ions in/out of the cell. In this case, pumps act as symports or antiports,

coupling the drug efflux to the downhill transport of an ion along a concentration gradient.

With the exception of SMR family, which has been so far only described in prokaryotes, the rest of efflux systems have been found in both prokaryotes and eukaryotes. As most transporters have been recently described, it is possible that in coming years many other pumps of families belonging to both types of cells will be discovered.

4.1 ABC Transporters

ATP-binding cassette (ABC) transporters operate in all known phyla and constitute one of the largest protein superfamilies (Jones and George 2004). They are divided into three main functional categories (Davidson et al. 2008):

Importers: mediate the uptake of nutrients into the cell. The transported substrates include ions, amino acids, peptides, sugars, and other molecules mostly hydrophilic. They are only found in prokaryotic cells. Eukaryotes do not posses any importers.
Exporters or effluxers: are present both in eukaryotes and prokaryotes. They extrude toxins and drugs out of the cell.
Members of a third subgroup of ABC proteins do not function as transporters. They are involved in translation and DNA repair processes.

4.1.1 Structure

The common structure of all ABC transporters obtained by X-ray crystallography, include two transmembrane domains (TMDs), also known as membrane-spanning domains (MSDs) or integral membrane (IM) domains and two cytosolic ATP-binding cassettes, known as nucleotide-binding domains (NBDs). These four domains can comprise one, two, or four polypeptide chains, encoded by the same or different genes, which assemble into monomers, homo-, or heterodimers, or tetramers. The TMD undergoes conformational changes to span the lipid bilayer and transport substrates across the membrane. Its structure consists of multiple alpha helices embedded in the membrane bilayer with different sequences and architecture depending on the substrates that can be translocated. The NBD domain is located in the cytoplasm and is the site for ATP binding and hydrolysis. Contrary to the TMD its sequence is highly conserved. Bacterial ABC importers also contain a periplasmic binding protein that captures substrate and delivers it to the transporter. The TMDs also contain cytosolic regions known as intracytoplasmic loops (ICLs), which are thought to coordinate ATP binding and hydrolysis with substrate binding

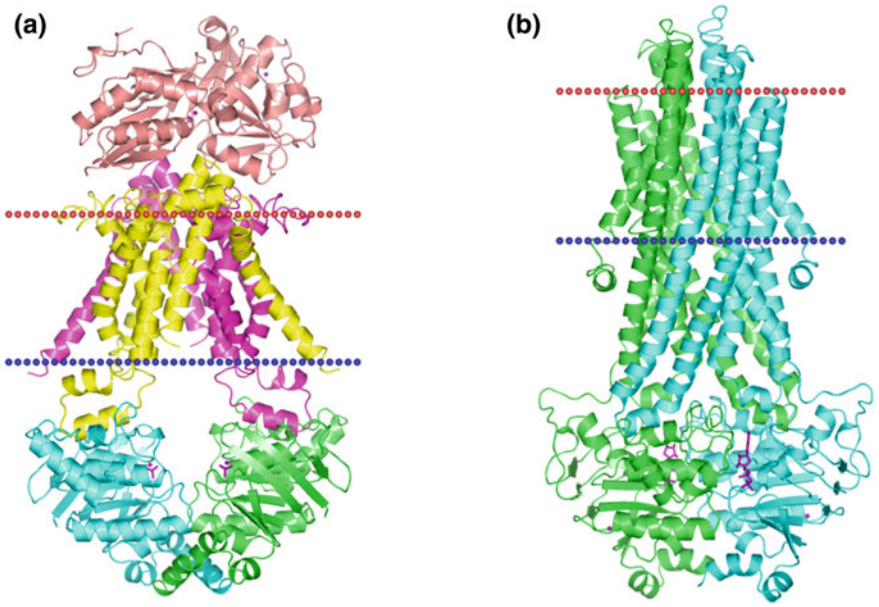

Fig. 1 Crystal structures of **a** Molybdate importer ModBC from *Methanosarcina acetivorans* (2onk.pdb) and **b** *Staphylococcus aureus* multidrug exporter Sav1866 (2HYD.pdb). *Red line* corresponds to the outside and *blue line* corresponds to the inside of the plasma membrane (RCSB Protein Databank)

and translocation (Oldham et al. 2008; Jones et al. 2009). Figure 1 represents the crystal structures of resolved ABC transporters ModBC and Sav1866

4.1.2 Transporter Mechanism

The exact mechanism of ABC transporters has not been fully elucidated yet. It has been proposed that the protein undergoes an ATP-dependant conformational change that causes the substrate to be pumped across the membrane. This hypothesis has been supported by recent X-ray crystallographic studies. These studies show that both import and export proteins oscillate between two conformations: one with the substrate-binding site open to the cytoplasm, and one with the substrate-binding site open to the periplasm. In this model, ATP binding would favor the outward-facing orientation, while ATP hydrolysis would return the transporter back to the inward facing conformation, thus enabling the transport of a substance against its concentration gradient (Davidson and Maloney 2007). Figure 2 shows a schematic representation of the proposed mechanism for ABC efflux pumps.

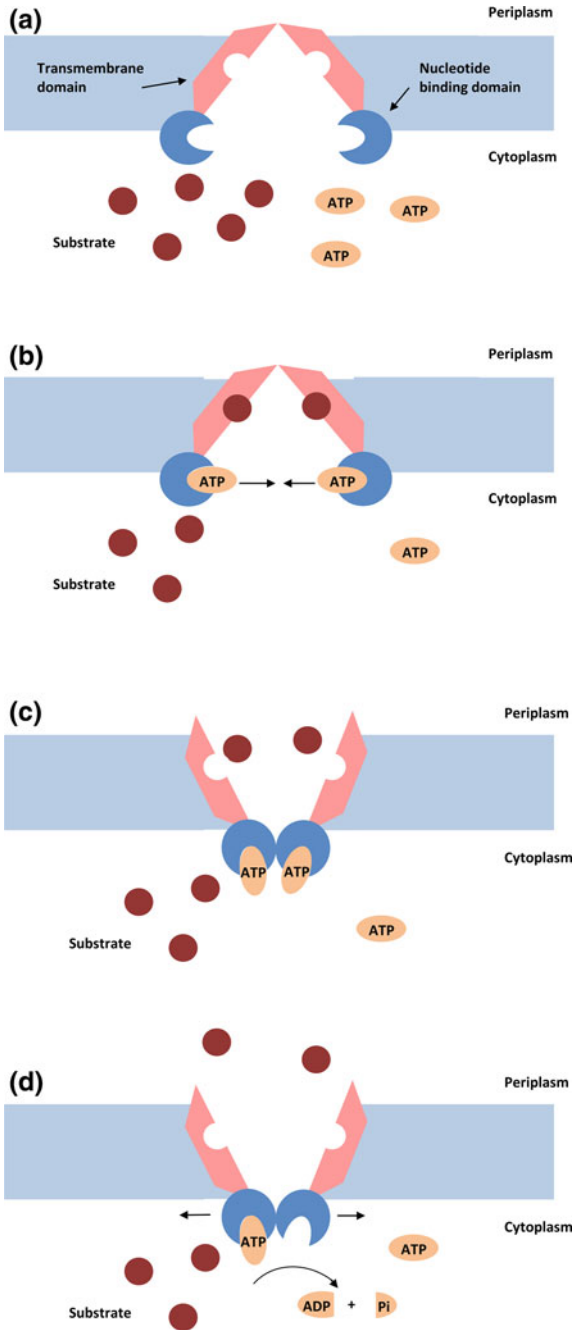

Fig. 2 Schematic illustration of the proposed mechanism for ATP efflux transporters. **a** The two transmembrane domains of the functional protein are attached to nucleotide-binding domains that are widely separated. **b** ATP and the substrate bind to their domains, this making the nucleotide-binding regions containing ATP to undergo a conformational shift which brings them close together. **c** This causes a conformational change in the substrate-binding region, prompting its opening to the outside of the cell, which allows efflux of the substrate. **d** ATP is hydrolyzed to ADP and PPi and the protein returns to its original state. Figure adapted from (Cox 2010)

4.2 Major Facilitator Superfamily (MFS)

The MSF is an ancient and ubiquitous transporter family found in all kingdoms of life. It is the largest group of secondary active transporters with more than 10,000 identified proteins which transport a wide spectrum of substrates including ions, carbohydrates, lipids, amino acids and peptides, nucleosides and other molecules (Pao et al. 1998). MFS proteins include both facilitators and secondary active transporters. Facilitators are uniporters that catalyze facilitative diffusion of a substrate down its concentration gradient, whereas the secondary active transporters harness the electrochemical potential of ions or solutes to achieve the energetically unfavorable transport of a substrate against its gradient. MFS active transporters catalyze solute:cation (H^+ or Na^+) symport and/or solute:H^+ or solute:solute antiport (Marger and Saier 1993). Although half of the sequenced molecules are of unknown or putative function, others are known to be vital in multitude of physiological processes. And some subfamilies of exporters play a major role in multidrug resistance in bacteria and fungi (Hassan et al. 2011; Prasad and Rawal 2014).

4.2.1 Structure and Transport Mechanism

Although proteins from the known families show low sequence similarity, distinct substrate specificities and different transport coupling mechanisms, they all share a common structural fold known as MFS fold. The average MFS fold comprises 12 transmembrane helices (TMs), organized into two discretely folded domains, the N and C domains, each containing six consecutive TMs. Different TMs play different roles in the transport. Some of them are essential for substrate binding and co-transport coupling, some are involved in the interactions between N and C domains of the transporter, and others are important for structural integrity (Yan 2013).

As seen above for the ABC transporters, the transport mechanism of all membrane transporters fits a general alternating access model, in which the substrate-binding site is alternately exposed to the cytoplasm or the periplasm of the cell. During each cycle, the transporter presents different conformational states: outward facing, occluded and inward facing. In order to validate the alternating access model, crystal structures of all conformations may be obtained. But unlike ABC transporters, obtaining multiple conformations of an MFS protein has not been possible yet. Not a single MFS protein has yielded crystal structures of more than one conformation so far (Yan 2013). Figure 3 represents the outward open state of *E. coli* FucP importer and the occluded state of *E. coli* EmrD exporter.

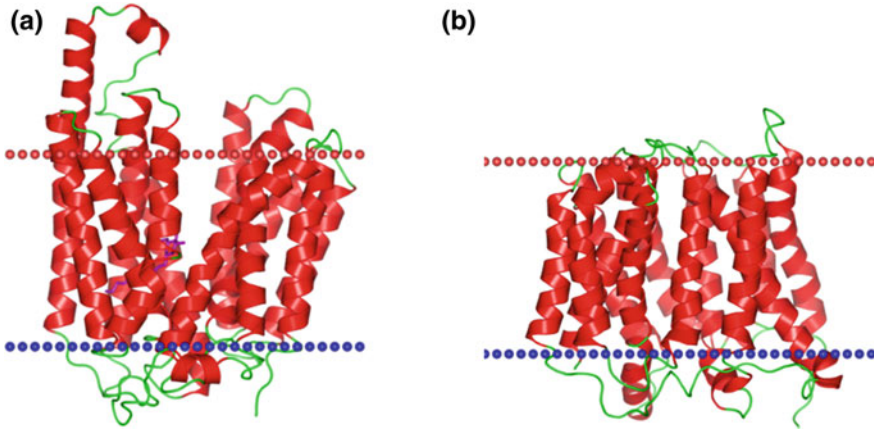

Fig. 3 Crystal structures of **a** outward open state of *E. coli* FucP importer (307Q.pdb) and **b** occluded state of *E. coli* EmrD exporter (2GFP.pdb). *Red line* corresponds to the outside and *blue line* corresponds to the inside of the plasma membrane (RCSB Protein Databank)

4.3 Multidrug and Toxic Compound Extrusion Family (MATE)

Previously thought to belong to the MFS superfamily because of similar membrane topology, the proteins of the MATE family are now recognized as a separate family of transporters since they show no sequence homology to MFS proteins (Kumar and Schweizer 2005; Brown et al. 1999). Members of the MATE family are distributed

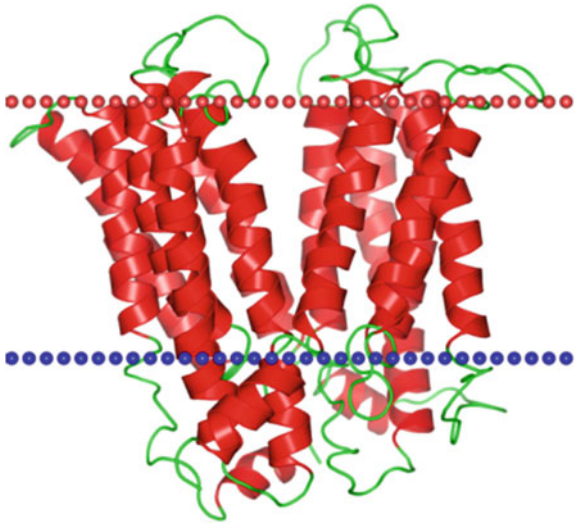

Fig. 4 Crystal structure of *Vibrio cholerae* multidrug efflux transporter NorM (3mkt pdb). *Red line* corresponds to the outside and *blue line* corresponds to the inside of the plasma membrane (RCSB Protein Databank)

in all living organisms and their predicted structure is similar to the above described for MFS proteins. Substrates for the MATE transporters identified up to now are various and have unrelated chemical structures (Kuroda and Tsuchiya 2009).

Figure 4 shows the multidrug efflux transporter NorM from *Vibrio cholerae*, which mediates resistance to multiple antimicrobial agents, such as norfloxacin, ethidium bromide, and some aminoglycosides. X-ray structure of NorM has been reported (He et al. 2010) revealing an outward-facing conformation with two portals open to the outer leaflet of the membrane and a unique topology of the predicted 12 transmembrane helices distinct from any other known multidrug resistance transporters. NorM seems to function as drug/Na^+ antiporter which is the first example of Na^+-coupled multidrug efflux transporter discovered (Morita et al. 2000).

4.4 Small Multidrug Resistance Family (SMR)

The small multidrug resistance (SMR) family is a prokaryotic multidrug transporter family. As the name suggests, SMR proteins are composed of only four transmembrane short alpha-helices that confer resistance to a broad range of antiseptics and detergents, a variety of antibiotics and lipophilic quaternary ammonium compounds (QAC). Similar to MFS superfamily proteins, SMR proteins also demonstrate drug efflux via an electrochemical proton gradient and have been classified as proton-dependent multidrug efflux systems (Bay et al. 2007).

Based on SMR sequence alignments and phylogenetic analysis it has recently been proposed that this family could be expanded into three subclasses: the small multidrug pumps (SMP), suppressor of *groEL* mutation proteins (SUG), and the paired small multidrug resistance proteins (PSMR) (Bay and Turner 2009).

Although no accurate crystal structure of SMR has been obtained so far, it has been reported that all members of the family consist of four predicted TM alpha-helices, based on hydropathy plot analyses and secondary structure predictions. But the arrangement of TM strands within the protein complex has not yet been resolved.

The transport mechanism of SMR proteins also remains unresolved, although numerous models have been proposed according to the general alternating model. Since SMP subclass proteins in particular confer host cell resistance to a broad range of substrates, they are believed to possess a generic substrate-binding site (Bay et al. 2007).

4.5 Resistance Nodulation Division Superfamily (RND)

Characterized members of the RND superfamily all probably catalyze substrate efflux via an H^+ antiport mechanism. These proteins are found ubiquitously in bacteria, archaea, and eukaryotes (Transporter classification database). Efflux pumps of the

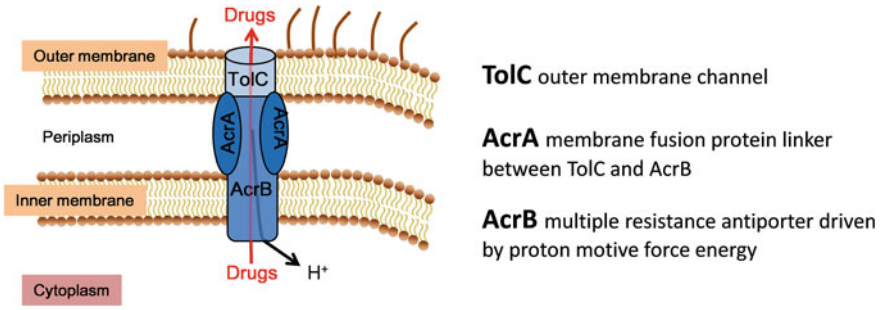

Fig. 5 Schematic representation of *E. coli* tripartite multidrug efflux system AcrAB-TolC

RND family are major contributors to intrinsic resistance in Gram-negative bacteria, where they extrude a broad spectrum of antibiotics and biocides, including fluoroquinolones, beta-lactams, tetracyclines, oxazolidinones, and beta-lactamase inhibitors from the periplasm to the outside of the cell (Opperman and Nguyen 2015; Padilla et al. 2010). In Gram-negative bacteria those systems are typically linked to accessory protein components of the outer membrane called TolC (Koronakis et al. 2004), which allow the extrusion of toxic substances from the cell directly into the medium bypassing periplasm and the outer membrane. TolC proteins are a type of proteins called porines which are also found in ABC, MATE, and MFS systems.

In *E. coli*, the tripartite efflux system AcrA/AcrB/TolC (Fig. 5) extrudes multiple antibiotics, dyes, detergents and bile salts (Pos 2009). AcrB, a member of the RND family, resides in the inner membrane and is responsible for substrate recognition and energy transduction. Drugs are extruded in a coupled exchange with protons through the TolC component, which forms a pore in the outer membrane extended by a long periplasm conduit. AcrA component mediates contact between AcrB and TolC. The presence of all three components is required for the MRD phenotype.

5 Impact of Efflux in Resistance

Intrinsic resistance of many bacteria to antimicrobials depends on inducible or constitutive expression of different efflux systems, which extrude toxic compounds. Some experiments have demonstrated that efflux pumps by themselves confer low resistance levels to the bacterial cells, reason why their relevance has been questioned by some authors. However, such bacteria are better equipped to survive antibiotic pressure and develop further mutations in genes encoding the target sites of antibiotics (Webber and Piddock 2003). In general, efflux systems work in cooperation with other mechanisms to confer higher resistance levels and broader spectrum of activity (Marchetti et al. 2011).

Gram-negative bacteria tend to be more resistant to lipophilic and amphiphilic chemotherapeutic agents than Gram-positive bacteria. This intrinsic resistance has

often been attributed entirely to the presence of the outer membrane. This barrier decreases the rate of transmembrane diffusion of lipophilic solutes due to the low fluidity of the lipopolysaccharide leaflet and the narrow size of its porin channels. However, some studies suggest that the outer membrane cannot be the whole explanation. It has been described that *E. coli* AcrAB pumps confer intrinsic resistance only to long chain lipophilic compounds such as erythromycin, fusidic acid or detergents, but small antibiotic such as tetracyclines, chloramphenicol, or fluoroquinolones, go across porin channels very fast. Multiresistance to all the above-mentioned compounds can only be achieved by the synergic action of both outer membrane and efflux systems (Nikaido 1996).

A typical example of cooperation is the efflux of penicillin and the action of beta-lactamases. The efflux pumps prevent the enzymatic system from saturation conferring high levels of resistance to low beta-lactamases producing bacteria. Some bacteria even present the combined action of three different mechanisms: mutation studies performed with *P. aeruginosa* show that its intrinsic penem resistance is generated from the interplay among the outer membrane barrier, the active efflux system and a beta-lactamase (Okamoto et al. 2001).

A clinical implication of the broad range of structurally dissimilar substrates which can be transported by some efflux pumps is the appearing of cross-resistance. Exposure to an agent that belongs to the substrate profile of a pump would favor overexpression of that pump and consequent cross-resistance to all its possible substrates. These may include clinically structurally unrelated relevant antibiotics such as macrolides and fluoroquinolones (Webber and Piddock 2003).

The bacterial resistance due to efflux pumps can be easily disseminated among phylogenetic unrelated strains, as the genetic elements codifying pumps expression can be found in plasmids and in conjugative or transformable transposons located either in plasmids or in the chromosome (Van Bambeke et al. 2003).

Nevertheless, it is important to highlight that the unlimited overexpression of efflux systems in the membrane, has got potential disadvantages for the bacteria. Besides the extrusion of toxic compounds, overexpression of pumps could promote the exit of essential growth nutrients out of the cell (Marchetti et al. 2011).

The clinical impact of multiresistance due to efflux pumps is not easy to establish. There are many publications describing efflux systems in different microorganisms but very few about its clinical importance.

6 Strategies to Overcome Resistance by Efflux

There is increasing evidence of the role of efflux pumps in bacterial resistance to antimicrobials. Although they do not confer high levels of resistance by themselves, their overexpression combined with other resistance mechanisms is of clinical importance. For this reason, some strategies to overcome resistance by efflux are being studied (Bohnert and Kern 2005; Chevalier et al. 2004; Hannula and Hänninen 2008; Hendricks et al. 2003; Sáenz et al. 2004; Schumacher et al. 2006):

1. **Modification of the existing antibiotics**: As differences in transport can be observed between related molecules belonging to an antibiotic class, optimizing the structure of such compounds by taking into account susceptibility to resistance mechanisms is an important design element. The newer molecules developed from existing antibiotics exhibit in general less susceptibility to be extruded by efflux. Examples of this are third and fourth generation quinolones versus first and second generation quinolones, ketolides versus macrolides or glycylcyclines versus tetracyclines (Van Bambeke et al. 2003; Li and Nikaido 2004).
2. **Biological inhibition of active efflux**: This approach is used to study the role of efflux pumps on antibiotics exposure in vitro, but not applicable for therapeutics. It consists of blocking either the proteins using neutralizing antibodies, or interfering with the transcription or translation of the corresponding genes using antisense oligonucleotides of small interfering RNA or other antisense molecules. The inhibition of the AcrAB efflux pump in *E. coli* using this strategy has led to a patent (Oethinger and Levy 2004) whose application can be broadened to every pump of known sequence or regulatory mechanism. The research leading to the aforementioned patent demonstrated that deletion of the *acrAB* gene in *E. coli* restores its sensitivity to a series of antibiotics (Oethinger et al. 2000) while a mutation in its regulator has the opposite effects (Kern et al. 2000).
   Pharmacological inhibition of active efflux: the use of efflux pumps inhibitors (EPIs) is a widely studied strategy with proved clinical utility when combined with specific antibiotics. Although little is known about the exact mode of action of most EPIs, the induction of a flux-competition may probably be the most general mechanism. The efflux pump inhibitor-antibiotic combination is expected to increase the intracellular concentration of antibiotics that are expelled by efflux pumps, decrease the intrinsic bacterial resistance to antibiotics, reverse the acquired resistance associated with efflux pumps overexpression, and reduce the frequency of the emergence of resistant mutant strains. In recent years, different classes of EPIs have been described and tested, including analogues of antibiotics and new molecules (Zechini and Versace 2009).

One of the first discovered pump inhibitors and the most popular one is reserpine (Fig. 6), an indole alkaloid that has been used for the control of high blood pressure and for the relief of psychotic symptoms. Its activity, fortuitously discovered, has been proved in combination with fluoroquinolones to fight *Streptococcus pneumoniae* and *Staphylococcus aureus* (Gibbons and Udo 2000). But other drugs such as phenothiazines (Molnar et al. 1997), calcium channels antagonists (Choudhuri et al. 1999), selective inhibitors of serotonin re-uptake (Kaatz et al. 2003), or proton pump inhibitors (Aeschlimann et al. 1999), have been described to have similar effects as the observed for reserpine. A limitation of the use of pharmacological inhibitors in combination with antibiotics is that usually the concentrations of the inhibitor needed to attain the desired effect are much higher than the ones needed to exert its typical pharmacological effects, this making its use dangerous.

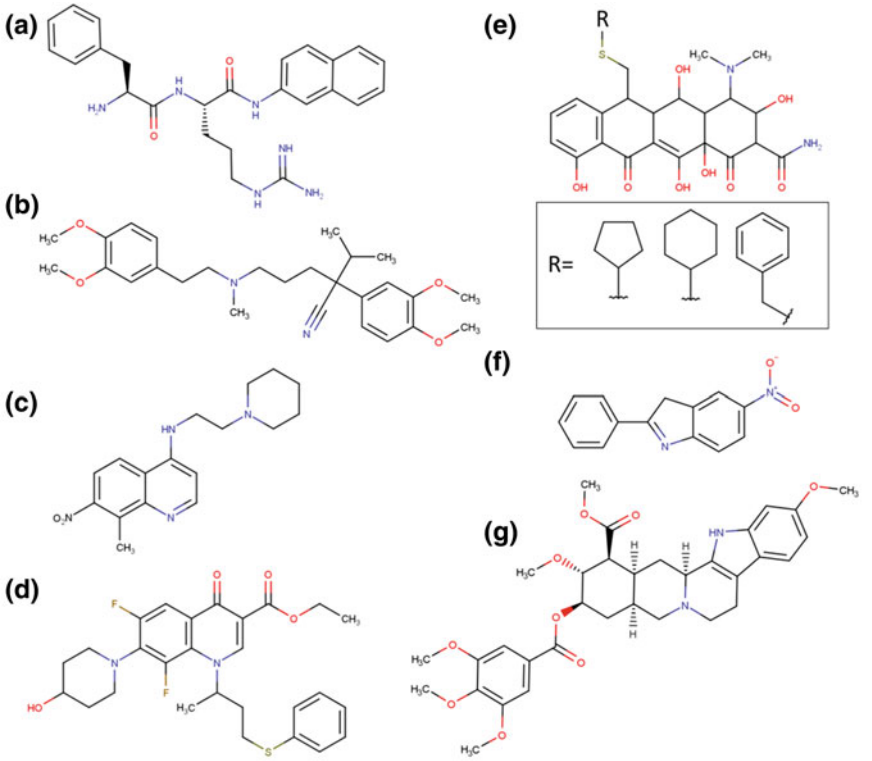

Fig. 6 Chemical structures of some efflux pump inhibitors: **a** Peptidomimetic inhibitor MC 207,110 active when combined with antibiotics of all classes and with proved activity against a wide spectrum of bacteria including *P. aeruginosa*, *B. pseudomallei*, *A. baumannii*, *S. maltophilia*, *Y. enterocolitica*, *S. enterica*, *E. aerogenes,* and *E. coli.* **b** Calcium channel blocker Verapamil. **c** Alkylaminoquinoline derivative 814 with demonstrated activity against *E. aerogenes* when combined with norfloxacin, chloramphenicol, and tetracycline. **d** Fluoroquinolone analogue with proved activity against *S. aureus* when combined with fluoroquinolones. **e** Tetracycline analogue showing activity against resistant strains of *S. aureus*, *E. faecalis,* and *E. coli* when combined with tetracyclines. **f** Indole derivative IFN55 active against *S. aureus* and *S. pneumoniae* in combination with ciprofloxacin and ethidium bromide. **g** Indole alkaloid reserpine, active against *S. pneumoniae* and *S. aureus* in combination with fluoroquinolones

Pump inhibitors can be divided into two groups according to their structure:

1. Antibiotics with low antibacterial effect which are analogues to antibiotics of clinical use. Three families of analogues have been patented to date: analogues of tetracyclines (Levy 1998), aminoglycosides (Nelson and Alekhsun 2004) and quinolones (De Souza et al. 2002) which lower the efflux of the corresponding clinically useful antibiotics.
2. New molecules, not related to any known antibiotic. Some of them have been reported to inhibit multidrug efflux pumps. Patented molecules include indoles, ureas, and aromatic amides (Markham et al. 2000); arylbenzo and diarylthiophenes

(Lemaire et al. 2006); piperidine-carboxylic acid derivatives (Grossman 2005); diverse quinolines (Pages et al. 2003); peptidomimetics (Chamberland et al. 1999, 2000, 2001) and substituted disiloxanes (Varga et al. 2001).

Pump inhibitors belonging to both classes are shown in Fig. 6. Although there are some exceptions, in general, pump inhibitors show a chemical structure based on aromatic rings and ionizable moieties. This is consistent with the fact that efflux pumps preferentially transport amphiphilic substrates (Van Bambeke et al. 2000) and possess affinity binding pockets with amino acid side chains prone to establish hydrophobic and aromatic bonds and van der Waals interactions (Yu et al. 2003).

Some pump inhibitors also modulate eukaryotic multidrug transporters. This can affect the pharmacokinetics of antibiotics in the host, resulting in adverse effects. Such is the case of the calcium channel blocker verapamil (Fig. 6), which inhibits drug binding to P-glycoprotein (Cornwell et al. 1987). In some cases, this property could be advantageous to study treatments for intracellular infections. But in general, the use of inhibitors that do not interact with eukaryotic transporters is clinically preferable because it minimizes adverse effects due to the inhibition of physiological functions of eukaryotic efflux pumps (Van Bambeke et al. 2010).

7 Current Outlook on Multidrug Resistance

Although there is much literature on the characterization of efflux systems, very few has been reported about their clinical importance. The lack of statistical studies on the subject makes difficult to compare this resistance mechanism with others and to establish its real impact (Marchetti et al. 2011).

Some studies on multiresistance have been performed in different countries with indigenous pathogenic strains, most of them evaluating the role of efflux pumps belonging to the RND family. Research is mostly focused on using pump inhibitors, blocking the overexpression of pumps and studying the prevalence of multiresistant strains in human and animal isolates. The most studied pathogenic microorganisms include species of *Campylobacter* (Randall et al. 2003; Lin and Martínez 2006; Hannula and Hänninen 2008), *Pseudomonas* (Lomovskaya et al. 1999; Hendricks et al. 2003), *Enterobacter* (Chevalier et al. 2004), *Klebsiella* (Hasdemir et al. 2004) and *Salmonella* (Thorrold et al. 2007).

But multiresistance is not only a matter of pathogenic microorganisms. Resistance genes can be acquired or selected either in pathogenic or commensal bacteria. Many multiresistant commensal strains have been isolated from animals and humans exposed to environments where the use of antibiotics is very extensive, such as farms or hospitals (Marchetti and Mestorino 2013). The emergence of resistance in microorganisms belonging to the normal microbiota is an imminent risk for the efficiency of antimicrobials since, as mentioned above, the bacterial resistance due to efflux pumps can be disseminated among phylogenetic unrelated microorganisms, including pathogenic strains (Van Bambeke et al. 2003).

E. coli is one of the commensal microorganisms with higher potential to generate resistances in the human and veterinary field. Hence its study is of great importance to determine which antimicrobials are likely to fail, to lower treatment failure and to avoid risks in public health and economic losses to farmers. Several studies evaluate the effect of pump inhibitors on *E. coli* strains from human isolates (Schumacher et al. 2006), farms (Marchetti and Mestorino 2013), and in experimental mutants (Elkins and Mullis 2007).

In such a scenario of increasing bacterial resistance to antibiotics, two strategies of search for alternatives to currently existing drugs can be met:

1. The discovery of new antibiotics directed to bacterial targets not affected by existing resistance mechanisms. But the approval of such molecules for therapeutic uses is a very laborious procedure and development of resistances is inevitable.
2. The development of inhibitors of resistance mechanisms. This strategy allows extending the utility of existing antibiotics with well-known pharmacological and toxicological properties.

Therefore, research on efflux pump inhibitors is a promising strategy toward new antibacterial therapies.

The examples presented in this chapter have shown excellent results in in vitro experiments, but nevertheless there is a need for accurate preclinical and clinical studies to asses that efflux pump inhibitors could be a safe and useful alternative to overcome bacterial resistances in vivo.

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Bacterial Proteases as Targets to Control Bacterial Growth

Ricardo V. Ullán and Carlos Barreiro

Abstract Proteases (PRs) catalyze the cleavage of peptide bonds by hydrolysis in proteins and peptides playing crucial functions in organisms all over the phylogenetic tree. These enzymes are present in all types of bacteria and are involved in critical processes such as acquisition of nutrients for growth and proliferation, facilitation of dissemination, colonization and evasion of host immune defenses or tissue damage during infection. Bacterial pathogens use their PRs to acquire or activate the function of host PRs to help them in their growth or progression of disease. Research into bacterial PRs and their substrates will allow the development of novel PRs inhibiting compounds that could potentially be used to limit host virulence or to block cell growth. The emergence resistances to traditional antibiotics have created clinical difficulties for nosocomial treatment on a global scale. Thus the pharmacological development of new PRs inhibitors that target essential proteins in the bacterial pathogen is of great interest, and it is the focus of this review.

1 Introduction

Proteolytic enzymes also known as proteases (PRs), peptidases, or proteinases are one of the largest functional groups of proteins, with more than 4000 members actually described (Rawlings et al. 2012). PRs catalyze the cleavage of peptide bonds by their hydrolysis in proteins and peptides playing crucial functions in organisms all over the phylogenetic tree, starting from viruses, bacteria, protozoa,

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metazoa, fungi, and ending with plants and animals. PRs enzymes are extensively applied in several sectors of industry and biotechnology (Rao et al. 1998; Kirk et al. 2002; Rani et al. 2012; Ray 2012). Therefore, several research applications require the use of PRs, including peptide synthesis, digestion of unwanted proteins during nucleic acid purification and the production of Klenow fragments, use of proteases in cell culture experiments and in tissue dissociation, preparation of recombinant antibody fragments for research, peptide sequencing, proteolytic digestion of proteins in proteomics diagnostics and therapy, exploration of the structure–function relationships by structural studies, and removal of affinity tags from fusion proteins in recombinant protein techniques.

PRs are present in all types of bacteria and are involved in critical processes such as acquisition of nutrients for growth and proliferation, facilitation of dissemination, colonization and evasion of host immune defenses or tissue damage during infection (Miyoshi and Shinoda 1997; Travis and Potempa 2000). Bacteria produce a variety of PRs and their roles in virulence factors of the various bacteria genera are well known. Bacterial PRs have the ability to destroy important host defense proteins, such as those present in the complement system, which is the main part of a host's innate immunity (Popadiak et al. 2007), as well as degrade the functional and structural proteins present in the host organism (Maeda 1996). Besides, it should be noted the ability of bacteria to use the PRs it produces to pass the proteinaceous barriers present inside a host organism, which is a major part of bacterial virulence. Bacterial pathogens use its PRs to acquire or activate the function of host PRs to help in its growth or progression of disease (Lantz 1997).

Bacterial pathogens rely on proteolysis for variety of purposes during the infection process and they are involved in the interruption of the cascade activation pathways, disruption of cytokine network, excision of cell surface receptors, and inactivation of host protease inhibitors (Maeda 1996; Miyoshi and Shinoda 1997; Rice et al. 1999; Travis and Potempa 2000). Thus, the proteolysis phenomena have been adopted by bacterial pathogens at multiple levels to ensure their success in contact with the host. It goes without saying that bacterial PRs may represent very attractive targets for the development of novel types of antibiotics (Travis and Potempa 2000).

Bacterial PRs inhibition would presumably lead to the death of the invading pathogen. Moreover, consider the specific role that bacterial PRs play in such critical steps for the successful invasion of the host. Taking into account the constant emergence of antibiotic resistance (Rolain et al. 2012; Bond 2015), it would be extremely beneficial to develop bacterial PRs inhibitors as a second antibiotic generation. Research into bacterial PRs and their substrates will allow the development of novel PRs inhibiting compounds that could potentially be used to limit the action of destructive PRs produced during the bacterial infection process

(Zindel et al. 2013; Drag and Salvesen 2010). Though targeting PRs for creating PRs inhibitors has proven to be a useful idea for therapeutic intervention against pathogenic bacteria, utilizing these PRs or taking advantage of their functions to help in drug delivery techniques has not been widely tried.

In a general way, the inhibitor binds to the enzyme and decreases its activity; the classification of inhibitors is based on where and how such binding occurs, as well as, the type of binding (Fersht 1985; Otto and Schirmeister 1997). PRs inhibitors as enzymatic inhibitors bind either into the active site as active site-directed inhibitors or at another site as allosteric inhibitors. An active site-directed inhibitor can either bind covalently or noncovalently. Covalent inhibitors are electrophiles that are attacked by the active site to form a covalent bond which prevents further enzymatic reactions. Noncovalent inhibitors must depend on other interactions in order to remain in the binding site. Electrostatic interactions such as hydrogen bonds, salt bridges, and pi–pi interactions between aryl groups are the most important for protein recognition and binding with high specificity. Noncovalently bound inhibitors are reversible and the enzyme can be reactivated. Covalently bound inhibitors are either irreversible or reversible. Finally, the irreversible inhibitor reacts with the active site and blocks further modifications (Fersht 1985; Gohlke and Klebe 2002).

According to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology, PRs belong to the hydrolase class of enzymes (EC 3) and are grouped into the subclass of the peptide hydrolases or peptidases (EC 3.4) (Table 1). Depending on the reaction they catalyze, proteases are divided into exopeptidases which specifically cleavage protein substrates from the carboxyl- or amino-termini and endopeptidases which preferentially hydrolyse peptide bonds in the inner regions of peptide chains. Aminopeptidases (Table 1) can liberate single amino acids (EC 3.4.11), dipeptides (dipeptidyl peptidases, EC 3.4.14), or tripeptides (tripeptidyl peptidases EC 3.4.14) from the N-terminal end of their substrates. Single amino acids can be released from dipeptide substrates by dipeptidases (EC 3.4.13) or from polypeptides by carboxypeptidases (EC 3.4.16, EC 2.4.17, EC 3.4.18) (Table 1), while peptidyl dipeptidases (EC 3.4.15) liberate dipeptides from the C-terminal end of a polypeptide chain. Finally, as mentioned above, endopeptidases (Table 1) cleave peptide bonds within and distant from the ends of a polypeptide chain [serine endopeptidases (EC 3.4.21), cysteineendopeptidases (EC 3.4.22), asparticendopeptidases (EC 3.4.23), metalloendopeptidases (EC 3.4.24), threonineendopeptidases (EC 3.4.25) and endopeptidases of unknown catalytic mechanism (EC 3.4.99)]. Seventh catalytic types of PRs have been recognized according to the nature of the catalytic residues [MEROPS—the peptidase database (http://merops.sanger.ac.uk/) (Rawlings et al. 2010)]: serine—(first described in 1993), cysteine—(1993), aspartic—(1993), metallo—(1993), threonine—(1997), glutamic acid—(2004), and asparagine-protease—(2010) (Kohei 2012).

Table 1 General classification of proteases according to the recommendations of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology on the nomenclature and classification of enzymes by the reactions they catalyze (http://www.chem.qmul.ac.uk/iubmb/enzyme/)

Protease	Enzyme commission (EC) code	Mechanism
Aminopeptidases	3.4.11	Release N-terminal amino acid residues from polypeptides and protein
Dipeptidases	3.4.13	Exopeptidases specific for dipeptides
Dipeptidyl peptidases and	3.4.14	Release of an N-terminal dipeptide from a polypeptide
Tripeptidyl peptidases	3.4.14	Release of an N-terminal tripeptide from a polypeptide
Peptidyl dipeptidases	3.4.15	Release of free C-terminus liberate a dipeptide
Serine-type carboxypeptidases	3.4.16	Release of a single residue C-terminal from a polypeptide and have an active center serine involved in the catalytic process
Metallocarboxypeptidases	3.4.17	Release of a single residue C-terminal from a polypeptide using a metal ion in the catalytic mechanism
Cysteine-type carboxypeptidases	3.4.18	Release of a single residue C-terminal from a polypeptide and have a cysteine in the active center
Omega peptidases	3.4.19	Remove terminal residues that are linked by isopeptide bonds
Serine endopeptidases	3.4.21	Cleave internal bonds in polypeptide chains. Have an active center serine involved in the catalytic process
Cysteine endopeptidases	3.4.22	Cleave internal bonds in polypeptide chains. Have a cysteine in the active center
Aspartic endopeptidases	3.4.23	Cleave internal bonds in polypeptide chains having an aspartic acid residue for their catalytic activity
Metalloendopeptidases	3.4.24	A metal ion (often, but not always, Zn^{2+}) is involved in the catalytic mechanism for cleaving internal bonds in polypeptide chains
Threonine endopeptidases	3.4.25	Cleave internal bonds in polypeptide chains having a threonine residue for their catalytic activity
Endopeptidases of unknown catalytic mechanism	3.4.99	Acting on peptide bonds (peptide hydrolases)

2 Bacterial PRs Functions and Their Inhibitors

2.1 Bacterial PRs for Growth and Proliferation

It is well known that during the process of bacterial cell division and growth, they utilize several different PRs for the assembly and disassembly of the bacterial cell wall (Humann and Lenz 2009). Peptidoglycan (PGN) synthesis and its hydrolysis are processes required by almost all bacteria for growth; nevertheless, the involved pathways for cell division and growth are function of the shape and Gram type of the bacterium (Humann and Lenz 2009; Margolin 2009).

The structure of PGN in both Gram-positive and Gram-negative bacteria comprises repeating disaccharide backbones of *N*-acetylglucosamine (NAG) and β-(1-4)-*N*-acetylmuramic acid (NAM) that are cross-linked by peptide stem chains attached to the NAM residues (Fig. 1) (Bourhis and Werts 2007). PGN is concealed by an outer membrane in Gram-negative bacteria, or by layers of proteins and glycopolymers in Gram-positive bacteria (Fig. 2). During turnover of the bacterial cell wall for bacterial growth and division, bacteria employ several classes of PGN-hydrolyzing enzymes that participate in the assembly and disassembly of this cell wall. Bacterial PGN-hydrolyzing enzymes degrade the PGN of the producing organism or degrade intact bacterial cells.

These bacterial PRs are defined by their catalytic specificities and are classified as autolysins. Two classes of enzymes are involved in the PGN glycan backbone digestion: *N*-acetylmuramidases and *N*-acetylglucosaminidases. While the

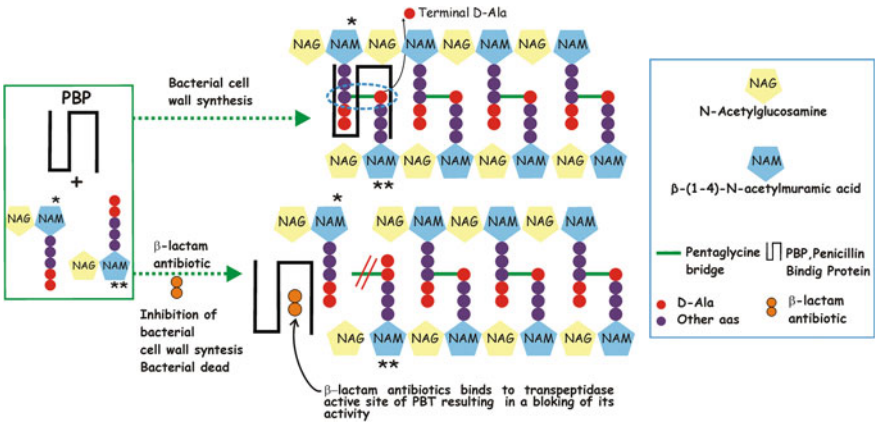

Fig. 1 PGN biosynthesis and mode of action of β-lactam antibiotics. As illustrated, in absence of β–lactam antibiotics, PBPs work in PGN biosynthesis creating a rigid cell wall. Nevertheless, in the presence of β-lactam antibiotics, they bind to the transpeptidase site of PBP inhibiting cell wall synthesis, resulting in a weakened cell wall which ends with bacterial death

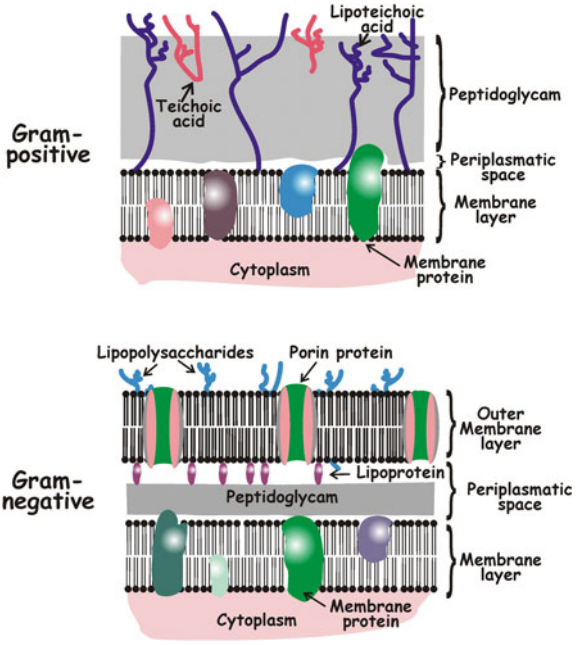

Fig. 2 Scheme of bacterial cell wall composition in Gram-positive and Gram-negative bacteria (Based on: Santos-Beneit et al. 2014)

N-acetylmuramidases cleave PGN between the *N*-acetylglucosamine (NAG) and β-(1-4)-*N*-acetylmuramic acid (NAM) bond upstream of NAM; N-acetylglucosaminidases cleave the NAM–NAG bond.

Another group of autolysins, *N*-acetylmuramyl-L-alanine amidases, separates the PGN sugar backbones from the stem peptide chain concretely these enzymes cleave between NAM and the first alanine of the peptide chain (Fournier and Philpott 2005; Scheurwater et al. 2007). Finally, the lytic transglycosylases cleave between the *N*-acetylmuramic acid and *N*-acetylglucosamine sugar chains to facilitate cell growth among other functions (Zahrl et al. 2005; Scheurwater et al. 2008; Cloud-Hansen et al. 2008).

Bacilluis subtilis is the most studied microorganism in autolysin-related research, since it is a nonpathogenic model system for investigating the roles of autolysins in the cell wall metabolism (Smith et al. 2000). This knowledge, which was obtained through the study of PGN fine structure, the analysis of multiply inactivated mutants by sequencing of the entire *B. subtilis* genome, has become possible to define the roles played by individual autolysins in a number of important cellular processes (Rogers et al. 1984; Kunst et al. 1997; Smith et al. 1996). Cell separation in *B. subtilis* relies on the activity of the autolysins LytF, LytE, and CwlS.

All autolysins are cysteine proteases inside of the NlpC/p60 endopeptidase family (Smith et al. 2000; Yamamoto et al. 2008). LytN is another autolysin produced by *Staphylococcus aureus* that works as amidase and endopeptidase during the synthesis of PGN (Sugai et al. 1998). These bacterial PRs contribute to the

release of the staphylococcal protein A (SpA) by removing amino sugars [i.e., NAM–NAG] from attached PGN (Becker et al. 2014). In a general way, autolysins and in particular *B. subtilis* and *S. aureus* are essential for the synthesis of PGN because its deletion in these microorganisms results in cell growth defects and altered growth morphology (Hashimoto et al. 2012; Frankel and Schneewind 2012).

Penicillin-binding proteins (PBPs) are a distinct group of autolysins that play an important role in cell morphology and viability. PBPs process D-amino acid bonds during PGN synthesis (Wise and Park 1965) by the degradation of the D-alanine–D-alanine bonds (Fig. 1). In addition, PPBs catalyze the terminal stages of PGN synthesis by creating crossbridges between the stem peptides (Frère and Page 2014). Taking into account that not all of the PBPs produced by bacteria are essential, as it was checked in an *Escherichia coli* mutant (Denome et al. 1999), they are validated targets for the β-lactam antibiotics in the antibacterial therapy.

2.2 *β-Lactam Antibiotics as Tools for Bacterial Growth Inhibition*

β-lactam antibiotics (cephalosporins, cephamycins, cephabacims, olivanic acids, thienamycins, epithienamycins, monolactams (nocardicins and monobactams), and clavams) (Fig. 3) are the most frequently prescribed antibiotics worldwide used to treat bacterial infections (Fernández-Aguado et al. 2014).

The general antimicrobial mechanism of action of β-lactam antibiotics consists of the inhibition of PGN biosynthesis, which weakens the bacterial cell wall during the cellular division, leading to cytolysis and death. These antibiotics covalently bind to the active site of PBPs (Fig. 1, detail), which catalyze the linking of PGN molecules in bacteria in the last step of the bacterial cell wall biosynthesis. It is due to the structural similarity between β-lactam antibiotics and the last two amino acids (acyl-D-alanine-D-alanine) of the pentapeptide that links the PGN molecule.

PBP enzymes, including transglycosylases (PBP1 complex), transpeptidases (PBP3), and carboxypeptidases (PBP4, PBP5, and PBP6) are irreversibly inhibited by β-lactam antibiotics, no longer catalyzing the linking reaction. These covalent complexes block the normal transpeptidation reaction inhibiting cell wall biosynthesis. In addition, these antibiotics lead to cell stress responses by the activation of bacterial cell wall hydrolases and autolysins, which result in bacterial cell lysis (Tomasz 1979).

The activity of β-lactam antibiotics is initially higher against Gram-positive bacteria (Fig. 2). In these microorganisms, PBPs are located on the cytoplasmic membrane exposed to the environment, unlike Gram-negative bacteria, where PBPs are present in the periplasmic space protected by the external outer membrane, which acts as a barrier for different molecules (Fig. 2). However, the incorporation of new molecules to the penam and cephem nuclei has given rise to the synthesis of semisynthetic antibiotics with a higher activity against Gram-negative microorganisms. Currently, many used β-lactams have very broad spectrum activity against most

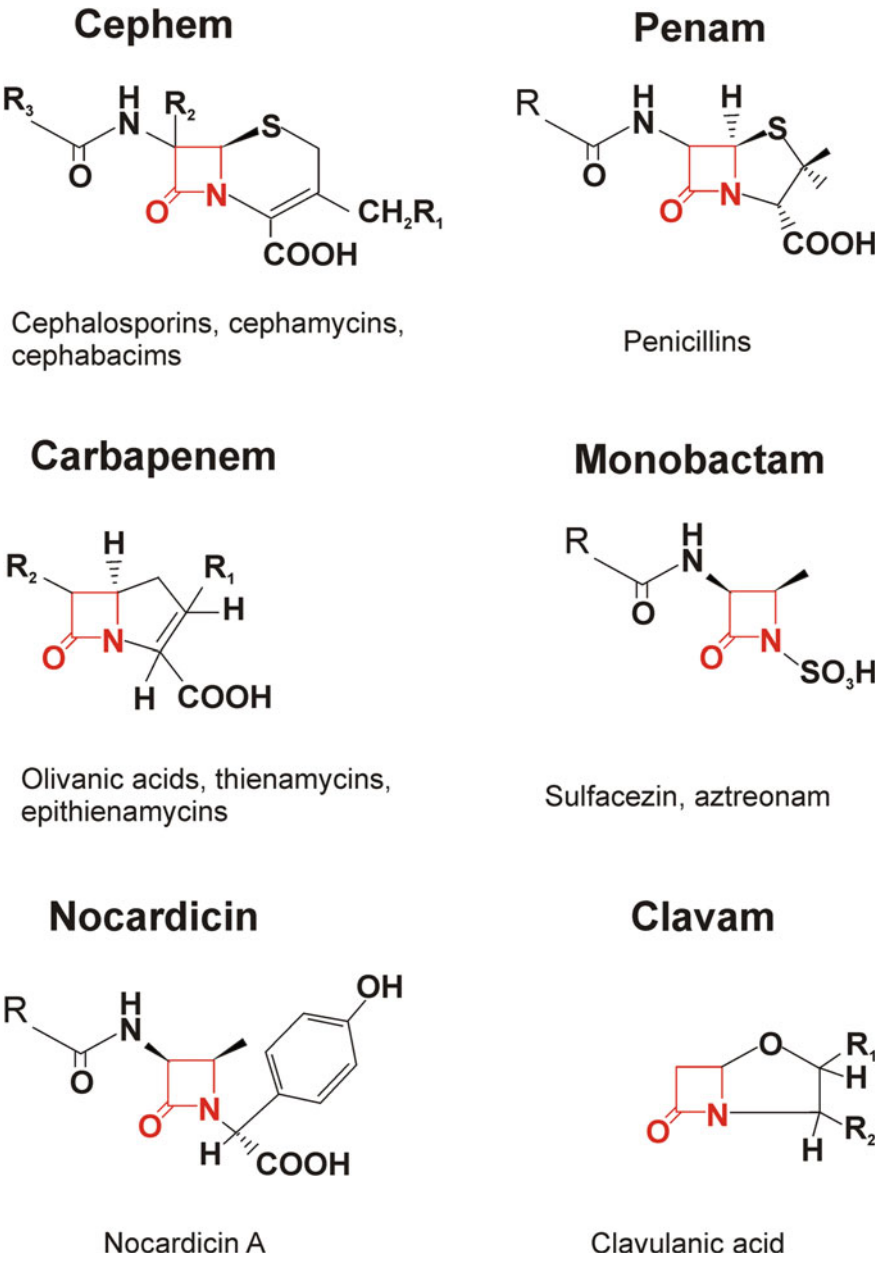

Fig. 3 Chemical structure, classification, and family generic names of β-lactam antibiotics. β-lactam ring is indicated in *red*

aerobic and anaerobic Gram-positive and Gram-negative bacteria, as well as, low-toxicity profiles making them popular first line antibiotics. Nevertheless, bacteria have acquired resistance to β-lactams mainly through three different strategies: active expulsion of β-lactams via efflux pumps, production of a specific β-lactamases, and presence of low-affinity PBPs (Poole 2004; Worthington and Melander 2013).

The first β-lactam antibiotic to be discovered was penicillin in 1932 (Clutterbuck et al. 1932) though it was not introduced to the clinic until the early 1940s. This antibiotic discovery started by an accidental experiment performed by Sir Alexander Fleming in his lab in 1928. Fleming evaluated the antimicrobial activity generated by a fungus culture, later identified as *Penicillium notatum* that contaminated a Petri dish with *Staphylococcus* sp. (Fleming 1929).

Penicillin (Fig. 3) and more specifically benzylpenicillin (penicillin G) is obtained by submerged fermentation of industrial strains of *the* filamentous fungus *Penicillium chrysogenum*, recently renamed as *Penicillium rubens* (Fernández-Aguado et al. 2013, 2014). Nowadays, several reports indicated the presence of a penicillin-resistant *Staphylococcus aureus* that produces enzymes that inactivate the β-lactam antibiotic by hydrolyzing the β-lactam core (Jovetic et al. 2010). These enzymes are known as β-lactamases. Later in 1959, the isolation of 6-aminopenicillanic acid (6-APA) from the *P. chrysogenum* fermentation end product [penicillin G or penicillin V (phenoxymethylpenicillin)] allowed the development of numerous semisynthetic penicillins that are resistant to the staphylococcal β-lactamases as a result of steric protection of the β-lactam ring. Methicillin-susceptible *S. aureus* produces five kinds of PBPs: PBP1, PBP2, PBP2B, PBP3, and PBP4, for which the genes have been cloned and sequenced (Boyle-Vavra et al. 2003). Nevertheless, Staphylococci have developed another mechanism for resistance to β-lactam antibiotics by the production of a new penicillin-binding protein 2a (PBP2a). These bacterial strains have been designated as methicillin-resistant *S. aureus* (MRSA), from 1960s to present. β-lactam resistance in MRSA is primarily due to the expression of *mec*A gene which encodes the low-affinity penicillin-binding protein PBP2 (Wu et al. 2001; Smith 2007). Bioinformatic analysis of PBP2a protein revealed a clear similarity to those of the shape-determining protein (PBP2) and septum forming (PBP3) of *E. coli* (Song et al. 1987).

These beta-lactam resistance gene *mecA* of *S. aureus* is carried on a large mobile genetic element known as SCCmec, which integrates it into the chromosome of MRSA strains (Katayama et al. 2000; Ito et al. 2001; Jansen et al. 2006). The mechanism of resistance of PBP2a producers is the outcome of the limited accessibility of the antibiotics to the active site which results in a reduced rate constant for acylation (3–4 orders of magnitude) as compared to other PBPs, and an increased dissociation constant for the preacylation complex. In contrast to the low accessibility of the PBP2a active site to β-lactam antibiotics, the native PGN substrate is still able to access the active site. It is believed as a consequence of conformational changes brought about by allosteric binding of PGN to the enzyme, resulting in effective PGN cross-linking and subsequent cell wall viability (Fuda et al. 2004, 2005; Worthington and Melander 2013).

It could be considered that PBP2a is an essential element for the prevalence of MRSA. Likewise in *Streptococcus peneumoniae* resistance to b-lactams is commonly caused by expression of a variety of low-affinity PBPs (Walsh 2003). These β-lactam resistances have proliferated dramatically and have created clinical difficulties for nosocomial treatment on a global scale. Strains of *S. aureus* exhibiting either PBP2a production or β-lactamase (or both) have established a considerable ecological niche among human pathogens which constitutes a serious health problem.

Inside the β-lactam antibiotics cephalosporins (Fig. 3) are widely and successfully used in medicine in the treatment of different bacterial infections. The history of cephalosporins started in 1945, when the filamentous fungus *Cephalosporium acremonium* (renamed as *Acremonium chrysogenum*) was isolated by Giuseppe Brotzu from the bay water at Cagliary (Italy) (Brotzu 1948). *A. chrysogenum* was found to produce at least three types of antimicrobial compounds, which were isolated and identified (Hamilton-Miller 2000). The first compounds isolated in 1949 were members of the cephalosporin P complex, tetracyclic triterpenes chemically related to helvolic acid (fumigacin). Later the same year, a second compound active against Gram-negative and Gram-positive bacteria, which was penicillin with a D-α-aminoadipic side chain was isolated and named penicillin N. Finally, the third compound isolated and identified in 1953 was cephalosporin C. This antibiotic was active against Gram-negative and Gram-positive bacteria and it was not hydrolyzed by penicillinase, a feature especially relevant due to the appearance of penicillin-resistant bacteria. Cephalosporin C is produced by *Acremonium chrysogenum* and a few other filamentous fungi (Ullán et al. 2002, 2007, 2010).

Early generations of semisynthetic cephalosporins improved their cellular penetration to increase their spectrum of activity against Gram-negative pathogens and to enhance their pharmacokinetics. Next generations have become increasingly focused on combating β-lactam resistance also by chemical side chain modifications on the cephem nucleus (Fig. 3). Actually, in their fifth generation, an extensive search for cephalosporins with efficacy against MRSA bacteria has been made. As a result of this search, new cephalosporin derivate drugs have been found like ceftobiprole that inhibits MRSA at 1–2 mg/L under standard conditions (Livermore 2006; Zhanel et al. 2008).

Following this line, ceftaroline as cephalosporin has the ability to bind to PBP2a to inhibit the bacterial growth of MRSA strains (Zhanel et al. 2009). Another novel class of anti-MRSA semisynthetic-cephalosporins are propenylamide and propenylsulfonamide cephalosporins that act against MRSA strains with minimum inhibitory concentrations as low as 1 μg/mL (Pohlmann et al. 2010). However, nowadays new resistances to ceftaroline and ceftobiprole cephalosporins have been reported (Kelley et al. 2015; Chan et al. 2015; Schaumburg et al. 2015).

In addition to the classical β-lactam compounds, many nonconventional β-lactam structures of scientific and industrial interest have been discovered and characterized since 1970. As shown in Fig. 3, these nonconventional β-lactams contain a β-lactam ring and they usually have a distinct bicyclic structure. The second ring

in the molecule of clavulanic acid and other clavams is an oxazolidinic ring that includes oxygen instead of the sulfur atom occurring in classical β-lactams. The members of carbapenem family have a carbapenem ring containing a carbon atom instead of sulfur. Carbapenem members attack a wide range of PBPs, have low toxicity, and they are much more resistant to β-lactamases than the penicillins or cephalosporins. Monolactams (nocardicins and monobactams) contain a monocyclic structure (the β-lactam ring) and different side chains. Nonconventional β-lactams are also inhibitors of PGN biosynthesis (monolactams), others are potent β-lactamase inhibitors with weak antibiotic activity (clavulanic acid), or have antifungal activity (some clavams) (Brakhage et al. 2005; Coulthurst et al. 2005). β-lactamase inhibitors bind irreversibly to β-lactamases but do not have good activity against PBPs. These β-lactam compounds are combined with β-lactams in clinical treatments.

Besides fungi, some Gram-positive (actinomycetes such as *Streptomyces clavuligerus* or *Amycolatopsis lactamdurans*) and Gram-negative bacteria (e.g., *Lysobacter lactamgenus*) also synthesize hydrophilic β-lactam structures including cephalosporins (mainly as intermediates of biosynthetic pathways), cephamycins, cephabacins, clavams, carbapenems, and monolactams (Liras 1999). Cephamycins are 7-methoxy-cephalosporins active against penicillin-resistant bacteria. Cephabacins contain a formylamino group at C-7 and an oligopeptide side chain at C-3. The C-7 formylamino substituent of cephabacins and the C-7 metoxyl group of cephamycins confer to these antibiotics their characteristic β-lactamase resistance.

2.3 Inhibition of PBPs by Non-β-Lactam Antibiotics

An alternative to β-lactam antibiotics, to impede bacterial growth by inhibition of PBPs, are the noncovalent inhibitors. This way, Toney et al. (1998) developed a methodology for the identification of several non-β-lactam inhibitors, which exhibit IC50 values between 10 and 30 mM. Besides, the noncovalent inhibitors arylalkylidene rhodanines and arylalkylidene iminothiazolidin-4-ones can interfere with bacterial growth across the inhibition of PBPs (Zervosen et al. 2004). Recently, Turk et al. (2011) found new noncovalent inhibitors of PBPs after carrying out a screening of more than 250 compounds. As a result, among others, these authors identified a compound which inhibited PBP2a with a promising IC50 of 97 mM.

Other identified potential inhibitors of PBPs are the 4-quinolones (Shilabin et al. 2012) and the penicilloic acids (van Berkel et al. 2013). Inside this research line, a new option that has emerged recently to treat systemic MRSA infection are the biodegradable antimicrobial polycarbonates (Pascual et al. 2015; Cheng et al. 2015). Its antimicrobial mechanism is disrupting the bacterial membrane.

Studies involving FtsZ (Filamenting temperature sensitive mutant Z) indicated that it is an essential protein required for cell division in prokaryotes. (Addinall and Holland 2002). Tan et al. (2012) described that the combination of the FtsZ-specific

inhibitor PC190723 and β-lactam antibiotics restored MRSA susceptibility to β-lactams. These kinds of FtsZ inhibitors act synergistically with β-lactams. Therefore, Merck has developed a patent for the combination of FtsZ inhibitors with β-lactam antibiotics to target methicillin-resistant *Staphylococcus epidermidis* and MRSA stains [Merck Sharp & Dohme Corp. WO2011112435 (2011)]. Nevertheless in general, vancomycin is the drug of choice to treat MRSA.

2.4 Role of PRs in Bacterial Virulence—PRs Inhibitors

In pathogenic bacteria, many secreted bacterial PRs are virulence factors that aid in bacterial invasion into host cells by the degradation of host-associated proteins (Table 2). During the infective process bacterial PRs involved in growth and proliferation contribute to bacterial virulence indirectly since they are necessary to the bacterium survival within the host environment.

There are several examples of targets of bacterial PRs such as coagulation factors, fibrinogen, and fibrin. The degradation of these proteins that are involved in blood clot formation in the host, may lead to disease states causing, among others, disseminated intravascular coagulation and the formation of small blood clots in the blood vessel (Imamura et al. 1997, 2001; Komori et al. 2001; Massimi et al. 2002). The role of bacterial PRs in disseminated intravascular coagulation is based on the experimental evidences obtained by Komori et al. (2001) who observed that *Pseudomonas aeruginosa* LasA (elastase A) PRs together with its fibrinogenolytic activity affects endothelial cells and destroys the basement membrane of blood vessels to cause hemorrhage in mice.

Therefore, LasA enhances the virulence activity of the metalloendopeptidase LasB (elastase) in the establishment of a *P. aeruginosa* infection. LasA (Table 2) cleaves a wider range of glycine-containing proteins, including, glycine-rich synthetic peptides, specific sequences present in elastin and tropoelastin-derived pentapeptides. This metalloprotein endopeptidase known as LasA also enhances the activity of several other host elastolytic proteases, including, human neutrophil elastase, human leukocyte elastase, and other proteases (Hoge et al. 2010). During the infective process the combination of both *P. aeruginosa* PRs LasA and LasB result in the degradation of elastin that is a component of the connective tissue of vertebrates that give them elastic properties (Morihara 1964; Kessler et al. 1997; Cowell et al. 2003). The key role of LasB in pseudomonal virulence makes it a potential target for the development of antimicrobial agents to attenuate virulence processes without bactericidal action and to avoid the emergence of resistant strains. This way, Cathcart et al. (2011) described the ability of *N*-mercaptoacetyl-Phe-Tyr-amide ($K(i)$ = 41 nM) to block the pseudomonal virulence processes across the inhibition of LasB. Besides, diethylene triamine pentaacetic acid (pentetic acid) suppresses elastase production by *P. aeruginosa* (Gi et al. 2014). The advantage of this compound resides in the fact that nowadays, it is clinically used as a contrast agent for diagnostic imaging. Nevertheless,

Table 2 General classification of proteases according to the recommendations of the nomenclature indicated in Table 1

Name of bacterial PRs	Type of catalytic activity	Targets	EC code	Bacterial pathogen
Staphopain A (ScpA)	Cysteineendopeptidases	Kininogen, AMPs (cathelicidins)	3.4.22.48	*Staphylococcus aureus*
Staphopain B (SspB)	Cysteineendopeptidases	Kininogen Fibrinogen Fibronectin	3.4.22.48	*Staphylococcus aureus*
LasA protease, pseudolysin (LasA), staphylolysin, Staphylolytic protease	Metalloendopeptidase	Elastin Fibrinogen	3.4.24.26	*Pseudomonas aeruginosa*
LasB protease Pseudolysin (LasB), elastase	Metalloendopeptidase	Elastin, collagen (types III and IV), fibronectin, IgA, IgB AMPs (LL-37), Chemokines (RANTES, MCP-1), Cytokines (IL-6, IFN- γ)	3.4.24.26	*Pseudomonas aeruginosa*
Anthrax lethal factor (LF)	Metalloendopeptidase	Mitogen-activated protein kinase (MAPK) kinases, Nlrp1	3.4.24.83	*Bacillus anthracis*
Gingipain R (Rgp) Arg-gingipain. Argingipain. Gingipain-1	Cysteineendopeptidase	Kininogen, Coagulation factors (IX, X), Cytokines (TNF-α, IFN-γ, IL-1β, IL-6, IL-12 etc.), AMPs (LL-37, histatin-5, dermaseptin, brevinin etc.), Chemokines (IL-8)	3.4.22.37	*Porphyromonas gingivalis*
Gingipain K (Kgp) Lys-gingipain. PrtP proteinase	Cysteineendopeptidase	Neurotensin, Haemoglobulin, Bradykinin, Chemokines (IL-8), Cytokines (TNF-α)	3.4.22.47	*Porphyromonas gingivalis*
Botulinum neurotoxins (BoNT) Bontoxilysin	Metalloendopeptidase	Synaptobrevin	3.4.24.69	*Clostridium botulinum*

identification of new antimicrobial agents may not always result in clinical application due to concerns associated with safety for use in the human body.

2.4.1 Lethal Factor Inhibitors

Bacillus anthracis is a spore-forming bacterium that secretes, among others, the metalloendopectidase known as lethal factor (LF) (Table 2). This toxin is lethal to the host through disruption of signaling pathways, cell destruction, and circulatory shock. Treatment against *B. anthracis* must be given early after infection, delay of treatment reduces survival of infected patients because antibiotics can eliminate an anthrax infection but not combat the LF-mediated toxemia that persists even after the bacteria have been eliminated by antibiotics. The discovery of efficient LF inhibitors can increase the probability of host survival by blocking late stage effects of LF in post-exposure anthrax cases (Forino et al. 2005). Thus Shoop et al. (2005) described a hydroxamate LF inhibitor that can be administrated with conventional antibiotics to treat the anthrax infectious disease. Hydroxamate chelates the Zn^{++} from the LF active site resulting in reduced protease activity of LF in the mitogen-activated protein kinase (MAPK) cleavage assay (Li et al. 2012). Other LF inhibitors are the catecholate siderophores enterobactin and the cationic polyamine from salmon sperm, protamine. However, protamine, exhibits the best results in the LF inhibition assays (Thomas and Castignetti 2009). In the same research line, Dell'Aica et al. (2004) described that the green tea polyphenols epigallocatechin-3-gallate and catechin gallate also inhibit the LF and show reduced MAPK kinase cleavage activity. Benzylamine-derived inhibitors, PT8420 and PT8541 are also LF inhibitors, provide protection against *B. anthracis* in combination with other treatments (Moayeri et al. 2013).

Another target of LF inhibitors is the LF exocites which contribute to the catalysis process and participate in the efficient binding of substrate at other points of the active site. Their inhibition action results in an LF conformational alteration and, therefore, in a reduction of LF activity. As result, Bannwarth et al. (2012) screened a library of LF exosites inhibitors, which allowed the identification and evaluation of the depsidone stictic acid as a possible inhibitor. Lethal toxin (LT) is the combination of LF and protective antigen, it has been reported that the organogold compound auranofin inhibited LT-mediated toxicity in mouse macrophages. Auranofin, due to its anti-inflammatory activity, acts downstream of MAPKK cleavage by inhibiting the caspase-1 activation (Newman et al. 2011).

2.4.2 Periodontium Infective Process, Gingipain Inhibitors

Another target of bacterial PRs virulence factors are the proteins of the host connective tissue. A clear example is the periodontium infective process by *Porphyromonas gingivalis*, where the proteolytic activity of the secreted gingipains results in periodontal connective tissue destruction as a result of host protein

degradation (Potempa et al. 2000). The gingipain family of *P. gingivalis* comprises three related cysteineendopeptidases that hydrolyze peptide bonds at the carbonyl groups of lysine (Lys-Xaa) and arginine (Arg-Xaa) residues. The Lys-specific gingipain, Lys-gingipain (Kgp), is encoded by the *kgp* gene; whereas the homologous arginine-specific gingipains, RgpA and RgpB, are products of two related genes *rgpA* and *rgpB* (Fitzpatrick et al. 2009). Proteolytic activities of these PRs play a fundamental role in the attachment and colonization, acquisition of nutrients, evasion of host defense and tissue invasion, and dissemination of *P. gingivalis* (Guo et al. 2010).

Discovery and characterization of gingipain inhibitors could prevent or slow down the progression of this dental disease. In a review article of Olsen and Potempa (2014) described a battery of gingipain inhibitors that include gingipain N-terminal prodomains, synthetic compounds, inhibitors from natural sources, antibiotics, antiseptics, antibodies, and bacteria. The use of nonpathogenic bacteria as antagonists of pathogenic bacteria to combat tooth decay, deserves special mention. Thus, Tenorio et al. (2011) isolated and identified 19 bacterial strains from the subgingival plaque from 20 patients who were diagnosed as having periodontitis. Some of the identified isolates decreased the gingipain activity and interfering with the growth of *P. gingivalis* in vitro. Besides, Camelo-Castillo et al. (2014) isolated and identified several bacterial strains from the supragingival dental plaque from adult individuals who had never suffered dental caries. Two of these isolates [*Streptococcus dentisani* Str. 7746 (CECT83135, DSM 27089) and *Streptococcus dentisani* Str. 7747T (CECT 8312T, DSM 27088)] are considered for these authors as potential probiotics for human oral health.

2.4.3 Immune System PRs Inhibitors

Another virulence factor of some pathogenic bacterial PRs is its role in the "escape response" to several host defense mechanisms (Potempa and Pike 2009). Pathogens cause immunosuppression in their infected host that shows depressed immune responses to antigens in general, including those of the infecting pathogen. Thus, the contact system (also known as the intrinsic pathway of coagulation or the kallikrein/kinin system) of host delivers antimicrobial peptides (AMPs) derived from kininogen and traps bacteria in the thrombus. Furthermore, the bradykinin peptide causes spreading of macrophages and induces an influx of neutrophils into the surrounding host tissue (Frick et al. 2007).

Immunoglobulins (Igs) play a key role in the immune defense system of humans. Igs recognize, in a specific way, the invading microorganisms and mediating their killing by professional phagocytes or the complement system, or both. Igs consist of antigen-recognizing Fab (fragment antigen-binding) regions, which are linked through a flexible hinge region with the constant Fc (fragment crystallizable) effector region (von Pawel-Rammingen and Björck 2003).

Streptopain [also called streptococcal pyogenic exotoxin B (speB), SCP] is a cysteine protease encoded by the *speB* gene in the Gram-positive bacterial pathogen

Streptococcus pyogenes (Elliott 1945). The enzyme is secreted as a zymogen and autocatalyzes cleavage into the mature SpeB. It is involved in host–pathogen interactions and increases the invasive action of *S. pyogenes* by hydrolysis of human fibronectin and vitronectin (Kapur et al. 1993). SpeB prevents normal functions of the human immune system by cleavage of M proteins and C5a peptidases. SpeB cleaves the human antibody IgG between residues Gly236 and Gly237 and also other antibodies, such as IgA, IgD, IgE, and IgM (von Pawel-Rammingen and Björck 2003). *S. pyogenes* also secretes the cysteine protease IdeS (also called IgG-degrading enzyme of *S. pyogenes* or Mac-1), distinct from streptopain, which cleaves human IgG in the hinge region with a high degree of specificity (von Pawel-Rammingen et al. 2002). IdeS is secreted by *S. pyogenes* as a mature enzyme of 339 residues and cleaves IgG antibodies bound to bacterial surface structures, thereby inhibiting the killing of pathogen by phagocytic cells. Degradation of IgG by IdeS and SpeB could be complementary under certain conditions (Fig. 4).

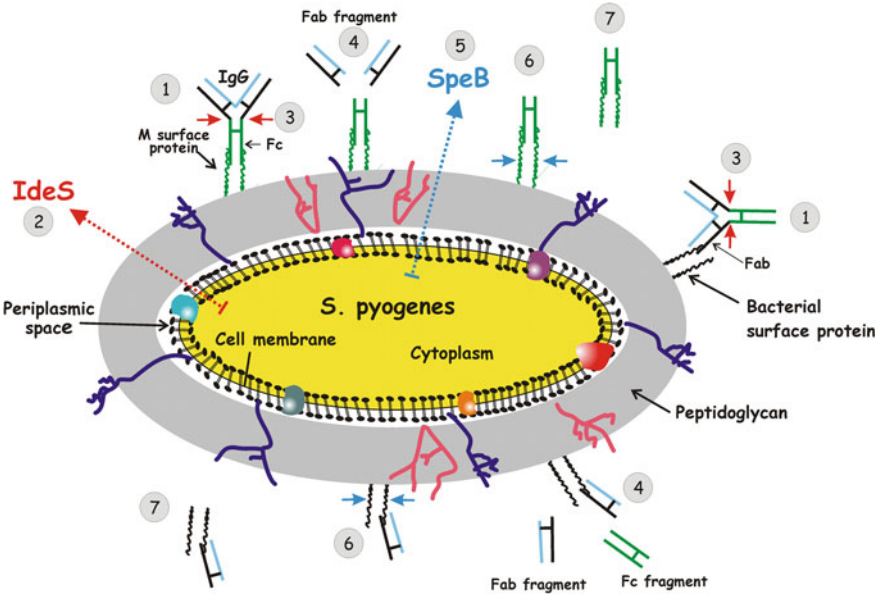

Fig. 4 Schematic picture showing the action of IdeS and SpeB secreted by *S. pyogenes* against human IgG antibodies (adapted from von Pawel-Rammingen and Björck 2003) (*1*) Y-shaped IgG binds to bacterial surface proteins and M surface proteins. Fab (fragment antigen–binding) fragments bound to specific bacterial surface proteins and Fc (fragment crystallizable) fragments bound by M proteins. (*2*) *S. pyogenes* secretes IdeS. (*3*) IdeS cleavages IgG in the hinge region (indicated by *red arrows*). (*4*) Unbound Fab and Fc fragments are released. (*5*) Secretion of SpeB. (*6*) SpeB degrades bacterial/M surface proteins (indicated by *blue arrows*). (*7*) Complexes of bacterial/M surface proteins and Fc and Fab fragments of IgG are released

Already nowadays IdeS has potential medical applications to block arthritis development induced by IgG due to its PRs activity against this immunoglobulin (Nandakumar et al. 2007). Studies in rabbits revealed that IdeS due to its highly selective degradation of IgG, may be used as an immunosuppressant drug used to prevent rejection after renal transplantation. In addition, autoimmune conditions where IgG labels endogenous compounds as pathogenic may be treated by IdeS injected into the circulatory system (Johansson et al. 2008).

Other examples of Igs degradation capacity are, among others, the pathogenic bacteria *Neisseria* spp., *Streptococcus pneumoniae*, *Ureaplasma urealyticum,* and *Haemophilus influenza* that secrete IgA1 proteases (degrade the heavy chain of human IgA1 in the hinge region) to avoid the host defense mechanisms at sites of infection and to promote disease. These bacterial PRs interfere with the protective functions of the principal mediator of specific immunity on mucosal surfaces, and especially in the upper respiratory tract (Plaut 1983; Poulsen et al. 1998). Gingipains (mentioned above) and staphopains (A and B) are cysteineendopeptidases produced by *P. gingivalis* and *S. aureus,* respectively (Table 2). Both bacterial PRs degrade kininogen to produce kinin which induces vascular permeability and promote an influx of plasma-containing nutrients into the site of infection (Imamura et al. 2005). In addition, it is well known that invasion of the systemic circulation by *P. aeruginosa* across the pseudomonal extracellular PRs production facilitates septicemia as well as toxemia through activation of the bradykinin-generating cascade (Sakata et al. 1996).

S. aureus produces the staphylococcal PRs inhibitors staphostatin A and B, respectively. Similar to the cystatins, the staphostatins interact specifically with their target PRs forming tight and stable noncovalent complexes, staphostatin A with staphopain A and staphostatin B with staphopain B. (Rzychon et al. 2003). The squamous cell carcinoma antigen (SCCA) 1 is an epithelial-derived serpin that inhibit the staphopains in an efficient way (Kantyka et al. 2011). Kantyka and Potempa (2011) indicated that the high association rate constant (k(ass)) for inhibitory complex formation (1.9×10^4 and 5.8×10^4 M^{-1} s^{-1} for staphopain A and staphopain B interaction with SCCA1, respectively) argues that SCCA1 can restrain staphopain activity in vivo at epithelial sites colonized by *S. aureus*.

Production of AMPs and proteins is an important means of host defense in eukaryotes against bacterial pathogens. In fact they are an important part of the innate immune system of all complex organisms as well as some microbes, with antibacterial, antifungal, antiparasitic, and antiviral activity (Jenssen et al. 2006; Guilhelmelli et al. 2013). For example, the antimicrobial activity of the human AMP LL-37 (member of the cathelicidin family of AMPs of the innate immune system) is mediated by disruption of the target cell lipid bilayer via toroidal pore formation, leading to osmotic lysis and cell death (Henzler-Wildman et al. 2004). Nevertheless, several bacterial PRs can inactivate these AMPs by their degradation. In this way, the human pathogens *Proteus mirabilis,* Group A Streptococci and *Enterococcus faecalis* have been demonstrated to target and inactivate LL-37 thought PRs secretion (Kindrachuk et al. 2010). Likewise, gingipain R of *P. gingivalis* and LasB of *P. aeruginosa* are able to degrade the AMP LL-37

(Schmidtchen et al. 2002; Carlisle et al. 2009). Furthermore, *S. aureus* can cleave and inactivate LL-37 in a time- and concentration-dependent manner by the production of the metalloproteinase aureolysin (Sieprawska-Lupa et al. 2004).

Other targets of these bacterial PRs are cytokines and chemokines that play an essential role in regulating both immunity adaptive and innate systems. The interaction between bacterial PRs and these small proteins made by host cells in the immune system disturb the communication network in the host which affects the bacterial pathogenicity (Mikolajczyk-Pawlinska et al. 1998; Baba et al. 2002; Leidal et al. 2003; Matheson et al. 2006; Sheets et al. 2008). For example, during the infective process of the human host by *P. aeruginosa* in lung disease, LasB PRs degrade RANTES and MCP-1 resulting in a loss of chemotactic activity, which suggests that this pathogen may alter the relative amounts of critical immunomodulatory cytokines in the airway (Leidal et al. 2003).

Nevertheless, further research into interaction between bacterial PRs and these host immune communication signals is necessary to extrapolate the in vitro experimental results to the situation in infected tissue. In summary, for the pathogenic bacteria the secretion of bacterial PRs at the site of infection is essential to progress in the infective process of the host, giving them an evolutionary advantage over non-protease-secreting bacteria.

3 Plants as Source of Bacterial PRs Inhibitors

Relying on the fact that PRs play an important role in the protection of plant tissues from pest and pathogen attack; Rakashanda et al. (2012) isolated the serine PRs inhibitor LC-pi I from the seeds of the plant *Lavatera cashmeriana*. This PRs inhibitor showed strong antibacterial activity against the pathogenic bacteria *Klebsiella pneumoniae* and *P. aeruginosa.* These kinds of bacteria can cause urinary tract infection, pneumonia, and septicemia in humans. Besides, the Soap Nut Trypsin inhibitor isolated from *Sapindus trifoliatus* exhibited antibacterial activity against *B. subtilis*, *S. aureus*, *E. coli,* and *Proteus vulgaris* (Rachel et al. 2013).

As mentioned above *B. anthracis* produces de metalloendopeptidase LF during the invective process. The plant derivate curcumin (diferuloylmethane) and its chemically modified 4-phenylaminocarbonylbis-demethoxycurcumin inhibit LF by both increasing its substrate affinity and decreasing its catalytic capacity (Antonelli et al. 2014). Curcumin is the yellow pigment associated with the curry spice of the curcuminoid family that was originally isolated from the Indian spice turmeric (*Curcumin longa*) being an important component in the daily diet of several Asian countries.

Another source of plant PRs inhibitors is *Coccinia grandis*, which have antibacterial activity by killing or inhibiting the growth of *B. subtillis* [minimal bactericidal concentration (MBC) of 1.25 mg⁄mL; minimal inhibitory concentration (MIC) of 1 mg⁄mL], *S. aureus* (MBC of 1.2 mg⁄mL; MIC of 1 mg⁄mL), *E. coli* (MBC of 1 mg⁄mL; MIC of 0.63 mg⁄mL), *P. vulgaris* (MBC of 0.5 mg⁄mL; MIC of

0.2 mg⁄mL), and *K. pneumoniae* (MBC of 0.5 mg⁄mL; MIC of 0.01 mg⁄mL) (Satheesh and Murugan 2011). The authors purified and isolated the PRs inhibitor and proposed that the mechanisms of action of this natural compound would be the formation of a channel in the bacterial cell membrane that will originate the cell death as a result of the out of the cellular contents.

Finally, in the *Brassica chinensis* seed there is a napin-like polypeptide with trypsin inhibitor activity. This compound inhibits the bacterial growth of *Pseudomonas fluorescens* [concentration that inhibits bacterial growth by 50 % (IC50) of 66 μM], *Bacillus megaterium* (IC50 of 215 μM), *Mycobacterium phlei* (IC50 of 146 μM), *Bacillus cereus* (IC50 of 222 μM), and *B. subtilis* (IC50 of 236 μM) (Ngai and Ng 2004). It becomes clear that further research with these natural PRs inhibitors is necessary to elucidate both its mechanism of action and its possible application from a pharmacological point of view.

4 Conclusions and Future Perspectives

It is well known that animals and plants have been developed defense mechanisms against pathogenic microorganisms (fungi, bacteria, and viruses) by biochemical compounds production. Between these biochemical compounds there is a production of proteins and other metabolites, which inhibit bacterial pathogen-secreted proteins by different mechanisms. They are known as bacterial PRs inhibitors that in some cases could limit host virulence or, in others, should block cell growth.

Targeting virulence proteins with PRs inhibitors to prevent colonization of the host and enable removal of bacteria by the host immune system represents an amazing opportunity to develop new antimicrobial compounds with novel mechanisms of action. Unlike classical antibiotics, it is expected a reduction of the risk of development of resistant strains in a clinical setting. The inhibition of virulence pathways will apply only a mild evolutionary pressure since these pathways are not essential for normal bacterial growth (Rasko and Sperandio 2010).

In fact, MRSA strains, as mentioned previously, or even the totally drug-resistant *Mycobacterium tuberculosis* (Velayati et al. 2009) make necessary the discovery, that is, a development of new antibiotics to combat these resistances. Nowadays, in the market there are several PRs inhibitors for the treatment of a range of diseases. For example, telaprevir (Incivek; Vertex Pharmaceuticals) and boceprevir (Victrelis; Merck) are PRs inhibitors that target the hepatitis C virus (HCV) NS3-4A protease2,3 (Vermehren and Sarrazin 2011). Another example of PRs inhibitor is the oral anticoagulant rivaroxaban (marketed as Xarelto by Bayer) that inhibits the factor Xa in the coagulation cascade (Eriksson et al. 2008). Nevertheless, this class of enzymes has not yet been exploited in the treatment of bacterial infections notwithstanding the pharmaceutical successes with the above-mentioned PRs modulators.

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Quorum Sensing as Target for Antimicrobial Chemotherapy

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Abstract The increasing antibiotic resistance of human pathogens and their tolerance mechanisms to antimicrobial agents highlight the need for new therapeutic strategies. The phenomenon of quorum sensing (QS) is mediated by small signaling molecules, and plays major roles in bacterial physiology and ecology. Since its discovery more than 40 years ago, QS has been shown in many microorganisms, including animal and plant pathogens, and extensive studies have revealed that, among other physiological properties, virulence is often controlled by QS mechanisms. These findings prompt a move for novel ways to control microbial populations, changing the focus from traditional bacteriostatic or bacteriolytic agents to new compounds targeting QS systems. Consequently, natural and synthetic QS-interfering compounds (QSIs) that neither destroy nor inhibit the growth of pathogens, but modulate microbial phenotypes by attenuating virulence, are now being characterized. QSI agents can be potentially utilized to reduce the spread of infection or to improve the removal of pathogens. Moreover, the pharmacological targeting of nonessential functions, such as virulence factors or biofilm formation, may alleviate the development of resistance by lessening the need of using conventional agents which, by themselves, select for resistance. The usefulness of these kinds of compounds against infection does not just rely on being a therapy but also in representing a prophylactic approach to avoid invasion of materials where infection can initiate. Some relevant developments on these topics are discussed.

1 Introduction

Besides metabolic cooperation, interacting competition, and DNA exchange, microbial intercellular signaling may also occur by secreted diffusible molecules. Quorum sensing (QS) is a common regulatory system in unicellular microorganisms which allows individual cells to come together and to act in a synchronized

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fashion, behaving as a collective unit. In essence, this process rests on the production, release, and detection by a group of cells of self-generated extracellular signaling molecules. Often, the gene encoding the enzyme that synthesizes the signaling molecule is activated by QS, leading to the concept of autoinducer (AI) (Keller and Surette 2006). AIs are usually small molecules that either diffuse freely across the cell membrane or are actively transported out of the cell. By using these diffusible mediators, bacteria can regulate their social behavior according to cell number. Despite differences in molecular mechanisms and regulatory components, the fundamental steps involved in detecting and responding to fluctuations in population density are similar in all known QS systems (Fig. 1).

This process allows bacteria to switch between two distinct gene expression programs: one favored at low cell density for individual cells, characterized by individual asocial behaviors, and another prompted at high cell density for social group performance (Rutherford and Bassler 2012). The functional basis of this phenomenon relies on the principle that when a single bacterium releases AIs into the environment, their concentration is too low to be detected. At these conditions, AIs diffuse away into the medium and are present at concentrations below the threshold required for detection. However, at high cell population density sufficient bacteria are present to accumulate local high concentration of AIs, allowing bacteria to sense the existence of a critical cell mass by appropriate cytoplasm or membrane-anchored receptors, thus triggering a subsequent response. The detection

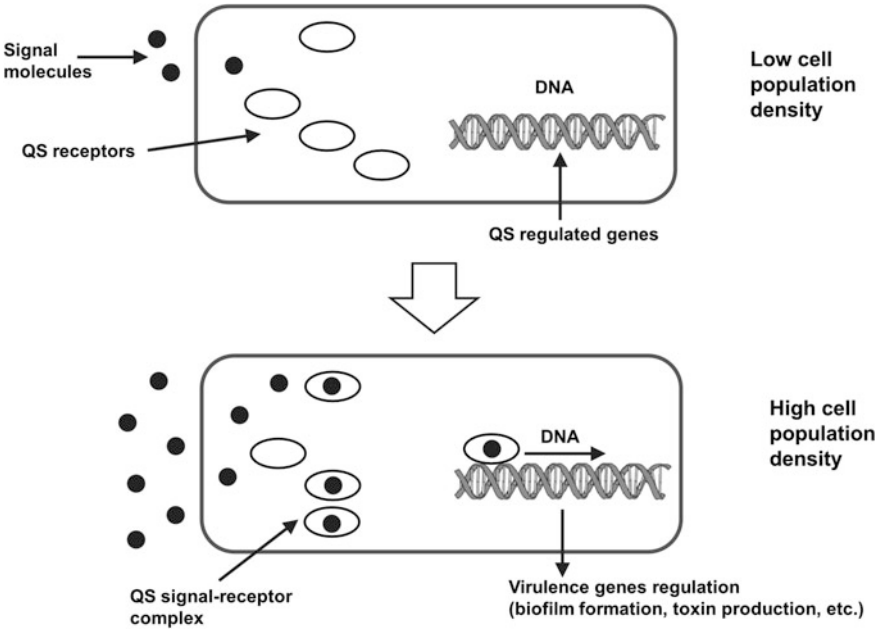

Fig. 1 Simplified view of QS. Accumulation of signaling molecules enables single cell to sense the number of bacteria (cell density) and to perform changes in the gene expression patterns

of stimulatory threshold concentrations of AIs results in changes in gene expression. Also, in addition to activating or repressing target genes necessary for cooperative behaviors, the perception of AIs may result in activation of their own production, prompting a loop that likely promotes synchrony in the population. AIs specifically bind to, and activate transcriptional regulator proteins that target DNA sequences to enhance or block the transcription of regulated genes, resulting in the synchronous activation of certain phenotypes in a bacterial population (Solano et al. 2014).

2 Biological Context

The discovery that bacteria are able to communicate by a chemical language changed the longtime considered perception that individual cells just try to find nutrients and to grow during their cell cycle. The existence of cell-to-cell communication revealed that bacteria show coordinated activities once believed to be restricted to multicellular organisms. Although the nature of the chemical signals, the transduction mechanisms, and the target genes controlled by QS systems may differ, in all cases the ability to communicate allows bacteria to coordinate gene expression, and therefore to regulate the behavior of the whole community. This process awards bacteria with some properties of higher organisms and opens the possibility that might have been one of the early steps toward multicellularity (Miller and Bassler 2001; Keller and Surette 2006).

Although QS studies were initially limited to a few microorganisms, the QS phenomenon forms part of the regulatory machinery of many bacterial species. QS systems represent not only signaling pathways within species, but also between species, and even between prokaryote and eukaryote representatives (Atkinson and Williams 2009). Notably, some bacteria can modulate their behavior in response to cell-to-cell signals that they are not able to synthesize, thus listening secretly on the communication of other organisms. As determinants of cell population density, multiple microbial QS systems are often integrated with each other and within global regulatory networks under the influence of particular environmental conditions. Congruent with this, most bacteria with operative QS systems are somewhat associated with animals or plants in relationships that can be mutually positive (symbiotic bacteria) or antagonistic (pathogenic bacteria). For instance, QS appears to be more common in plant-associated *Pseudomonas* spp. than in free-living soil *Pseudomonas* spp. (Elasri et al. 2001), an observation that suggests that QSs are important in bacterial relationships with eukaryotes. Processes controlled by this type of signaling include bioluminescence, sporulation, motility, competence for DNA uptake in natural transformation, biofilm development, secretion of key virulence factors, production of exopolysaccharides, and biosynthesis of secondary metabolites as antibiotics. In particular, QS has also a major impact on the susceptibility to antibiotics, either by increasing tolerance in biofilms, by regulating

endogenous resistance genes or by controlling the acquisition of exogenous genes for antibiotic resistance by transformation (Rampioni et al. 2014).

Bacterial QS is not only ecologically important but also medically relevant because there is evidence that QS controls the production of virulence factors. In this context, the ability of a collective behavior represents a mechanism that optimizes cooperation by delaying expression of a social behavior until obvious benefits are reached. In general, it seems no beneficial for bacterial survival to produce virulence factors before reaching a certain population density. Activation of virulence genes at low cell population density might result in triggering host defense responses and early disadvantages for the establishment of the bacteria. However, survival is enhanced if the host defense responses are made when the bacterial population is high to increase the likelihood of successful prospects. Moreover, coordinated expression of bacteria avoids wasting energy by synthesis of compounds that are not required for bacterial growth at high cell population density. Changes in the expression of genes can also result in the establishment of symbiotic or pathogenic relationships with eukaryotic hosts, in controlling the growth of other species or in the protection from deleterious environments. Many bacterial species use QS to coordinate not only biofilm development but also dispersion, that becomes essential for bacteria to escape and colonize new niches when nutrient resources become limited and waste products accumulate (Nadell and Bassler 2011; Solano et al. 2014).

On the other hand, eukaryotes are capable of interfering with bacterial communication by producing molecular signals that interact with the bacterial QS systems (Teplitski et al. 2004). Production of natural and synthetic compounds targeted toward a specific bacterial population include molecules that are either antagonistic to the AIs or that mimic their functional structure. In addition, QSI can potentially target other components of the system, such as the stability and function of the regulator proteins or the corresponding synthases (Manefield et al. 2002; Keshavan et al. 2005). All these types of QSIs have been investigated in the last years as potential agents of microbial control (Hirakawa and Tomita 2013; Rampioni et al. 2014).

Drugs attempting to attenuate virulence rather than bacterial growth and based on disruption of the QS define a strategy that is often named quorum quenching (QQ). The alarming spread of bacterial resistance to traditional antibiotics warrants the engineering of nanoparticles to silence bacterial QS communication as a method of antimicrobial therapy that might provide a scaffold and critical stepping stone for more pointed developments (Miller et al. 2015). Unfortunately, identical QS systems controlling virulence and conserved across all pathogens have not been identified, so that the prospect of broad spectrum interfering agents is unlikely (Rampioni et al. 2014). Successful QQ requires a good knowledge of the architectural features of the particular transduction pathways and the specific members involved in the QS to be targeted. A short view of major types follows below.

3 *Quorum Sensing* Regulatory System and AIs

Structurally, AIs are effectors of QS-dependent genes that belong to a wide range of chemical classes including *S*-adenosyl-methionine (SAM) derivates, like homoserine lactones (HSLs) or AI-2, oligopeptides, and *cis*-unsaturated fatty acids.

Gram-negative and Gram-positive bacteria use different types of AIs and regulatory mechanism of QS systems and each bacterial species may harbor more than one different circuitry. In general, binding of AI to the receptor feedback activates expression of AI (Rutherford and Bassler 2012). Gram-negative bacteria use small molecules as AIs, mainly HSLs, or other molecules whose production depends on SAM as a substrate (Wei et al. 2011). These AIs are produced in the cell and freely diffuse across the inner and outer membranes. Once a threshold concentration is reached, the AI interacts and stabilizes a cytoplasmic protein that has dual roles: as HSL cognate receptor and as transcriptional regulator. The AI-bound receptor enables it to bind to specific DNA sequences and regulates expression of target genes (e.g., LuxI/LuxR in *Vibrio fischeri*, LasI/LasR, and RhlI/RhlR in *Pseudomonas aeruginosa* and in more than 100 QS systems of Gram-negative bacteria). In some cases of bacterial Gram-negative QS, AIs are detected via membrane-bound two-component type sensor histidine kinase/phosphatase (e.g., CqsS and LuxPQ in *Vibrio cholerae*) or type sensor histidine kinase/AI biosynthesis regulator (e.g., RpfC in *Xanthomonas campestris*).

Gram-positive bacteria use specially oligopeptides as AIs. Once produced in the cell as precursors, these autoinducing peptides (AIPs) are processed and secreted. At high cell population density, AIPs are detected by membrane-bound two-component signal transduction systems (composed by histidine kinase receptor and cytoplasmic response regulator) to activate transcription of the QS-target genes (e.g., *Staphylococcus aureus* Agr system or *Streptococcus pneumoniae* ComD/ComE system). In other cases of bacterial Gram-positive systems, once secreted, AIPs are transported back into the cell where they interact with transcription factors to modulate the transcription factor's activity and, in turn, modulate gene expression changes (e.g., *Bacillus cereus* PapR/PlcR system).

3.1 Quorum Sensing *by SAM Derivatives*

The most common class of AIs used by Gram-negative bacteria in QS is acyl homoserine lactones (A-HSL). They contain a conserved homoserine lactone ring derived from SAM and a specific fatty acid side chain whose length provides specificity for the signals and varies within different species. Although new classes of HSL with unusual chains have been reported (Schaefer et al. 2008; Lindemann et al. 2011), the fatty acyl groups are usually short and may show terminal modifications by methyl branches, presence of various substituents, or different unsaturation degrees. A same AI of this type can regulate different phenotypes in

different bacterial strains. For instance, the first SAM derivative AI described in *Vibrio fischeri* (Eberhard et al. 1981) activates bioluminescence but this molecule also regulates exopolysaccharide production in *Erwinia stewartii* (Watson et al. 2002). This cross talk makes the development of indicator strains to detect the presence in a given sample of A-HSLs feasible (Llamas et al. 2004).

3.1.1 LuxI/LuxR, LasI/LasR and RhlI/RhlR Systems

Gram-negative bacteria typically use the so-called LuxI/LuxR type QS systems (composed by AI synthase/AI receptor). In these systems, LuxI-like protein is a synthase that catalyzes acylation and lactonization reactions between SAM and an acyl carrier protein (ACP) to produce a freely diffusible A-HSL AI (Ng and Bassler 2009). At high concentrations, A-HSL AIs bind to cytoplasmic receptor LuxR-like transcription factors. When not bound by the AI, the receptor is degraded, but the binding stabilizes the LuxR-type protein, and forms hydrogen bonds between amino acids in the protein and the A-HSL molecule. Then the receptor protein suffers a conformational change in the C-terminal domain enabling it to bind to a DNA sequence upstream of the target gene's promoter, termed "lux box," which favors transcription (Fig. 2). Typically, the AI-bound LuxR-type protein also activates *luxI* expression, forming a feed-forward autoinduction loop that floods the population with AI.

Numerous Gram-negative pathogens control biofilm and virulence factors production using Lux type systems for QS. As an example, *P. aeruginosa* harbors two complete circuits, composed of a LuxI type synthase, responsible of A-HSL synthesis (LasI and RhlI), and a LuxR-type receptor (LasR and RhlR). LasI synthesizes 3-oxo-C_{12}-HSL and RhlI synthesizes C_4-HSL. In addition, *P. aeruginosa* has two orphan LuxR homologues, VqsR and QscR. QscR responds not only to the LasI signal but also to multiple other A-HSLs (Chugani and Greenberg 2014). Also, *P. aeruginosa* presents a third non-LuxI/LuxR type system called the *Pseudomonas* quinolone signal (PQS), which is interconnected with the LasI/LasR and RhlI/RhlR signaling circuits (Williams and Camara 2009). The Las system positively regulates

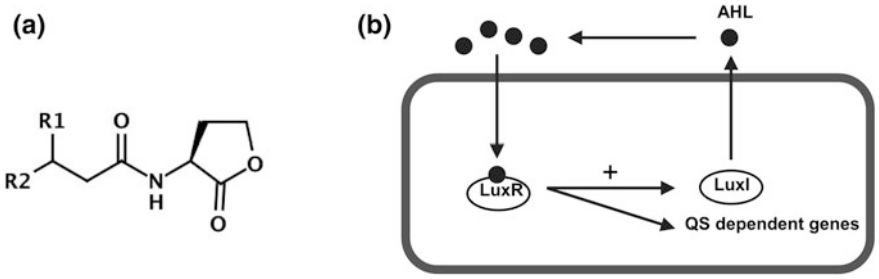

Fig. 2 **a** Structural features of AHL-type compounds. **b** Generalized view of an AHL-based QS system. Producer and receiver cells are represented here as one single cell

the Rhl system, and together regulate PQS (see below). One important element in *P. aeruginosa* biofilm dispersal is the synthesis of rhamnolipids which is controlled by both types of signaling. These biosurfactants maintain channels among multicellular structures in biofilms promoting bacterial motility, nutrients distribution, and removal of waste products (Pamp and Tolker-Nielsen 2007).

3.1.2 CqsA/CqsS and LuxS/LuxPQ Systems

Vibrio cholerae produces two autoinducers, CAI-1 and AI-2, which are detected via membrane-bound two-component sensors using two parallel QS circuits. CAI-1 is synthesized by CqsA using SAM and decanoyl-coenzyme A as substrates (Wei et al. 2011). Since homologs of CqsA have been identified in all *Vibrio* species, CAI-1 has been proposed as an intergenus communication molecule, allowing communication between other CqsA-encoding vibrios (Miller et al. 2002). The second autoinducer, AI-2, is synthesized by LuxS (Chen et al. 2002). This type represents a non-species specific signal that was originally characterized as AI in the regulation of bioluminescence and later discovered in other bacteria regulating QS signaling within species (bioluminescence, secretion, virulence factors) and among species (formation of mixed-species biofilms) (McNab et al. 2003). Different forms of the AI-2 deriving from the same precursors have been recognized as signal molecules in *Vibrio* spp. (Chen et al. 2002), *Salmonella typhimurium* (Miller et al. 2004), *Sinorhizobium meliloti* (Pereira et al. 2008), or *Yersinia pestis* (Kavanaugh et al. 2011). As AI-2 is produced by many species from different bacterial groups, this AI appears to function as a global signal for interspecies communication, allowing other bacteria that contain luxS in a specific environment to contribute to the overall cell density information (Xavier and Bassler 2005). It is produced and detected not only by Gram-negative but also by Gram-positive bacteria such as *Streptococcus mutans* (Huang et al. 2009).

By using both CAI-1 and AI-2, *V. cholerae* is presumably able to detect both the number of other vibrios and the total number of other bacteria in the environment. CAI-1 and AI-2 signal throughout two parallel membrane-bound two-component histidine kinases, CqsS and LuxPQ, respectively. At low cell population density these receptors function as kinases. Upon autophosphorylation, the phosphotransfer LuxU is phosphorylated and then transfers phosphate to the response regulator LuxO. Once phosphorylated, LuxO activates expression of genes encoding four sRNAs (Qrr1-4) that regulate target mRNAs by base pairing. The principal targets of the Qrr1-4 sRNAs are the mRNAs encoding the QS master regulators HapR and AphA. At low cell density, Qrr1-4 sRNAs repress *hapR* expression, activate *aphA* expression and subsequently, promote production of virulence factors and biofilm development. At high cell density, AIs accumulate and after binding to their receptors the complexes act as phosphatases so that unphosphorylated LuxO cannot activate the transcription of *qrr1-4* and *hapR* mRNA is therefore translated. HapR represses *aphA* to shut down virulence production and promotes detachment of cells from biofilms formed at low cell density (Rutherford and Bassler 2012;

Solano et al. 2014). In this way, at low cell density *V. cholerae* remains attached to host tissue when virulence factors are expressed, competing for nutrients in the host, whilst at high cell density, QS repression of biofilms allows dispersal of *V. cholerae* into the environment, maximizing its capacity to reach other hosts (Nadell and Bassler 2011). Interestingly, in the close relative *V. vulnificus*, activation of the HapR homologue promotes disassembly of the biofilm at high cell population density by upregulating the synthesis of capsule exopolysaccharides that have antibiofilm properties (Valle et al. 2006; Rendueles et al. 2013). Thus, similar to *P. aeruginosa, Vibrio* may utilize QS to repress the synthesis of biofilm matrix compounds and induce the synthesis of molecules with surfactant properties.

3.2 Quorum Sensing *by Autoinducing Peptides (AIPs)*

In Gram-positive bacteria most AIs are encoded as precursor forms and later processed and secreted by specialized transporters. These signaling molecules are linear or cyclized oligopeptide with diverse amino acid sequence. The AIPs are detected by membrane-bound two-component signal transduction systems (Ji et al. 1995; Solomon et al. 1996). Binding to a cognate membrane-bound two-component histidine kinase receptor usually activates the receptor's kinase activity by autophosphorylation at conserved histidines residues with ensuing transfer of phosphate from the histidine to a conserved aspartate on a cognate cytoplasmic response regulator protein. The phosphorylated response regulator then activates transcription of the QS-target genes (Rutherford and Bassler 2012).

3.2.1 Agr System

The *Staphylococcus aureus* Agr (accessory gene regulator) system has been extensively studied (Rutherford and Bassler 2012; Solano et al. 2014). This pathway consists of a membrane-bound protein (AgrB) that modifies and exports the AIP (AgrD), and a classical two-component signal transduction system, composed

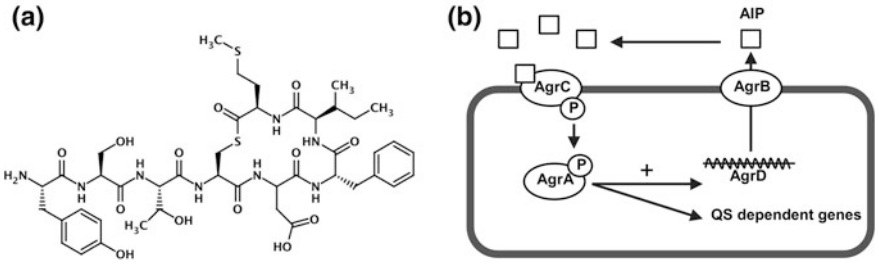

Fig. 3 **a** AIP-I from *S. aureus*. **b** Simplified view of the Agr system

of the sensor histidine kinase (AgrC) and its cognate response regulator (AgrA) (Fig. 3). There are four specific Agr compatibility types of *S. aureus* depending on the AIP (I-IV) of the strain (see Sect. 4.3). The regulatory cascade reduces biofilm development and the expression of surface adhesins (such as protein A, fibronectin binding proteins or coagulase) and additional toxins. Also, it increases the production of secreted virulence factors (such as δ-hemolysin or α-toxin), apparently switching the bacterium from a colonizing commensal to an invasive pathogen (Antunes et al. 2010). In addition, phosphorylated response regulator AgrA activates virulence genes encoding modulins, a specific class of secreted peptides with surfactant properties that mediate the main impact of Agr system in biofilm dispersion (Queck et al. 2008; Periasamy et al. 2012). Modulins play a dual function in biofilm development depending on their aggregation state: as monomers, they promote biofilm detachment, but when polymerized they favor biofilm formation (Schwartz et al. 2012). At low cell population density biofilm formation gives *S. aureus* conditions to increase the population density and thereby to produce virulence factors. Then, to facilitate its dispersal, *S. aureus* finishes biofilm production and decreases surface adhesins (Boles and Horswill 2008).

The four types of AIPs produced by *S. aureus* are detected by a corresponding cognate sensor. The presence of a nonappropriate AIP results in inhibition of the QS and, consequently, the first strain to establish its QS cascade in the host causes infection (Fleming et al. 2006).

3.2.2 ComD/ComE System

Induction of competence, biofilm formation, and bacteriocin production by *Streptococcus mutans* and *S. pneumoniae* is controlled by the ComD/ComE two-component regulatory system (Martin et al. 2013). Competence is induced in exponentially growing cultures by a secreted competence-stimulating peptide (CSP) that is produced as a pro-AIP encoded by *comC*, exported by the ComAB transporter and cleaved to generate the mature peptide. CSP constitutes, together with the membrane-bound histidine kinase ComD and the cognate response regulator ComE, the master competence switch (Pestova et al. 1996). Induced genes include *comAB* and *comCDE* themselves, resulting in a positive feedback loop that promotes synchronized development of competence throughout the population in response to increasing levels of CSP. ComE also induces expression of *comX*, which encodes an alternative sigma factor that stimulates expression of a larger group of late-phase genes, including those required for DNA uptake and recombination (Luo et al. 2003).

3.2.3 PapR/PlcR System

In some QS systems of Gram-positive bacteria, the pro-AIP is secreted and processed by extracellular proteases into the mature AIP. The active AIP is imported

back into the cytoplasm by a permease system and binds to a transcription factor, changing its conformation and activity, which regulates transcription of target genes. The PapR/PlcR system of *B. cereus* group has been extensively described (Rocha-Estrada et al. 2010). Peptide-activating PlcR (PapR) is an unmodified, linear signal peptide that is secreted and activated via proteolytic processing by NprB and then re-internalized via Opp system. At high cell density, the processed PapR binds to the sensor/transcription factor PlcR which controls expression of most of *B. cereus* virulence factors. Similar to *S. aureus*, the *B. cereus* group can be divided into four phenotypes based on the sequence and the specificity of the PlcR–PapR pair. Some phenotypes contain different species, and can therefore communicate across species, whereas in other cases, different isolates of the same species fall into different subgroups and are prevented from communicating with one another (Slamti and Lereclus 2005).

3.3 Quorum Sensing *by Cis-Unsaturated Fatty Acids*

3.3.1 DSF Signal in RpfC/RpfG System

The signal molecule known as DSF (diffusible signal factor) is a cis-unsaturated fatty acid first described in the plant pathogen *X. campestris* (Wang et al. 2004). Members of this family of signals may differ in the presence of branches, the presence of a second cis double bond, and in chain length. This type of molecule is detected by the RpfC/RpfG two-component system, where RpfC is a hybrid receptor histidine kinase that has dual role as sensor and biosynthesis regulator of the diffusible signal factor. At low cell population density, unphosphorylated RpfC promotes the formation of a complex with RpfF that limits the production of DSF. However, under cell concentration, these molecules accumulate, triggering the autophosphorylation of RpfC and thus the release of RpfF, resulting in increased DSF production (He et al. 2006). The cognate regulator RpfG is a phosphodiesterase involved in the degradation of the second messenger cyclic di-GMP. The phosphorylation of RpfG and consequent alterations in the level of cellular cyclic di-GMP affect the synthesis of virulence factors, extracellular enzymes, extracellular polysaccharide, biofilm dispersal and motility.

Synthesis of DSF is not restricted to *Xanthomonas*. New findings indicate a broader significance for this signaling molecule in the bacterial world. Related cis-unsaturated fatty acid molecules are produced by human pathogenic bacteria such as *Burkholderia cenocepacia* and *P. aeruginosa* and regulate a range of biological functions that includes cell growth, biofilm dispersal, antibiotic tolerance and virulence (Ryan and Dow 2011). Moreover, one bacterial species may produce more than one molecule pertaining to this signal family and, hence, these signal mediators are involved in intraspecies, interspecies and interkingdom communication, thus modulating microbial behavior. Different molecules of the DSF family have a role in communication between *B. cenocepacia* and *Stenotrophomonas*

maltophilia, and the interaction of *P. aeruginosa* with these two organisms is very intriguing because they are found together in a number of environmental niches such as infections of the cystic fibrosis lung. In fact, *P. aeruginosa* responds to these exogenous signals by modulating biofilm formation and polymyxin tolerance. Therefore, coinfection could modify the efficacy of polymyxins, which are being reintroduced into clinical practice as agents for treatment of *P. aeruginosa* infections. Molecules of this family have an impact not only in the dispersion of *P. aeruginosa* biofilms but also of those formed by a variety of other bacteria and even by *Candida albicans* (Davies and Marques 2009; Ryan and Dow 2011).

Multiple DSFs related to this family of signals, but showing some structural differences, have been detected among different bacteria, ranging from plant pathogens to the human oral cavity inhabitant *Streptococcus mutans*, whose signal molecule also acts as an interkingdom signal inhibiting the yeast–hyphal transition in *C. albicans* (Ryan and Dow 2011).

3.4 Quorum Sensing *by Alkyl-Quinolones (AQs)*

3.4.1 PQS System

To control virulence factor gene expression, *P. aeruginosa* also uses an additional non-Lux type QS system (Sect. 3.1). The transcriptional regulator PqsR is required to produce low-molecular weight derivatives of the 4-hydroxy-2-alkylquinolines family including the intercellular signals HHQ and PQS, which function in the transcriptional regulation of multiple virulence genes (Déziel et al. 2005; Heeb et al. 2011). These two primary AQ molecules, HHQ and PQS, function as PqsR ligands (Fig. 4). Notably, PqsR, LasR, and RhlR are interlinked. While PqsR controls operons which encode proteins mediating HAQ biosynthesis, the final step of PQS production, HHQ hydroxylation, appears to require a LasR-regulated gene (Xiao et al. 2006). Hence, evidence supports that the PQS circuit is intimately tied to the Las and Rhl QS systems to influence virulence (Rutherford and Bassler 2012).

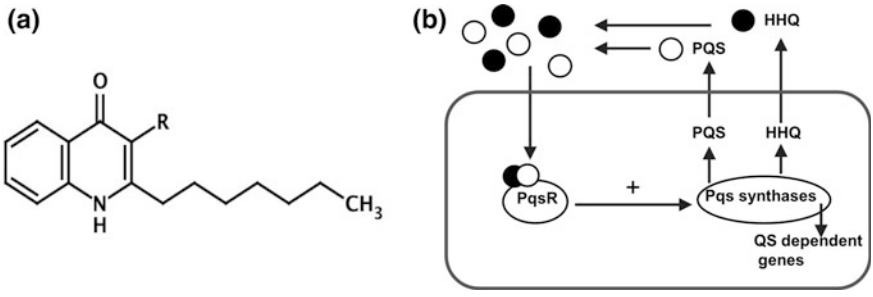

Fig. 4 **a** General structure of AQs signaling molecules. **b** Overview of an AQ-dependent QS

AQs produced by *P. aeruginosa* share the basic 4-quinolone backbone of commercially synthesized quinolone antibiotics. Indeed, *P. aeruginosa* produces an AQ that possesses antibiotic activity although its mechanism of action differs from synthetic quinolone antibiotics (Huse and Whiteley 2011).

3.5 Quorum Sensing *by Other Types of AIs*

Nodulation in the bradyrhizobia is modulated by a cell density factor designated bradyoxetin, and cycle dipeptides have been identified in several Gram-negative bacteria for their ability to activate A-HSL sensors, although at a much higher concentration than A-HSLs, which adds complexity and diversity to the QS language (González and Keshavan 2006).

Recently, a new QS signal molecule, called IQS, has been identified in *P. aeruginosa* that connects Las system and phosphate stress response mechanism to the downstream PQS and Rhl systems (Lee et al. 2013; Lee and Zhang 2015).

Some bacteria also produce inter-domain signals. For instance, AI-3 is an epinephrine-like molecule involved in host-bacteria interactions. The pathogen *Escherichia coli* serotype O157:H7 produces and responds to AI-3 but also responds to host hormones epinephrine/norepinephrine to modulate virulence and motility, possibly via QseC sensor kinase (QseB/QseC two-component signaling system regulated by LuxS), and alternative adrenergic receptors (Karavolos et al. 2013). The AI-3-dependent QS signaling cascade is present in all *Enterobacteriaceae* (Franzin and Sircili 2015). However, the identity of the corresponding synthase is currently unknown.

4 *Quorum Sensing* Components as Therapeutic Targets

The use of compounds to combat bacterial infection by conventional approaches results in substantial stress of the target bacteria, which rapidly select for resistance (Werner et al. 2008). In fact, antibiotic resistance is one of the greatest challenges in current chemotherapy. However, the increasing understanding of bacterial pathogenesis and intercellular communication opens ways for novel strategies to develop drugs against infectious diseases. A promising alternative is to target virulence rather than bacterial growth (Clatworthy et al. 2007; Cegelski et al. 2008; Barczak and Hung 2009; Rasko and Sperandio 2010). In this sense, QS systems are attractive targets for new antimicrobials because they control virulence and no human homologues to QS components are known. Antibiotics target viability, while anti-virulence agents target functions necessary for infection, thus disarming bacteria and allowing the immune system to overcome infection. However, when considering QS inhibition as an anti-infective strategy, the emergence of mutant strains in key QS genes in clinical samples should be considered, so that the activity

of QSIs against fresh clinical isolates rather than laboratory reference strains should be evaluated (Rampioni et al. 2014). Presumably, QSIs should exert less selective pressure for developing resistance since most virulence traits are not essential for bacterial survival. In addition, inhibiting QS may not only reduce virulence but also restore susceptibility to conventional antimicrobials (Rampioni et al. 2014). Hence, such agents could be administered in combination with traditional agents to increase their efficacy.

The dependence of QS on small molecule signals has inspired the design of molecules that can intercept these signals and thereby perturb bacterial group behaviors. On the other hand, as QS-mediated processes are often involved in interactions with hosts, it is not surprising that higher organisms have developed mechanisms to block QS. Thus, signal interference mechanisms existing in nature can also be exploited for preventing bacterial infections.

Numerous small-molecule inhibitors of bacterial QS systems have been identified using high-throughput screening of chemical libraries. Many strategies use modeled antagonists based on signal and/or receptor molecular structures (Smith et al. 2003a, b; Suga and Smith 2003) or on enzymes that degrade the QS signal (Dong et al. 2001). Broadly, interference with QS includes signal synthesis inhibition, prevention of signal reception (antagonist for receptor, signal trapping), signal degradation, suppression of QS circuit and promotion of immune response to signals.

4.1 Interference with QS Systems of Pseudomonas Aeruginosa

Pseudomonas aeruginosa is a typical model organism in QS studies. As an opportunistic pathogen, it may cause serious infectious in immune-compromised patients and become a major cause of nosocomial infections; often, chronic infections associate with cystic fibrosis and wound infections after burns. In all cases, this bacterium uses QS to produce a wide range of virulence factors that promote its disease-causing ability. As a whole, the QS systems of this bacterium control the expression of more than 10 % of genes for multiple virulence factors, secondary metabolites, swarming motility, and biofilm development (Williams and Camara 2009). *P. aeruginosa* has two main A-HSL-mediated QS systems. The first includes Las and Rhl (Sect. 3.1), and the second one the PQS system (Sect. 3.4) plus the IQS system (Sect. 3.5). Because LasR sits at the top of the *P. aeruginosa* QS cascade, identifying LasR inhibitors has been a major focus of interest. In general, analogs are potential antagonists of the native ligand (McInnis and Blackwell 2011).

Several LasR inhibitors have been modified to act as competitive inhibitors of RhlR, and some resulting molecules are potent inhibitors of both receptors. Examples include C_{10}-CPA and mBTL (Ishida et al. 2007; O'Loughlin et al. 2013). Screens of small synthetic molecule libraries have revealed additional inhibitors like the compounds TP-5, V-06-018, and PD-12 (Müh et al. 2006a, b). These molecules

show promising in vitro results in preventing pathogenesis but their activity in animal models remains to be assessed (Schuster et al. 2013).

Some natural compounds also behave as A-HSL inhibitors. Halogenated acyl-furanones from the marine microalga *Delisea pulchra* structurally resemble the HSLs, suggesting that they may target LuxR-type proteins, likely in a nonagonist fashion by accelerating LuxR-type protein turnover (Manefield et al. 2002; Kim et al. 2008). Natural furanones have a limited inhibitory effect on the QS of *P. aeruginosa*, but the synthetic analogs C-30 and C-56, which lack the alkyl side chain, exhibit interference in the LasR-mediated *lasB* gene expression (encoding elastase associated with virulence), render *P. aeruginosa* biofilms susceptible to clearance by detergent and antibiotics, and show significant efficacy in a murine infection model (Wu et al. 2004; Christensen et al. 2012). Other furanones derivates have also been shown with increased effectiveness (Taha et al. 2006; Kim et al. 2008). In any case, despite initial promises, halogenated furanones are highly reactive and toxic in vivo for clinical development. However, the onset of the infection is Las-dependent suggesting that at least LasR inhibitors might be used as prophylactics (Rutherford and Bassler 2012).

Other natural products have A-HSL-dependent QSI activity, although their mechanism of action is often unknown. Among them, protoanemonin, isolated from plants of the *Ranunculaceae* family inhibits QS signaling of *P. aeruginosa* in naturally occurring concentrations (Bobadilla Fazzini et al. 2013). Also, solenopsin A, a venom alkaloid from the ant *Solenopsis invicta*, exhibits moderate structural similarity to 3-oxo-C_{12}-HSL. In *P. aeruginosa*, solenopsin efficiently disrupts QS signaling, likely by targeting the C_4-HSL-dependent Rhl QS system (Park et al. 2008). Similarly, the flavan-3-ol catechin from *Combretum albiflorum* interferes with the perception of C_4-HSL by RhlR (Vandeputte et al. 2010). Other natural QSIs can compete with *P. aeruginosa* AHLs for binding to LasR or RhlR, as for instance, ajoene from garlic and iberin from horseradish extracts (Jakobsen et al. 2012a, b). These are structurally unrelated linear sulfide compounds, whereas the mycotoxins penicillic acid and patulin have structures somewhat similar to natural furanone compounds (Rasmussen et al. 2005). Patulin accelerates LuxR turnover like furanone C-30. Besides, ajoene and patulin enhance the susceptibility of *P. aeruginosa* biofilms to tobramycin and promote faster clearing of the bacterium in mouse lung infection models.

AHLs-degrading organisms (bacteria, fungi, plants, and mammalians) are widespread in nature and can inactivate or modify A-HSL by three known classes of QQ enzymes: lactonases, acylases, and oxidoreductases (Chen et al. 2013; LaSarre and Federle 2013). Most A-HSL-degrading enzymes are either lactonases that cleave the ester bond in the HSL ring or acylases that irreversibly hydrolyze the amide bond between the HSL moiety and the acyl chain. Lactonases are generally metalloproteins with very broad A-HSL substrate specificity, likely due to the fact that the targeted HSL ring is conserved among all A-HSLs. Some bacteria, as *Bacillus* spp., express lactonases that degrade the HSL ring. Transgenic plants expressing the *Bacillus* A-HSL lactonase show resistance to QS-dependent bacterial infection (Dong et al. 2001). Thus, the enzymatic inactivation of A-HSL by cloning

QQ genes could be an effective approach to control bacterial infections. In addition, a set of mammalian enzymes termed paraoxonases also have A-HSL lactonase activity (Amara et al. 2011). Unlike lactonases, acylases exhibit substrate specificity. The first A-HSL acylase to be characterized was obtained from *Ralstonia eutropha* (Lin et al. 2003) and its heterologous expression in *P. aeruginosa* was shown to abolish the accumulation of both Las and Rhl signals. In particular, one A-HSL acylase has been used to develop a dry, stable, and inhalable powder formulation for the treatment of pulmonary *P. aeruginosa* infections (Wahjudi et al. 2013). The least common class of QQ enzymes, the oxidoreductase, does not degrade but rather modify the AHL to an inactive form by oxidizing or reducing the acyl side chain (Uroz et al. 2005).

Alternate strategies for attenuating A-HSL QS systems of *P. aeruginosa* may involve sequestration of the signal or the application of QS anti-activators or suppressors (Kato et al. 2006; Kaufmann et al. 2006; Piletska et al. 2011). Another approach is to develop catalytic antibodies capable of degrading AIs and rendering them inactive (De Lamo et al. 2007). On the other hand, screening of QS anti-activators or suppressors includes agonists and antagonists of the LuxR homolog QscR (Sect. 3.1), which represses the Las and Rhl systems (Mattmann et al. 2008). In this context, propanoyl-HSL is particularly interesting since inhibits LasR and simultaneously activates QscR. Such dual activity might make this compound a potent inhibitor of QS in *P. aeruginosa*, since the tandem inhibition of LasR and activation of QscR should be synergistic (Mattmann and Blackwell 2010).

A new via to identify QSIs with clinical applications is searching for anti-QS activity among the drugs already approved for other clinical use in humans. Thus, anthelmintic drug niclosamide strongly inhibited Las and Rhl QS systems, production of virulence factors, biofilm development, and prevented *P. aeruginosa* pathogenicity in a model of acute infection (Imperi et al. 2013). Treatment at subinhibitory concentrations with macrolides, generally ineffective against Gram-negative bacteria, improves respiratory function in chronic lung infectious diseases caused by *P. aeruginosa* (Southern et al. 2011), probably influencing their QS systems. In particular, azithromycin may repress the activity of both the levels of LasI and Rhl and the expression of dependent proteins such as elastase, rhamnolipid, and pyocyanin (Hirakawa and Tomita 2013). Azithromycin has been actually applied routinely to cystic fibrosis patients as an anti-QS treatment in several centers around the world (Wu et al. 2015). Despite its success in reducing *P. aeruginosa* virulence, macrolides are no ideal because excessive usage is associated with bacterial resistance.

In addition to Las and Rhl systems, *P. aeruginosa* employs PQS system (AQs) to cell communication (Sect. 3.4). In particular, 2,4-dioxygenase Hod downregulates PQS-controlled genes and reduces the virulence of *P. aeruginosa* in a plant leaf infection model (Pustelny et al. 2009). On the other hand, the structure of PqsD, which catalyzes the last and key step in HHQ formation, has inspired the design of AQs biosynthesis inhibitors (Weidel et al. 2013). Antagonists of PqsR receptor display reduced PqsR-antagonistic activity when evaluated in *P. aeruginosa* (Klein et al. 2012; Zender et al. 2013). Also, quinazolinone derivatives strongly reduce AQ

biosynthesis, virulence gene expression and biofilm development (Ilangovan et al. 2013), whereas a modified HHQ analog agonist in *P aeruginosa*, is a potent in vivo anti-virulence compound and targets PqsR (Lu et al. 2014).

4.2 *Interference with QS Systems of* Vibrio Cholerae

V. cholerae integrates two separate SAM-derived QS signals, CA-1 and AI-2 (Sect. 3.1). The enzyme 5′-methylthioadenosine nucleosidase (MTAN) is directly involved in the biosynthesis of both AIs. Thus, inhibition of MTAN provides a method for blocking not only CAI-1 production, but also AI-2. Two general classes of MTAN inhibitors tested are derived from immucillin (Gutierrez et al. 2009). These analogs also reduce biofilm formation and bind to the catalytic active site of the protein producing hydrophobic stacking interactions. On the other hand, high-throughput screening has led to the identification of molecules able to suppress *V. cholerae* virulence factor expression inhibiting the ATPase activity of the response regulator LuxO (Ng et al. 2012; Faloon et al. 2014).

Vibrio cholerae QS is crucial in switching from an infection/virulence state at low cell density to a dissemination state at high cell density. Hence, molecules that activate QS have the potential to repress its virulence; in other words, therapeutic treatment of cholera would require QS agonism while bacteria are at low cell density to promote repression of virulence factors and facilitate premature exit from the intestine. Exposure of *V. cholerae* to spent culture supernatants from a strain of *E. coli* expressing CqsA (CAI-1 synthase) or coculture with this strain decreased cholera toxin production by the pathogen. Furthermore, CAI-1 expressed by the *E. coli* strain colonizing the intestines of mice, significantly attenuated *V. cholerae* infection (Duan and March 2010). These results not only provide encouraging outlook for the treatment of cholera, but also demonstrate the potential power of probiotic bacteria as transmission vectors of signaling molecules (Lasarre and Federle 2013).

In a recent study, oil sophorolipids have been shown to act as QSIs with anti-biofilm activity against *V. cholerae* (Mukherji and Prabhune 2014).

4.3 *Interference with QS Systems of* Staphylococcus Aureus

Staphylococcus aureus is a dangerous opportunistic pathogen with strains resistant to most antibiotics. *S. aureus* has multiple virulence factors including extracellular toxins, such as α-hemolysin encoded by *hla*, and cell surface adhesion factors, such as protein A encoded by *spa* (Chan et al. 2004). Their expression is coordinated through several key regulators, of which the effector RNAIII of Agr QS system is

central (Sect. 3.2). An RNAIII-inhibiting peptide (RIP) inhibits *S. aureus* biofilm formation and toxin production in laboratory animals (Cirioni et al. 2007). On the other hand, the fungal metabolite ambuic acid inhibits the AIP biosynthesis in Gram-positive bacteria, including *S. aureus* (Nakayama et al. 2009).

Polymorphism of the *agr* locus allows to distinguish four types of Agr system with distinct AIPs and AIPs-receptors designated AgrC-I, -II, -III, and -IV. Apart from AIP-I and AIP-IV, which are able to cross-activate, all other types cross-inhibit suggesting that QS in *S. aureus* may function not only as mechanism to control gene expression but also as tool to eliminate competitors and favor more efficient host colonization. These findings have led to the discovery of AIP antagonists, and also to immunotherapy-based QQ with the generation of anti-AIP antibodies which interfere with this system in vitro and in vivo. Thus, administration of a mAb specifically inhibited Agr system in AIP-IV strains, reduced virulence factors, and prevented skin abscess in a mouse skin infection model. Moreover, this antibody provided complete protection against a lethal *S. aureus* challenge (Park et al. 2007).

While the tail of the AIP is essential for Agr activation, the macrocyclic ring is responsible for antagonistic activity. On these ideas, synthesis of inhibitors based on truncated AIPs has been performed (Lyon et al. 2002). However, there are only few reports of natural antagonists to this system (Mansson et al. 2011). Ngercheumicims isolated from a marine *Photobacterium* strain, inhibit transcription of the RNAIII in *S. aureus*, although to a lesser extent than solonamides, which are cyclodepsipeptides from the same strain (Kjaerulff et al. 2013). Like solonamides, these molecules have structural similarity to the AIPs of *S. aureus* so that may interfere with QS pathways present in the marine environment or even act as alternative QS molecules. Recently, it has been shown that one solonamide also interferes with the binding of AIPs to AgrC receptor (Nielsen et al. 2014). Some non-peptidic agents have been identified as inhibitors of AgrC including Las signal of *P. aeruginosa* (Murray et al. 2014). Moreover, a screening assay conducted to assess the effect of compounds or extracts on three key virulence genes expression (*hla, spa*, and *rnaIII*), identified two xanthones as novel QSIs of the Agr system (Nielsen et al. 2010).

Staphylococcus aureus can produce two types of biofilm matrices, one utilizing surface proteins, regulated by Agr system (see above), and other based on synthesis of exopolysaccharide, which is negatively regulated by AI-2 (Sect. 3.1) in an *icaR*-dependent manner (Yu et al. 2012). Although a functional luxS gene has been found (Zhao et al. 2010), the QS cascade that connects AI-2 with IcaR expression is not yet known.

4.4 Interference with QS Systems of Streptococci

Some members of the genus *Streptococcus* are endogenous opportunistic agents (dental caries) while others are exogenous pathogens that cause infections ranging

from mild skin or respiratory diseases to serious infections (pneumonia and septic shock). Streptococci biofilm formation is controlled by at least two QS systems, the ComD/ComE (CSP) (Sect. 3.2) and the LuxS/LuxPQ (AI-2) (Sect. 3.1). A CSP derivative carrying a single substitution suppresses *S. pneumoniae* virulence both in vitro and in a mouse infection model. Also, when DNA-encoding antibiotic resistance is supplied, the derivative blocks the ability of the bacteria to transform into an antibiotic-resistant strain (Zhu and Lau 2011). These results illustrate the potential of CSP analogs not only to inhibit virulence but also to block horizontal transfer of antibiotic resistance (Rampioni et al. 2014).

Moreover, synthetic furanones C-30 and C-56 have been widely studied since they can inhibit QS controlled behaviors (Sect. 4.1). They decrease the production of extracellular virulence factors and biofilm formation by *S. mutans* (He et al. 2012). The mechanisms by which furanone affects biofilms remain unknown, but they likely interfere QS circuits, mainly LuxS/LuxPQ, in a similar way to LuxI/LuxR. It has been reported that metabolic derivatives of the flowering plant *Petiveria alliacea*, which can reduce *P. aeruginosa* biofilm formation and QS (Cady et al. 2012), also affect biofilm development of oral bacteria, including *S. mutans* and *S. sanguinis*, probably by interfering with AI-2-based cell–cell communication (Kasper et al. 2014).

4.5 *Interference with PapR/PlcR System of* Bacillus Cereus *Group*

The *B. cereus* group consists of six highly related species of Gram-positive, spore-forming bacteria that includes some relevant human pathogens, like *B. cereus* and *Bacillus anthracis*. The expression of various virulence factors is activated by the pleiotropic regulator, PlcR, specific to the *B. cereus* group. The activity of PlcR depends on the presence of the small signaling peptide PapR that acts as a QS effector (Sect. 3.2). When *Bacillus subtilis* SC-8 and *B. cereus* are cocultured, PapR is assumed to stimulate antibiotic production by *B. subtilis* SC-8 that has a narrow antagonistic activity against bacteria in the *B. cereus* group (Yeo et al. 2014). Recent findings focusing on QS paths of these microorganisms show lessons that can be learned for domestication of their sociality and competence (Pollak et al. 2015; Jakobs and Meinhardt 2015).

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The Case of Lipid II: The Achilles' Heel of Bacteria

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1 Introduction

Lipid II (Fig. 1) is a membrane-bound lipid involved in bacterial cell wall synthesis, which is the target for a variety of antimicrobials, including the glycopeptide vancomycin. It is a complex molecule composed of a sugar moiety, a lipid portion, and a pentapeptide. The full name of this compound is *N*-acetylmuramoyl-L-alanyl-D-isoglutaminyl-L-lysyl-D-alanyl-D-alanine-diphosphoundecaprenyl-*N*-acetylglucosamine. This structure allows the molecule to locate in the lipid matrix of the cellular membrane in vivo, and with organic solvents in vitro. Lipid II could represent a suitable target for different biomolecules, including human beta-defensin 3 (Sass et al. 2010) and oyster defensins. These compounds specifically inhibit peptidoglycan synthesis in Gram-positive bacteria, by directly binding to Lipid II, resulting in accumulation of the UDP-*N*-acetylmuramoyl-pentapeptide cell wall precursor (Schmitt et al. 2010). According to Schneider and Sahl (2010a): "*Lipid II represents a particular 'Achilles' heel' for antibiotic attack in bacteria.*" There are a variety of compounds that can use lipid II as their specific target and some of them will be addressed in this chapter.

Barr and Rick (1987) discovered lipid II while studying the role of lipids in the synthesis of the enterobacterial common antigen. They named the new compound lipid II because it is a lipid I derivative that contains *N*-acetyl-D-glucosamine and pyrophosphorylundecaprenol. The processes in which these two lipids are involved can be inhibited by tunicamycin, an antibiotic used, since the mid-1970s, in

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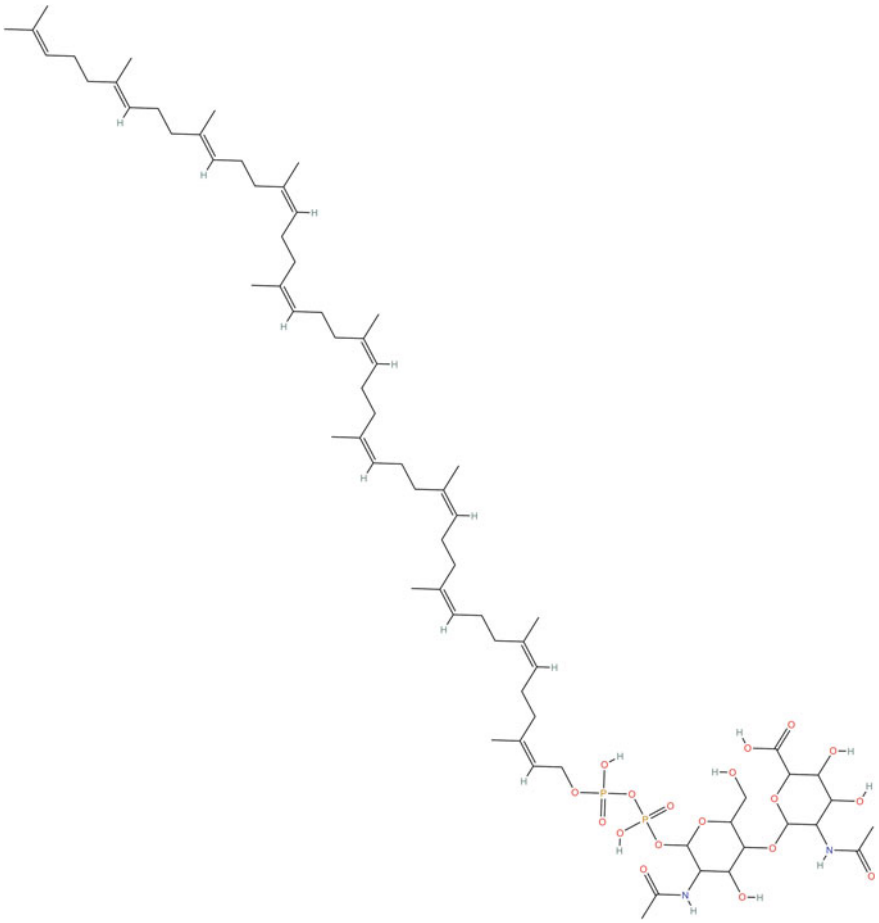

Fig. 1 Structure of lipid II, according to Barr and Rick (1987)

structural studies of eukaryotic glycoproteins (Kuo and Lampen 1974; Lehle and Tanner 1976; Sánchez et al. 1982; Huffaker and Robbins 1982). The *rff* genes in *Salmonella typhimurium* are involved in both the formation of membrane lipids and the synthesis of the enterobacterial common antigen. In particular, the rff-726 mutation affects the structural transferase gene, an enzyme that catalyzes the transfer of *N*-acetyl-D-mannosaminuronic acid from UDP-*N*-acetyl-D-mannosaminuronic acid to lipid I, to generate lipid II (Barr et al. 1988). In fact, re-examination of the mutant strain revealed that it was unable to synthesize lipid III, due to a defect in the structural gene coding for the enzyme 4-acetamido-4,6-dideoxy-D-galactose transferase (Meier-Dieter et al. 1990). Hence, lipid II generates lipid III by addition of fucose (Barr et al. 1989).

The amount of lipid II present in the bacterial cell wall must be closely controlled for the cell wall to function properly, as excessive accumulation of this compound, due mutation, can produce deleterious effects. For example, there are lipid II-accumulating mutant *Escherichia coli* strains that are sensitive to bile salts, while others have abnormal synthesis of outer membrane proteins, such as LamB, which affects the strain's lambda bacteriophage-sensitivity. The fact that excessive lipid II accumulation creates abnormal *E. coli* phenotypes, suggests that abnormal quantities of lipid II can perturb the structure of the bacterial cell (Danese et al. 1998).

Lipid II is important for bacteria, even in organisms, such as the intracellular *Wolbachia* species, that lack a cell wall. These organisms lack the enzymes required to synthesize peptidoglycan, but maintain the capacity to produce lipid II. But, if the microorganisms lose the ability to produce lipid II, their bacterial growth is disturbed and the peptidoglycan-associated lipoprotein is redistributed to other cellular locations (i.e., lipid II is required for normal bacterial growth; Vollmer et al. 2013). Lipid II, however, is the target of certain colicins, such as colicin M, a molecule produced by *E. coli*, *Pseudomonas*, and other bacteria to fight bacterial competitors (Touzé et al. 2012). This raises the possibility of using colicins, either alone or in combination with lantibiotics targeting lipid II, in the clinical treatment of broad-spectrum-resistant bacteria. Grinter et al. (2012) reported that syringacin M is capable of degrading lipid II. This molecule is produced by the phytopathogen *Pseudomonas syringae* pv. *tomato*, and surprisingly, it is a compound that shares an unexpected evolutionary relationship with colicin M-related bacteriocins. Syringacin M kills susceptible bacterial cells through a highly specific phosphatase activity that targets lipid II, thus interrupting normal peptidoglycan synthesis. The peptide binds Ca^{++}, which is coordinated by a conserved aspartic residue, and is essential for the catalytic activity.

Lipid II can be targeted by small molecules, either natural or synthetic, which act as peptidoglycan biosynthesis inhibitors. Derouaux et al. reported, in 2011, that two small molecule analogues (2-chlorophenyl) methyl-2-methyl-5-methylsulfanylindol-3-yl ethanamine and 3,4-dichlorophenyl)methyl-2-methyl-5-methylsulfanylindol-3-yl ethanamine) exhibited antibacterial activity, mainly against Gram-positive bacteria.

The peptidoglycan, in the cell wall of *Staphylococcus aureus* contains a high degree of crosslinking, is almost free of carboxyl groups (due to amidation of D-glutamic) and is highly negatively charged. Münch et al. reported, in 2012, that *Staphylococcus* mutants with a reduced degree of amidation show increased susceptibility to methicillin. These authors identified two genes (GatD and MurT) that are required for amidation, resulting in a physically stable bi-enzyme complex. In this Gram-positive bacterium, both genes are organized in an operon and are both essential for proper cell wall formation. *S. aureus* contains an additional lipid II-derived molecule (lipid II Gly_5), synthesized by the enzyme lipid II: glycine glycyltransferase (EC 2.3.2.16) that catalyzes the transfer of glycine from a charged tRNA to *N*-acetylmuramoyl-L-alanyl-D-isoglutaminyl-L-lysyl-D-alanyl-D-alanine-diphosphoundecaprenyl-*N*-acetylglucosamine (Schneider et al. 2004).

Lipid II can be chemically synthesized in sufficient amounts to test the action of lipid II-interacting molecules, hence that could putatively be used as antibacterials. In this line, Meng et al., reported (2011) the synthesis of polyprenyl *N*-glycolyl lipid II, as a substrate for mycobacterial transglycosylase, in order to evaluate biomolecules to combat tuberculosis. All things considered, and as indicated by Martin and Breukink (2007): "*lipid II is an essential cell-wall precursor required for the growth and replication of both Gram-positive and Gram-negative bacteria. Compounds that use lipid II to selectively target bacterial cells for destruction represent an important class of antibiotics*." This group of compounds also includes the "last-resort" class of antibiotics, with vancomycin as the best known and perhaps the most relevant drug. These represent a group of drugs that are acquiring even greater relevance, as antibiotic-resistant bacteria continue rising in prevalence all over the world. Lantibiotics (see below) are lipid II-targeting molecules that represent a great hope for future generations of novel antibiotics and deserve a relevant place in this review. The downside of these molecules is that their oxidation can render them totally ineffective. This process was described for nisin (Wilson-Stanford et al. 2009), the first lantibiotic approved for use in foodstuffs and clinically. The term lantibiotic was coined to define a group of antibiotics containing the structural group lanthionine. Lanthionines are composed of a single sulfur atom that is linked to the beta-carbons of two alanine moieties, with the peculiarity that the sulfur is amenable to oxidation. Lantibiotics are ribosomally synthesized antimicrobial peptides, as opposed to either glycopeptide or lipopeptide antibiotics, which are synthesized via the specific action of biosynthetic enzymes. Lantibiotics exert their antimicrobial action by docking onto lipid II, inhibiting bacterial cell wall biosynthesis and forming a stable pore in the microbial cell membrane (Islam et al. 2012a).

2 Lipid II-Acting Lantibiotics

Lantibiotics are prokaryotic polypeptides active, at low (nanomolar) concentrations, against other prokaryotes (Drider and Rebufatt 2011). They are ribosomally synthesized as precursor peptides, which must undergo posttranslational modifications to attain their mature, biologically active, form. Lantibiotics contain the modified amino acids lanthionine and methyllanthionine, produced by dehydration of Ser/Thr residues, followed by the reaction of the resulting dehydro amino acids with cysteines to form thioether linkages.

Nisin represents the first (Rogers and Whittier 1928) and, to date, the most important lantibiotic, and is mainly used in food science (for a recent review, see Bierbaum and Sahl 2009). Mattick and Hirsch purified nisin, in 1947, from the culture media of *Lactococcus lactis*. The second lantibiotic, subtilin, was described by Jansen and Hirschmann (1944); this was followed by cinnamycin (Benedict et al. 1952). Sahl (1991) organized the lantibiotics into two groups. Type A includes molecules with long flexible structures that tend to form pores in the cellular membranes; while those belonging to type B are globular and usually inhibit cell

wall synthesizing enzymes. Lantibiotics do not always lyse their target bacteria, indeed the bacterial cells can continue growing in the presence of the lantibiotic, albeit at lower rate. This is the case for lacticin 481 (O'Sullivan et al. 2002), which induces enzyme leakage (lactate dehydrogenase (LDH) and post-proline dipeptidyl aminopeptidase) in the target bacterium. This property of lacticin is used in the dairy industry to improve cheese quality and flavor. Similarly, Martínez-Cuesta et al., in 2006, demonstrated that lacticin 3147 enhances aldehyde formation in *L. lactis* IFPL730 cultures, hence enhancing cheese flavor.

Lipid II, as well as other bactoprenol-bound cell wall precursors, could represent important targets for controlling bacteria proliferation. In fact, a number of natural products inhibit bacterial cell wall biosynthesis by targeting lipid II (Schneider and Sahl 2010b). These substances include glycopeptides, lipopeptides, lipodepsipeptides, and lantibiotics, in particular those belonging to group II (or type B). As indicated above, lantibiotics are antibiotic peptides that contain rare thioether amino acids; they act on the lipid II of Gram-positive bacteria, but are inactive on the producing strains, due to the presence of immunity systems (Lan I proteins). Christ et al. (2012) studied the structure of the lantibiotic immunity protein, SpaI, from *Bacillus subtilis* ATCC6633. SpaI is a 16.8 kDa lipoprotein attached to the outside of the cytoplasmic membrane via a covalent diacylglycerol anchor.

Mersacidin is a lantibiotic, first described by Ganguli et al. (1989) in some *Bacillus* strains, capable of acting on other Gram-positive bacteria (unless they harbor the appropriate resistome; Kingston et al. 2013). Its mode of action was investigated by Niu and Neu (1991), and compared to the action of the glycopeptide vancomycin and the peptolide daptomycin. This lantibiotic spans four intra-chain thioether bridges, formed by 3-methyllanthionine and S(2-aminovinyl)-3-methylcysteine residues (Chatterjee et al. 1992). Mersacidin was crystallized by Schneider et al., in 2000. It possesses a globular structure (thus, it belongs to the type B lantibiotics group, see above), with enzyme-inhibiting abilities and a low degree of flexibility (Fig. 2). In 1997, Brötz et al. reported that mersacidin inhibited

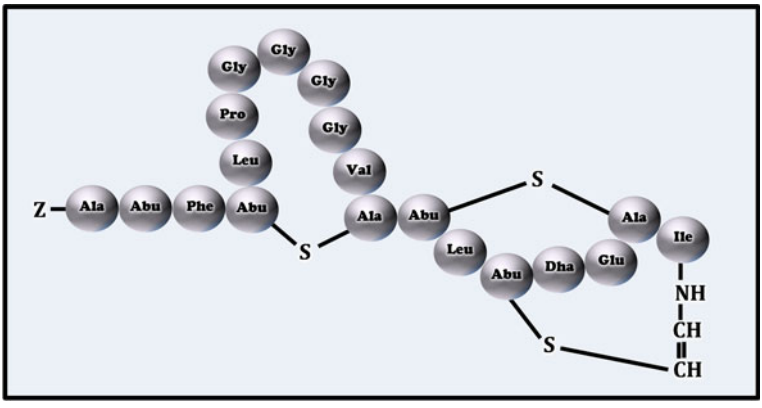

Fig. 2 Structure of mersacidin. The abbreviations used are: *Abu* = Aminobutyric acid; *Dha* = Dehydroalanine

peptidoglycan biosynthesis at the level of transglycosylation. The mechanism of action of mersacidin was found to be different from that of vancomycin, since the former inhibited peptidoglycan formation from UDP-*N*-acetylmuramoyl-tripeptide in vancomycin-resistant *Enterococcus faecium* strains. This means that mersacidin could represent a viable alternative for treating infections caused by vancomycin-resistant *S. aureus*. Indeed, electron micrographs of mersacidin-treated staphylococci displayed a thinner cell wall, thus indicating that this drug can inhibit cell wall synthesis, which eventually leads to bacterial death (Brötz et al. 1995; Molitor et al. 1996).

Brötz et al. (1998) demonstrated that mersacidin inhibits peptidoglycan synthesis by targeting Lipid II; they showed that addition of purified lipid II to the culture broth of *S. aureus* efficiently antagonized the bactericidal effect of the lantibiotic. Additionally, the formation of the complex lipid II–mersacidin does not involve the C-terminal D-alanyl–D-alanine moiety of the lipid intermediate, but it appears to recognize a target different from that of vancomycin and related antibiotics. Indeed, mersacidin can eradicate methicillin-resistant *S. aureus* in a mouse rhinitis model (Kruszewska et al. 2004). It is worth mentioning that the bacteria capable of synthesizing lantibiotics (Gram-positive) also have the capacity of carrying out their posttranslational modifications (encoded by a set of clustered genes) and, of course, they can also produce the necessary compounds to protect themselves from their own antibacterial molecules (Guder et al. 2002). Mersacidin is produced by the ribosome as a 68 amino acid long peptide, but further processing results in the elimination of a 48 amino acid leader sequence, producing a mature polypeptide of 1824 Da (Schneider et al. 2000).

The gene cluster associated with mersacidin was described by Altena et al., in 2000; it encompasses a 12.3 kb locus, located at 348° on the *Bacillus* sp. strain HIL Y-85, 54,728, chromosome. This locus contains 10 open reading frames, which include the lantibiotic production gene (*mrsA*), as well as all the necessary genes for posttranslational modification and transportation of the antibiotic. The structural gene for mersacidin production is transcribed by the housekeeping sigma factor A, and is subjected to autoregulation (Schmitz et al. 2006). In addition, the cluster contains three regulatory genes (for a comprehensive review, see Altena et al. 2000). A peculiar fact is that synthesis of active mersacidin requires a FAD-dependent flavoprotein (MrsD), which belongs to the homo-oligomeric, flavin-containing, Cys decarboxylases family (Majer et al. 2002).

Hsu et al. (2003) published a very interesting article using nuclear magnetic resonance (NMR) to study the interaction between mersacidin and lipid II, in dodecylphosphocholine micelles. They showed that the lantibiotic's conformational changes are the key to its antimicrobial activity. The junction between the residues Ala-12 and Abu-13 serves as a hinge for the opening and closure of ring-like structures and this, in turn, modulates the surface charge distribution. The authors suggested that: "*the observed conformational adaptability of mersacidin might be a general feature of lipid II-interacting antibiotics/peptides.*" Böttiger et al. (2009) investigated the effect of Ca^{++} on mersacidin activity, and found that these ions

appear to facilitate the interaction between the lantibiotic and the cellular membrane, rather than being an essential part of a peptide–lipid II complex.

Szekat et al. (2003) used site-directed mutagenesis to study the structural gene for mersacidin, and found that replacing Dha-16 for isoleucine drastically reduced the lantibiotic's activity. Similarly, replacement of either Glu-17 for alanine, or an aromatic for an aliphatic hydrophobic residue at position 3, also resulted in low biological activity. Appleyard et al., in 2009, constructed a saturation mutagenesis library of the residues of mersacidin not involved in cycle formation. In this way, they obtained a variety of mersacidin analogues with biological activity, and this approach should allow the future generation of a whole family of mersacidin-related antibiotics with different antibacterial activity. Herzner et al. (2011) were able to generate, by genomic DNA transfer, a *Bacillus amyloliquefaciens* FZB42 strain with the ability to produce mersacidin. These authors conclude: "*the new mersacidin producer strain enables us to use the full potential of the biosynthetic gene cluster for genetic manipulation and downstream modification approaches*." There are, however, good natural mersacidin producers, such as the *B. amyloliquefaciens* strain reported by Hao et al., in 2012, so it may not become necessary to use transgenic strains to produce this lantibiotic.

Actagardine, also known as **gardimycin**, is another lantibiotic structurally related to mersacidine that also displays lipid II-binding ability. Actagardine was denominated gardimycin by Arioli et al., who in 1976 described it as an antibiotic with in vitro antibacterial activity against Gram-positive bacteria, as well as against Gram-negative microorganisms such as *Neisseria gonorrhoeae*. This lantibiotic is produced by *Actinoplanes garbadinensis* and *Actinoplanes liguriae*, and in vivo parenteral or rectal administration of this drug confers good protection against pathogens. Coronelli et al. (1976) partially characterized this molecule as a peptide antibiotic. This new antibacterial was later shown to specifically inhibit peptidoglycan synthesis in the cell wall of *B. subtilis*, resulting in the accumulation of both uridine 5′-diphosphate-*N*-acetylmuramylpentapeptide and lipid II, the intermediate in peptidoglycan synthesis (Somma et al. 1977). The structure of actagardine was finally elucidated by Kettenring et al. (1990), as a tetracyclic 19-residue peptide lantibiotic This was later on corrected by Zimmermann et al. (1995), who showed that the molecule contains one lanthionine bridge and three overlapping beta-methyl lanthionine bridges (Fig. 3). There are currently several actagardine derivatives, such as Ala(0)-actagardine, Lys(0)-actagardine and Ile(0)-actagardine, and some of them possess a higher biological activity than the native actagardine (Vértesy et al. 1999). Recently, Boakes et al. (2012) used saturation mutagenesis to generate a number of actagardine mutants displaying novel properties, as compared to the parental strain. In particular, one of them (V15F) demonstrated improved activity against a variety of Gram-positive pathogens, including *Clostridium difficile*. Boakes et al., in 2009, studied the genes encoding actagardine biosynthesis. The gene cluster includes the structural gene, *gar*A, which encodes the actagardine prepropeptide, *gar*M, involved in the dehydration and cyclization of the prepeptide, several putative transporter and regulatory genes, as well as a monooxygenase gene, named *gar*O. Cloning and heterologous expression of this gene cluster resulted in

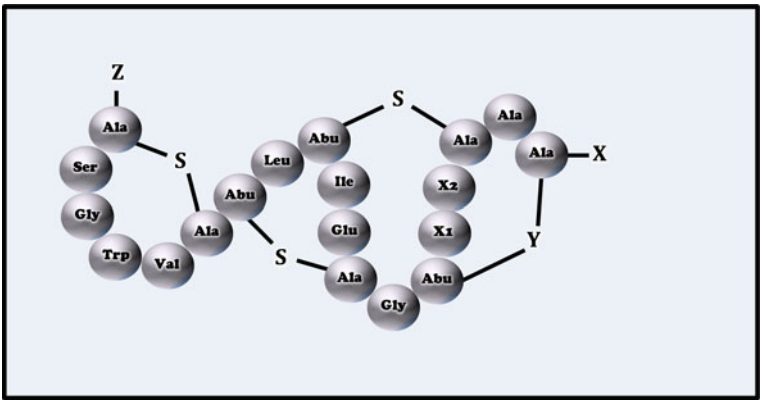

Fig. 3 Basic structure of actagardine. The symbols used are: *X1* = Leu or VaI; *X2* = Leu or VaI

bacterial strains, such as *Streptomyces lividans* (Boakes et al. 2009) and *E. coli* (Shi et al. 2012), expressing the lantibiotic.

Mannopeptimycin and ramoplanin These two compounds are not true lantibiotics but, due to their cyclic nature, they merit to be noted here. They can be used, in combination with mersacidins, for the treatment of staphylococci and/or streptococci. He et al., in 2002, isolated mannopeptimycin and ramoplanin from *Streptomyces hygroscopicus* LL-AC98, as novel compounds with activity against both methicillin-resistant staphylococci and vancomycin-resistant enterococci. These antibiotics, designated mannopeptimycins α-Ɛ (1–5), are glycosylated cyclic hexapeptides, characterized by containing two stereoisomers of a rare amino acid (α-amino-β-[4′-(2′-iminoimidazolidinyl)]-β-hydroxypropionic acid). The core of this antibiotic is attached to a mannosyl monosaccharide moiety in 2, and to mannosyl monosaccharide and disaccharide moieties in 1, 3, 4, and 5. A year later, Singh et al. (2003) demonstrated that the *S. hygroscopicus* LL-AC98 strain produced α, β, Γ, δ, and Ɛ mannopeptimycins, with γ, δ, and Ɛ isovaleryl substitutions at various positions on the terminal mannose of the disaccharide moiety, and that this structural modification resulted in mannopeptimycins with maximal antimicrobial activity. Ruzin et al. (2004) used a radioactive mannopeptimycin labeled with a photoactivation ligand, to demonstrate that this lantibiotic interacts with the cell wall precursor lipid II in a different manner than that of either vancomycin or mersacidin. Additionally, these authors found that mannopeptimycins nonspecifically bind to lipoteichoic acid, and that this may facilitate the accumulation of the lantibiotic on the bacterial cell surface. The lipoglycodepsipeptide ramoplanin (Breukink and de Kruijff 2006) is another antibiotic belonging to the "not true lantibiotics" group. It also inhibits bacterial cell wall synthesis by inhibiting the transglycosylation step in the synthesis of peptidoglycan.

Planosporicin is produced by *Planomonospora* sp. as a 2194 Da polypeptide (Castiglione et al. 2007). It contains 24 amino acids (Fig. 4), in addition to lanthionine and methyllanthionine, with five intramolecular thioether bridges. Like all

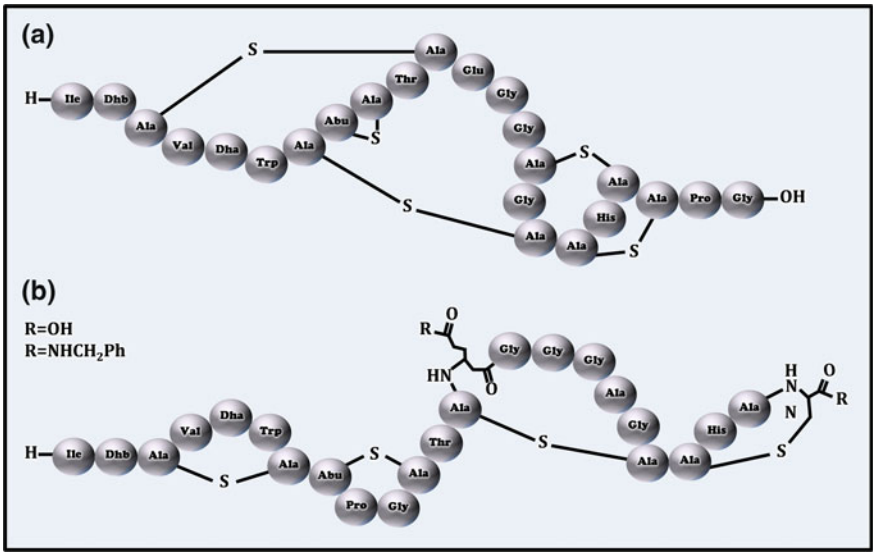

Fig. 4 Planosporin structure, as proposed by Castiglione et al. 2007 (**a**), and Maffioli et al. 2009 (**b**)

lantibiotics, planosporicin is ribosomally synthesized and posttranslationally modified to introduce methyllanthionine bridges. This compound is structurally related to mersacidin and actargadine, and therefore belongs to the B lantibiotic subgroup, characterized by pore formation and accumulation of UDP-linked peptidoglycan precursors. The above authors conclude that, due to its microbial activity both in vitro and in vivo, planosporicin potentially represents a novel antibiotic to treat emerging pathogens. Maffioli et al. reviewed the structure of this compound in 2009, and found that planosporicin is not a group B lantibiotic, but actually belongs to the nisin subgroup, as it has a shift of two amino acids and a reorganization of two thioether bridges. Sherwood et al., in 2013 studied the planosporicin produced by *Planomonospora alba* and characterized it as a type AI lantibiotic, structurally similar to those which bind lipid II. Genome mining revealed that planosporicin production is encoded by a minimal cluster of 15 genes, and, by reverse transcription PCR, the gene cluster was transcribed into three operons (Sherwood et al. 2013). Finally, Sherwood and Bibb (2013) found that planosporicin regulates its own synthesis, and that its gene expression is transcriptionally controlled by three regulatory proteins (an extra-cytoplasmic function σ factor, its cognate anti-σ factor, and a transcriptional activator with a C-terminal helix-turn-helix DNA-binding domain).

Amylolysin was first reported as a potent antilisterial bacteriocin produced by *Bacillus amyloliquefaciens* GA1 (Halimi et al. 2010). Later, Argüelles-Arias et al. (2013) characterized the genome of this bacterium and identified a putative gene cluster for lantibiotic production. This gene cluster included a structural gene and a

modification gene, as well as auxiliary genes for transport, regulation and immunity. Analysis of the purified amylolysin led to the conclusion that this compound represented a new type B lantibiotic, capable of binding lipid II. Amylolysin displays no antimicrobial activity against either ascomycetous or basidiomycetous yeasts, but it is effective against *E. faecium* and, to a lesser degree, against *Enterococcus faecalis*. The lantibiotic is also useful against Gram-positive bacteria and methicillin-resistant *S. aureus*, as well as on pathogens responsible for food poisoning, such as *Listeria monocytogenes* and *Bacillus cereus*. On the other hand, this lantibiotic is ineffective against lactic acid bacteria, such as *Weissella* sp. and *Lactobacillus plantarum*. As expected, amylolysin is greatly affected by protease treatment, but it is very thermostable (even when heated at 100 °C) and can withstand wide pH variations (Argüelles-Arias et al. 2013).

Bovicin (Fig. 5) is a peptide isolated, from rumen streptococci, by Whitford et al. (2001), who named it "bovicin 255." The antibacterial active principle is a 6 kDa peptide, with characteristics similar to those described for class II bacteriocins from Gram-positive bacteria. A year later, Mantovani et al. (2002) discovered that the *Streptococcus bovis* HC5 strain produced a high antibacterial activity against nisin-resistant *S. bovis*. The bovicin obtained from HC5 was inactivated by both pronase E and trypsin, but it was resistant to heat, proteinase K and alpha-chymotrypsin. This peptide exhibited a molecular mass of 2440 Da, and contained a terminal amino acid sequence: VGXRYASXPGXSWKYVXF. Although the protein did not contain cysteine residues, bovicin HC5 otherwise shared significant similarity to the lantibiotic precursor from *Streptococcus pyogenes* SF370. Xiao et al. (2004) found another *S. bovis* strain (HJ50) that produces a lantibiotic; this compound has a molecular mass of 3428 Da and contains a disulfide bridge. The structural gene encoding bovicin HJ50 produces a 58 amino acid prepeptide, consisting of an N-terminal leader sequence of 25 residues and a C-terminal propeptide domain of 33 amino acids. Xiao et al. (2004) also reported that bovicin HJ50 shares structural similarity with type AII lantibiotics.

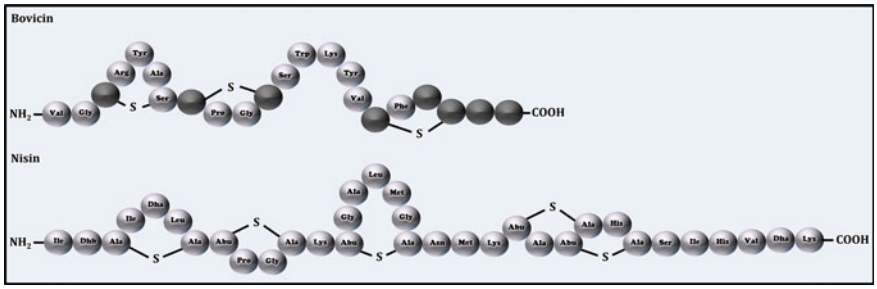

Fig. 5 Comparison of the primary structures of the lantibiotics bovicin and nisin. The *solid circles* in bovicin represent amino acids different from the 20 commonly found in proteins; these amino acids reflect posttranslationally modified residues (for further details, see Paiva et al. 2011)

Bovicin HC5 was used in field experiments to protect cattle from *L. monocytogenes* infection (the cattle acquired the bacteria when consuming contaminated plant materials, silage, or from the soil), and this lantibiotic showed considerable promise in this application. Indeed, at slightly acidic pH, bovicin HC5 caused an almost complete efflux of the bacterial intracellular potassium, as well as a decrease in the ATP values, although the lantibiotic was less effective at higher pH values (Mantovani and Russell 2003, 2008). Houlihan and Russell (2006) studied the factors affecting the release, stability and binding of bovicin HC5 to sensitive bacteria. They found that bovicin HC5 remains associated with the producing cells until the pH of the media is <5.0, and that acidic NaCl can easily dissociate the drug from the cell surface. Additionally, the biomolecule is highly stable in acidic environments, binding to target bacteria when the pH value is lower than 6.0.

Bovicin HC5, as is the case for all lantibiotics, targets the peptidoglycan precursor lipid II, leading to the formation of pores in the bacterial membrane. Paiva et al. (2012a) used tryptophan fluorescence and circular dichroism spectroscopy in artificial membrane systems that allow pore formation only when bovicin binds to lipid II. They found that binding of bovicin to lipid II created pores in the artificial systems, just as it happens in natural membranes, and that this phenomenon was accompanied by changes in fluorescence emission. Moreover, the interaction between bovicin HC5 and lipid II was highly stable even at pH 2.0.

Paiva et al. (2012b) tested the potential cytotoxic and hemolytic effects of bovicin HC5 on Vero cells in vitro, by measuring the release of hemoglobin. They also studied the effect of cholesterol on the drug's activity (using nisin as reference), by measuring the inhibition of growth in Gram-positive bacteria. These authors found that cholesterol does not affect the lantibiotic and that bovicin HC5 only exerts cytotoxic effects at concentrations far higher than those needed for biological action. Paiva et al. (2013) further the knowledge into the in vivo effects of bovicin HC5, by studying the histological and immunostimulatory effects of orally administrated bovicin in a Balb/c mice model. They observed that treatment with bovicin HC5 resulted in reduced weight gain and caused alterations in the small intestine of the mice, although goblet cell numbers and mucopolysaccharide production were not affected by the lantibiotic. On the other hand, they found Paneth cell hypertrophy, as well as an increase in the number of mitotic cells. These authors concluded that bovicin HC5 only has minor effects on intestinal permeability, and that it does not elicit an allergic response. The latter makes this lantibiotic a good candidate for enteral applications.

Nukacin ISK-1 The bacterium *Pediococcus* sp. ISK-1, reclassified as *Staphylococcus warneri* ISK-1 by Sashihara et al., in 2000, produces a bacteriocin known as nukacin ISK-1 (Fig. 6). The structural gene (*nukA*) encodes a peptide with 57 amino acids, including the 30 residues that constitute the leader region. Nukacin ISK-1 is highly homologous to lactacin. Sashihara et al. (2001) found that osmotic stress effectively stimulates nukacin ISK-1 production; this is partly due to transcriptional regulation of the structural gene. Aso et al. (2004a, b) characterized the nukacin gene cluster and found that it contains six genes, the structural gene nukA, five other genes (-M, -T, -F, -E, and -G), and two open reading frames

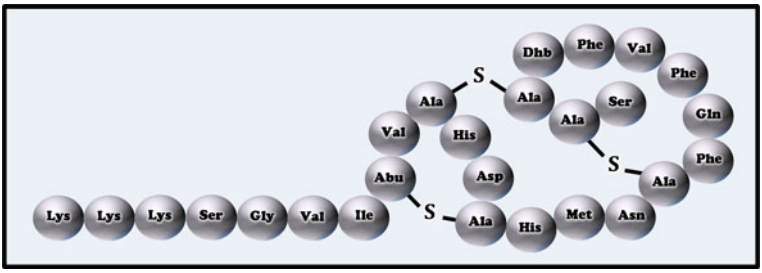

Fig. 6 Structure of nukacin ISK-1

(ORF1 and ORF7). Genes M and T are likely involved in posttranslational modification and secretion of nukacin ISK-1, while -F, -E, and -G are predicted to be membrane bound and provide self-protection. Aso et al. (2005) sequenced two *S. warneri* plasmids, pPI-1 (30.2 kb) and pPI-2 (2.8 kb) and found that the first plasmid contains the biosynthetic genes for Nukacin ISK-1. They also reported that, the gene organization in the nukacin ISK-1 biosynthesis and immunity gene clusters was different from that found in other lacticin-481 type gene clusters.

Asaduzzaman et al. (2009) described that nukacin ISK-1 is not responsible for either membrane potential dissipation or efflux of either ATP or K^+ ions. They concluded that this lantibiotic should, in fact, be considered a bacteriostatic agent, since it does not cause cytoplasmic leakage in bacteria. Okuda et al. (2013) further studied this matter and found that nukacin ISK-1 only displayed a bacteriostatic effect on planktonic cells from a methicillin-resistant *S. aureus* strain, but it did not have any effect on the same bacteria in biofilms. As shown in Fig. 6, the ring A of nukacin ISK-1 resembles a lipid II-binding motif (TxS/TxD/EC), similar to that present in mersacidin (see above), which suggests a putative lipid II-binding role for this lantibiotic. In fact, nukacin ISK-1 inhibits cell wall biosynthesis, and produces accumulation of lipid II precursor inside the cell (Islam et al. 2012b)

Haloduracin (Fig. 7) was first identified in the genome of the Gram-positive alkaliphilic bacterium *Bacillus halodurans* C-125. This is a two-component (α/β) lantibiotic with two precursor peptides (HalA1 and HalA2). These precursor peptides must be fully processed, by the appropriate enzymes (HalM1 and HalM2, respectively), to produce the active lantibiotic. In fact, haloduracin's biological activity depends on the presence of both modified peptides (McClerren et al. 2006). Lawton et al. (2007a) sequenced the complete genome of *B. halodurans* and found that the haloduracin gene cluster contains 11 genes. These include genes involved in the production, processing and resistance to this, otherwise typical, lantibiotic. This represented the first report of a two-peptide lantibiotic in a *Bacillus* species.

Oman and van der Donk (2009) demonstrated that haloduracin is active against a wide range of Gram-positive bacteria, including *Bacillus anthracis*. They also showed that the two peptides display optimal activity in a 1:1 stoichiometry, and that this compound is considerably more stable than nisin at pH 7. Haloduracin, as

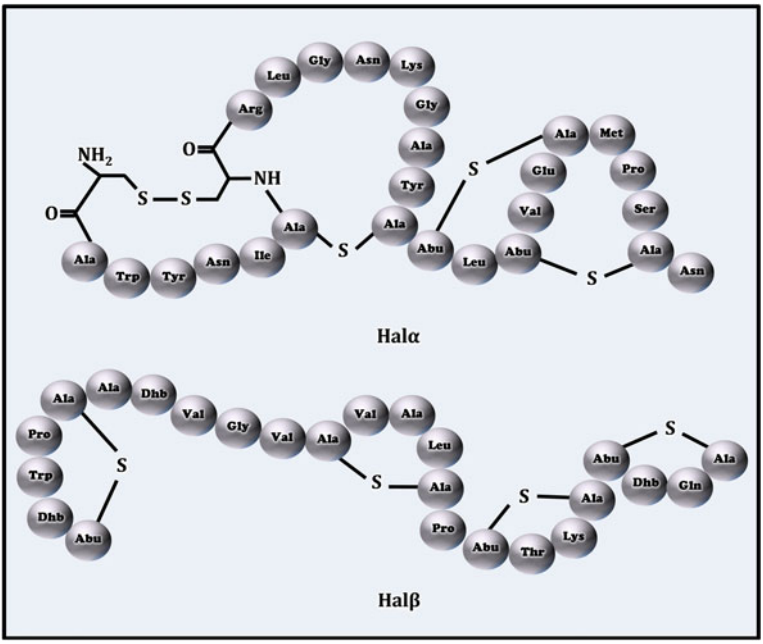

Fig. 7 Structure of haloduracin (α/β), a lantibiotic produced by the alkaliphilic *Bacillus halodurans*

is the case for all other lantibiotics, binds lipid II, thus inhibiting the bacterial cell wall synthesis (Oman et al. 2011).

Macedovicin represents one of the latest lantibiotic described (Georgalaki et al. 2013), and it is produced by *Streptococcus macedonicus* ACA-DC 198. This bacterium produces two important lantibiotics: macedocin (the major one) and macedovicin (the minor one). Georgalaki et al. (2013) purified this compound and characterized it, by mass spectrometry, as a polypeptide with a molecular weight of *ca.* 3.4 kDa, highly homologous to both bovicin HJ50 and thermophilin 1277, produced by *S. bovis* and *Streptococcus thermophilus*, respectively. These authors concluded that, although the gene clusters of macedovicin, thermophilin 1277 and bovicin HJ50 contained almost identical nucleotide sequences, there were enough differences in their predicted genes and proteins to justify considering macedovicin a new lantibiotic.

Clausin, a lantibiotic produced by *Bacillus clausii,* was originally described in a patent application submitted by Bressollier et al., in 2007, and published 2 years later (Bouhss et al. 2009). This is a type A lantibiotic with a 75 % sequence homology to mutacin-1140, and a lower homology (30 %) to nisin, although the three of them contain the classical characteristic two A/B lanthionine rings. The putative interaction of clausin with bacterial lipids was analyzed in dodecylphosphocholine surfactant micelles, with the conclusion that both lipid I, and lipid II

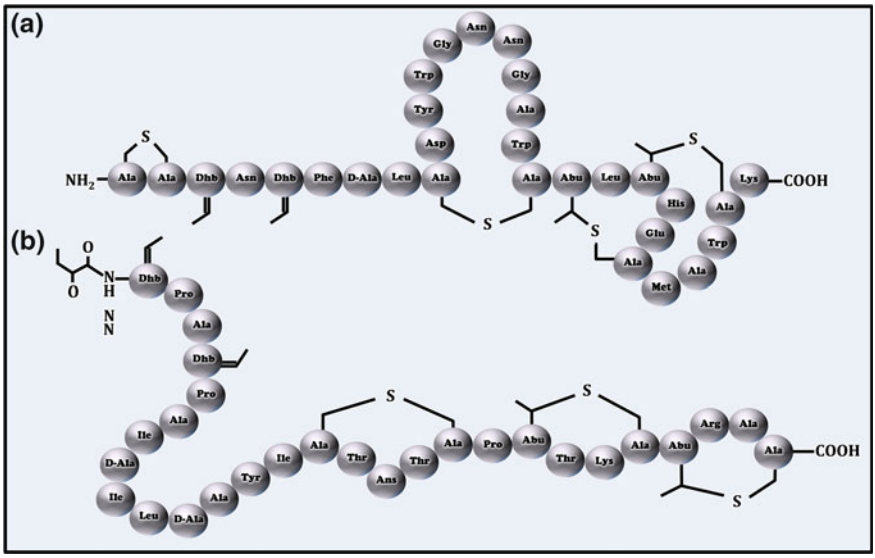

Fig. 8 Structure of the two lacticin 3147 peptides (**a** A1 polypeptide; **b** A2 polypeptide), as published by Martin et al. (2004)

were indeed potential targets for this compound. Bouhss and coworkers described that the interaction between the lantibiotic and the lipids was enthalpy-driven. They conclude: "*For the first time, to our knowledge, we evidenced the interaction between a lantibiotic and C(55)-PP-GlcNAc, a lipid intermediate in the biosynthesis of other bacterial cell wall polymers, including teichoic acids*."

Lacticin 3147 (Fig. 8) was described by Rea and Cogan, in 1994, in a *L. lactis* strain isolated from kefir grains, traditionally used in Ireland to prepare buttermilk. This lantibiotic, as is the case for nisin, displays a broad spectrum of activity against a vast variety of Gram-positive bacteria. Hickey et al. (2001) found that the active compound is a two-component peptide system encoded by a plasmid in *L. lactis* subspecies *lactis* DPC3147, and this plasmid can be transmitted to non-producing strains by bacterial conjugation. Martin et al. (2004) described the two polypeptides involved as A2 (containing the specific lanthionine bridge and thus resembling a globular lipid II-interacting type B lantibiotic) and A1 (an elongated structure similar to membrane-interacting type A lantibiotics). Although lacticin 3147 shares similarities with nisin, it requires the two peptides to carry out its antibacterial effect. The A1 peptide, on its own, is not capable of inhibiting bacterial cell wall biosynthesis in vitro, and it requires the A2 peptide for substantial K^+ leakage (McAuliffe et al. 1998). In the presence of lipid II, the lantibiotic is capable of producing 0.6 nm pores, both in natural and in artificial membranes (Wiedemann et al. 2006a). These pores are selective for K^+ ions and inorganic phosphate, and the ion leakage lowers, or even abrogates, the cellular membrane potential, eventually leading to cell death (McAuliffe et al. 1998). Long before the lacticin 3147 structure

was elucidated, this compound was regularly used in the manufacture of cheddar cheese. In this process, the lantibiotic remained active for a period of at least 6 months, with the concomitant significant reduction in the levels of non-starter lactic acid bacteria (Ryan et al. 1996). The compound was also successfully used in fresher cheeses, such as cottage cheese, as a way of controlling post-ripening contamination by *L. monocytogenes* (McAuliffe et al. 1999). Lacticin 3147 was even successfully used in the treatment of mastitis in dairy cattle (Ryan et al. 1998, 1999a; Twomey et al. 2000; Crispie et al. 2004). Lacticin 3147 has even found a place in the control of common Gram-positive foodborne pathogens, as well as for maintaining the bacteriological quality of minimally processed dairy foods, since the combination of low amounts of this lantibiotic with high pressure treatment greatly enhances bacterial death (Morgan et al. 2000).

Ryan et al. (1999b) described that the A1 peptide contains 30 amino acids, whereas A2 spans 29 residues and that both polypeptides encompass the thioether amino acid lanthionine. A peculiarity of these peptides is that their alanine content is above the amount predicted from the nucleotide sequence of genes encoding them. Cotter et al. (2005) explained that the excess of D-alanine results from extensive posttranslational modification of serine residues in the primary translation product, and it is vital for optimal activity of lacticin 3147. The plasmid pMRC01 (60.2 kb) encodes the production and immunity of lacticin 3147, in a locus spanning 12.6 kb and containing 11 genes, arranged in two clusters with opposite orientations (McAuliffe et al. 2000). McAuliffe et al., identified, in 2001, the lacticin 3147 transcription initiation sites and promoters, and found that this lantibiotic is indeed a two-operon system. They also found that the transcriptional repressor, LtnR, regulates lacticin 3147 immunity, by binding to a DNA region overlapping the Pimm promoter.

O'Connor et al., described, in 2006, an interesting application for this lantibiotic. These authors found that food grade preparations of lacticin 3147 could inhibit oral streptococci. This means that lacticin 3147 could represent an efficient anticariogenic agent, particularly in the field of functional foods for the improvement of oral health. Dobson et al., corroborated these findings in 2011. Additionally, Cotter et al. (2006) concluded: "*Given that lantibiotics are ribosomally synthesized and amenable to site-directed mutagenesis, they have the potential to serve as biological templates for the production of novel peptides with improved functionalities*." They could constitute a "blue print" for the design of novel antimicrobials with applications in all instances of infections involving bacterial pathogens. Gardiner et al., reported in 2007 that lacticin 3147 was degraded, in vitro, by enzymes such as α-chymotrypsin, with a resultant loss in biological activity. They also tested the lantibiotic in vivo, in a pig model, and found that, following oral ingestion, no lacticin 3147 peptides were detected in the gastric content, jejunum, ileum, or pigs' feces. These authors concluded that, as is the case for nisin: "... *lacticin 3147 ingestion is unlikely to have adverse effects, since it is probably inactivated during intestinal transit*" (for potential uses of these two-system lantibiotics, such as lacticin 3147, staphylococcin C55, plantaricin W, Smb, BHT-A, and haloduracin, in both human and veterinary medicine, see review by Lawton

et al. 2007b). Lacticin 3147 has been successfully used to control *Clostridium difficile* infections that cause diarrhea, the most common hospital-acquired diarrhea, and also infectious gastroenteritis in elderly patients (Rea et al. 2007); with low side effects on normal colon microbiota and, hence, low levels of collateral damage (Rea et al. 2011). Additionally, lacticin 3147, in combination with nisin, has been successfully used to combat mycobacterial infections (Carroll et al. 2010). This lantibiotic displayed high activity against all mycobacterial strains tested, producing MIC90 (Minimum Inhibitory Concentration required to inhibit the growth of 90 % of organisms) values as low as 60 mg/L for *Mycobacterium kansasii*. The compound was even more effective against *M. tuberculosis* H37Ra (MIC-90 7.5 mg/L). Draper et al. (2013) recently used this lantibiotic against Gram-negative bacteria, including *Cronobacter* and *E. coli*, and found that this therapy is effective when administered in combination with polymyxins A or E, producing a synergistic effect capable of controlling the growth of Gram-negative bacteria Lacticin 3147 has been the subject of bioengineering research, to check the effect of ring substitution/elimination in both the A and B polypeptides. Suda et al. (2010) found that an intact N-terminal lanthionine bridge (Ring A) is required to enhance the molecule's resistance against thermal damage and proteolytic degradation. Field et al., in 2013, subjected the lantibiotic to site-saturation mutagenesis, in order to determine the functional importance of the amino acids and the optimal sequence for maximal antibacterial activity.

Silkin et al. described, in 2008, a bizarre application for lacticin 3147. The molecule displays spermicidal activity on bovine, equine, boar, and rat sperm. Although the exact mechanism for this activity remains unknown, it has been suggested that the lantibiotic can generate pores on the sperm's membranes causing cell death (sperm contains high concentrations of anionic phospholipids, such as phosphatidylglycerol and phosphatidylserine).

Plantaricins C and W González et al., in 1994, discovered plantaricin C in *L. plantarum* LL441 strain, and described it as a small bactericidal polypeptide of

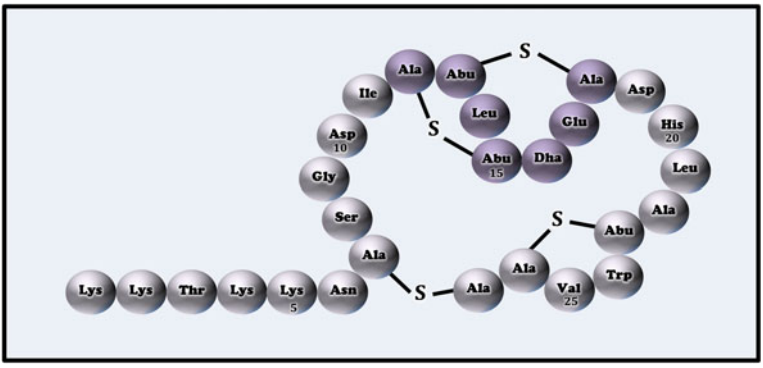

Fig. 9 Structure of plantaricin C. The abbreviations used are: *Dha* = dehydroalanine; *Ala-S-Ala* = lanthionine; *Abu-S-Ala* = β-methyllanthionine (Wiedemann et al. 2006b)

3.5 kDa (Fig. 9). This lantibiotic can retain biological activity after being subjected to high temperatures, such as boiling, long-term storage, and a wide range pH conditions. Later on, González et al. (1996) found that the molecule belongs to the pore-forming bacteriocin group, which does not require a specific membrane receptor. Turner et al., in 1999, finally claimed the molecule as a lantibiotic and, as is the case for all the other lantibiotics, it exerts its antibacterial activity by targeting the bacterial lipid II (Wiedemann et al. 2006b).

Holo et al. (2001) discovered plantaricin W, a new two-peptide lantibiotic, in the supernatant of *L. plantarum* LMG 2379, a bacterial strain used to ferment Pinot Noir wine in Oregon. Plantaricin W contains two peptides, α and β; either of these peptides, by itself, exhibits low antibacterial activity but, when present in a 1:1 stoichiometry, the lantibiotic displays high antimicrobial activity. Indeed, both peptides can be considered lantibiotics, as they both contain the typical modified amino acids. Additionally, polypeptide α contains two unmodified cysteines and one serine residue, whereas polypeptide β only possesses one cysteine residue. Holo et al. (2001) found that plantaricin structural genes encode prepeptides with sequences similar to other two-peptide lantibiotics, and that the conserved residues (serines, threonines and cysteines) at the C-terminus of the molecules are likely involved in intramolecular thioether bond formation.

Ruminococcin A Ramare et al. (1993) discovered ruminococcin A in a strain of *Peptostreptococcus* sp. of human origin (later reclassified as *Ruminococcus gnavus* E1; Dabard et al., 2001) that displayed activity against a variety of Gram-positive bacteria, including *C. difficile*. Gomez et al. (2002) purified the active principle and named it ruminococcin A (Fig. 10). They described it as a trypsin-dependent 2.6 kDa polypeptide, harboring a 21 amino acid lanthionine structure, highly homologous to other type IIA lantibiotics. This represented the first report of strictly anaerobic bacteria, isolated from human feces, which produced a lantibiotic with bactericidal activity; thus protecting the human gut against a variety of pathogenic bacteria, such as *C. difficile* and *B. cereus.* Gomez et al. (2002) described that the ruminococcin gene cluster spans 13 genes (including three, almost identical, structural genes), organized in three operons, with predicted functions in lantibiotic biosynthesis, signal transduction regulation, and immunity.

Cinnamycin, Duramycin, and Ancovenin Cinnamycin was first reported by Benedict et al., in 1952, as an antibiotic produced by *Streptomyces cinnamoneus*

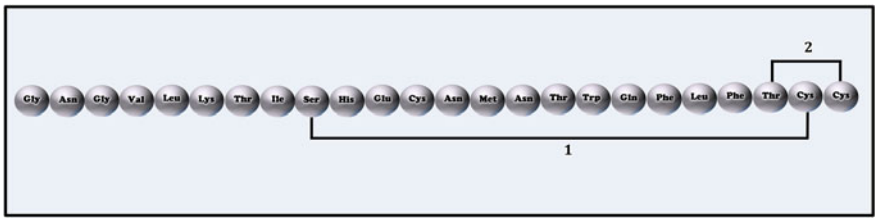

Fig. 10 Structure of ruminococin A. The number *1* indicates the Lan structure, while *2* refers to Me-Lan structures (Dabard et al. 2001)

nov. sp.. Unfortunately, 2 years later Ambrose (1954) found that this compound, when injected either intraperitoneally or subcutaneously, produced acute toxicity in mice. This negative result is probably responsible for the long hiatus in the research into this group of lantibiotics, that inhibit human phospholipase A2 at concentrations of 10^{-6} M, until finally, Fredenhagen et al., reviewed the structures of these lantibiotics in 1990. Although Gross and Brown had already published a partial structure of duramycin in 1976, and the first complete structure of ancovenin, a new peptide inhibitor of angiotensin I-converting enzyme, was presented by Wakamiya and colleagues in 1985. As seen in Fig. 11, all these lantibiotics share the same basic structure (19 amino acids), with minor variations in some of the amino acidic residues. Phospholipase A2 (EC 3.1.1.4) plays an important role in the release of arachidonic acid, from phospholipids in cellular membranes; which can be later converted into prostaglandins or leukotrienes that either mediate inflammation or are involved in allergic processes (Zipser and Laffi 1985). Hence, despite the acute toxicity reported for these compounds (Ambrose 1954), these lantibiotics could still find a specific therapeutic niche, such as their use for lung mucus clearance in cystic fibrosis. Grasemann et al. (2007) reported that duramycins promote chloride secretion in lung epithelial cells, and Märki et al. (1991) describe the use of cinnamycin in the treatment of atherosclerosis. Cinnamycin is a 19 amino acid lantibiotic with antimicrobial activity against Gram-positive rods, such as bacilli, *Clostridium botulinum* and *Mycobacterium* spp. (Lindenfelser et al. 1959). The compound can also reach the cytosolic part of the mammalian cellular membranes, where phosphatidylethanolamine is located. Widdick et al. (2003) reported that the cinnamycin biosynthetic gene cluster, in *S. cinnamoneus* DSM 40005, contains 21 open reading frames (spanning *ca*. 17 kbp), and includes all the genes required for

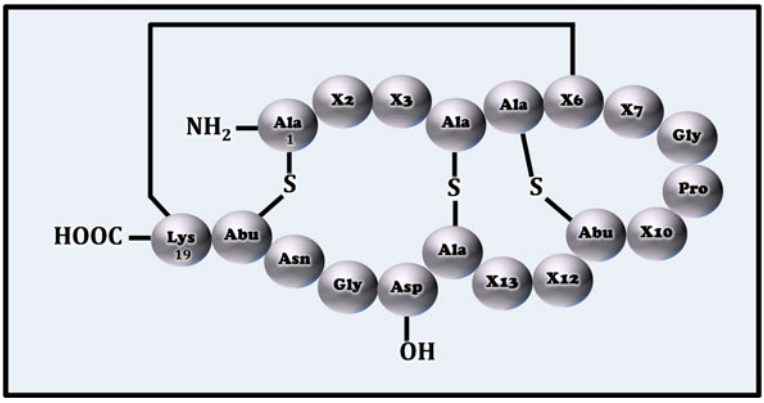

Fig. 11 Primary structure proposed by Fredenhagen et al. (1990) for the lantibiotics duramycin B and C. The symbols used are: *Duramycin* = leucopeptin: *X2* = Lys, *X3* = Gln, *X6* = Ala, *X7* = Phe, *X10* = Phe, *X12* = Phe, *X13* = Val; Duramycin B: *X2* = Arg, *X3* = Gln, *X6* = Ala, *X7* = Phe, *X10* = Leu, *X12* = Phe, *X13* = Val; Cinnamycin: *X2* = Arg, *X3* = Gln, *X6* = Ala, *X7* = Phe, *X10* = Phe, *X12* = Phe, *X13* = Val; Ancovenin: *X2* = Val, *X3* = Gln, *X6* = Dehydroalanine, *X7* = Phe, X10 = Leu, *X12* = Trp, *X13* = Ser

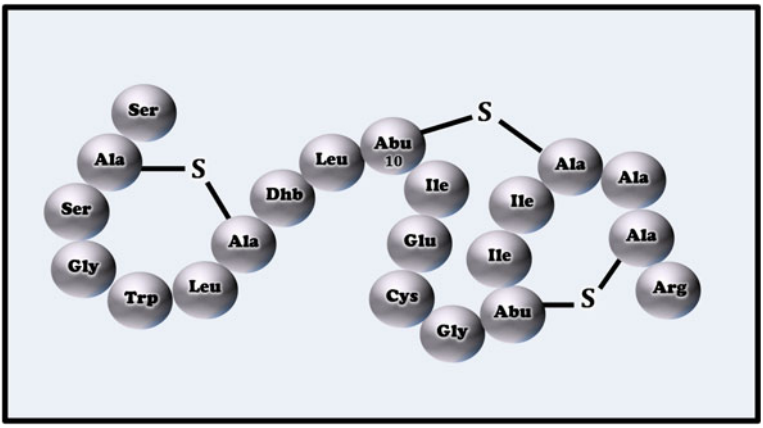

Fig. 12 Primary structure of michiganin A, as published by Holtsmark et al. (2006). Abu-S is the threonine-derived moiety of a methyllanthionine ring. Ala-S represents either the serine-based half of a lanthionine ring or the cysteine-derived moiety

cinnamycin production, regulation, and self-resistance. The cinnamycin precursor peptide, CinA, contains a C-terminal core region of 19 amino acids, and a long N-terminal leader sequence of 59 residues. The C-terminal 19 residues require 9 posttranslational modifications to originate the active form of cinnamycin (Ökesli et al. 2011). These authors suggest that: "*Understanding the biosynthetic pathway of cinnamycin may be used to generate analogues with potentially improved properties for treatment of cystic fibrosis or clostridium infections*."

Michiganin A This is a new lantibiotic (Fig. 12), similar to actagardine (see above), named michiganin A because it was isolated from the tomato pathogen *Clavibacter michiganensis* subsp. *michiganensis* (Holtsmark et al. 2006). The mature unmodified peptide consists of 21 amino acids, which must undergo a series of modifications to achieve biological activity. In this manner, all the threonine residues undergo dehydration, and three of those amino acids interact with cysteine residues, via thioether bonds, to form the methyllanthionine bridges that are a typical structure of the type B lantibiotics. According to these authors, the predicted molecular mass for the mature michiganin A molecule is 2144.6 Da, whereas the mass for the unmodified gene product, without the leader peptide, is 2216.6 Da (the 72 Da are lost by threonine dehydration). This lantibiotic inhibits the growth of *Clavibacter michiganensis* subsp. *sepedonicus*, the causal agent of bacterial ring rot of potato.

3 Lipid II-Acting Antibiotics

Only a few antibiotics, such as telavancin, vancomycin, and related antibiotics, belong to this group. They are included here because of the assumption that they require lipid II to exert their antibacterial activity. However, lipid II does not constitute their primary target, as is the case for some of the compounds mentioned above. On the other hand, some other peptides, such as human alpha and beta defensins and moenomycins, do target lipid II and therefore deserve a place in this chapter.

Telavancin (Fig. 13) is a nonribosomal lipopeptide and semisynthetic antibiotic that is a vancomycin derivative (King et al. 2004; Leadbetter et al. 2004). It has a bactericidal activity that only requires a single daily dose, which was approved, in 2009, for use in skin infections caused by methicillin-resistant *S. aureus* (after lengthy phase III trials that started in September 2004; Pace and Judice 2005). In 2013, this antibiotic was approved for the treatment of hospital-acquired infections. It was soon found that this compound plays a multifunctional role and that it represents an update on an old successful class of antibiotics (Higgins et al. 2005;

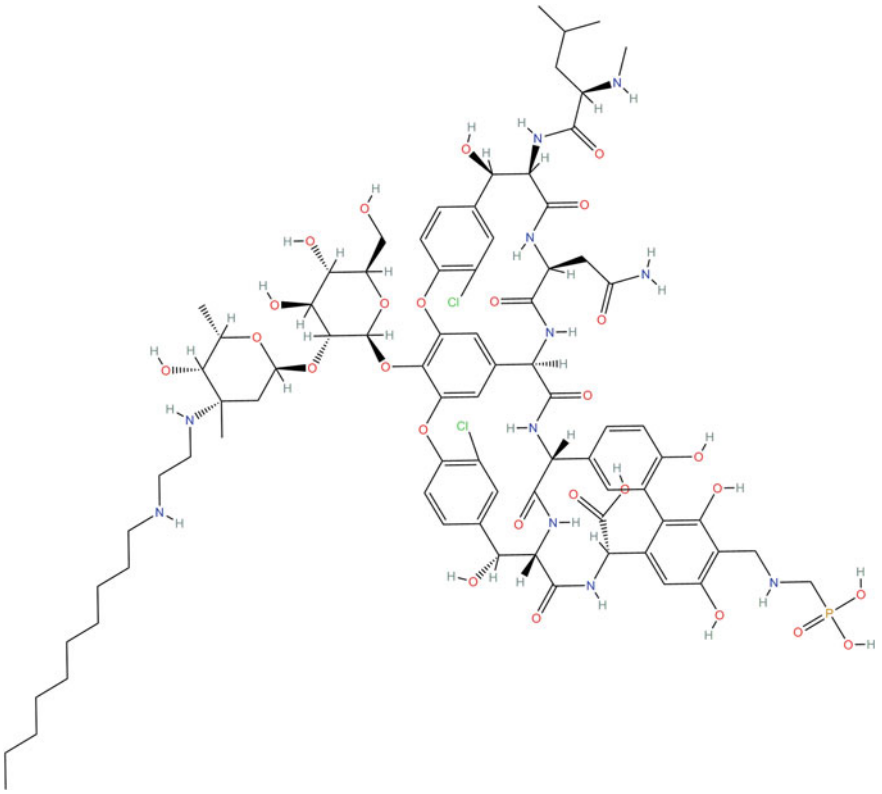

Fig. 13 Primary structure of telavancin

Pace and Yang 2006). Telavancin displays, at least, two mechanisms of action. As observed with vancomycin, telavancin inhibits late-stage peptidoglycan biosynthesis, in a substrate-dependent fashion, and it also affects the plasma membrane potential, causing leakage of cellular ATP and K^+ and therefore membrane permeability. The importance of this novel antibiotic was the subject of several reviews, such as that by Laohavaleeson et al. (2007), that demonstrated that the in vitro activity of telavancin is superior to that of vancomycin and at least comparable to, if not greater than, the antimicrobial activity of compounds such as linezolid, daptomycin, and other novel lipoglycopeptides. The antibiotic is effective against Gram-positive pathogens that cause soft tissue infections, including endocarditis, pneumonia and bacteremia. Telavancin, as is the case for vancomycin, inhibits bacterial cell wall synthesis by binding to the D-Ala-D-Ala terminus of the peptidoglycan molecule in the growing bacterial wall. Additionally, telavancin also induces cell membrane depolarization, in a process that requires the antibiotic's

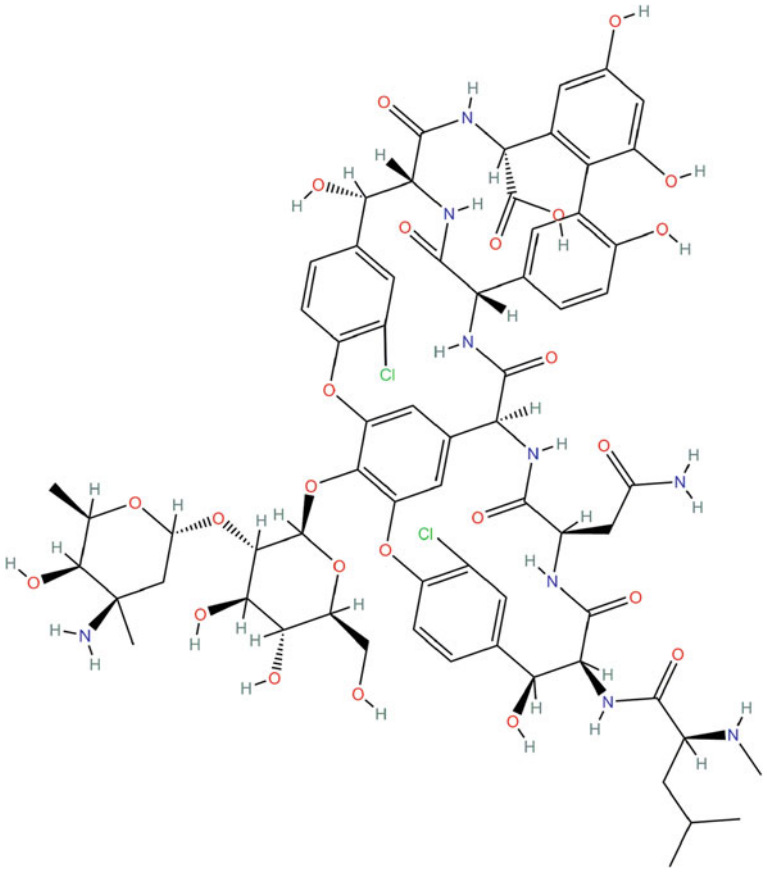

Fig. 14 Structure of vancomycin

interaction with lipid II, thus contributing to the compound's bactericidal effect (Lunde et al. 2009; Nannini et al. 2010)

Vancomycin is a nonribosomal glycopeptide antibiotic (a branched tricyclic glycosylated nonribosomal peptide) produced by the bacterium *Amycolatopsis orientalis* (formerly *Nocardia orientalis*). It was first isolated in 1953, by the Eli Lilly pharmaceutical company, from a soil sample collected deep in the interior jungle of Borneo. The structure of this antibiotic is shown in Fig. 14. The mechanism of action of vancomycin, as is the case for telavancin, originates from its ability to form hydrogen bond interactions with the terminal D-alanyl-D-alanine moieties of the NAM/NAG peptides, thus inhibiting the synthesis of peptidoglycan; again, this activity requires the presence of lipid II. For a comprehensive review on the synthesis of vancomycin and related antibiotics, see van Wageningen et al. (1998).

In 2006, van Bambeke provided an excellent review on the importance of glycopeptides and glycodepsipeptides in clinical development. The author compares the antibacterial spectrum, pharmacokinetics and clinical efficacy of both naturally produced (*i.e.,* ramoplanin, unstable in the bloodstream and used orally to treat resistant *C. difficile* infections) and semisynthetic glycopeptides (such as telavancin, oritavancin, and dalbavancin).

Plusbacin Plusbacin-A3 is a cyclic lipodepsipeptide that exhibits antibacterial activity against multidrug-resistant Gram-positive pathogens. This antibiotic is believed not to enter the cytoplasm, but to remain in the bacterial cellular membrane, through insertion of its isotridecanyl side chain into the membrane bilayer, thus involving lipid II (Kim et al. 2013). These authors synthesized analogues of pb-A3, [(2)H]pb-A3 and deslipo-pb-A3, to test their membrane insertion mechanism and mode of action. The compound [(2)H]pb-A3 has an isotopically (2) H-labeled isopropyl subunit of the lipid side chain, whereas deslipo-pb-A3 is missing the isotridecanyl side chain; but both analogues have the pb-A3 core structure. They showed that the isotridecanyl side chain is crucial for antibacterial activity, as the deslipo-pb-A3, lacking the isotridecanyl side chain, produced loss of antimicrobial activity. However, rotational-echo double-resonance nuclear magnetic resonance characterization of [(2)H]pb-A3 bound to [1-(13)C]glycine-labeled *S. aureus* cells, demonstrated that the isotridecanyl side chain does not insert into the lipid membrane but, instead, is found in the staphylococcal cell wall, positioned near the pentaglycyl cross-bridge of the cell wall peptidoglycan. Addition of [(2)H] pb-A3 during the growth of *S. aureus* resulted in the accumulation of Park's nucleotide, consistent with the inhibition of the transglycosylation step of peptidoglycan biosynthesis.

Human defensins and moenomycins In this chapter, we have reviewed several compounds that cause bacterial lysis, mainly in Gram-positive bacteria, by interacting with lipid II. These include telavancin, vancomycin-related antibiotics, and cyclic peptides. Here we refer to a fourth class of compounds that display sufficient antimicrobial activity to be considered as potential future drugs to treat bacterial infections. These compounds include the primate, and in particular the human, defensins (Brogden et al. 2003; Lehrer 2004; Selsted and Ouellette 2005; Bevins

2006). Since the publication by de Leeuw et al., in 2010, describing the functional interaction between the human defensin peptide HNP1 with lipid II, many reports have confirmed that lipid II is indeed the target for these compounds. Members of this family include the fungal defensins plectasin, oryzeacin, and eurocin (Schneider et al. 2010), in addition to lucifensin (from the blowfly *Lucilia sericata*) and gallicin (from the *mussel Mytilus galloprovincialis*).

Varney et al. (2013) raised the interesting fact that glycopeptides, defensins, lantibiotics, and cyclic peptides do not share structural homology with defensins (either alpha or beta), and yet all of them specifically target lipid II. Surprisingly, no synthetic compounds (except for telavancin and related compounds, mentioned above) have yet been developed that can interfere with lipid II. This situation is starting to change, thanks to the pioneering work of Varney et al. 2013, with the synthesis of BAS00127538, a compound that targets the aminosugar moiety of the lipid II molecule and displays efficacy, in vivo, against Gram-positive microorganisms that cause sepsis. These authors conclude: "*Studies like these on defensin mimetics and on plectasin may provide insight for future development, design and synthesis of efficient, defensin-derived compounds specifically targeting Lipid II as promising therapeutic leads.*"

Cheng et al. (2010), and more recently Sinko et al. (2014), reported the use of undecaprenyl diphosphate synthase inhibitors as novel antibacterial agents against methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant enterococci (VRE). Of the 450,000 compounds tested, only IC50 was active against MRSA, *L. monocytogenes*, *Bacillus anthracis,* and VRE, with a minimal inhibitory concentration (MIC) value between 0.25 and 4 μg/mL. Taking into account all that is known about these compounds, and considering the current rate of antibiotic resistance, there is great hope for this novel group of compounds, and their research may spur the quest towards new antibacterial compounds in all types of novel ecological niches.

It is almost 17 years (Subramaniam-Niehaus et al. 1997) since the identification of the phosphoglycolipid moenomycin (aka flavomycin, bambermycin, and flavophospholipol), an antibiotic produced by *Streptomyces ghanaensis*, *Streptomyces bambergiensis*, *Streptomyces ederensis*, and *Streptomyces geysiriensis*, and used as growth promoter in animal feeds. This compound displays strong antibacterial activity against both Gram-positive and Gram-negative microorganisms, and acts by inhibiting the transglycosylation process involved in peptidoglycan synthesis, thus preventing polymerization of lipid II into linear peptidoglycan (Halliday et al. 2006). These authors indicated that: "*Despite moenomycin's success, no developments of direct transglycosylase enzyme inhibitors were reported for over 30 years.*" Perhaps, the time has come now for scientist worldwide to start reconsidering these compounds as putative tools to fight bacterial infections (for a comprehensive and exhaustive review, see Ostash and Walker 2010). The moenomycins are a group of antibiotics described in 1965 (Huber et al. 1965; Wallhausser et al. 1965) that are 10- to 1000-fold more potent than vancomycin and have a long half-life in the bloodstream (9 days for mice; Huber 1979). They all possess a characteristic oligosaccharide chain linked, via a phosphoric acid diester and a glyceric acid unit, to a

C_{25}-hydrocarbon chain, which makes them amphiphilic compounds (Volke et al. 1997). These molecules exhibit many residues were novel radicals can be attached, thus extending the potential applications of this old antibiotic. In particular, Goldman et al. (2000) reported that the manipulation on the trisaccharide unit of these antibiotics creates novel moenomycins, with a different spectrum of activity to that displayed by the parent molecule. Kurz et al. elucidated the three-dimensional structure of moenomycin in 1998. Even at this stage, we know very little about the clinical potential and usefulness of other members of the moenomycin family, such as pholipomycin 12, a compound produced by *Streptomyces lividoclavatus* (Arai et al. 1977). In summary, the moenomycin family of antibiotics has a great potential in the development of broad-spectrum antibiotics with low side effects, which target the bacterial lipid II. As concluded by Ostash and Walker (2010): "*Now, when powerful tools are available to model, generate and assay novel moenomycins, we anticipate that progress … will further boost the efforts to develop moenomycin-based molecules for human use*."

3.1 Recent Advances in Lipid II-Interacting Antibiotics

Using a co-culture technique of bacteria and fungi, Essiq et al., have reported in 2014 the isolation of a new peptidic antibiotic, copsin, that is produced by the fungus *Coprinopsis cinerea* that binds specifically to the peptidoglycan precursor lipid II, therefore interfering with the cell wall biosynthesis. The third position of the lipid II pentapeptide is essential for effective copsin binding (Essig et al. 2014). Copsin was effective against Gram-positive bacteria, such as *B. subtilis*, *Listeria* spp., and *Enterococcus* spp., including a vancomycin-resistant *E. faecium* strain, although most potent activity was against *L. monocytogenes* with MIC values of 0.25–0.5 μg/ml (Essig et al. 2014).

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Microbiome Control in the Prevention and Early Management of Cancer

Paul A. Nguewa, Tomás G. Villa and Vicente Notario

Abstract Data accumulated during the past 15–20 years have established that chronic infections may contribute to the development of tumors in humans. Although the impact of certain viruses on human carcinogenesis has been known for some time, the involvement of bacteria in such process was not demonstrated until *Helicobacter pylori* was confirmed as an etiological agent for stomach cancer. Later, other bacterial species (i.e., *Borrelia*, *Campylobacter*, *Chlamydia*) have been associated with different human malignancies. The availability of antibiotics to these pathogens boosted eradication as the main prevention and therapeutic strategy to manage bacterially-promoted cancers. However, more recently, the not surprising emergence of antibiotic-resistant cases, along with recent information on the remarkable qualitative and quantitative changes that take place in the normal tissue/organ-specific microbiota at different stages of the carcinogenic process, have suggested the possibility of modifying or restoring the microbiota for cancer prevention strategies and, either alone or in combination with conventional anticancer agents, for therapeutic approaches. This chapter focuses on *Helicobacter* species (*H. pylori* in particular) as biological tumor-inducing agents, the proposed mechanisms underlying the oncogenic processes which they contribute to initiate, such as gastric cancer, colorectal adenocarcinoma, lung cancer, gastric MALT lymphoma, gastric diffuse large-B-cell lymphoma (DLBCL), and biliary tract cancer. Eradication versus microbiota manipulation alternatives are discussed in this context.

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1 Introduction

The International Agency for Research on Cancer (IARC), the specialized cancer agency of the World Health Organization, revealed global cancer statistics based on 28 types of cancer in 184 countries worldwide. World cancer burden rose to 14.1 million new cases and 8.2 million cancer deaths in 2012, compared with 12.7 and 7.6 million, respectively, in 2008. The prevention and early management of cancer development remains one of the most important goals in global health. Solid tumors (e.g., colorectal adenocarcinoma, lung and gastric cancers) and some lymphomas exhibit significant interest in public health due to their prevalence and death rates (Ferlay et al. 2013, 2015). Prevalence estimates for 2012 indicated that 32.6 million people (over the age of 15 years) were alive five years after being diagnosed a cancer. Globally, the most frequently diagnosed tumors were lung (1.8 million), breast (1.7 million), and colorectal (1.4 million) cancers. Regarding cancer-related deaths, the most common causes were lung (1.6 million), liver (0.8 million), and stomach (0.7 million) tumors (Ferlay et al. 2013, 2015). Data analysis also demonstrated that, in 2012, more than half of all cancers (56.8 %) and cancer deaths (64.9 %) were reported in developing countries (Ferlay et al. 2013, 2015).

On the other hand, the emergence of treatment resistant infectious agents has become another source of public health, as well as scientific, concern. In fact, the eradication of bacterial infections is far to be achieved. The epidemiology of resistance shows that it is now a worldwide problem. It affects not only Africa and developing countries, but also industrialized areas, Europe (with the highest rate in the Mediterranean countries), North America (mostly higher in the United States than in Canada), East Asia, and South America (with the worst resistance patterns). Therefore, such a long-term exposure to these pathogens may lead to dramatic consequences. There is growing evidence showing bacteria as biological causes of cancers as well as virus and parasites (Chang and Parsonnet 2010; Schwabe and Jobin 2013).

In this chapter, we present some *Helicobacter* species (*H. pylori* in particular) as biological tumor-inducing agents as well as the mechanisms proposed to understand their contribution to the oncogenic processes. It is clear that a good understanding of these events besides the microbiome control may shed some light on new strategies to reduce cancer evolution. Due to the growth and aging of the global population, the prevalence is expected to increase to 19.3 million new cancer cases by 2025. By that time in less developed regions of the world, the estimations of the proportions of all cancers (>56 % in 2012) and cancer deaths (>64 % in 2012) will also increase further (Ferlay et al. 2013, 2015). Although the proportion of tumors initiated as a consequence of bacterial infections is relatively small, the fact that the initial etiologic agents are known offers the possibility of designing targeted strategies that may contribute to reduce the overall incidence of cancer in the human population. In this regard, a critical question that remains unsolved to date relates to the nature of the disease management approaches: eradication of the infectious

pathogens by the use of antibiotics or manipulation of the microbiota of the affected tissue or organ to maintain, or regain, its normal characteristics, and restore microbiota-host symbiotic interactions that guard general homeostasis.

Recent culture-independent technological advances (i.e., next-generation sequencing) have moved the field of microbiota analysis far beyond the limitations of traditional culture-based identification methods (Engstrand and Lindberg 2013; Arora et al. 2015; Schulz et al. 2015), thus facilitating the quantitative and qualitative characterization of the normal human microbiota as well as our understanding of the dynamic changes in tissue/organ-specific bacterial populations that accompany the onset and progression of cancer and other disease. The possibility of manipulating the human microbiota for cancer prevention purposes and, either alone or in combination with conventional anticancer agents, to design therapeutic strategies is becoming a tangible option, which could make the use of antibiotics to eradicate the infectious bacteria, with all its possible negative consequences, an obsolete approach.

2 *Helicobacter* spp. and Tumorigenesis

The genus *Helicobacter* includes around 33 validity proposed members. Species within the *Helicobacter* genus are non-spore-forming gram-negative bacteria. Their cellular morphology may be curved, spiral, or fusiform, being typically 0.2–1.2 μm in diameter and 1.5–10 μm long. The percent G + C content of their chromosomal DNA ranges from 30 to 48. Some *Helicobacter* species (e.g., *H. pylori*, *H. acinonychis*, *H. bizzozeronii*, *H. felis*, *H. mustelae*, *H. salomonis*) are associated with gastric mucosa, whereas others (e.g., *H. bilis*, *H. canis*, *H. hepaticus*, *H. rodentium*) associate with the intestinal mucosa (Enterohepatic). *H. pylori* has emerged as one of the most important members of the genus, and is known to colonize the human stomach in about 50 % of the population of the world. It is responsible of more than 90 % of duodenal ulcers and up to 80 % of gastric ulcers.

Helicobacter pylori is a spiral-shaped gram-negative bacterium, 2.5–5.0 μm long and 0.5–1.0 μm wide, with 4–6 polar-sheathed flagella (Goodwin and Armstrong 1990). After colonizing the human stomach, it can generate a long-term infection of the gastric mucosa leading in adults and children to chronic gastritis, peptic ulcer disease, and cancers (gastric and lymphoma). The number of Americans suffering from peptic ulcer disease during their lifetime is estimated to be around 25 million. During the past decade (2006), the annual ulcer-related new cases and hospitalizations were approximately 500,000–850,000 and 1 million, respectively. The risk of developing gastric cancer and mucosal-associated-lymphoid-type (MALT) lymphoma is increased two to sixfold in infected patients compared to uninfected subjects. *H. pylori* and other *Helicobacter* species have therefore been related to an increased risk of cancers such as mucosa-associated lymphoid tissue (MALT) lymphoma, gastric adenocarcinoma, colorectal adenocarcinoma, and biliary tract cancer.

2.1 Gastric Cancer

Gastric cancer can be divided into cardia and noncardia gastric adenocarcinoma (NCGA). Although, the global incidence of NCGA has declined, its death rate in patients is extremely high. Currently, gastric cancer is the fifth most common cancer in the world after lung, breast, colon, rectum, and prostate. However, according to Globocan 2012, it is the third leading cause of death in both sexes (723,000 deaths) from cancer (Bray et al. 2013). More than 70 % of this cancer occurs in developing countries. Interestingly, for 60 % of total cases was reported in three East Asia countries (China, Japan, and Korea). Several studies demonstrated the relation between long-term infection with *H. pylori* and the development of gastric cancer. In 1994, the IARC definitively listed *H. pylori* as a human oncogenic agent. In this section, we have summarized some mechanisms and factors involved in *Helicobacter*-induced gastric cancer.

2.1.1 Chronic Inflammation, Host Genetic Polymorphisms and Impairment of DNA Repair System

In human stomach, chronic infection by *H. pylori* ultimately leads to chronic inflammation. After the infection several processes, such as persistent cell necrosis and regeneration, alterations in cell differentiation, dramatically change the gastric mucosa. Inflammation is a mechanism induced by the host to eliminate the invading *H. pylori*. The epithelial cells then release the reactive oxygen and nitrogen oxide species (ROS and RNOS) to combat pathogens, interleukin-8 (IL-8), Gro-a, and chemokines that attract and activate lymphocytes, neutrophils, and macrophages (Ernst et al. 1997). Accompanying this recruitment is the induction of a Th1-predominant cellular immune response and subsequently the secretion of some pro-inflammatory cytokines (IL-1 beta, TNF-alpha, and gamma interferon—IFN-γ—) (Kraft et al. 2001). Recently, oipA (Outer inflammatory protein), also called HopH, that plays a role in the gastric mucosa colonization (Islami and Kamangar 2008) and is involved in the bacterial adherence to gastric epithelia, was demonstrated to be a pro-inflammatory outer membrane protein. It is present in about 97.5 % of patients with gastric or duodenal ulcer. It strongly correlates with mucosal IL-8 production by gastric epithelial cell lines. In contrast, IL-8 levels are substantially reduced when oipA expression is inhibited (oipA knockout mutants). The oipA status is also highly linked to *cag*PAI (cagA), *vacA*, and *babA2* genotypes (Kiviat et al. 1985; Kraft et al. 2001). By phosphorylating multiple signaling cascades interacting with *cag*PAI (CagA)-related pathways, oipA therefore induces inflammation and actin dynamics (Klein and Silverman 2008; Kondo et al. 2009). And such chronic inflammatory process may lead to tumorigenesis.

Host genetic polymorphisms are also involved in gastric cancer development. This process also links chronic inflammation to the oncogenic phenomenon. Several gene polymorphisms associated with increased risk for gastric cancer have

been described. For example, the pro-inflammatory cytokines (TNF-alpha, IL-8, and IL-17), the anti-inflammatory cytokine (IL-10), the hypochlorhydria factor IL-1 that reduces gastric acid secretion (El Omar et al. 2000; Lu et al. 2005; Wang et al. 2007). Globally, the free radicals and secondary products derived from ROS and RNOS may damage DNA, proteins, and cell membranes and indirectly induce cell repair (Coussens and Werb 2002). Inflammation process stimulates regenerative cell division that may lead to point mutations, deletions, or translocations since there is an impairment of the DNA repair system. The damaged and aberrant DNA is then transmitted to next generations by subsequent cell divisions and may result in oncogenesis. For example, the initial oxidative damage by ROS increases the levels of 8-Hydroxyguanine (8HdG) that causes G–T and A–C substitutions in the DNA (Cheng et al. 1992). Another oxidative damage marker, cyclooxygenase-2, displays a gene expression upregulation. In *H. pylori*-infected patients, those levels of the mutagenic metabolite 8HdG and cyclooxygenase-2 confirmed to be high, returned to baseline once eradicated *H. pylori* (Burkitt et al. 2009). An additional hypothesis suggests that *H. pylori* may cause tumor by activating mechanisms mediated by the proto-oncogenes c-*fos* and c-*jun*, both mitogenic signal transduction pathways (Meyer-ter-Vehn et al. 2000).

2.1.2 Hormones. Human Gastrin Hormone

There is growing evidence linking hormones to cancers. Some studies have postulated that chronic infection with *H. pylori* may also cause carcinogenesis through hypergastrinemia, a mechanism particularly related to Gastrin. This hormone is one of the most important peptide hormones in the human stomach. It is produced by neuroendocrine G cells in the antrum of the stomach. Gastrin and its precursors are involved in gastrointestinal tumors including gastric cancer. *H. pylori* harbors, at the tip of the T4SS pilus, a ligand (CagL) that activates the gastrin promoter by interacting with αvβ5-integrin and integrin $\alpha_5\beta_1$ receptor of the gastric epithelial cells via integrin linked kinase (ILK) signaling complex and via Arg-Gly-Asp (RGD) motif, respectively (Abramson et al. 2005). Therefore, besides the previously known function of CagL as an activator of epidermal growth factor receptor (EGFR), Raf cascade and MAP kinase (EGFR/Raf/MAP/Erk signaling pathway), it has been also shown to increase gastrin expression. Gastrin then binds to cholecystokinin 2 receptors and activates directly acid secretion or indirectly by previously stimulating histamine release that further binds to histamine 2 receptors (Aly et al. 2004; Anderson et al. 2008). The subsequent hypergastrinemia induced by *H. pylori* may trigger gastric precancerous conditions. *H. pylori* infection together with gastrin hormone can promote the secretion of heparin-binding epidermal growth factor (HB-EGF) and other growth factors as inducers of cell cycling and proliferation (Varro et al. 2002; Dickson et al. 2006). Finally, some findings also show that CagL, the mentioned activator of gastrin promotor, is essential for injection of the oncoprotein CagA into gastric epithelial cells. This issue will be discussed further.

2.1.3 Oncogenic Determinants

In this section, we describe the contribution of *H. pylori* oncoproteins to cell transformation. In particular, we will focus on CagA and VacA.

CagPAI and CagA: *Cag*PAI is a 40 kb region of chromosomal DNA, harboring approximately 31 genes. It encodes a functional type IV secretion system (T4SS) that forms a pilus to deliver the oncoprotein CagA into the cytosol of gastric epithelial cells through a rigid needle structure (Covacci and Rappuoli 2000; Rohde et al. 2003; Backert and Selbach 2008). *CagA* is a polymorphic gene with different numbers of repeated sequences in its C-terminal region. Each of these repeated regions contains Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs with a tyrosine phosphorylation site (Hatakeyama 2004). Four distinct EPIYA segments (EPIYA-A, EPIYA-B, EPIYA-C, and EPIYA-D), each of which contains a single EPIYA motif, have been identified in the EPIYA-repeat region (Fig. 1). The EPIYA-repeat region of CagA from Western *H. pylori* isolates is A–B–C-type CagA and the EPIYA-C segment is frequently one to three times repeated in tandem (Fig. 1). Whereas CagA from East Asian *H. pylori* isolates is A–B–D-type and the EPIYA-D segment is unique to East Asian CagA (Higashi et al. 2002; Hatakeyama 2009) (Fig. 1). In the host target cells, this effector oncoprotein is phosphorylated by Src and Abl kinases (Miehlke et al. 2000) at EPIYA motif located in the 3′ region of CagA. Phosphorylated CagA then deregulates SHP2 phosphatase activity through the interaction with this eukaryotic protein (Higashi et al. 2002; Backert and Selbach 2005).

SHP-2 specifically binds to the tyrosine-phosphorylated EPIYA-C (light union) or EPIYA-D (strong binding) segment. Therefore, East Asian CagA (A–B–D-type) exhibits stronger SHP-2 binding than does Western CagA (A–B–C-type). A consequence of this process is the possible production of precancerous lesions and gastric cancer (Higashi et al. 2002; Hatakeyama 2009). Similarly, in Western CagA species, those with a greater number of EPIYA-C segments display higher interaction with SHP-2. These findings may explain the higher incidence of gastric cancer in East Asia and the geographic variability in the incidence of this tumor in Western countries. For example, the incidence of this cancer is higher in Colombia (with 57 % of the isolates exhibiting two EPIYA-C segments) than in USA (with only 4 % of the isolates showing two EPIYA-C segments) (Yamaoka 2010; Yamaoka and Graham 2014). CagA-deregulated SHP2 activates Erk MAP kinase signaling (in both Ras-dependent and Ras-independent pathways) promoting proliferation, and also inhibits focal adhesion kinase (FAK) activity related to cell-extracellular matrix interaction (Higashi et al. 2004; Tsutsumi et al. 2006). Phosphorylated CagA also induces the activation of pro-inflammatory cytokines, cell proliferation, motility, elongation (Wessler and Backert 2008) as well as the dysregulation of β-catenin signaling (Franco et al. 2005; Murata-Kamiya et al. 2007), an increase of cell motility and oncogenic transformation (Suzuki et al. 2005; Franco et al. 2008). Recent studies indicated that *cag*PAI activates gastric interleukin-8 (IL-8) production, a potent neutrophil-inducing chemokine (Brandt et al. 2005) and that

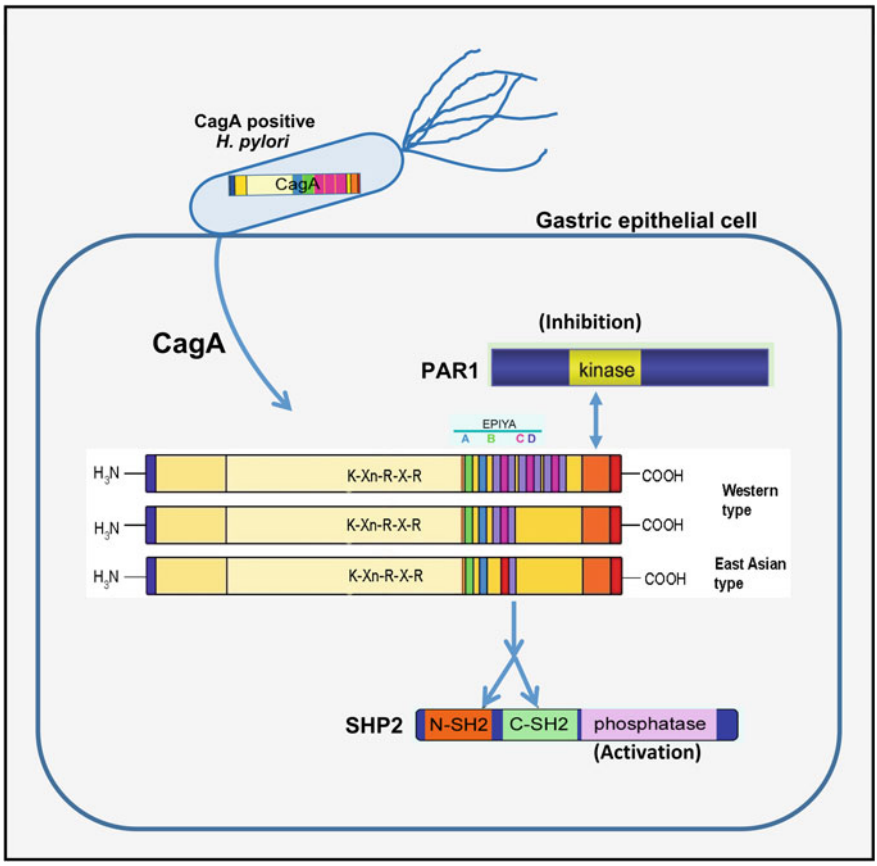

Fig. 1 Schematic representation of the structure of the most common *Helicobacter pylori* CagA variants of the western and East Asian types. Two important CagA protein-protein interactions are shown. Upon entry into gastric epithelial cells, CagA interacts and activates the SHP2 phosphatase, in a tyrosine phosphorylation-dependent manner. In addition, CagA interacts with PAR1 and inhibits its serine/threonine kinase activity. These interactions result in profound morphological alterations and polarity changes in the host cells, thus contributing to their neoplastic transformation. This figure was designed by combining portions of (1) a figure from Morales-Guerrero et al. (2013) (© 2012 Morales-Guerrero SE, Mucito-Varela E, Aguilar-Gutierrez GR, López-Vidal Y, Castillo-Rojas G) that was originally published under CC BY 3.0 license (available from: http://dx.doi.org/10.5772/53136), and (2) of a figure from an article (Yamahashi and Hatakeyama, 2013, available online from: http://wwww.tandfonline.com/ doi:10.4161/cam.21936) published by Taylor & Francis in the Jan–Feb 2013 issue of *Cell Adhesion and Migration*, licensed to VN (no. 3742711162415, on Nov 5, 2015)

CagL plays an important role in the injection of CagA into gastric epithelial cells (Tegtmeyer et al. 2010).

Vacuolating Cytotoxin Gene (*vacA*): The gene encoding VacA is present in all *H. pylori* strains. Upon the secretion of the cytotoxin VacA from the bacteria, this large polypeptide (140-kDa) is trimmed at both ends and latter delivered as an

active form to host cells. VacA exerts multiple cellular activities: alteration in the endosomal maturation leading to epithelial cell vacuolation, induction of membrane-channel formation, release of cytochrome c from mitochondria and activation of a pro-inflammatory response through the interaction with cell membrane receptors (Amieva and El Omar 2008). VacA toxins exhibit distinct capability of inducing vacuolation in epithelial cells (Atherton et al. 1995; Blaser and Atherton 2004) and its cytotoxic activity varies among strains (Amieva and El Omar 2008; Wroblewski et al. 2010). VacA has been associated with an increased risk of developing gastric epithelial injury and gastric cancer (Atherton et al. 1995; Basso et al. 2008; Lopez-Vidal et al. 2008; Yamaoka 2010).

2.1.4 Antigens. Blood Group Antigen-Binding Adhesion (*BabA*)

The adhesin BabA belongs to a family of highly conserved OMPs (Outer-Membrane Proteins) and binds to ABO histo-blood group antigens and corresponding Le^b antigens (also called MUC5AC) expressed on gastric human epithelial cells (Ilver et al. 1998). There are three *bab* alleles (*babB*, *babA1*, *babA2*). BabA2 is the only one required for Le^b binding activity (Abadi et al. 2013). *BabA2* prevalence in gastric cancer patients was found to be 95 % in some regions of the Middle East (Toller et al. 2011). There is a co-expression between *babA2* and *cagA*, and *vacA* (Zambon et al. 2003). They exert their activity synergistically fostering the inflammation. The bacterial adhesion via *babA* has been suggested to participate in the DNA double-strand breaks (DSB) induced upon *H. pylori* infection (Toller et al. 2011). DSB phenomenon may finally provoke genetic instability and the frequent chromosomal alterations found in gastric cancer.

2.1.5 Toxic Bacterial Metabolites—Nitrosamines

Bacterial cells could also produce toxic metabolites that may damage host cell DNA initiating oncogenic process. Nitrosamines are one of the most powerful mutagens. They methylate oxygen and nitrogen atoms of the DNA. *H. pylori* promotes the local production of radicals, like *N*-nitroso compounds, that play an important role in the initiation of gastric cancer. There is a higher susceptibility of the infected gastric mucosa cells to these DNA-damaging agents because the effect induced by nitrosamines is synergistic to that caused by the infection. The excess of ammonia, a product of *H. pylori* urease, in gastric cells may be involved in this process. In fact, by stimulating neutrophils and phospholipases, ammonia may induce indirect mucosal injury. Furthermore, by degrading phospholipids and producing ulcerogenic precursor factors, it may damage the gastric mucosa. A posited mechanism of action is that the interaction between *H. pylori*-induced free radicals and the gastric mucosa cell membranes subsequently might generate several products of lipid peroxidation that finally destroy the integrity of the gastric epithelium (Figura 1997; Arabski et al. 2006). Another mechanism that may allow also the development of

gastric cancer is related with the finding that *N*-nitroso compounds are higher during chronic atrophic gastritis. Interestingly with gastric cancer, *H. pylori* sometimes caused hypochlorhydria. Therefore, such conditions prompt the overgrowth of diverse bacteria that deliver nitrosamines (Carboni et al. 1988).

2.2 Colorectal Adenocarcinoma

Colorectal cancer was the third most common cancer in the world in 2012. That year there were approximately 1.4 million new cases diagnosed. The adenocarcinoma subtype represents nearly 95 % of the colorectal cancers. Other possible subtypes are mucinous carcinomas and adenosquamous carcinomas. The highest incidence of colorectal cancer was in Oceania and Europe and the lowest incidence in Africa and Asia. More than 50 % of colorectal cancer cases were detected in industrialized countries. In the United States, from data available in 2010, 131,607 people (67,700 men and 63,907 women) were diagnosed with colorectal cancer and 52,045 persons (27,073 men and 24,972 women) died. It is the third most common cancer diagnosed in the United States and globally (both sexes combined), it is the second leading cause of cancer-related deaths. Overall, the lifetime risk of developing colorectal cancer is around 5 % (1 in 20). For past year (2014), the estimations of new cases were 96,830 for colon cancer and 40,000 for rectal cancer. On the other hand, 50,310 deaths were expected (American Cancer Society 2014). Several studies have demonstrated the presence of *H. pylori* in colorectal adenomas and colorectal cancer tissues (Kapetanakis et al. 2013; Kountouras et al. 2013, 2014). There is an increased risk of colorectal cancer due to *H. pylori* infection (Zhao et al. 2008; Kapetanakis et al. 2013). Herein, we suggest some mechanisms and factors involved in the described association between *H. pylori* infection and colorectal neoplasia.

2.2.1 Inflammation, Proliferation, Stem Cells Recruitment

Recent data indicate that *H. pylori* colonizing colonic malignant tissue may increase cell proliferation and impair apoptotic cell death mainly in tumor specimens. This process may also lead to colon cancer progression (Kountouras et al. 2003). In colorectal adenomas and colorectal cancer tissues, the presence of *H. pylori* was accompanied with immunohistochemical expression of proliferation marker Ki-67, anti-apoptotic Bcl-2, CD45 (assessing T and B lymphocytes locally) and stem cells marker CD44 (indicator of cancer stem cells—CSCs—, and/or bone marrow-derived stem cells—BMDSCs—) (Kountouras et al. 2013, 2014).

2.2.2 Gastrin Hormone and Its Receptor CCK-2

CCK2 (cholecystokinin-2), encoded by the CCKBR gene, is a G protein-coupled receptor for gastrin and cholecystokinin (CCK), regulatory peptides of the brain and gastrointestinal tract. Both gastrin and its receptor, cholecystokinin-2 (CCK-2) receptor, exhibited high levels in many premalignant colonic lesions. These proteins seem to be important in colorectal tumorigenesis, and tumor growth (Chao et al. 2010). Four carboxy-terminal amino acids of gastrin are essential for activation of the CCK2 receptor (Hollestelle et al. 2008). *H. pylori* induced gastrin release that may act as a promoter of cell proliferation and differentiation. These cell processes are mainly promoted by inducing COX-2 overexpression and PI3-kinase-mediated tyrosine phosphorylation of E-cadherin and β-catenin) in the colon and different gastrointestinal tract sites (Kountouras et al. 2000). Subsequently and as aforementioned, the bone-marrow-derived stem cell recruitment observed during *H. pylori* infection also plays a role during colon cancer progression (Kountouras et al. 2006).

2.3 Other Cancers

2.3.1 Lung Cancer

A hypothesis gaining widespread acceptance suggests that *H. pylori* infection increases the risk of lung cancer. Several authors have postulated that *H. pylori* may induce lung carcinogenesis by mechanisms and factors similar to those described for gastric cancer. In lung cancer patients, the levels of serum gastrin are higher compared to controls. Furthermore, such levels correlate with tumor stage (Zhou et al. 1992). Gastrin has been shown to be co-expressed with COX-2 (Subramaniam et al. 2008). Therefore, both may stimulate tumor growth and angiogenesis (Gocyk et al. 2000). As previously mentioned, CagA is initially phosphorylated by Src. The activation of FAK and Src leads to the injection of CagA. A substrate of Src kinase is p130cas. Once activated by Src, p130cas can recruit a Crk (v-crk sarcoma virus CT10 oncogene homolog)/DOCK180 (dedicator of cytokinesis) complex (Gu et al. 2001). The Crk/p130cas complex plays a critical role in *H. pylori*-infected gastric epithelial cells, promoting bacteria-induced migration and invasive growth of gastric epithelial cells (Schneider et al. 2008). In cancer cells including lung tumors, p130cas is involved in carcinogenesis (tumor cell growth and migration, cell cycle progression) (Huang et al. 2012). It also mediates cell survival and delays the death of cancer cells (Wei et al. 2002). P130cas is implicated in tumor prognosis, and in lung cancer samples its overexpression is also correlated with poor overall survival (Huang et al. 2012). All these data suggest that p130cas may be involved in *H. pylori*-mediated carcinogenesis in the lung. The surface enzyme of *H. pylori* called Urease has shown to be involved in bacterial infection and survival. Furthermore, urease is being highly expressed in gastric cancer specimens

(Wu et al. 2007) likely promoting cell proliferation and gastric mucosal hyper-proliferation. The detection of *H. pylori* urease proteins in lung (Herndon et al. 2013) support the hypothesis that *H. pylori* urease may induce proliferation and carcinogenesis of the pulmonary mucosa.

2.3.2 Gastric MALT Lymphoma

As aforementioned, chronic infection with *H. pylori* may also generate an environment allowing lympho-proliferation and gastric MALT lymphoma. The most common site (approximately 50 % of all cases) for MALT lymphoma to develop is in the stomach (Gastric MALT lymphoma). Gastric MALT lymphoma represents 5 % of all primary gastric cancers (tumors starting in the stomach) and therefore remains an unusual type of stomach cancer. MALT lymphoma may start in a context of chronic inflammation, especially in an *H. pylori* infection background (Swerdlow et al. 2008). In more than 80 % of cases, a robust association between chronic *H. pylori* infection and MALT gastric lymphoma has been observed. It is well demonstrated in this tumor pathogenesis that the aforementioned bacterial infection plays a key role (Stolte et al. 2002; Psyrri et al. 2008; Swerdlow et al. 2008). Chronic *H. pylori* infection induces the antigenic stimulus, leading to the clonal proliferation of lymphoid cells arising to the MALT lymphoma. Some particular strains of *H. pylori* are important in this process, those expressing CagA protein since they carry the major histocompatibility complex (MHC) class II T cell epitope. After an infection with this specific strain, CD4+T cells activation occurs and it has been postulated that such conditions may therefore allow tumor development (Arnold et al. 2011). On the other hand, in most of patients (75–80 %) with early stage MALT lymphoma, the eradication of *H. pylori* was followed by a complete regression of such low-grade disease (Stolte et al. 2002; Yoon et al. 2004; Jezersek et al. 2006; Nakamura et al. 2008). This approach was particularly successful in patients exhibiting infection only in gastric mucosa or submucosa. However, elsewhere location of infection (muscularis propria or serosa, nodal disease) correlated with the decrease of complete response rates (Levy et al. 2002; Stolte el al. 2002; Nakamura et al. 2003, 2008; Yoon et al. 2004; Chen et al. 2005; Jezersek et al. 2006; Fischbach 2010).

2.3.3 Burkitt Lymphoma (BL)

Burkitt lymphoma (BL) is an aggressive mature B cell neoplasm. This form of non-Hodgkin's lymphoma is highly prevalent in children and young adults. The WHO classification describes three types of BL. All three variants exhibit chromosomal rearrangement of c-MYC oncogene. This biological phenomenon leads to some modifications of cell cycle regulation, cellular metabolism, adhesion, differentiation, and apoptosis driving to cancer development (Pagano et al. 2009). Studies performed in the past decade suggested the role of *H. pylori* in BL. In fact,

it has been reported the complete remission of BL after the administration of *H. pylori* eradication therapy (Baumgaertner et al. 2009).

2.3.4 Gastric Diffuse Large-B-Cell Lymphoma (DLBCL)

Gastric Diffuse Large-B-Cell Lymphoma (DLBCL) is another form of extra-nodal non-Hodgkin's lymphoma detected in gastric tissue. The mechanisms related to *H. pylori* infection in primary DLBCL and leading to tumor development are similar to those described above (for Gastric MALT lymphoma).

3 Disease Management Strategies

Evidence for the tumor-promoting effects of the bacterial microbiota has been obtained from studies in both animals and humans (Cho and Blaser 2012; Schwabe and Jobin 2013). Tumor incidence in germ-free rodents exposed to a variety of carcinogenic insults was generally lower than that observed in normal animals under the same conditions. In addition, fewer tumors developed in antibiotic-treated animals than in untreated controls. Similarly, eradication of bacterial pathogens with antibiotic treatment caused a reduction in cancer progression, or even regression, in the treated patients (Schwabe and Jobin 2013; Bultman 2014). Although the preponderant evidence supports the tumor-promoting effects of the bacterial microbiota, the correlation is not perfect. Nevertheless, *H. pylori* has become the best example of the capacity of an infectious bacteria to drive the carcinogenic process in gastric tumors (McColl 2010; Polk and Peek 2010; Wroblewski and Peek 2013) and, consequently, the prime model for the evaluation of diverse strategies to manage the infection and, particularly, to provide anticancer benefits.

An important consideration to make relates to the fact that, in addition to being associated with increased cancer risk and demonstrated greater stomach cancer incidence, the presence of *H. pylori* (particularly of CagA-positive strains) in the gastric environment correlates with parallel changes in the nature and number of other phylotypes present not only in the stomach itself, but also in the lower esophagus as well as in the upper intestinal tract (Khurana 2012; Engstrand and Lindberg 2013; Sheh and Fox 2013, Arora et al. 2015; Nardone and Compare 2015; Schulz et al. 2015). These observations support the notion that the alteration of the normal microbiota may contribute to carcinogenic progression by having additional effects on host factors and, thus, further changing the environment. However, it is not clear whether *H. pylori* causes these microbiota alterations, or whether these changes come first and enhance the pro-carcinogenic activity of *H. pylori*. It seems still possible that the microbiota changes by themselves may be the initiating event in the carcinogenic process, as complete tumor regression has not been achieved with antibiotics to *H. pylori* alone.

Regardless of whether *H. pylori* colonization of the gastric epithelium or the observed microbiota alterations came first, bacterial eradication with effective antibiotics seems to be the most intuitive and immediate course of action to prevent cancer development or to treat already established stomach tumors. However, there are several limitations to the eradication approach. For instance, eradication does not have uniform beneficial effects at all stages of stomach cancer development (Arora et al. 2015). Although significant gastric cancer risk reductions were observed when eradication treatments were performed on normal individuals or in patients with non-atrophic gastritis (NAG) or on those with atrophic gastritis without intestinal metaplasia (IM), there was no benefit for cases with IM or for those with dysplastic changes (Arora et al. 2015). Furthermore, not only *H. pylori* eradication efforts have not been as widely beneficial as expected, but it has increased the incidence of inflammatory bowel disease (IBD) and, most importantly, of esophageal cancer. In fact, these data are so significant that a protective beneficial role against esophageal adenocarcinoma has been attributed to *H. pylori* (Blaser 2008; Sheh and Fox 2013; Abadi 2014). According to this notion, *H. pylori* would have dual protective and pathogenic roles (Blaser 2008; Atherton and Blaser 2009; Dorer et al. 2009; Sheh and Fox 2013). Finally, there is always the risk that inappropriate use of antibiotics may result in the selection of *H. pylori* antibiotic-resistant isolates, as it was the case for other diseases caused by bacterial pathogens. For all these reasons, eradication must be carried out, if at all, in a well-balanced fashion (Blaser 2008).

A second disease management alternative is based on the notion that protocols designed to minimize the negative effects of *H. pylori* colonization on the stomach mucosa is more appropriate than eradication approaches. The goal is to manipulate the microbiota to maintain that of normal individuals in such a way that protection against *H. pylori* and/or against the negative effects of *H. pylori* eradication may be provided (Arora et al. 2015; Schulz et al. 2015). Protocols for microbiota restoration that are being developed with prophylactic purposes include the use of dietary prebiotics (i.e., fiber sources or polyphenols) and, most frequently, of probiotics (Cho and Blaser 2012; Bultman 2014; Patel et al. 2014), including fecal transplantation approaches (Bowman et al. 2015). An important aspect regarding the use of probiotics relates to the use of either single probiotic strain or several strains in combination, as results to date do not seem to fully support one way or the other (Schulz et al. 2015). The goal of ongoing research efforts is to get to the point of having "designer probiotics" for the personalized treatment, prophylactic or therapeutic, of individual patients suffering from diverse diseases, including cancer. In the case of therapeutic protocols, a pre-therapeutic characterization of probiotic strains before use in combination seems to be essential (Patel et al. 2014; Schulz et al. 2015).

In the context of *H. pylori*-associated cancers, an important limitation of the microbiota manipulation strategy derives from the fact that it has not yet been conclusively demonstrated whether the pro-carcinogenic events result from the direct action of *H. pylori*, to the alterations of the stomach microbiota, or to both. Consequently, there are important considerations to take into account when deciding how to modify the stomach microbiota. Four suggestions may be useful in

this regard: (1) Identification (and replacement with) bacterial strains present in the stomach prior to *H. pylori* infection; this is something that can be accomplished by individuals getting a personalized microbiome "identity card" that should be updated regularly; (2) Usage of probiotics to induce a general gastric normalization process, not only in the stomach but also in the lower esophagus and the upper intestine; (3) Minimization of the action of host factors known to contribute to *H. pylori* pathogenesis; and (4) Inclusion of bacterial strains that may compete with *H. pylori* and thereby decrease its cancer malignancy promoting action. For example, as it seems consistently clear that the promotion of stomach cancer is primarily due to CagA-positive *H. pylori* cells, one could consider the possibility of including CagA-negative strains in microbiota replacement protocols. The competition of CagA(−) with CagA(+) cells might minimize their disease-promoting effects and contribute to establish an overall level of *H. pylori* in the stomach that will be compatible with a more balanced microbiota and will not increase the risk of esophageal cancer.

4 Perspectives and Conclusions

The field of microbiome research is currently advancing at a great pace. Nevertheless, we still need a deeper understanding of the underlying cellular and molecular mechanisms of bacterial-induced carcinogenesis and the role of host-derived contributing factors to reach the point of being able to applying microbiome manipulation strategies at the clinical level. From the perspective of cancer detection, the advantage of *H. pylori* related cases is that infection with *H. pylori*, and indeed with other Helicobacter species, causes the appearance of gastritis symptoms early in the process. The early detection of *H. pylori* may allow to establish an "active surveillance" strategy to time disease stage-specific microbiome remodeling interventions. Personalized microbiome modulation is a likely possibility in the not so distant future for the management of cancer and other human disease states.

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Targets Against *Helicobacter pylori* and Other Tumor-Producing Bacteria

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Abstract People are prone to cancer when infected with infectious agents. *Helicobacter pylori* was the only infectious agent well characterized for stomach cancer and the rest of the cancers are yet to be characterized. Literature review has provided information on other *Helicobacter* species such as *Helicobacter felis, Helicobacter salmonis, Helicobacter bizzozeronii* and *Helicobacter heilmannii* (known for causing stomach cancer), *Helicobacter bilis* (known for causing biliary tract cancer), and *Helicobacter hepaticus* (known for causing liver cancer). Non-*Helicobacter* species such as *Neisseria gonorrhoeae, Campylobacter jejuni,* and *Chlamydophila pneumoniae* are also known for causing prostate cancer, stomach cancer, and lung cancer, respectively. In this chapter, we focused on reviewing the mechanism of pathogenesis and drug targets available till date for cancer-causing bacteria like *H. pylori, H. felis, H. salmonis, H. bizzozeronii, H. heilmannii, N. gonorrhoeae, C. jejuni,* and *C. pneumoniae.*

1 Introduction

People are more prone to cancer due to chemical and infectious agents. World's 15–20 % cancers are caused by infectious agents, leading to stomach, colon, liver, prostate, lung, gall bladder cancer, etc. (Table 1; Fig. 1). Infectious agents when infected cause cancer only in a subset of the population and not to all people of a population. Till day, *Helicobacter pylori* infection leading to stomach cancer was the only cancer characterized and the rest of the cancers are yet to be characterized in detail. Other *Helicobacter* species which are known for causing cancer upon infection are *Helicobacter bilis, Helicobacter hepaticus, Helicobacter felis, Helicobacter salmonis, Helicobacter bizzozeronii,* and *Helicobacter heilmannii.*

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Table 1 List of *Helicobacter* and non-*Helicobacter* species causing inflammation, tumor, and cancer

S. No	Tumor-causing bacteria	Inflammation	Tumor	Type of cancer	References
***Helicobacter* species**					
1	*Helicobacter pylori*	Yes	Yes	Stomach and duodenal linings cancer	Neelapu et al. (2014)
2	*Helicobacter bilis*	Yes	–	Liver cancer	Matsukura et al. (2002)
3	*Helicobacter hepaticus*	Yes	Yes	Colorectal/liver cancer	Ward et al. (1994)
4	*Helicobacter feili*	Yes	Yes	Stomach cancer	Hasegawa et al. (2004), Fritz et al. (2006)
5	*Helicobacter salmonis*	Yes	–	Stomach cancer	De Bock et al. (2005)
6	*Helicobacter bizzozeronii*	Yes	–	Stomach cancer	De Bock et al. (2005)
7	*Helicobacter heilmannii*	Yes	–	Stomach cancer	Bahadori et al. (2014)
***Non-Helicobacter* species**					
8	*Campylobacter jejuni*	Yes	Yes	Stomach cancer	(Moran 2010)
9	*Bacteroides fragilis*	Yes	Yes	Colon cancer	Toprak et al. (2006)
10	*Neisseria gonorrhoeae*	Yes	–	Prostate cancer	Cheng et al. (2010)
11	*Chlamydophila pneumoniae*	Yes	–	Lung cancer	Zhan et al. (2011)
12	*Salmonella typhi*	Yes	–	Gallbladder cancer	Gonzalez-Escobedo et al. (2011)

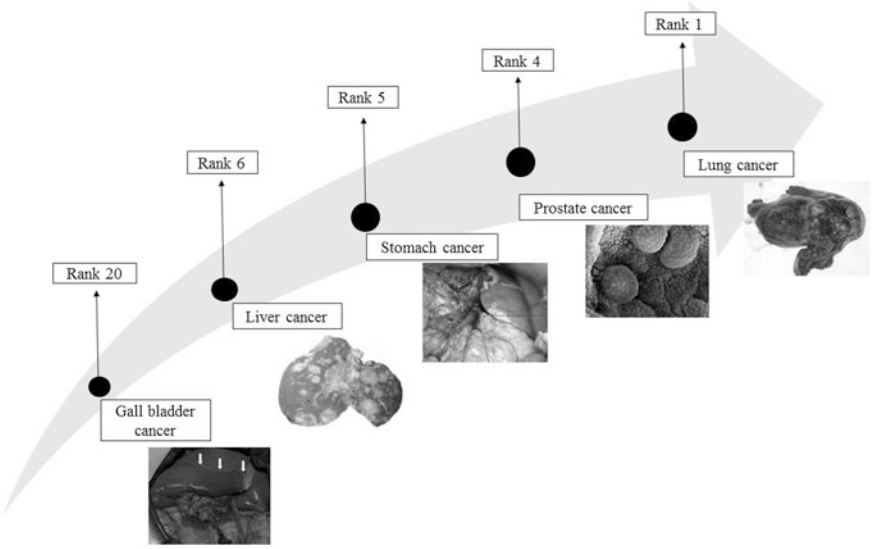

Fig. 1 Different cancers and their respective ranks

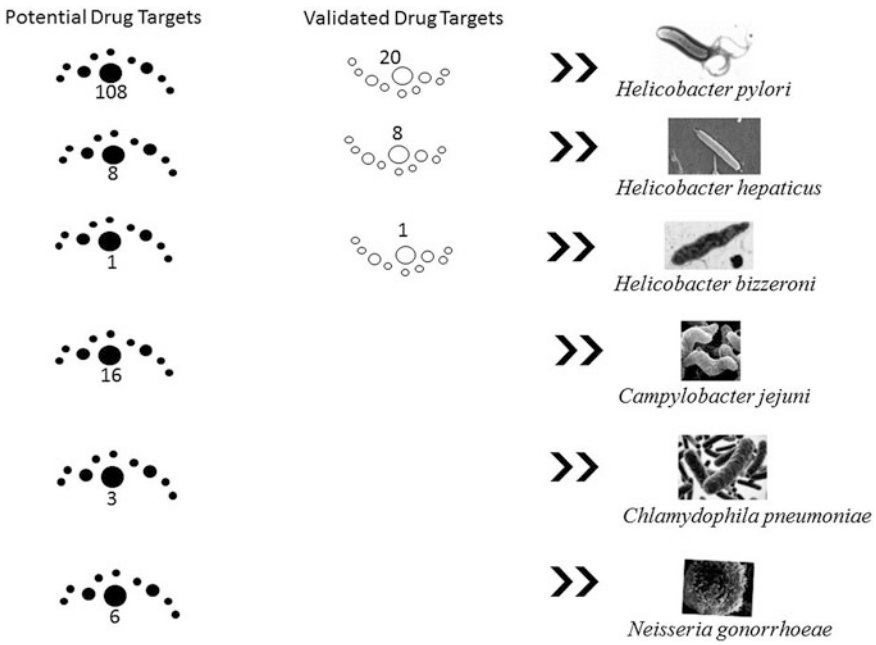

Fig. 2 Number of potential and validated drug targets identified for various tumor causing bacteria

Non-*Helicobacter* species which are also known for causing cancer are *Bacteroides fragilis, Neisseria gonorrhoeae, Campylobacter jejuni, Chlamydophila pneumoniae,* and *Salmonella typhi* (Fig. 2).

2 Identification and Validation of Drug Targets

Identification and validation of novel drug targets is a key process for discovery of new compounds. Various methods and approaches are available for discovery and validation of drug targets for infectious diseases. Neelapu et al. (2013) postulated an integrated approach and paradigm for drug target discovery and validation. The approach starts with identifying innovation drivers in the literature, groups, and organizations working on diseases, implementing crowd sourcing as a platform to connect the groups and organizations working on diseases, then establishing the drug target networks for the drugs and drug targets helps in revealing more prospects of a particular disease. Later drug targets can be identified using in silico, in vitro, and in vivo approaches simultaneously. In silico approaches like genome analysis, grid technology, metabolome analysis, microarray data analysis, proteome analysis, and reverse docking are used for identification of drug targets. Genomes,

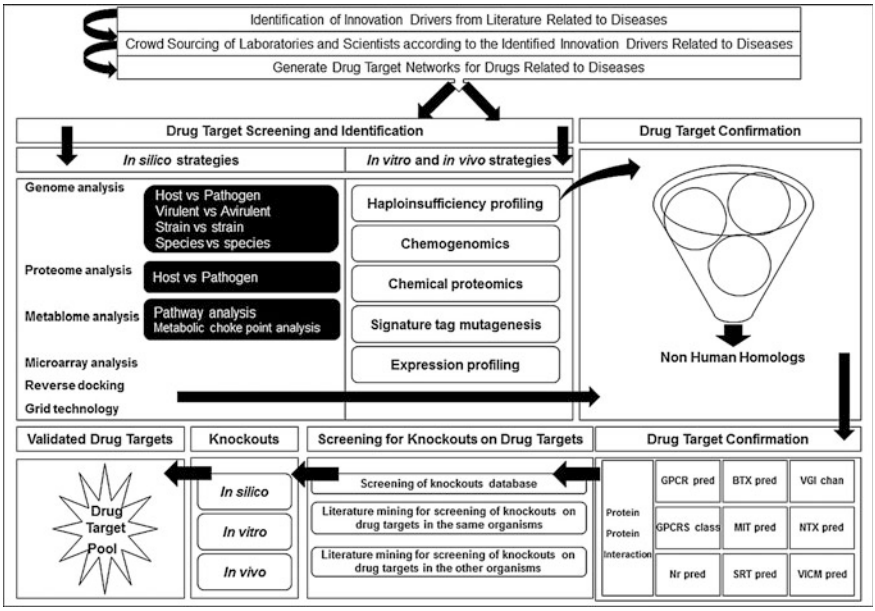

Fig. 3 Schematic representation of identification, confirmation, and validation of drug targets

proteomes, metabolomes of the above-identified molecules can be validated by comparing with that of the host to get nonhomologue of humans. Validated drug targets can be subjected to property analysis for drug target properties such as enzymes, virulence, toxins, outer membrane, etc. Further, validation can be done by screening of drug targets in the knockouts database and by mining of literature for knockouts in the same or similar organisms. Simultaneously, in vitro and in vivo methods like chemogenomics, chemical proteomics, expression profiling, haploinsufficiency profiling, and signature tag mutagenesis can be implemented for identification and validation of drug targets. In silico knockouts and gene knockout technology can be used for drug target validation (Fig. 3).

3 Targets for *Helicobacter pylori*

3.1 Helicobacter pylori *Infection, Tumor, and Gastric Cancer*

Gastric inflammation, ulcer, and cancer are induced by *H. pylori* infection. Coordinated cascade manner of infection helps *H. pylori* to colonize the host. Colonization of *H. pylori* is initiated in the stomach by making itself amicable to the harsh acidic environment (Neelapu et al. 2014). *H. pylori* senses the pH in the stomach using chemoreceptor TlpB and moves toward the less acidic region (Goers

Sweeney et al. 2012). In addition periplasm and cytoplasm of *H. pylori* are neutralized by producing urease (Wen et al. 2003). *H. pylori's* next impediment is gastric mucosal barrier in the stomach and it has the potential to surpass this gastric mucosal barrier (Burrows 2010). Toxin VacA, cytokines, and gastrin are used by pathogens to weaken the gastric mucosal barrier to colonize in the mucous or submucous or epithelial cells. The gastric mucosal barrier is weakened either by loosening/disrupting the mucous layer or by modifying the mucous glycoproteins (Neelapu et al. 2014). *H. pylori* with the help of various adhesion molecules like AlpA, AlpB, BabA, HopZ, OipA, and SabA adhere to the epithelial lining of the stomach after the gastric mucosal barrier is weakened (Petersen and Krogfelt 2003; Ilver et al. 1998; Mahdavi et al. 2002; Moodley et al. 2009). Later, several toxins are used by pathogen to establish interaction with the host leading to inflammation, a condition known as gastritis. T4SS system coded by cytotoxin-associated gene pathogenicity island (cag PAI) injects CagA, peptidoglycan, and VacA into the host. Cag A changes the expression of host cells; induces elongation of cell, loss of cell polarity and cell proliferation; decreases acid secretion; and degrade cell–cell junctions (Yamaoka 2010). Cytokines (IL-1, IL-6 and IL-8), chemokines (CXCL8, CCL3, CCL4), metalloproteinases (MMPs), prostaglandin E2 (PGE2), and reactive oxygen nitrogen species (RONS) in turn augment and prolong the inflammatory cascade. Prolonged inflammation induces G cells to secrete the hormone gastrin stimulating loads of acid damaging duodenum, a condition known as ulcers (Blaser and Atherton 2004; Schubert and Peura 2008). *H. pylori* is unprotected from acid environment, peristalsis, oxidative stress, and phagocytes leading to DNA damage (Olczak et al. 2002) and the acquired DNA repair mechanism is used by *H. pylori* for successful infection (Michod et al. 2008). NF-κβ and β-catenin signaling pathways directly or indirectly induce double-stranded breaks (DBS) and defective mitotic checkpoints (DMC); deregulate HR pathway of DSB repair and DNA repair enzymes leading to microsatellite instability (MSI) and chromosomal instability (CI) (Colotta et al. 2009). MSI and CI leads to genetic diversification randomly leading to activation of oncogenes and inactivation of tumor-suppressor genes RUNX3, p53, ASPP2, TFF1, TFF2, TFF3, GK1, and GKN2 leading to gastric cancer (Neelapu et al. 2014).

3.2 *Drug Targets for* Helicobacter pylori

Dutta et al. (2006) used subtractive genomics for identification of essential genes in *H. pylori* strain HpAG1, Hp26695, and J99 (Table 2). Kiranmayi et al. (2009) identified essential transporter genes in *H. pylori* using bioinformatics approaches (Table 2). Neelapu and Pavani (2013) identified 17 novel drug targets in *H. pylori* strains HpB38, HpP12, HpG27, HpShi470, HpSJM180 using in silico genome and proteome analysis, whereas in a similar type of analysis carried out in the strain HpAG1 29 novel drug targets were identified (Neelapu et al. 2015) (Table 2). Nammi et al. (2015) used comparative genomics, proteomics, etc., for 23 *H. pylori*

strains to identify 29 novel drug targets (Table 2). Mandal and Das (2014) used in silico approach for identifying drug targets in *H. pylori.* Sarkar et al. (2012) used metabolic pathway analysis to identify drug targets in *H. pylori*. Cai et al. (2006) used reverse docking to identify drug target in *H. pylori.*

Literature review provided a total of 108 drug targets identified so far for *H. pylori* belonging to different metabolic categories. Of these membrane fusion protein of the hefABC efflux system; hydrogenase expression/formation protein HypD gene; cag pathogenicity island protein X gene (Neelapu and Pavani 2013); superoxide dismutase (SOD); HtrA protease/chaperone protein; heat-inducible transcription repressor HrcA; HspR, transcriptional repressor of DnaK operon; cobalt–zinc–cadmium resistance protein CzcA (Neelapu et al. 2015); menaquinone via futalosine step 1 gene; type III restriction modification system methylation subunit; type I restriction modification system specificity subunit S, DNA-binding protein HU, dipeptide transport system permease protein DppABC, oligopeptide transport system permease protein OppC, HoxN/HupN/NixA family nickel/cobalt transporter gene, D-alanine–D-alanine ligase B, flagellar biosynthesis protein FliP, Na^+/H^+ antiporter, potassium channel protein, and YafQ toxin protein (Nammi et al. 2015) are the drug targets validated in the wet bench. Therefore, designing an inhibitor for these proteins would lead to therapeutic intervention for gastric pathogen *H. pylori.*

3.2.1 Drug Targets Influencing Resistance of Antibiotics and Toxic Compounds of the Pathogen

Neelapu and Pavani (2013) identified membrane fusion protein of the hefABC efflux system as a drug target for *H. pylori* in strains of HpB38, HpP12, and HpShi470. HefABC, hefDEF, and hefGHI belonging to the family of restriction–nodulation–division (RND) efflux systems use proton motive force to control the flow of antibiotics and antimicrobial compounds thus providing resistance to the organism (Moore et al. 1995; Saier et al. 1994; Paulsen et al. 1996). Studies in pathogens *Escherichia coli* (Ma et al. 1995), *Haemophlius influenzae* (Sanchez et al. 1997), *H. pylori* (Bina et al. 2000), *Neisseria gonorrhoeae* (Haman et al. 1995), and *Psuedomonas aeruginosa* (Li et al. 1995) established the role of RND multiple drug efflux systems. Expression of this efflux system leads to antibiotic resistance, whereas inactivation makes bacteria susceptible to antimicrobials (Li et al. 1995; Paulsen et al. 1996; Kohler et al. 1997). Therefore, designing an inhibitor to the inactive membrane fusion protein of the hef ABC efflux system makes bacteria susceptible to antimicrobials.

Table 2 List of drug targets in *Helicobacter pylori*

S. No	Name of the drug targets	Metabolic category	References
1	Membrane fusion protein of the hefABC efflux system	Resistance to antibiotics and toxic compounds	Neelapu and Pavani (2013)
2	50S ribosomal protein L33	Protein biosynthesis	Neelapu and Pavani (2013)
3	Peptidyl-prolyl cis–trans isomerase ppiD	Protein metabolism	Neelapu and Pavani (2013)
4	LSU ribosomal protein L36p	Protein metabolism	Nammi et al. (2015)
5	Hydrogenase expression/formation protein HypD	Central carbohydrate metabolism	Neelapu and Pavani (2013)
6	Phospho trans acetylase	Central carbohydrate metabolism	Neelapu and Pavani (2013)
7	Cag pathogenicity island protein X	Central carbohydrate metabolism	Neelapu and Pavani (2013)
8	Apolipoprotein N-acyl transferase	Central carbohydrate metabolism	Neelapu and Pavani (2013)
9	DNA adenine methylase	DNA repair	Neelapu and Pavani (2013)
10	Type II adenine specific DNA methyl transferase	DNA repair	Neelapu and Pavani (2013)
11	Putative histidine kinase sensor protein	DNA repair	Neelapu and Pavani (2013)
12	Molybdopterin converting factor, subunit-1	Folate and pterines	Neelapu and Pavani (2013)
13	Dipeptide transport system permease protein	ABC transporters	Neelapu and Pavani (2013)
14	Proline and betaine transporter	Proline and 4-hydroxy proline	Neelapu and Pavani (2013)
15	Histone like DNA-binding protein HU	Miscellaneous	Neelapu and Pavani (2013)
16	Peptidoglycan associated lipoprotein	Miscellaneous	Neelapu and Pavani (2013)
17	Na^+/H^+ antiporter	Miscellaneous	Dutta et al. (2006), Neelapu and Pavani (2013), Nammi et al. (2015)
18	SSU ribosomal protein S14p (S29e) ## Zinc dependent	Miscellaneous	Nammi et al. (2015)
19	Hydroxy ethyl thiazole kinase	Miscellaneous	Neelapu and Pavani (2013)
20	Thiamine phosphate pyrophosphorylase	Miscellaneous	Neelapu and Pavani (2013)
21	Cytochrome c-type biogenesis (DsbD) analog	Respiration	Neelapu and Pavani (2013)
22	Putative cytochrome C-type biogenesis protein	Respiration	Neelapu and Pavani (2013)
23	Cytochrome c-oxidase subunitCcoN	Respiration	Neelapu and Pavani (2013)

(continued)

Table 2 (continued)

S. No	Name of the drug targets	Metabolic category	References
24	Cytochrome c-oxidase subunitCcoO	Respiration	Neelapu and Pavani (2013)
25	Cytochrome c-oxidase subunitCcoP	Respiration	Neelapu et al. (2015)
26	Cytochrome c-oxidase subunitCcoQ	Respiration	Neelapu et al. (2015)
27	NADH-ubiquinone oxidoreductase chain J	Respiration	Nammi et al. (2015)
28	Peptide chain release factor 1	Stress response	Neelapu et al. (2015)
29	Similarity with glutathionylspermidine synthase group 2	Stress response	Neelapu et al. (2015)
30	Ferroxidase	Stress response	Neelapu et al. (2015)
31	Iron- binding ferritin-like antioxidant protein	Stress response	Neelapu et al. (2015)
32	Nonspecific DNA-binding protein in Dps	Stress response	Neelapu et al. (2015)
33	Superoxide dismutase[Fe]	Stress response	Neelapu et al. (2015)
34	Cytochrome c551 peroxidase	Stress response	Neelapu et al. (2015)
35	HtrA protease/Chaperone protein	Stress response	Neelapu et al. (2015)
36	Carbon stravation protein	Stress response	Neelapu et al. (2015)
37	O-methyl transferase	Stress response	Neelapu et al. (2015)
38	Heat-inducible transcription repressor HrcA	Stress response	Neelapu et al. (2015)
39	HspR, transcriptional repressor of DnaK operon	Stress response	Neelapu et al. (2015)
40	Miab family protein, possibly involved in tRNA or rRNA modification	Stress response	Neelapu et al. (2015)
41	Ribosomal RNA small subunit methyltransferase E	Stress response	Neelapu et al. (2015)
42	Ribosomal protein L11 methyltransferase	Stress response	Neelapu et al. (2015)
43	Tetrapyrrole(corrin–porphyrin) methylase family protein UPF0011	Stress response	Neelapu et al. (2015)
44	Thioredoxin reductase	Sulfur metabolism	Neelapu et al. (2015)
45	Ferric siderophore transport system, periplasmic-binding protein TonB	Virulence	Neelapu et al. (2015)
46	Haemin uptake system ATP-binding protein	Virulence	Neelapu et al. (2015)
47	Cation efflux system protein CusA	Virulence	Neelapu et al. (2015)
48	Cobalt–zinc–cadmium resistance protein CzcA	Virulence	Neelapu et al. (2015)
49	Probable Co/Zn/Cd efflux system membrane fusion protein	Virulence	Neelapu et al. (2015)

(continued)

Table 2 (continued)

S. No	Name of the drug targets	Metabolic category	References
50	Ferric siderophore transport system, biopolymer transport protein ExbB	Virulence	Neelapu et al. (2015), Nammi et al. (2015)
51	Membrane fusion protein of RND family multidrug efflux pump	Virulence, disease and defense	Nammi et al. (2015)
52	Heavy metal RND efflux outer membrane protein, CzcC family	Virulence, disease and defense	Nammi et al. (2015)
53	Menaquinone via futalosine step 1	Cofactors, vitamins, prosthetic groups, pigments	Nammi et al. (2015)
54	Molybdopterin guanine dinucleotide biosynthesis protein MobA	Cofactors, vitamins, prosthetic groups, pigments	Nammi et al. (2015)
55	3-polyprenyl-4-hydroxybenzoate carboxy-lyase UbiX	Cofactors, vitamins, prosthetic groups, pigments	Nammi et al. (2015)
56	Pantoate–beta-alanine ligase	Cofactor biosynthesis	Nandode et al. (2012)
57	Dihydrodipicolinate reductase	Cofactor biosynthesis	Nandode et al. (2012)
58	Type III restriction modification system methylation subunit	DNA metabolism	Nammi et al. (2015)
59	Type I restriction modification system, specificity subunit S	DNA metabolism	Nammi et al. (2015)
60	DNA-binding protein HU ## epsilonproteobacterial type	DNA metabolism	Nammi et al. (2015)
61	Dipeptide ABC transporter, periplasmic dipeptide-binding protein (DppA)	Membrane protein	Dutta et al. (2006)
62	Dipeptide transport system permease protein DppB	Membrane transport	Nammi et al. (2015)
63	Dipeptide transport system permease protein DppC	Membrane transport	Dutta et al. (2006), Nammi et al. (2015)
64	Oligopeptide transport system permease protein OppC	Membrane transport	Nammi et al. (2015)
65	HoxN/HupN/NixA family nickel/cobalt transporter	Membrane transport	Nammi et al. (2015)
66	Rare lipoprotein A (rlpA)	Membrane protein	Dutta et al. (2006)
67	Iron (III) dicitrate transport protein (fecA)	Membrane protein	Dutta et al. (2006)
68	Toxin-like outer membrane protein	Membrane protein	Dutta et al. (2006)
69	Signal-transducing protein histidine kinase	Membrane protein	Dutta et al. (2006)
70	Cell division membrane protein (ftsX)	Membrane protein	Dutta et al. (2006)
71	Ribonuclease BN	RNA metabolism	Nammi et al. (2015)
72	Soluble lytic murein transglycosylase precursor	Cell wall and capsule	Nammi et al. (2015)

(continued)

Table 2 (continued)

S. No	Name of the drug targets	Metabolic category	References
73	D-alanine–D-alanine ligase B	Cell wall and capsule	Nammi et al. (2015)
74	Flagellar biosynthesis protein FliP	Motility and chemotaxis	Nammi et al. (2015)
75	(3R)-hydroxymyristoyl-[acyl carrier protein] dehydratase	Fatty acids, lipids, and isoprenoids	Nammi et al. (2015)
76	Inner membrane protein YihY, formerly thought to be RNase BN	Clustering-based subsystems	Nammi et al. (2015)
77	rRNA small subunit 7-methylguanosine (m7G) methyltransferase GidB	Clustering-based subsystems	Nammi et al. (2015)
78	Membrane metalloprotease	Clustering-based subsystems	Nammi et al. (2015)
79	Hydrolase (HAD superfamily)	Clustering-based subsystems	Nammi et al. (2015)
80	YafQ toxin protein	Regulation and cell signaling	Nammi et al. (2015)
81	Potassium channel protein	Potassium metabolism	Nammi et al. (2015)
82	Chorismate synthase	Amino acid bioysnthesis	Nandode et al. (2012)
83	Bifunctional riboflavinkinase/FMN Adenylyltransferase	Flavin biosynthesis	Nandode et al. (2012)
84	dTDP-4 dehydrorhamnose3,5-epimerase	dTDP-Lrhamnose biosynthesis I	Nandode et al. (2012)
85	Uracil-DNA glycosylase	DNA replication machinery	Nandode et al. (2012)
86	N-acetylmuramoyl-Lalanine amidase	Cell wall synthesis	Nandode et al. (2012)
87	lipid A biosynthesis (KDO)2-(lauroyl)-lipid IVA acyltransferase	(KDO)2-lipid A biosynthesis I	Nandode et al. (2012)
88	Menaquinone specific isochorismate synthase	Tryptophan biosynthesis	Nandode et al. (2012)
89	Alanine racemase	Alanine and cell wall synthesis	Nandode et al. (2012)
90	Aspartate Kinase	Homoserine biosynthesis	Nandode et al. (2012)
91	Aspartate-semialdehyde dehydrogenase	Homoserine biosynthesis	Nandode et al. (2012)
92	Indole-3-glycerol-phosphate synthase	Tryptophan biosynthesis	Nandode et al. (2012)
93	Indole-3-glycerol-phosphate lyase	Tryptophan biosynthesis	Nandode et al. (2012)
94	Phosphoribosylanthranilate isomerase	Tryptophan biosynthesis	Nandode et al. (2012)

(continued)

Table 2 (continued)

S. No	Name of the drug targets	Metabolic category	References
95	Ketol-acid reductoisomerase	Valine biosynthesis	Nandode et al. (2012)
96	Dihydroxy-acid dehydratase	Valine biosynthesis	Nandode et al. (2012)
97	3-deoxy-7-phosphoheptulonate synthase	Chorismate biosynthesis	Nandode et al. (2012)
98	3-dehydroquinate synthase	Chorismate biosynthesis	Nandode et al. (2012)
99	3-dehydroquinate dehydratase	Chorismate biosynthesis	Nandode et al. (2012)
100	D-sedoheptulose 7-phosphate isomerase	Carbohydrate biosynthesis	Nandode et al. (2012)
101	3-deoxy-8-phosphooctulonate synthase	Carbohydrate biosynthesis	Nandode et al. (2012)
102	UDP-N-acetylglucosamine 1 carboxyvinyltransferase	Cell wall Synthesis	Nandode et al. (2012)
103	glutamate racemase	Cell wall synthesis	Nandode et al. (2012)
104	UDP-N-acetylmuramate dehydrogenase	Cell wall synthesis	Nandode et al. (2012)
105	UDP-N-acetylmuramate—Lalanine ligase	Cell wall synthesis	Nandode et al. (2012)
106	Thiamine phosphate diphosphorylase	Thiamin biosynthesis I	Nandode et al. (2012)
107	Beta-ketoacyl-acyl-carrier-protein synthase I	Fatty acid synthesis	Nandode et al. (2012)
108	Lipid IV(A) 3-deoxy-Dmanno-octulosonic acid transferase.	Fatty acid synthesis	Nandode et al. (2012)

3.2.2 Drug Targets Influencing Carbohydrate Central Metabolism of the Pathogen

Neelapu and Pavani (2013) identified hydrogenase expression/formation protein HypD gene and Cag pathogenicity island protein X gene as drug targets in *H. pylori* strain HpSJM180. Nickel-dependent enzymes like ureases, CO dehydrogenases, or hydrogenases require the activities of hyp proteins—hypA, hypB, hypC, hypD, hypE, and hypF—for incorporation of nickel into the metal center (Jacobi et al. 1992; Lutz et al. 1991; Menon et al. 1994). Olson and Maier (2002) studied cooperative action and role of hyp proteins in colonizing *H. pylori*. In addition, Jacobi et al. (1992) showed that maturation of hydrogenases 1, 2, and 3 is possible for HypB, HypD, HypE, and HypF (Jacobi et al. 1992). Therefore, inactivation of hyp D would decrease the colonization rate and thus affect the survival of the organism.

H. pylori strains containing cag pathogenicity islands (cag PAI genes) are associated with early colonization, virulence, and clinical outcomes. Marchetti and Rappuoli (2002) studied the role of cag genes in the mouse model of infection and observed that the ability to colonize host was affected when cag genes were

mutated. Thus, colonization and survival of *H. pylori* would be compromised if pathogen lacks cag pathogenicity island protein X.

3.2.3 Drug Targets Influencing Virulence of the Pathogen

Cation efflux system protein CusA, Cobalt/Zinc/Cadmium efflux system membrane fusion protein, Cobalt, Zinc, and Cadmium resistance protein CzcA genes, and heavy metal RND efflux outer membrane protein CzcC are the drug targets identified for *H. pylori* (Neelapu et al. 2015; Nammi et al. 2015). Neelapu et al. (2015) identified cation efflux system protein CusA, Cobalt/Zinc/Cadmium efflux system membrane fusion protein, Cobalt, Zinc, and Cadmium resistance protein CzcA genes as drug targets in *H. pylori* strain HPAG1. Nammi et al. (2015) identified heavy metal RND efflux outer membrane protein CzcC as a drug target in *H. pylori* strains Hp51, Hp52, Hp908, HpB8, HpCuz20, HpF16, HpF32, HpF57, HpG27, HpAG1, HpIndia7, HpJ99, HpP12, HpB38, Hp83, HpSJM180, HpShi470, Hp26695, HpF30, Hpsat464, Hp35A, and Hp2017.

Bacteria like *H. pylori* require Cobalt, Zinc, and Cadmium in traces for its growth and when in excess they are toxic to bacteria (Blindauer et al. 2001). Pathogen when detects excess amounts of Cobalt, Zinc, and Cadmium induces genes like cation efflux system protein CusA, Cobalt/Zinc/Cadmium efflux system membrane fusion protein and Cobalt, Zinc, and Cadmium resistance protein CzcA, heavy metal RND efflux outer membrane protein CzcC (Nies 2003) for efflux of Co/Zn/Cd; and the interactions among these proteins were established in *H. pylori*. Knockout studies on CzcA and CzcB genes observed that *H. pylori* was not able to colonize the stomach effecting efflux of trace elements. Mutational studies in *H. pylori* on cation efflux system protein CusA a homolog to *czcA* gene showed copper sensitivity (Waidner et al. 2002). Therefore, inhibitors for the above genes would allow excess amounts of trace elements into a pathogen, thereby making it toxic and lethal to the pathogen.

3.2.4 Drug Targets Influencing Oxidative Stress of the Pathogen

Neelapu et al. (2015) identified SOD, heat-inducible transcription repressor (HrcA), a transcriptional repressor of DnaK operon (HspR), and serine protease high temperature requirement A (HtrA) as drug targets for *H. pylori* strain HpAG1.

SOD catalyzes the dismutation of superoxide radicals into oxygen and hydrogen peroxide. Seyler et al. (2001) demonstrated that *H. pylori* mutants deficient of SOD are sensitive to oxidative stress and thereby influence colonization of the host. Therefore, pathogens devoid of SOD activity are vulnerable to oxidative stress and are incapable of colonizing the host.

Transcriptional repressor proteins heat-inducible transcription repressor (HrcA) and transcriptional repressor of DnaK operon (HspR) negatively regulate expression of the *groES–groEL* and *hrcA-grpE-dnaK* operons. Roncarati et al. (2007)

Table 3 List of drug targets in other *Helicobacter* species

S. No	Name of the drug targets	Metabolic category	References
Helicobacter bilis			
1	Cag A	Virulence	Maurer et al. (2005)
Helicobacter hepaticus			
2	Fla A	Motility	Sterzenbach et al. (2008)
3	Fla B	Motility	Sterzenbach et al. (2008)
4	CdtA	Virulence	Proposed
5	CdtB	Virulence	Proposed
6	CdtC	Virulence	Proposed
7	Urease	Virulence	Ge et al. (2008), Fox et al. (2011)
8	Catalase (Kat A)	Oxidative stress resistance	Hong et al. (2007)
9	Hydrogenase (Hya)	Oxidative stress resistance	Mehta et al. (2005)
Helicobacter felis			
10	Urease	Virulence	Mohammadi et al. (1996)
Helicobacter salmonis			
11	Urease	Virulence	Proposed
Helicobacter bizzozeronii			
12	Urease	Virulence	Hänninen et al. (1996)
Helicobacter heilmannii			
13	Urease	Virulence	Dieterich et al. (1998)

compared transcriptomes of wild-type strain, *hspR* and *hrcA* singly and doubly deficient strains and identified HspR- and HrcA-mediated stress response and motility functions in *H. pylori.* Inhibitors for HspR and HrcA block flagellar synthesis leading to reduced motility and thereby colonization of the pathogen.

High-temperature requirement A (HtrA) cleaves the tumor suppressor E-cadherin leading to apoptosis, changes in cell adhesion, invasive mesenchymal transformation, progression of malignant adenocarcinoma, and signaling (Hoy et al. 2010; Chan 2006; De Wever et al. 2007). Löwer et al. (2011) performed virtual ligand screening for HtrA and identified inhibitors that prevent epithelial invasion. Therefore, a suitable inhibitor would inactivate HtrA making *H. pylori* defective in host colonization (Tables 3 and 4).

3.2.5 Drug Targets Influencing Cofactors, Vitamins, Prosthetic Groups, Pigments of the Pathogen

Nammi et al. (2015) identified menaquinone via futalosine step 1 gene as a drug target in *H. pylori* strains Hp35, Hp51, Hp52, Hp908, HpB8, HpCuz20, HpF16, HpF32, HpF57, HpG27, HpAG1, HpIndia7, HpJ99, HpSJM180, HpShi470, Hp26695, HpF30, Hpsat464, Hp2017, and Hp2018. Menaquinone via futalosine step 1 is an alternative pathway for electron transfer present in *H. pylori* and *C. jejuni.* Kim et al. (2014) observed bacteriostatic growth when menaquinone via

Table 4 List of drug targets in non-*Helicobacter* species

S. No	Name of the drug targets	Metabolic category	References
Campylobacter jejuni			
1	Citrate synthase	Carbohydrate metabolism	Tilton et al. (2014)
2	FlaA gene	Motility	Nuijten et al. (1990)
3	Flab gene	Motility	Nuijten et al. (1990)
4	Peb1A	Virulence	Ziprin et al. (1999), Konkel et al. (1999)
5	CadF	Virulence	Ziprin et al. (1999), Konkel et al. (1999)
6	CiaB	Virulence	Ziprin et al. (1999), Konkel et al. (1999)
7	Cdt A	Virulence	Proposed
8	Cdt B	Virulence	Proposed
9	Cdt C	Virulence	Proposed
10	SodB	Oxidative stress	Pesci et al. (1994)
11	GroESL	Stress response	Konkel et al. (1998)
12	DnaJ	Stress response	Konkel et al. (1998)
13	DnaK	Stress response	Konkel et al. (1998)
14	ClpB	Stress response	Konkel et al. (1998)
15	DNA gyrase	Virulence	Changkwanyeun et al. (2015)
16	Serine peptidase	Virulence	Karlyshev et al. (2014)
17	Menaquinone	Virulence	Kim et al. (2014)
Neisseria gonorrhoeae			
18	Fructose-1,6-bisphosphate aldolase	Glycolysis/pentose phosphate pathway/fructose and mannose metabolic pathways	Barh and kumar (2009)
19	Putative two-component system transcriptional response regulator	Two-component system	Barh and kumar (2009)
20	Phosphotransferase system	Two-component system	Barh and kumar (2009)
21	Anthranilate phosphoribosyltransferase	Two-component system	Barh and kumar (2009)
22	Indole-3-glycerol-phosphate synthase	Two-component system	Barh and kumar (2009)
23	Tryptophan synthase subunit	Tryptophan biosynthesis pathways	Barh and kumar (2009)
Chlamydophila pneumoniae			
24	Ribonucleotide diphosphate reductase subunit beta	Pyrimidine metabolism	Ravindranath et al. (2013)
25	Holliday junction DNA helicase RuvB protein	DNA repair mechanism	Reddy (2008)
26	Heat shock protein (HSP)—60	Stress response	Rajan et al. (2014)

futalosine pathway is disrupted. So, an inhibitor for menaquinone via futalosine step 1 would affect the growth of pathogen.

3.2.6 Drug Targets Influencing DNA Metabolism of the Pathogen

Nammi et al. (2015) identified type III restriction modification system methylation subunit, type I restriction modification system specificity subunit S, and DNA-binding protein HU as the drug targets for *H. pylori*. Type I restriction modification system specificity subunit S is the drug target for the *H. pylori* strains HpF30 and HpF52. Type III restriction modification system methylation subunit is the drug target for *H. pylori* strains HpF30 and HpF52. DNA-binding protein HU is a unique drug target for strain HpB38.

H. pylori has the capability to take free DNA/plasmids into the cell (Suerbaum et al. 1998) and DNA from bacteriophages (von Heinegg et al. 1993). Uptake of DNA leads to accumulated missense and frameshift mutations, sometimes even turning off R-M system genes such as endonuclease and methylase genes. *H. pylori's* R-M system protects genome from mutation caused due to bacteriophages/free DNA/plasmids. Mutants deficient of this R-M system are sensitive to DNA damaging agents leading to hypermutability and hyperrecombination. Therefore, an inhibitor for type I restriction modification system specificity subunit S and type III restriction modification system methylation subunit result in alteration of the genetic material decreasing the rate of survival of *H. pylori.*

Bacterial cell envelope protects cells, and controls the exchange and communication "from and to" the cell. Hup mutants demonstrated altered composition in the outer membrane and were sensitive to common features of *H. pylori* like acid and oxidative stress. Wang et al. (2012) used a mouse infection model and observed noncolonization of pathogen when infected with hup mutant strain, whereas colonization was observed when hup mutant strain was complemented with the functional hup gene. Therefore, organisms lacking histone like DNA-binding protein HU make organisms more susceptible to oxidative and acid stress resulting in noncolonization of *H. pylori*.

3.2.7 Drug Targets Influencing Membrane Transport of the Pathogen

Dutta et al. (2006) identified dipeptide transport system permease protein DppC as the drug target for *H. pylori*. Nammi et al. (2015) identified dipeptide transport system permease protein DppB, dipeptide transport system permease protein DppC, oligopeptide transport system permease protein OppC, and HoxN/HupN/NixA family nickel/cobalt transporter as drug targets for *H. pylori.* Dipeptide transport system permease protein DppB was identified as a drug target in the following strains Hp2017, Hp2018, HpF32, Hp51, and Hp52. Dipeptide transport system permease protein DppC was identified as a drug target in the strains Hp2017 and Hp51. Oligopeptide transport system permease protein OppC was identified as a

drug target in the strain Hp35A. HoxN/HupN/NixA family nickel/cobalt transporter was identified as a drug target in the strains HpF30, HpF32, and Hp51.

Dipeptides are transported by dipeptide *DppABCDF* genes belonging to a class ABC-type transporter. Oligopeptides are transported by oligopeptide *OppABCD* genes belonging to a class of ABC-type transporter. Weinberg and Maier (2007) demonstrated that mutants of *H. pylori* deficient with dipeptide and oligopeptide system were not able to use dipeptides, hexapeptides, and nonapeptides. In addition, it was observed that mutants compromised either permease- or substrate-binding domains. So, growth and survival of *H. pylori* would be compromised if pathogen lacks dipeptide transport system permease protein DppBC and oligopeptide transport system permease protein OppC genes.

H. pylori requires nickel-dependent enzyme urease for its colonization and to survive in the gastric mucosa with acidic conditions. Nickel [Ni (II)]/cobalt [Co (II)] is imported into bacterial cell by Nickel/cobalt transporters and nickel is incorporated into the metal center of nickel-dependent enzymes like urease. Bauerfeind et al. (1996) demonstrated that *nixA* mutants of *H. pylori* showed reduced nickel transport and urease activity. So, nickel transport, urease activity, and colonization of *H. pylori* would be compromised if pathogen lacks HoxN/HupN/NixA family nickel/cobalt transporter gene.

3.2.8 Drug Targets Influencing Cell Wall and Capsule of the Pathogen

Nammi et al. (2015) identified D-alanine–D-alanine ligase B as drug targets for *H. pylori* in the strain Hp26695. D-alanine is cross-linked with D-glutamate and diaminopimelate forming the stable backbone peptidoglycan against proteolytic degradation. D-alanine branch of peptidoglycan is synthesized by three enzymes, namely pyridoxal phosphate-dependent D-alanine racemase (Alr), the ATP-dependent D-alanine:D-alanine ligase (Ddl), and the ATP-dependent D-alanine:D-alanine-adding enzyme (MurF). Bruning et al. (2011) proposed D-alanine:D-alanine ligase (Ddl) as a drug target while observing weak cell walls and cell death due to the inhibition of enzymes involved in the pathway. So, D-alanine:D-alanine ligase (Ddl) is an attractive target for *H. pylori.* Singh et al. (2013) proposed analogs of quercitin for D-alanine:D-alanine ligase (Ddl) and demonstrated that quercetin and Ddl showed better interaction, affinity, and absorption through molecular docking analysis.

3.2.9 Drug Targets Influencing Motility and Chemotaxis of the Pathogen

Nammi et al. (2015) identified flagellar biosynthesis protein FliP as a drug target for *H. pylori* in the strains HpF30, HpF35A, HpF32, Hp51, and Hp52. Basal body component of flagella required for flagellar assembly is coded by *fliP* gene. Josenhans et al. (2000) demonstrated that mutants' deficient of *fliP* shutted down

flagellar assembly resulting in nonmotile *H. pylori.* Nonmotile mutants of *H. pylori* were not able to colonize in an animal model of infection (Eaton et al. 1992). Flagellar synthesis, motility, and colonization are hampered when *fliP* gene activity is inhibited with a suitable inhibitor.

3.2.10 Drug Targets Influencing Miscellaneous Metabolism of the Pathogen

Nammi et al. (2015) identified Na^+/H^+ antiporter as a drug target for *H. pylori* in the strains HpF35A, Hp2017, Hp2018, HpF32, Hp51, and Hp52. Extruding Na or Li when present in excess amounts or maintaining a lower cytoplasmic pH is the mechanisms that bacteria use for homeostasis. Na^+/H^+ antiporter using proton motive force regulates homeostasis (Kosono et al. 1999). Karasawa et al. (2005) demonstrated that defective HpNhaA mutants when compared to the wild type showed lower antiporter activity, whereas antiporter activity was reestablished when the functional HpNhaA gene is complemented with HpNhaA mutants when grown in alkaline pH range. Thus, if Na^+/H^+ antiporter is inactivated, then it can act as a suitable drug target for *H. pylori* by lowering antiporter activity leading to growth retardation.

3.2.11 Drug Targets Influencing Potassium Metabolism of the Pathogen

Nammi et al. (2015) identified potassium channel protein as a drug target for *H. pylori* in the strains Hp12, HpB38, and Hp83. Potassium (K^+) channel (HpKchA) protein is essential for colonization of the murine stomach. Stingl et al. (2007) demonstrated channel-mediated K^+ uptake in *H. pylori.* Mutants with defective potassium channel (Δ*hpKchA*) of *H. pylori* showed impediment in growth, at low K^+ concentration, whereas Δ*hpKchA* when compensated with additional KCl, retained the function of K^+ uptake system. Therefore, K^+ uptake, colonization, and survival of *H. pylori* can be compromised when an effective inhibitor was designed for HpKchA.

3.2.12 Drug Targets Influencing Regulation and Cell Signaling of the Pathogen

Nammi et al. (2015) identified YafQ toxin protein as a drug target for *H. pylori* in the strain Hp12. Locus *dinJ–yafQ* is the toxin–antitoxin (TA) systems (suicide modules) identified on chromosomes and plasmids of *H. pylori* to induce post-segregational killing. YafQ is toxin and DinJ is the cognate antitoxin. "…. During stress *yafQ* (endoribonuclease) cleaves mRNA at a particular frame and sequence, blocking elongation of translation inducing reversible cell growth arrest.

This temporary arrest of growth is vital to repair DNA…" (Nammi et al. 2015; Prysak et al. 2009). When stress prolongs cell death occurs. Antitoxin DinJ prevents YafQ toxin activity during environmental stress. Prysak et al. (2009) observed that coexpression or coinduction of *dinJ–yafQ* genes in a module have independently restored normal growth. So, short-term growth arrest, DNA repair are compromised if an effective inhibitor for YafQ toxin is designed affecting survival of *H. pylori*.

4 Targets for Other *Helicobacter* Bacteria and Their Cancers

Other *Helicobacter* species such as *H. felis, H. salmonis, H. bizzozeronii,* and *H. heilmannii* are known for causing stomach cancer upon infection in addition to *H. pylori*. *H. bilis* and *H. hepaticus* are known for causing biliary tract cancer and liver cancer, respectively.

4.1 Helicobacter bilis *and Biliary Tract Cancer*

H. bilis is a Gram-negative, microaerophilic, 0.5 by 4–5 mm in size, non-sporulating, nongastric, fusiform to slightly spiral in shape, with 3–14 flagella and periplasm fibers. *H. bilis* is isolated from hepatic, extrahepatic, intrahepatic, and bile tissue (Pandey et al. 2010). *H. bilis* was observed in bile juice of Chilean patients (Fox et al. 1998) and Japanese patients (Matsukura et al. 2002) confirming that *H. bilis* infection leads to the pathogenesis of biliary tract and cancer (Murata et al. 2004). Biliary tract cancer is a rare and fatal disease occurring in countries like Chile, India, and Japan (Misra et al. 2003; Nagakawa et al. 2002; Fox et al. 1998; Tominaga et al. 1979). *H. bilis* route of infection is enterohepatic. Virulence factors of *H. bilis* are broadly classified into three broad categories—colonization, persistence, and disease-inducing factors (Pandey et al. 2010). Maurer et al. (2005) proposed *H. bilis* induced carcinogenesis due to the virulence factor CagA interfering with signal transduction pathway leading to carcinogenesis. Therefore, we propose CagA as a drug target for *H. bilis* (Table 3). Myung et al. (2000) proposed that cytokines and other inflammatory mediators are released by host in response to *H. bilis* antigens, leading to carcinogenesis. Pandey et al. (2010) proposed that inflammation followed by proliferation of epithelia and alterations of signal transduction interfering the cell cycle can be the plausible explanation for carcinogenesis. However, the etiology and pathogenesis of biliary tract cancer remain unclear and further studies are required to demonstrate the pathogenesis of *H. bilis* in biliary tract cancer.

4.2 Helicobacter hepaticus *and Liver Cancer*

H. hepaticus is a Gram-negative bacteria, microaerophilic, non-sporulating, motile, slender, curved to spiral in shape, 0.2–0.3 μm by 1.5–5.0 μm in size, single bipolar flagella. *H. hepaticus* was isolated from the colons, ceca, livers of mice with active, chronic hepatitis (Fox et al. 1994). Several in vivo and in vitro experiments along with a comparison of genome with other bacterial species have provided us with new insights on how *H. hepaticus* infection was able to induce hepatitis. Though it was established that hepatitis is induced by *H. hepaticus* infection, mechanisms in detail, determining how host initially recognizes *H. hepaticus* and eventually leads to activation of immune response are yet to be characterized.

Literature review has provided various mechanisms developed by *H. hepaticus* and how flagella, enzyme urease, cytolethal distending toxin (Cdt), HHGI1 gene, and catalases are used by *H. hepaticus* to colonize and survive in the host. *H. hepaticus* akin to other *Helicobacter* sps. also possess flagella which is a prerequisite for colonization. *H. hepaticus* has major flagellin subunit *FlaA* which is encoded by FlaA1 (HH1364) and FlaA2 (HH1653) and regulated by FlaA (σ28) (Suerbaum et al. 2003). Sterzenbach et al. (2008) observed that mutations in *fliA* gene or both FlaA1 and FlaA2 genes resulted in non-synthesis of the flagella FlaA with nonmotility and noncolonization in a mice model of infection. So, *H. hepaticus* requires a bipolar-sheathed flagella for motility and colonization in the host. *H. hepaticus* in similar to other *Helicobacter* sps. also possesses enzyme urease and is required for colonization of the host. Ge et al. (2008) demonstrated that *H. hepaticus* is positive to urease activity. Urease-deficient mutants HhureNT9 of *H. hepaticus* 3B1 showed that urease was essential for colonization of liver, hepatitis, and producing proinflammatory cytokines IFN–γ and TN–α in a mice model of infection (Ge et al. 2008; Fox et al. 2011). Like any other bacteria, *H. hepaticus* also require some adhesion molecules which helps in adhering to the host and to establish an interaction with the host. But, unfortunately adhesion molecules related to *H. hepaticus* are yet to be explored.

Cytolethal distending toxin (CDTs) reported in *H. hepaticus* comprised of CdtA, CdtB, and CdtC subunits. CdtA and CdtC bind to CdtB to deliver CdtB and CdtB was known to have several activities like DNAase I like function, cell cycle arrest, phosphatase activity, and cell death. CDT induce DSB activating Myc, Nijemegen breakage syndrome 1 protein (Nbs1), check point kinase 2 (CHK2) in a cascade manner, leading to upregulation of Bcl2 or Bax and release of the proapopotic cytc C and activation of caspases 3 and 9. For the required action of the toxin Cdt *H. hepaticus* requires a set of virulence associated factors for pathogenesis. Pathogenesis-associated islands (PAI) were also identified on bacterial chromosomes of *H. hepaticus.* HHGI1 a 71 Kb region encodes for a set of 12 genes (HH0237 to HH0242, HH0242 to HH0252, HH0291) which is homologous to bacterial type VI secretion system (T6SS) (Suerbaum et al. 2003; Chow and Mazmanian 2010). Boutin et al. (2005) observed that HHGI1 mutant strain developed less hepatitis when compared with strain having HHGI1 in a mice model

of infection. The results demonstrate that HHGI1 has a role in pathogenecity of *H. hepaticus*. *H. hepaticus* requires to combat oxidative stress for effective colonization in the host. Catalase (KatA) and bacterial hydrogenase (Hya) are the enzymes identified in *H. hepaticus.* Hong et al. (2007) observed that *H. hepaticus* mutant, which is Kat A deficient was more sensitive to oxidative stress along with DNA fragmentation when compared with wild type of *H. hepaticus.* Bacterial hydrogenase *Hya ABCD* is coded by genes *HH0056* to *HH0059* in *H. hepaticus.* Mehta et al. (2005) observed that hydrogenase mutants are causing hepatitis in a mice model of infection. Studies on flagella, enzyme urease, Cdt, HHGI1 gene, catalases, and hydrogenases showed that directly or indirectly they induce proinflammatory substances like cytokines (IL), IFN–γ, TN–α, NF-κβ, and ERK pathways; and sometimes induce DBS leading to DMC activating carcinogenesis. Hence, the above studies prove that FlaA1 and FlaA2 coding for flagella; enzyme urease; CdtA, CdtB, and CdtC genes coding for the cytolethal distending toxin; HHGI gene; catalase (KatA) and hydrogenases (Hya) can be proposed as drug targets (Table 3) for *H. hepaticus*.

4.3 Helicobacter felis *and Stomach Cancer*

H. felis is a Gram-negative bacteria, microaerophilic, non-sporulating, motile, slender, spiral in shape measuring approximately 0.4 by 5–10 μm. *H. felis* is frequently found in the gastric mucous membrane of dogs and cats. Histological studies and PCR amplification well-characterized *H. felis* gastric pathology in South African population (Fritz et al. 2006). *H. felis* causes chronic infection and induces gastritis and mouse model of infection was used to establish this fact. Mohammadi et al. (1996) in a mouse model of infection observed that *H. felis* colonizes both fundus and antrum of animal. *H. felis* colonizes more in the antrum region than in the fundus. Further, *H. felis* possesses enzyme urease, which is required for colonization of the host. Mohammadi et al. (1996) showed urease positive activities with *H. felis* infections in a mouse model of infection. PCR amplification has also identified a fragment of the urease gene in *H. felis* demonstrating that urease plays an important role in colonization of the host. Inflammatory changes on the fundus and antrum of animal are noted after infection dominated by lymphocytes (Fox et al. 1991, 1993; Lee et al. 1990; Michetti et al. 1994). Therefore, enzyme urease can be proposed as the drug target (Table 3) for *H. felis*. The other observed inflammatory changes include the change in density of mononuclear cells, activated mononuclear cells, polymorphonuclear leukocytes, neutrophils, lymphocytes, etc. Mohammadi et al. (1996) observed significant changes in the mucosal architecture. The changes are variations in mucous thickness, glandular distortion, and necrosis leading to mucosal disruption, fibrosis of the lamina propria, and erosions of the surface epithelium. Thus, it can be concluded that *H. felis* upon infection has the capability to colonize the gastric epithelium leading to gastric inflammation.

4.4 Helicobacter salmonis *and Stomach Cancer*

H. salmonis is a Gram-negative, non-sporulating, loose spiral, motile, tufts of 10–23 sheathed flagella at one or both ends of the cell, without periplasmic fibrils and 0.8–1.2 pm wide by 5–7 pm long. *H. salmonis* cultures exhibited positive oxidase, catalase, and urease activities. As *H. salmonis* exhibited positive urease activity, therefore, for *H. salmonis* enzyme urease can be proposed as a drug target. *H. salmonis* was isolated from a gastric biopsy of a healthy pet dog (Jalava et al. 1997). *H. salmonis* colonizes the human stomach (Weber and Schmittdiel 1962). No substantial information is available about the species status of the infecting strain, making it difficult to link the species with certain pathologies.

4.5 Helicobacter bizzozeronii *and Stomach Cancer*

H. bizzozeronii is a Gram-negative, microaerophilic, gastric, non-sporulating, large, tight spirals, 5–10 microns long by 0.3 microns wide, 10–20 sheathed flagella, and without periplasmic fibrils. *H. bizzozeronii* was isolated from gastric biopsies of dogs with catalase, oxidase, and urease positive activity (Hanninen et al. 1996; Kondadi et al. 2013). As *H. bizzozeronii* exhibited positive urease activity, therefore, *H. bizzozeronii* urease gene can be proposed as a drug target (Table 3). *H. bizzozeronii* was isolated from human gastric biopsy tissue with chronic gastritis (Jalava et al. 2001; Kivisto et al. 2010). Human *H. bizzozeronii* probably was acquired from dogs and cats due to infection (Meining et al. 1998). Genome sequence of *H. bizzozeronii* is available where the genome can be compared with *H. pylori* and other *Helicobacter* species to establish the mechanisms of colonization in the niche; interaction with the host; evading the immune system; and inducing gastritis. Further, studies of gastric *H. bizzozeronii* provide new insights into pathogenesis of human gastritis.

4.6 Helicobacter heilmannii *and Stomach Cancer*

H. heilmannii, the gastric corkscrew-shaped *Helicobacter* species was seen in the gastric foveolae in 0.2–0.6 % of histological sections from the gastric mucosa of patients with dyspepsia in Western Europe. *H. heilmannii* was isolated from human gastric mucosa, fecal samples, liver, and gall bladder (Andersen 2001). *H. heilmannii* can naturally infect animals such as cats, dogs, pigs, and primates with frequency of infection being 80–100 %, whereas zoonosis might be the reason for infection in humans (Eaton et al. 1996; Lavelle et al. 1994; Queiroz et al. 1996; Stolte et al. 1994; Wegmann et al. 1991). Stolte et al. (1994) demonstrated that *H. heilmannii* infection in humans was due to close contact with animals. *H. heilmannii* colonization leads to mild to moderate gastritis (Eaton et al. 1996;

Neiger et al. 1998; Hilzenrat et al. 1995), gastric ulcers (Hilzenrat et al. 1995; Queiroz et al. 1996; Stolte et al. 1997; Yeomans and Kolt 1996), and even cancer (Morgner et al. 1995; Yang et al. 1995) in humans. Dieterich et al. (1998) amplified, cloned, and sequenced *H. heilmannii* urease B gene from gastric biopsies of human and cat and found that structural gene urease is highly similar in both humans and cat. Therefore, *H. heilmannii* urease B gene, which is specific for *Helicobacter* species can be proposed as a drug target (Table 3).

5 Targets for Non-*Helicobacter* Bacteria and Their Cancers

Non-*Helicobacter* species *N. gonorrhoeae, C. jejuni,* and *C. pneumoniae* are known for causing prostate, stomach, and lung cancers, respectively.

5.1 Neisseria gonorrhoeae *and Prostate Cancer*

N. gonorrhoeae (gonococcus), the human pathogen induces prostatitis and prostate cancer. *N. gonorrhoeae* adapts primarily by colonizing in the male urethra, female cervix and also vagina, pharynx, rectum, and conjunctiva of eye to survive within its sole human host. *N. gonorrhoeae* has different mechanisms for its survival and pathogenesis…."in the distinctly different microenvironments found within the male and female (uro)genital tracts" (Edwards and Apicella 2004). Pili, opacity-associated (Opa), porin and lipooligosaccharide (LOS) are the outer membrane constituents associated with invasion, colonization, and virulence of gonococci.

Pili helps in initial attachment of the gonococci with the host cells (Brodeur et al. 1977; Brooks 1985; Cohen et al. 1994; McGee et al. 1981; Kellogg et al. 1968; Swanson 1973) by modulating host cell signaling mechanisms like pilus-induced calcium flux (Källström et al. 1998); engaging in host cell cytoskeletal rearrangements (Griffiss et al. 1999; Merz et al. 1999, Merz and So 1997) thereby aiding in invasion of epithelia. Opa proteins promote urethritis in men, and studies with *N. gonorrhoeae* strain FA1090Opa mutant proved that Opa proteins are required for promoting urethritis (Edwards and Apicella 2004). Opa proteins Opa_{50} and Opa_{52} recognize host cell heparin sulfate proteoglycans (HSPG) and members of the carcinoembryonic antigen-related family of cell adhesion molecules (CEACAM), respectively (CEA/PSG Workshop Group 1999). Opa_{50}—HSPG interaction is mediated with a coreceptor integrin by $\alpha_v\beta_3$ or $\alpha_v\beta_5$ for vitronectin-mediated adherence or $\alpha_v\beta_1$ for fibronectin-mediated adherence (Duensing and van Putten 1997a, b; Gómez-Duarte et al. 1997; Dehio et al. 1998; van Putten et al. 1998) triggering a signal transduction cascade. Signal generates diacylglycerol via

phosphatidylcholine-dependent phospholipase C, in turn activating acidic sphingomyelinase resulting in ceramide production from spingomyelin modulating the cytoskeletal rearrangements required for endocytosis (Grassmé et al. 1996, 1997). Opa_{52}—CEACAM interaction activates the host cells Src tyrosine kinases, Hck, and Fgr (Skubitz et al. 1995), followed by Rac activation triggering cytoskeletal rearrangement (Hauck et al. 1998). Interaction of Opa is modulated by LOS, which lack the typical repeating O-antigen sugar of lipopolysaccharide (LPS), terminating in epitopes that mimic sugar moieties of mammalian glycosphingolipids—paragloboside (Harvey et al. 2001b; Mandrell 1992; Mandrell and Apicella 1993; Mandrell et al. 1988; Yamasaki et al. 1999; van Putten and Robertson 1995) providing the bacterium immune avoidance to enhance mucosal adherence (Gorter et al. 2003).

Water-filled channel porin, which traverses the outer membrane of gonococcus triggers variable functional responses within host cells. Porin is translocated at an intimate juxtaposition into host mitochondria (Blake and Gotschlich 1981, 1987; Lynch et al. 1984; Weel and van Putten 1991), forming a voltage-gated channel modulated by host cell ATP and GTP (Blake and Gotschlich 1987; Muller et al. 2000; Rudel et al. 1996; Weel and van Putten 1991). Porin induces calcium influx, consequently, calpain and caspase activity within these cells (Muller et al. 1999). Porin-induced apoptosis leads to cytotoxicity as observed in fallopian tube organ culture (FTOC) during mucosal infection (Dehio et al. 2000). Antiapoptotic events are observed in human male urethral epithelial cells which are required by the bacterium to proliferate within an intracellular, protective environment and, consequently, promote gonococcal colonization in the urethral epithelial cell (Binnicker et al. 2003). Porin facilitates the cytoskeletal rearrangements required for entry of the gonococcus into target host cell (Wen et al. 2000). Activation of polymorphonuclear leukocyte (PMN) results in oxidative burst (Hampton et al. 1998, Miller and Britigan 1997). Gonococci possess multiple gene products for survival in oxidative stress. Porin is known to inhibit PMN (Bjerknes et al. 1995) thereby gonococci survive under oxidative stress conditions and in vitro studies proved that some gonococci associated with PMN survive oxidative stress (Casey et al. 1986, 1979; Rest et al. 1994; Shafer and Rest 1989). In addition, antimicrobial peptides are produced locally in host as the first line of defense against gonococci. Therefore, host possesses both oxidative and nonoxidative mechanisms to kill gonococci. Gonococci are known to have the ability to acquire iron from human host (in men) either in the form of lactoferrin or transferrin for colonization in the urethra (Anderson et al. 2003; Cornelissen et al. 1998).

In men, LOS elicits chemokine interleukin-8 (IL-8), cytokines IL-6, and tumor necrosis factor alpha (TNF-α) in a primary urethral epithelial cells of urethral lumen progressing gonococcal disease (Harvey et al. 2002; Ramsey et al. 1995). Association between gonococcus and epithelium also can be due to the interaction of gonococcal LOS and asialoglycoprotein receptor (ASGP-R) followed by recycling of ASGP-R (Harvey et al. 2001a) and mediating endocytosis (Stockert 1995; Weigel and Oka 1998). Gonococcus having terminal lacto-*N*-neotetraose (LnnT) moiety on LOS can adhere to the ASGP-R (Harvey et al. 2001a), enhance

infectivity in humans (Schneider et al. 1991), can help in escaping immune recognition, and act as a sialic acid acceptor. Sialylated LNnT epitope impairs gonococci in invading epithelial cells (Harvey et al. 2001a; van Putten 1993, van Putten and Robertson 1995) and are subsequently phagocytosed by neutrophils (Kim et al. 1992; Rest and Frangipane 1992). Sialylated gonococci in proximity to any of these cells might then be expected to become desialylated through exogenous neuraminidase activity. It can be speculated that subsequent gonococcal adherence to the ASGP-R on sperm (Harvey et al. 2000) would then, in turn, facilitate disease transmission. Neuraminidase present on the surface of human sperm (McRorie and Williams 1974; Srivastava et al. 1970) and phagocytic cells (Cross and Wright 1991) desialylate favoring the progression of disease and also in adhering gonococci to the ASGP-R on sperm (Harvey et al. 2000) in turn, facilitating disease transmission from male to female.

Transmission of gonococci from male to female is followed by infection of lower and upper female genital tract. The sialylated gonococci are desialylated by neuraminidase of vaginal microflora in the female genital tract. Inflammatory response due to infection leads to gonococcal cervicitis in the lower genital tract of women. Antibodies, IL-1, IL-6, and IL-8 responses were not generated due to infection, suggesting that gonococci can evade the host immune system (Hedges et al. 1999), whereas elevated levels of IL-1, IL-6, and IL-8 were reported by Fichorova et al. (2001) contradicting the capability of gonococci to evade the host immune system.

Gonococci with the help of pilus bind to CR3 present on the ectocervix and endocervix in the lower female genital tract by producing and activating a complement. Complement C3b is deposited on gonococci; and affinity of porin for factor H and proximity of porin to sialyated LOS inactivate C3b to iC3b, where iC3b and porin are associated with I-domain of CR3 (Edwards et al. 2000, 2001, 2002, 2003, Swanson et al. 2001). Interaction of gonococci with upper female genital tract is mediated by lutropin receptor (LHr) of endometrial and fallopian tube epithelia (Bolan et al. 1999; Edwards et al. 2001; Hasty et al. 1994, Reshef et al. 1990), where ribosomal protein L12 acts as a ligand for adherence (Spence et al. 2002). LOS, peptidoglycan, and TNF-α bring in cytotoxicity (Cooper et al. 1986; Gregg et al. 1980, 1981; McGee et al. 1992; Melly et al. 1981, 1984) in ciliated cells of the fallopian tube epithelia, whereas in nonciliated cells gonococcus invade gaining access to subepithelial tissues and spaces into the blood stream (McGee et al. 1983). Inactivation of complement C4b and formation of a nonfunctional membrane attack complex (MAC) are the multiple mechanisms gonococci adapt to evade the immune system in the blood stream (Edwards and Apicella 2004). Barh and Kumar (2009) identified various drug targets in *N. gonorrhoeae* by in silico analysis. Genome and proteome of *N. gonorrhoeae* were compared by BLAST to identify unique genes, and DEG was used to know the essentiality of genes. Fructose-1,6-bisphosphate aldolase, two-component system like transcriptional response regulator proteins, phosphotransferase system, anthranilate phosphoribosyltransferase, indole-3-glycerol-phosphate synthase, tryptophan synthase subunit (Table 4) are the drug targets identified for *N. gonorrhoeae.*

Cytoplasmic glycolytic enzyme fructose-1, 6-bisphosphate aldolase (FBA) is highly conserved and present on the surface of bacteria showing non-glycolytic functions like adhering to the host cells in case of *N. meningitidis.* FBA-deficient mutant showed reduction in adhesion to host cell, compared to mutant complemented with a derivative of FBA (Tunio et al. 2010). Therefore, a suitable inhibitor for fructose-1, 6-bisphosphate aldolase may counter invasion of the pathogen.

Two-components system are required for bacterial virulence and regulate their own expression by binding upstream of the sensor and regulator genes, since these proteins are without cognate sensor. PmrA from *Francisella novicida* (Mohapatra et al. 2007) and DegU from *Listeria monocytogenes* (Gueriri et al. 2008) are the established regulators without a cognate sensor. Therefore, a suitable inhibitor for two-component system like transcriptional response regulator proteins, phosphotransferase system, anthranilate phosphoribosyltransferase, indole-3-glycerol-phosphate synthase, tryptophan synthase subunit may show an impact on the virulence of *N. gonorrhoeae*.

5.2 Campylobacter jejuni *and Stomach Cancer*

C. jejuni is a microaerophilic, curved, or spiral, Gram-negative, motile by means of unipolar or bipolar flagellae, non–spore-forming bacteria found in humans, cattle, swine, and birds growing best at 42 °C within a period of 72–96 h (Nachamkin 1995). *C. jejuni* invade and destroy epithelial cells to injure the sites of jejunum, ileum, and colon causing campylobacteriosis (tissue injury) in the gut. *C. jejuni* was linked to small bowel MALT lymphomas (Lecuit et al. 2004), peptic ulcer disease, acute idiopathic demyelinating polyneuropathy (AIDP), i.e., Guillain–Barré syndrome (GBS), and invasive/inflammatory diarrhea. Pathogenesis of *C. jejuni* was well studied and literature review provided us information on motility and chemotaxis; toxins; iron acquisition system; lipooligosaccharide (LOS) and lipopolysaccharide; superoxide and peroxide stress defense; and thermal tolerance in colonizing the intestine.

Motility and chemotaxis are essential for colonization of *C. jejuni* in intestine. Effective colonization of the intestine by *C. jejuni* requires a "corkscrew" unsheathed flagella coded by flaA and flaB genes (Nuijten et al. 1990). Mutational studies have shown that mutants of *C. jejuni* are nonmotile (Wassenaar et al. 1991, 1994) or with slightly decreased motility (Wassenaar et al. 1993). Chemotaxis is the other mechanism required by *C. jejuni* "to detect and move up or down the chemical gradients." Chemotaxis system in *C. jejuni* have the following components like chemoattractants (mucins, L-serine, and L-fucose), chemorepellants (bile acids), others genes cheY, cheA, cheV, cheW, and 10 chemoreceptor genes (containing methyl-accepting chemotaxis protein domains) (Hugdahl et al. 1988; Yao et al. 1997; Parkhill et al. 2000). Nonchemotactic *C. jejuni* mutants were not able to colonize the intestine in animal models (Takata et al. 1992). Flagella and chemotaxis help *C. jejuni* to move toward epithelial cells of the intestine. After reaching

epithelial cells *C. jejuni* uses flagella to bind, adhere, and gain entry in host cells (Wooldridge and Ketley 1997). Mutants of *C. jejuni* have shown reduced motility, reduced adherence, and no invasion (Yao et al. 1994) indicating that flagella are involved in motility, adherence, and invasion. Invasion of *C. jejuni* triggers proinflammatory cytokine interleukin 8 during inflammation (Everest et al. 1993; Hickey et al. 1999) leading to mucosal damage. The pilus-like appendage fimbriae are assumed to be involved in adhesion (Doig et al. 1996; Gaynor et al. 2001). Other adhesions identified in *C. jejuni* include PEB1, CadF, and Cia B. Mutants of peb1A, CadF, and Cia B showed decreased adherence and invasion (Ziprin et al. 1999, Konkel et al. 1999).

Toxic activity either by cytotoxins, enterotoxins, or both is reported in several bacterial pathogens (Wassenaar 1997). Pickett et al. (1996) reported high (cytolethal distending toxin) CDT activity in *C. jejuni* strains, whereas in cdt mutants of *C. jejuni* CDT activity was lost (Whitehouse et al. 1998; Purdy et al. 2000). *C. jejuni* contains two cdt genes with a haemolysin (haemolytic activity) and phospholipase (pldA) domains distending cell progressing into cell death. CDT might be involved in diarrhea the hallmark of *C. jejuni* by disturbing villus epithelial cells leading to a temporary erosion of the villus and a subsequent loss of absorptive functions (Whitehouse et al. 1998).

Iron is an essential nutrient for virulence and pathogenesis of *C. jejuni. C. jejuni* like all Gram-negative bacteria have a sophisticated iron acquisition system. *C. jejuni* expresses ferric iron acquisition systems in iron-restricted conditions. The iron acquisition system consists of the outer membrane (OM) receptor, a periplasmic binding protein, and an inner membrane (IM). OM receptor transports iron over OM from the energy channelized through the TonB–ExbB–ExbD complex, whereas ABC transporter transports iron with energy from ATP hydrolysis (Braun et al. 1998). The iron acquisition systems identified in *C. jejuni* are haemin/hemoglobin uptake system (chuABCD) (Rock et al. 1999), an enterochelin transport system lacking an OM receptor (ceuBCDE) (Richardson and Park 1995), cfrA (Guerry et al. 1997), fhuABD (Galindo et al. 2001), and FeoB protein. In addition, two divergent operons one encoding a periplasmic binding and IM transport system, another coding OM receptor and TonB–ExbB–ExbD complex (Parkhill et al. 2000) were identified in strain of NCTC 11168 of *C. jejuni* (Rock et al. 1999). In a mouse model of infection feoB mutants of *E. coli* and *H. pylori* did not colonize the intestine and stomach, respectively (Stojiljkovic et al. 1993; Velayudhan et al. 2000).

Gram-negative *C. jejuni* outer membrane LOS and LPS involved in adhesion, serum resistance, and endotoxicity (Fry et al. 1998; Wood et al. 1999). O-chain of LOS antigen are sialylated mimicking mammal ganglioside leading to serious autoimmune disorder of the peripheral nervous system known as Guillain–BarreÂ syndrome (Nachamkin et al. 1998) with symptoms of weakness in the limbs and respiratory muscle, and demyelination (Kuroki et al. 1993; Bersudsky et al. 2000; Endtz et al. 2000).

Microaerophilic bacteria *C. jejuni* have superoxide and peroxide stress defense to counter the oxidative stress in the host (Storz and Imlay 1999). Superoxide stress

defense is composed of SOD protein encoded by the *C. jejuni* sodB gene, which converts superoxides to hydrogen peroxide (Pesci et al. 1994; Purdy and Park 1994). Pesci et al. (1994) reported that the mutants of *C. jejuni* sodB exhibited decreased survival. Hydrogen peroxide produced during superoxide stress defense is removed by peroxide stress defense. Peroxide stress defense consists of two proteins, the catalase (KatA) and alkyl hydroperoxide reductase (AhpC or Tsa or TsaA) (Grant and Park 1995; Baillon et al. 1999). KatA converts hydrogen peroxide to water and oxygen, whereas AhpC protein reduces alkyl hydroperoxides to alcohols. *C. jejuni* responds to change in temperatures (thermal stress) when in avian gut (42 °C), human hosts (37 °C), during transmission in water, milk, or on meat (4 °C) or other temperatures by expressing heat shock proteins (HSPs). HSPs like GroESL, DnaJ, DnaK, and ClpB are known to provide thermotolerance in *C. jejuni* (Konkel et al. 1998; Thies et al. 1999a, b, c). Konkel et al. (1998) reported that mutants of dnaJ protein were not able to colonize chickens.

Studies by various groups have identified citrate synthetase, DNA gyrase, serine peptidase, and menaquinone as drug targets for *C. jejuni.* Tilton et al. (2014) based on the essential gene analysis and docking sites identified citrate synthetase of glyoxylate and dicarboxylate metabolism as a drug target in *C. jejuni.* Changkwanyeun et al. (2015) established DNA gyrase as a drug target for *C. jejuni* by studying the in vitro activity of quinolones against DNA gyrase. Karlyshev et al. (2014) proposed cj0511 encoding a serine peptidase in *C. jejuni* as a drug target. Mutant analysis identified serine peptidase as an essential gene for colonization in the chicken colonization model. Menaquinone is an important component of the electron transfer pathway. An alternative pathway is present in the human commensal intestinal bacteria *H. pylori* and *C. jejuni*. Disruption of menaquinone via futalosine pathway had shown inhibition of bacteriostatic growth (Kim et al. 2014). Therefore, designing an inhibitor for menaquinone via futalosine step 1 would affect the growth of *C. jejuni.* Therefore, based on the literature review we propose flaA, flaB, serine peptidase, DNA gyrase, citrate synthetase, mucins, cheY, cheA, cheV, cheW, PEB1, CadF, Cia B, menaquinone, and sodB genes as drug targets (Table 4) for *C. jejuni.*

5.3 Chlamydophila pneumoniae *and Lung Cancer*

C. pneumoniae, a Gram-negative obligate intracellular bacteria causes acute respiratory tract and chronic lung disease. Bronchitis, pneumonia, and sinusitis are acute respiratory tract disease whereas asthma is the chronic lung disease. *C. pneumoniae* successfully infects monocytes, macrophages, neutrophils, epithelial cells, smooth muscle cells, and endothelial cells (Shimada et al. 2009; Hammerschlag 2002; Yang et al. 1994); initially leading to wheezing, asthmatic bronchitis, and adult-onset asthma. This is followed by promotion of chronic inflammation, resulting in progeny of *C. pneumoniae* and finally leading to death of the host cell (Hahn et al. 1991). *C. pneumoniae* has the capability to reside

intracellularly for longer periods (Hogan et al. 2004). Infection of lung by *C. pneumoniae* and chronic inflammatory diseases may develop into lung fibrosis and inducible bronchus-associated lymphoid tissue (iBALT) (Foo and Phipps 2010). Macrophages sense and control bacterial infections and two types of macrophages namely classically activated macrophages (M1) and alternatively activated macrophages (M2) play a role in infection and tissue repair (Lawrence and Natoli 2011; Murray and Wynn 2011; Gordon and Martinez 2010). M1 macrophages also known as inflammatory macrophages induce T cell responses (Th1 or Th17) and tissue inflammation by producing cytokines (IL-12, IL-23) and proinflammatory mediators (iNOS, TNF-α) effectively controlling pathogen invasion (Duan et al. 2012; Qin et al. 2012). The prolonged inflammatory response may impede the wound healing and repair processes, and induce tissue damage (Anzai et al. 2012; Rahat et al. 2011). M2 macrophages increase growth factors concentration and release of anti-inflammatory cytokines (IL-10) into the tissues, playing a critical role in inducing IL-8, healing of wound, repair of tissue, and downregulating apoptosis of infected cells (Gordon and Martinez 2010). IL-8 an angiogenic factor promotes tumor growth resulting in human non-small cell lung carcinomas.

Holliday junction DNA helicase RuvB protein (Reddy 2008), ribonucleotide diphosphate reductase subunit beta (Ravindranath et al. 2013), and HSP—60 (Rajan et al. 2014) are identified as drug targets for *C. pneumoniae.* Reddy (2008) identified Holliday junction DNA helicase RuvB protein as a drug target by in silico analysis. Ravindranath et al. (2013) identified ribonucleotide diphosphate reductase subunit beta as a drug target in *C. pneumoniae* by metabolic pathway analysis and protein interaction network. Rajan et al. (2014) modeled and characterized the drug target HSP 60 of *C. pneumoniae.* Hema et al. (2015) identified 35 drug targets for *C. pneumoniae* by in silico analysis. Therefore, we propose Holliday junction DNA helicase RuvB protein, ribonucleotide diphosphate reductase subunit beta, and HSP—60 as drug targets (Table 4) for *C. pneumoniae.*

6 Conclusion

In conclusion, the review has provided a pool of drug targets that can be targeted for designing drugs to different cancers. Various studies have identified 108 drug targets for *H. pylori* among them 20 were validated drug targets. For other *Helicobacter* species such as *H. felis, H. salmonis, H. bizzozeronii, H. heilmannii,* and *H. bilis* one drug target for each species was available as per the literature review. Eight drug targets were available for *H. hepaticus.* For non-*Helicobacter* species like *N. gonorrhoeae, C. jejuni,* and *C. pneumoniae* six, seventeen, and three drug targets were available.

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Intestinal Microbiota: First Barrier Against Gut-Affecting Pathogens

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Abstract The complex microbial community of the gastrointestinal tract (GIT) plays an important role in GIT health and in whole body wellbeing by aiding digestion, producing nutrients, protecting against pathogens and in the maturation of the host immune system. A balanced intestinal microbiota and balanced microbe-microbe-host relationships is essential for the performance of all physiological, biochemical and enzymatic machinery in the GIT. Dietary nutrients are converted into metabolites, such as short-chain fatty acids (SCFAs) by the GIT microbiota that serve as biologically active molecules with regulatory functions in the host. When the intestinal microbiota gets unbalanced (dysbiosis) changes to this population can have major consequences. Probiotics may restore the balance of the composition of the GIT microbiota. Prebiotics can modulate the GIT microbiota inducing the growth of probiotic bacteria and can additionally produce beneficial effects on the host. Although there has been a great advance on the knowledge on the composition of the human gut microbiota, still more studies are needed to clarify the interaction of the host and the microbiota, determine the factors that govern host colonization and understand the ecological role of the common and diverse resident microbiota of the human gut.

1 Human Gut Microbiota

The human body has clusters of different microorganisms living in different parts of the body including the skin, hair, mouth, human gastrointestinal tract (GIT), and vagina (Alberts et al. 2002). Gut microbiota includes the microbe population living

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in the GIT. It contains about 10^{13}–10^{14} microorganisms (10 times more than human cells), including more than 1000 different species of known bacteria belonging to different orders as well as archaea, viruses and phages, fungi, yeasts and other Eukarya (such as Blastocystis and Amoebozoa). Reaching in total up to 1.5–2 kg of the fecal matter (Mai and Draganov 2009; Lozupone et al. 2012). All these microbial species account for more than 3 million genes (about 150 times more than human genes) (Qin et al. 2010; Gerritsen et al. 2011). In general, about 30 % of the gut microbiota is common to most people, while 60 % is specific to each individual and is maintained over a period of time, being like "an individual identity card" (Qin et al. 2010). Otherwise, the composition of the gut microbiota evolves throughout the entire life, being the result of age, diet, geography, drugs, antibiotics, alcohol, and phage populations (Gerritsen et al. 2011; Gabbard et al. 2014; Scarpellini et al. 2015). Gut microbiota has evolved with the host having several functions integrated in the host organism such as metabolic, immune, nutrients absorption, etc. (Guinane and Cotter 2013).

Among intestinal microbiota, Gram-negative Proteobacteria and Bacteroidetes, and Gram-positive Firmicutes and Actinobacteria, represent the major bacterial phyla (Table 1), methanogens as the predominant intestinal archaea (Dridi et al. 2011; Kamada et al. 2013) and the order *Saccharomycetales* includes the vast majority of yeast (Table 2). In general, in adult healthy individuals, *Bacteroidetes* and *Firmicutes* are the predominant phyla in adults, followed by *Actinobacteria* and *Proteobacteria* (Lozupone et al. 2012) (Fig. 1). In contrast to the knowledge on bacteria, less is known about commensal fungi, archaea and protozoa (Mai and Draganov 2009; Lozupone et al. 2012; Hoffmann et al. 2013; Dutilh et al. 2014).

Gut microbiota play an important role in the health of individuals through interactions microbe-microbe and host-microbe (Brestoff and Artis 2013; Brown et al. 2013; Kamada et al. 2013). The GIT harbors different amounts of microbiota and different species in the different locations and environments of the tract (O'Hara and Shanahan 2006; Zoetendal et al. 2008). The human GIT starts in the mouth with a viable count of 10^{8}–10^{10} colony forming units (CFU) per g saliva. The numbers are reduced in the stomach pH 2–2.5 ($<10^{3}$ CFU/g gastric juice). In the small intestine, duodenum—pH: 5.5–7 (10^{1}–10^{3} CFU/g content), jejunum pH: 6–7 (10^{3}–10^{4} CFU/g content), and ileum pH: 6.5–7.5 (10^{7}–10^{9} CFU/g content). In the large intestine, cecum (pH: 5–7), colon and rectum (pH: 6–7.5) (10^{10}–10^{12} CFU/g content) (Hakansson and Molin 2011) (Table 1).

Classical studies of the gut microbiome were based on traditional culture methods, although only 10–30 % of gut microbiota can be cultivated (Sokol and Seksik 2010; Tannock 2001). Traditionally, taxonomy was established based on studies of morphology and biochemical tests. Recently, molecular phylogenetics has been used to allow better elucidation of the evolutionary relationship of the species by analyzing their DNA sequences, mainly 16S ribosomal DNA. DNA-based methodologies such as PCR and sequencing put in evidence that gut microbial ecosystem is more complex than previously thought (Eckburg et al. 2005). The gut microbiome refers to the collection of genes of the gut microbiota including bacteria and other microorganisms such as fungi, viruses, and protozoan,

Table 1 Bacterial species conforming the GIT microbiota and their on the gastrointestinal tract

Location	Phylum—Class	Order—Families—Genera and/or species	References
Stomach pH 2–2.5 (<10^3 CFU/g gastric juice)	*Proteobacteria*	*Campylobacterales* (*Helicobacter pylori*)	Khosravi et al. (2014)
	Actinobacteria	*Bifidobacteriaceae* (*Bifidobacterium dentium*, *Scardovia inopinata*, and *Parascardovia denticolens*)	Maltarelli et al. (2014)
Small intestine pH:6−7 (10^1−10^9 CFU/g content)	*Firmicutes*	*Bacillales* (*Lactobacillales*–*Lactobacillus* spp., *Enterococcus* spp.), (*Streptococcaceae*–*Streptococcus* spp.)	Drasar (1991), Wang et al. (2005)
	Bacteroidetes	In the distal ileum	Wang et al. (2005)
Large intestine pH:5−7 (10^{10}–10^{12} CFU/g content)	*Bacteroidetes*, class *Bacteroidia*	*Bacteroidales* (*Bacteroides* spp., *Butyricimonas* spp., *Coprobacter* spp., *Parabacteroides* spp., *Prevotella* spp.)	Drasar (1991), Hoffmann et al. (2013)
	Phylum *Firmicutes*, class *Bacilli*	*Bacillales*	Hoffmann et al. (2013), Wang et al. (2005)
	Phylum *Firmicutes*, class *Clostridia* clusters XIVa and IV	*Clostridiales*	Wang et al. (2005)
	Proteobacteria	*Enterobacteriales, Pseudomonadales, Aeromonadales, Burkholderiales,*	Wang et al. (2005)
	Actinobacteria	*Actinomycetales* (*Actinomycetaceae*) *Bifidobacteriales*	Wang et al. (2005)

residing in the upper (stomach and duodenum) and lower GIT (jejunum, ileum, caecum, colon and rectum) (Dave et al. 2012). By analyzing the orphan genes, Kurokawa et al. (2007) identified 647 new gene families only present in human intestinal microbiomes. In this line, Stearns et al. (2011) performed a metagenomic study of the intestinal tract of 4 healthy subjects generating >32 million paired-end sequences of bacterial 16S rRNA genes (V3 region) representing >95,000 unique operational taxonomic units. This study is a comprehensive characterization of the microbiota found along the healthy human digestive tract (Fig. 1). Indeed, the Human Gut Microbiome Projects are shedding new light on the human GIT microbial composition. In the last decade, a huge effort to study and understand the role of the human microbiome launched initiatives like the International Human Microbiome Consortium (IHMC) via the American Human Microbiome Project (HMP) through the National Institutes of Health (NIH) and the European MetaHIT

Table 2 Bacterial microbiota in the human GIT

Phylum—Class	Order—Family—Genera and/or species	References
Bacteroidetes (Bacteroidia)	*Bacteroidales* (*Bacteroidaceae Bacteroides* spp., *Porphyromonadaceae—Butyricimonas* spp. *Coprobacter* spp., *Parabacteroides* spp., *Prevotellaceae—Prevotella* spp.)	Drasar (1991), Hoffmann et al. (2013)
Firmicutes (*class Bacilli*)	*Bacillales* (*Bacillaceae—Bacillus*) *Lactobacillales* (*Aerococcaceae—Abiotrophia* spp., Aerococcus spp.) *Carnobacteriaceae—Carnobacterium* spp., *Enterococcaceae—Enterococcus* spp., *Lactobacillaceae—Lactobacillus* spp., *Pediococcus* spp.)	Drasar (1991)
Firmicutes (*class Clostridia clusters XIVa and IV*)	*Clostridiales* (*Clostridiaceae—Clostridium* spp., *Haemophilus* sp., *and Sarcina* spp., *Eubacteriaceae—Eubacterium* sp., *Lachnospiraceae—Blautia* spp., *Butyricicoccus* sp., *Coprococcus* spp., *Dorea* spp, *Lachnospira* sp., *Roseburia* spp., *Peptococcaceae—Peptococcus* spp., *Peptostreptococcaceae Peptostreptococcus* spp., *Ruminococcaceae—Faecalibacterium* spp., *Ruminococcus* spp., *Sporobacter* spp., *Subdoligranulum* spp.)	Drasar (1991), Hakansson and Molin (2011), Eeckhaut et al. (2012), Guinane and Cotter (2013), Hoffmann et al. (2013), Brahe et al. (2015)
Actinobacteria	*Actinomycetales—Actinomyces* spp., *Propionibacterium* spp., *Bifidobacteriales—Bifidobacterium* spp.,	Drasar (1991)
	Corynebacteriales—Corynebacterium, *Nocardia*	Drasar (1991)
	Micrococcales (*Micrococcaceae—Arthrobacter* spp., *Kocuria* spp., *Micrococcus* spp.)	Drasar (1991)

(continued)

Table 2 (continued)

Phylum—Class	Order—Family—Genera and/or species	References
Proteobacteria		
I. *Gammaproteobacteria*	*Enterobacteriales* (*Enterobacteriaceae—Citrobacter*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Proteus*)	Drasar (1991)
	Pseudomonadales—Pseudomonas spp.	Drasar (1991)
	Aeromonadales—Aeromonas spp.	Drasar (1991)
II. *Betaproteobacteria*	*Burkholderiales* (*Alcaligenaceae—Alcaligenes* spp.)	Drasar (1991)
III. *Epsilonproteobacteria*	*Campylobacterales* (*Campylobacter* spp., *Helicobacter pylori*)	
Fusobacteria	*Fusobacteriales* (*Fusobacteriaceae—Fusobacterium* spp.)	Drasar (1991)
Verrucomicrobia, class Verrucomicrobiae	*Verrucomicrobiales* (*Akkermansiaceae—Akkermansia* sp.)	Dubourg et al. (2013)

project, financed by the European Commission under the seventh FP program. Both, the MetaHIT (Qin et al. 2010), and the HMP have contributed to the greater knowledge on the availability of the reference gene catalog (The Human Microbiome Project Consortium 2012a). Still, a lot of effort is needed in order to fully understand the role of the GIT microbiota as well as the interactions with the surrounding microbiota and with the host.

Culture-independent methodologies are giving evidence of microbiota residing in the GIT that cannot currently be cultured and that has not been previously described (Ott et al. 2004; Gupta and Prasad 2011; Koren et al. 2011). Wang et al. (2005) studied diversity of the mucosal biopsies from different locations of the human GIT, jejunum, distal ileum, ascending colon and rectum by sequencing of 347 PCR-amplified 16S rDNA clones finding six phylogenetic phyla of the domain *Bacteria*: *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Fusobacteria*, *Verrucomicrobia*, and *Actinobacteria.* They found *Firmicutes* and *Bacteroidetes* phyla accounted for most of the recovered sequences (88 %). At the genera level *Prevotella*, *Streptococcus*, *Veillonella*, *Rothia* and *Haemophilus* dominate the healthy human GIT (Nardone and Compare 2015). GIT locations were found to have specific microbiota associated. Thus, phylogenetic analysis of sequences showed that the jejunum library was dominated by sequences closely related to the *Streptococcus* genus, whereas in the distal ileum, ascending colon and rectum,

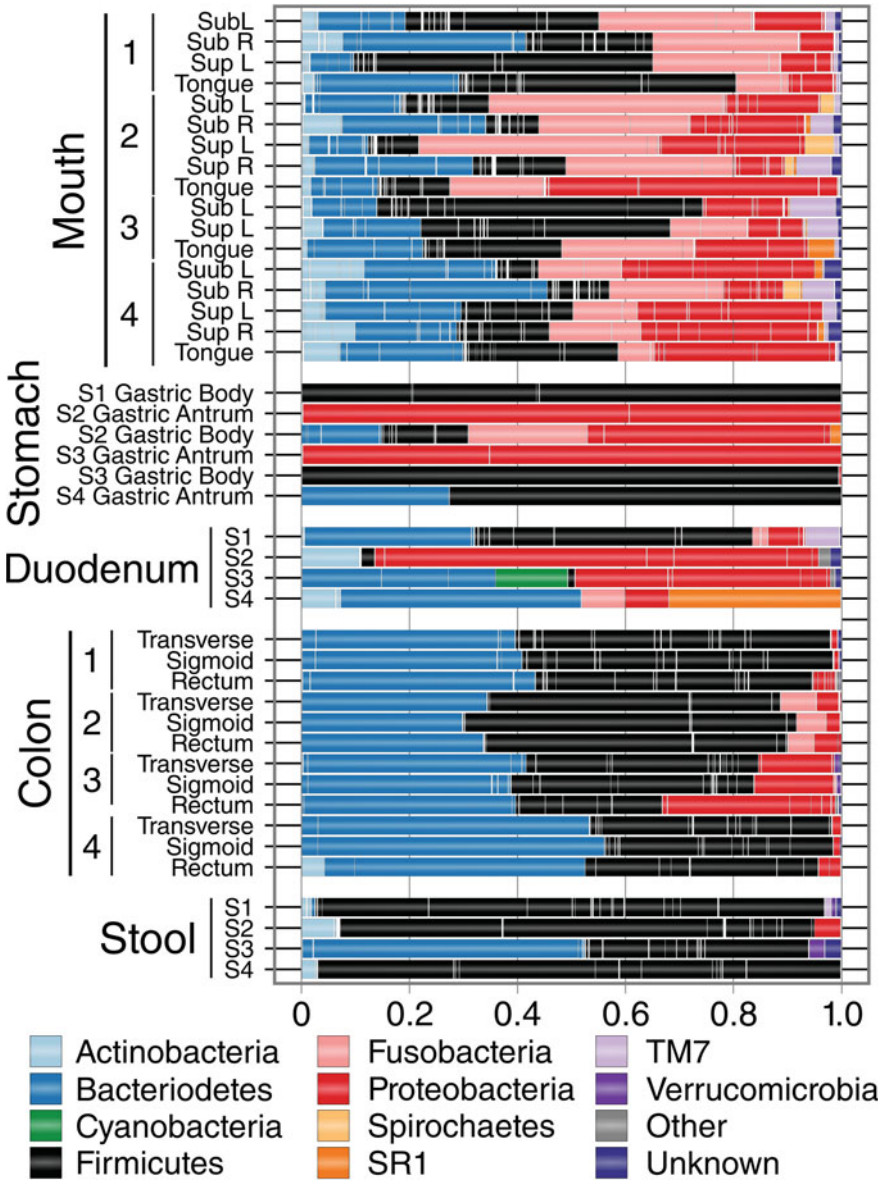

Fig. 1 Metagenomic study of the genera represented in the digestive tracts of four healthy subjects (S1-S4). Bars shown unique genera and are colored with the phylum level assignment for each group. Figure modified from Stearns et al. (2011)

Bacteroidetes, and *Clostridium* clusters XIVa and IV (Collins et al. 1994) were the predominant bacterial groups. Their results showed that 49, 27, and 43 % of the sequences retrieved from the distal ileum, ascending colon and rectum libraries,

respectively, belonged to the *Bacteroidetes* phylum. However, the composition of the GIT microbiota is dynamic and affected by such factors as diet, drugs, and diseases.

1.1 Superkindom Bacteria

As noted above, the GIT microbiota populations vary among individuals and within an individual part of the microbiota can vary over time and through changes in lifestyle, diet and drugs (Qin et al. 2010). Most of the bacteria described until recent years was based on culture methods from stools, being bacteria that grow quickly in classical high-nutrient growth media at mesophilic temperatures (Hugenholtz et al. 1998; Lagier et al. 2012). Culture-independent methodologies studies showed that there are six dominant phyla in the human gut. (1) Firmicutes are Gram-positive bacteria including many well-known genera such as *Bacillus*, *Listeria*, *Staphylococcus*, *Streptococcus*, *Enterococcus*, and *Clostridium* clusters XIVa and IV (Tables 1 and 2) that reside in different locations of the GIT. The Phylum Firmicutes is divided into class *Clostridia*, anaerobic, and class *Bacilli*, obligate or facultative aerobes. The group includes some notable pathogens. Many produce endospores, which can survive extreme conditions. The representing genera are *Clostridium* and *Bacillus*. (2) Bacteroidetes are Gram-negative, motile or non-motile bacilli, anaerobe, and are one of the dominant bacteria of the GIT representing about 30 % of all bacteria in the gut, and having an important role in the functioning of the host. (3) Actinobacteria or Actinomycetes are a group of Gram-positive bacteria, mostly aerobic, including *Bifidobacterium*, being abundant in the large intestine. There are a few pathogens such as *Mycobacterium*. (4) Proteobacteria are Gram-negative, mostly facultatively or obligately anaerobic with an outer membrane mainly composed of lipopolysaccharides and include a wide variety of pathogens, such as *Proteus, Escherichia, Salmonella, Vibrio, Klebsiella, Enterobacter, Citrobacter, Helicobacter,* and many other notable genera. (5) Fusobacteria are anaerobic non-sporeforming Gram-negative bacilli. This phylum has shown to be difficult to identify by traditional biochemical methods. However, the application of novel molecular biological techniques to taxonomy allowed the detection and differentiation of new species, and the differentiation of *Fusobacterium necrophorum* and *F. nucleatum*. Fusobacteria is known to be involved in a wide range of human infections causing tissue necrosis and septicemia (Bennett and Eley 1993). (6) Verrucomicrobia. The member present in the gut lumen is *Akkermansia* sp., Gram-negative and strictly anaerobic, constituting 0.1–3 % of the total GIT bacteria, closely associated with the mucus layer, it feeds on mucin (Derrien et al. 2004).

GIT bacteria have specific functions with a marked role in human health. For example, the butyrate was found to induce a specific transcriptional response in the human colon, affecting genes and pathways involved fatty acid oxidation, epithelial integrity, and apoptosis (Vanhoutvin et al. 2009). The butyrate-producing bacteria

group include *Akkermansia muciniphila*, *Faecalibacterium prausnitzii*, *Eubacterium hallii*, *Butyricicoccus*, *Roseburia intestinalis* and *Fusobacterium varium* (Barcenilla et al. 2000; Duncan et al. 2002; Pryde et al. 2002; Hold et al. 2002; Derrien et al. 2004; Potrykus et al. 2007). Members of the butyrate-producing clostridial clusters IV and XIVa have been reproducibly reported in the gut of Inflammatory Bowel Disease (IBD) patients (Sokol and Seksik 2010; Takaishi et al. 2008; Van Immerseel et al. 2010). Recent studies have identified butyric acid as a potential therapeutic agent for IBD, but in IBD patients, some of these strains are decreased. Restoring normal levels of butyrate-producing bacteria in the gut was found to improve the symptoms of IBD (Van Immerseel et al. 2010). Eeckhaut et al. (2012) reported that that a particular strain from clostridium cluster IV, *Butyricicoccus pullicaecorum*, was superior in decreasing lesions in a rat IBD model compared to the probiotic *F. prausnitzii*. Growth of specific beneficial bacteria such as butyrate producing, *Lactobacillus* spp. or *Bifidobacterium* may be stimulated by using prebiotics (Reuter 2001; Scott et al. 2014).

1.2 Superkingdom Archaea

Archaea, in particular methanogens, are anaerobic and represent about the 10 % of bacteria in the colon. Additionally, the diversity of the Archaea in the human body is considerably lower, including representatives of two phyla, Euryarchaeota and Thaumarchaeota. Some representing species isolated from the human gut include *Methanosphaera stadtmanae*, *Methanomicrococcus blatticola*, *Methanobrevibacter smithii*, *Nitrososphaera* spp. (Table 3) (Hoffmann et al. 2013; Mihajlovski et al. 2010; Gaci et al. 2014). Mihajlovski et al. (2010) studied the diversity of methanogenic Archaea in the human gut targeting the molecular metabolic marker of methanogenesis *mcrA*, detecting *M. smithii* and *M. stadtmanae* as well as a distant phylotype that does not cluster with any of the five methanogenic orders. Archaea perform H_2 depletion optimizing the fermentation and modifying the metabolic pathways of fermentative bacteria (Borrel et al. 2013; Aminov 2013; Gaci et al. 2014). Hydrogen is produced in the intestine by anaerobic fermentation of the undigested polysaccharide fraction of carbohydrates, being this H_2 the substrate for

Table 3 Superkingdom *Archaea* in the human GIT

Phylum—Class	Order—Family—Genera and/or species	References
Euryarchaeota—Methanobacteria	*Methanobacteriales* (*Methanobacteriaceae*—*Methanobrevibacter smithii*, *Methanosphaera stadtmanae*)	Hoffmann et al. (2013), Kamada et al. (2013), Mihajlovski et al. (2010)
Thaumarchaeota—*Nitrososphaeria*	*Nitrososphaerales*; (*Nitrososphaeraceae*—*Nitrososphaera* spp.)	Hoffmann et al. (2013)

methane production by intestinal methanogens. Archaea species seem to be relatively safe or even contribute to improve gut health by removing excess hydrogen via methanogenesis, sulfate reduction, or acetogenesis. Although methane is an inert gas, it seems to reduce propagation of the peristaltic movement in the intestine (Aminov 2013). Reports on presence of Archae in patients with altered intestinal motility concluded that in conditions with extended transit time in the intestine, such as constipation and diverticulosis, the incidence and rate of methane production are higher. In the same way, in fast transitions, such as in the diarrheal conditions, were observed lower numbers of methanogenic archaea and lower rates of methane production (Attaluri et al. 2010; Furnari et al. 2012; Aminov 2013). In fact, patients with delayed colonic transit, such as constipation, were found to breath methane (CH_4) positive (Lee et al. 2013).

1.3 Viruses

A virus is a small agent made up of a core of genetic material, either DNA or RNA, surrounded by a shell of protein (capsid). Viruses use the cells of the host as reproductive system to replicate. Then they leave the host cell, sometimes killing it in the process, and proceed to infect other cells within the organism. Viruses can infect plants, bacteria, and animals. Viruses that affect bacteria are called bacteriophages, or phages. Studying fecal samples it was found that bacteriophages are the most prevalent enteric viruses (Brabban et al. 2005; Breitbart et al. 2008; Focà et al. 2015). Phages replicate within the bacterium by the injection of their genome into its cytoplasm, affecting the bacterial genome architecture. Thus, two-thirds of all Gammaproteobacteria and low G-C Gram-positive bacteria harbor prophages, where the genome of the phage stays in the bacteria in a latent form (Canchaya et al. 2003; Casjens 2003). Different studies focused on the minimal bacterial cell concentration at which replication can still occur, finding that phages may replicate at concentrations as low as 10^2 cells/mL (Suttle and Chan 1994; Wiggins and Alexander 1985; Waterbury and Valois 1993). The human virome is essentially a collection of all the viruses that are found in or on human beings (Focà et al. 2015). Immense populations of viruses are present in the human gut and other body sites being 10 times higher than the microbiome. Understanding the role of these populations in the gut and increasing the knowledge of the human virome is important in order to decipher their implication in health and disease (Minot et al. 2011; Focà et al. 2015).

Gut viruses are not easily cultivable with common microbiological techniques. Thus, the development of nonculture-based metagenomic methods revealed a complex community that includes viruses studying fecal samples. Different studies have shown that the gut virome has a deep influence on the gut microbiome, contributing to the stability of the microorganisms that constitute the gut microbiota (Scarpellini et al. 2015; Waller et al. 2014). Moreover, the phage virome changes rapidly in the first week of life, creating the symbiotic relationships between host

and virome and evolving with the diet and other factors, thus creating a high interpersonal variation (Palmer et al. 2007; Waller et al. 2014; Reyes et al. 2012; Hofer 2013; Minot et al. 2011, 2013; Focà et al. 2015). These studies concluded that gut viruses are not only a source of pathogens but also a physiological component of the healthy human microbiota and they could be used for gut microbiota modulation. Different studies revealed that phages are important vehicles for horizontal gene exchange (via transduction) between different bacterial species leading to strain to- strain differences within the same bacterial species (Bouchard and Moineau 2000; Desiere et al. 2001; Lawrence et al. 2001; Boyd and Brussow 2002; Chibani-Chennoufi et al. 2004; Balcazar 2014). Thus, the transduction can occur from the environment to human and animal microbiomes and within body microbiomes (Muniesa et al. 2013).

Minot et al. (2011) analyzed fecal samples by metagenomic sequencing of DNA from virus-like particles (VLP) finding that the virome of individuals on the same diet had less variation than the one of individuals on a varied diet. In this sense, Holtz et al. (2014) studied the diversity of viral communities in stools from 87 children with diarrhea from two different geographic locations, Melbourne and Northern Territory (Australia). The samples were collected in the same time period. They found that many virus families were equally prevalent in the two cohorts, including viruses known to cause diarrhea such as norovirus (*Caliciviridae*) and astrovirus (*Astroviridae*) (Finkbeiner et al. 2008, 2009) as well as non-pathogenic enteric viruses, such as anelloviruses, viruses not associated with an enteric reservoir, viruses of plants, and novel viruses. They also detected sequences derived from the families *Reoviridae*, *Caliciviridae*, *Adenoviridae* or *Astroviridae* (Holtz et al. 2014). Methagenomic studies are giving evidence that the gut virome is populated by pathogens (e.g. *Norwalk*, *Rotavirus*, *Enterovirus*, etc.), as well as undetectable giant viruses (derived mainly from protozoa and parasites), plant-derived viruses and bacteriophages (Reyes et al. 2012; Lozupone et al. 2012).

Phages play a role in nutrient release through lysis of host bacteria. They reside in the thick mucosal layer that separates the intestinal epithelium from the lumen that conditions influence the nature of the interactions that occur between the human host, bacteria, and phage (Ogilvie and Jones 2015).

However, phages may be used as prophilactic or as therapeutic. Phages have been used for over 90 years as an alternative to antibiotics in the former Soviet Union and Central Europe, as well as in France. As therapeutics, phages have a high potential to reach and kill pathogenic bacteria without negatively modifying the environment where they are applied. In this sense, phage therapy may become the choice therapy against multi-drug-resistant strains of many bacteria (Keen 2012; Inal 2003). As prophilactics, they could be used to reduce harmful bacteria entering the food chain. However, to use them in the food chain production, some requirements should be fulfilled such as the production of virulent phages that can survive in extreme environments, having a broad host range for the target genus, and lacking bacterial virulence genes (Lu and Koeris 2011). Although phages have been approved to use in food and medical industries by several international agencies (such as FDA, GRAS, US-FSIS), it still exists obstacles which must be

overcome (as the evolution of bacterial resistance to phages, manufacturing challenges, systemic side effects of phage therapy and delivery) (Lu and Koeris 2011; Jassim and Limoges 2014). In fact, most phages do not represent a threat to human health unless they bear virulence factors (Brussow et al. 2004). Then, full genome sequencing of therapeutic or prophilactic phages should be used avoid the trasmision of virulence factors. One characteristic of phages is that they are specific to the host, infecting only a few strains of a bacterial species and, more rarely, they can infect more than one closely related bacterial genus. Thus, phages are safe for the gut microbiota and can even modulate it to favor a healthy quali—and quantitative composition (Mann 2008; Hyman and Abedon 2010; Gupta and Prasad 2011; Loc-Carrillo and Abedon 2011). Lytic bacteriophages could modulate gut bacterial composition, and bacteriophages alone or in combination with antibiotics could be used as therapeutics to help the overcome of bacterial antibiotic resistance (Scarpellini et al. 2015; Reyes et al. 2012).

1.4 Superkingdom Eukaryota—Fungi

Fungi are an important component of the intestinal microbiome (Table 4), but relatively low abundant and difficult to culture (Parfrey et al. 2011; Luan et al. 2015), and little is known about the mycobiome in human intestine and feces and their function. Culture-independent methods have given a new insight on the mycobiomes found in different body sites and their role in health and disease (Cui et al. 2013). Luan et al. (2015) studied the fungal microbiota in the intestinal

Table 4 Superkingdom *Eukaryota* including kingdom Fungi in the human GIT

Phylum—Class	Order—Family—Genera and/or species	References
Ascomycota (*Dothideomycetes*)	*Pleosporales* (*Pleosporaceae—Alternaria* spp.)	Hoffmann et al. (2013)
Ascomycota (*Saccharomycetes*)	*Saccharomycetales* (*Debaryomycetaceae—Candida* spp. *Debaryomyces* spp., *Pichiaceae* (*Pichia* spp. *Saccharomycetaceae—Kluyveromyces* spp., *Saccharomyces* spp.)	Hoffmann et al. (2013), David et al. (2014)
Ascomycota (*Eurotiomycetes*)	*Eurotiales* (*Aspergillaceae—Aspergillus* spp., *Penicillium* spp.)	Gouba et al. (2013)
Basidiomycota—Class *Agaricomycetes*	*Polyporales* (*Hapalopilaceae—Climacocystis* spp.)	Gouba et al. (2013)
Basidiomycota—Class *Tremellomycetes*	*Filobasidiales* (*Filobasidiaceae—Filobasidium* spp.)	
Dothideomycetes	*Pleosporales* (*Didymellaceae—Phoma* spp.)	Luan et al. (2015)
Zygomycota Mucoromycotina	*Mucorales—Mucor* spp.	Rodríguez et al. (2015)

mucosal of 27 patients with adenomas finding three dominant fungal phyla, Ascomycota, Glomeromycota and Basidiomycota, and two rare phyla (relative abundance <1 %), Chytridiomycota and Neocallimastigomycota. Their results also showed that two opportunistic pathogenetic fungi genera, *Phoma* and *Candida*, were abundant (45 %). Metagenomic analysis showed that fungi accounting for only 0.03 % of the fecal microbiota (Ott et al. 2008). Some genera like *Candida* spp., *Saccharomyces* sp. and *Malassezia* spp., etc. were thought to be the predominant commensal fungi species while *Aspergillus* spp., *Mucor* spp., *Cryptococcus* spp., *Rhodotorula* spp., *Trichosporon* spp., *Histoplasma* spp., *Coccidiodes* spp., *Paracoccidioides* spp. and *Blastomyces* spp. were classified as transient microbiota or exogenous pathogenic fungi (Wang et al. 2014). Recent studies found evidence of different yeast species (up to 50) living in the human gut (Gouba et al. 2013; Li et al. 2014; Luan et al. 2015). Gouba et al. (2013) studied stools from an obese young woman isolating a total eight different fungal species by PCR including *Aspergillus flavipes*, *Beauveria bassiana*, *Isaria farinosa*, *Penicillium brevicompactum*, *Penicillium dipodomyicola*, *Penicillium camemberti*, *Climacocystis* sp., and *Malassezia restricta.* Most of these fungal species detected apparently were originated from food. Recent studies on intestinal transplant recipients showed that they are often colonized with *Saccharomyces cerevisiae*, *Kluyveromyces waltii*, *Candida* spp., *Cryptococcus neoformans*, *Fusarium oxysporum* and *Aspergillus clavatus*, etc. (Li et al. 2012).

In a healthy body, gut microbiota maintains a healthy balance and a huge diversity of microorganisms coexists peacefully interacting with each other and with the host (Oever and Netea 2014). GIT disorders or under antibiotics, different drugs, food allergies, animal foods containing antibiotics, this balance may be altered, allowing *Candida* and other fungi to grow. Fungi thrive in an acidic environment; bacteria grow better in a weakly alkaline to neutral environment. Once the fungi population increases, they produce wastes that create an acidic environment and bacteria cannot grow. Thus, species such as *Candida albicans* that grow well in highly acidic environments like the gastric (Zwolinska-Wcislo et al. 2001; Wang et al. 2014) may increase the severity of gastric mucosal lesions. The intestinal mycobiome may contribute to the pathogenesis of several GI disorders such as IBD (Wang et al. 2014), obesity (Rodríguez et al. 2015) or diabetes (Gosiewski et al. 2014). Rodríguez et al. (2015) studied the mycobiome of obese and non-obese patients finding that *Candida*, *Nakaseomyces* and *Penicillium* were the most abundant genera detected in obese patients while *Mucor* was the most prevalent genus in non-obese patients, followed by *Candida* and *Penicillium*. After diet-induced weight loss, the relative abundance of *Mucor* spp. was increased. However, Mucorales are described to be pathogenic producing the disease mucormycosis mostly seen immunocompromised because of diabetic ketoacidosis, neutropenia, organ transplantation, and/or increased serum levels of available iron (Petrikkos et al. 2012).

Otherwise, although some fungi are pathogen, some fungal species have been a component of the human diet for thousands of years. In fact, yeast species such as *Saccharomyces cerevisae* and other Ascomycota have been included in human diet

for centuries or taken as nutritional supplements. Yeast possesses a cell wall polysaccharide, α-mannan, that seems to influence the ecology of the human gut microbiota. Cuskin et al. (2015) reported that yeast is a viable food source for the Gram-negative species *Bacteroides thetaiotaomicron*, a dominant member of the microbiota. Indeed, some emerging microbiological data on yeast composition and functions gave evidence of the use of yeast in the modulation of gut microbiota. One species, *Saccharomyces boulardii* is currently used as an efficient probiotic over placebo in the treatment of post-infectious and post-antibiotic diarrhea and the prevention and therapy of GIT disorders in general (Dinleyici et al. 2012; Kelesidis and Pothoulakis 2012).

1.5 Parasites

Intestinal parasites are parasites (helminths and protozoa) that can infect the GIT of humans and other animals. Protozoans, including *Cryptosporidium*, *Microsporidia,* and *Isospora* (Table 5). Parasites can get into the intestine through the mouth from uncooked or unwashed food, contaminated water or hands or by skin contact with larva-infected soil. Many species of parasites are being studied as emergent pathogens that can cause food-borne infectious diseases. Currently, about 300 helminths and 70 protozoan species are known to infect humans and animals (Lee et al. 2014; Evans and Mitre 2015). Usually parasites coexist with the gut microbiota in the healthy human gut and small numbers are safe and well tolerated, but when they overgrow, pathogenesis arises (Mortimer et al. 2006; Gaze et al. 2012; Giacomin et al. 2015). Whereas most infections and death from parasitic diseases affect people in developing countries (due mainly to some enteric protozoa such as *Entamoeba* spp., *Cryptosporidium* spp., and *Giardia* sp. causing diarrhea), they also affect people in developed countries (*Blastocystis* spp. and *Dientamoeba fragilis*) (Fletcher et al. 2012). The immune response to parasitic infection involves increased expression of the regulatory cytokines IL-10 and TGFβ, expansion of Foxp3+regulatory T cells, increased IL-22 and IL-5 expression and reductions in IL-23, IFNγ and IL-17A levels (Gaze et al. 2012; Mishra et al. 2014; Giacomin et al. 2015). Ferreira et al. (2013) observed that hookworm excretory/secretory products induced interleukin-4 (IL-4) + IL-10 + CD4 + T cell responses and suppress pathology in a mouse model of colitis.

However, some parasites such as helminths have been proposed as therapies for the treatment of inflammatory disorders of the human GIT, including inflammatory bowel disease, celiac disease, carcinogenesis and even autism (Schnoeller et al. 2008; León-Cabrera et al. 2014; Giacomin et al. 2015). Other works focused on two GIT parasitic nematodes, whipworms (*Trichuris* sp.) and hookworms (*Necator americanus*), to study strategies to treat inflammatory gut disorders (Reddy and Fried 2009; Giacomin et al. 2015).

Table 5 Superkingdom *Eukaryota*, including kingdom *Metazoa*, protozoans and helminths in the human GIT

	Phylum—Class	Order—Family—Genera and/or species	References
Protozoans	*Diplomonadida* (Fam. *Hexamitidae*)	(*Enteromonadidae—Enteromonas* spp., *Hexamitidae—Giardia* spp.)	Berrilli et al. (2012)
	Apicomplexa class *Coccidia*	*Eucoccidiorida* (Cryptosporidiidae—*Cryptosporidium* spp., *Eimeriidae—Isospora* spp.)	Berrilli et al. (2012)
	Amoebozoa	*Entamoebidae* (*Entamoeba histolytica)*	Berrilli et al. (2012)
	Stramenopiles	*Blastocystis hominis*	Denoeud et al. (2011)
Kingdom Metazoa	Phylum *Nematoda*, class *Enoplea*	*Trichocephalida* (*Trichuridae—Trichuris* spp.	Giacomin et al. (2015)
	Phylum *Nematoda*, class *Chromadorea*	*Rhabditida* (Fam. *Heligmosomatidae—Heligmosomoides* sp., Fam. *Trichostrongylidae—Ostertagia* sp.)	Giacomin et al. (2015)
Helminths	*Cestodes*	*Diphyllobothriidea* (*Diphyllobothriidae—Diphyllobothrium*) *Cyclophyllidea* (Fam *Taeniidae—Taenia*	Berrilli et al. (2012)
	Phylum *Platyhelminthes* class *Trematod*a	Order *Echinostomida* (Fam *Fasciolidae—Fasciolopsis*) *Order Opisthorchiida* (Fam *Heterophyidae—Heterophyes* spp. *Order Strigeidida* (*Fam* Schistosomatidae—*Schistosoma* spp.)	Berrilli et al. (2012)
	Nematodes geohelminths	Order Ascaridida (Fam *Ascarididae—Ascaris* spp.) *Rhabditida* (*Ancylostomatidae*, and *Strongyloides*) Order *Trichocephalida* (Fam *Trichuridae—Trichuris*	Berrilli et al. (2012)
	Nematodes	*Enterobius vermicularis*	Berrilli et al. (2012)

Different authors studied the interactions between parasites and microbial communities in the human gut (Torres et al. 2000; Berrilli et al. 2012) showing that the intestinal microbiota is essential for the pathogenicity but not for the multiplication of *Giardia duodenalis*. Andersen et al. (2015) studied the interaction between eukaryotic, *Blastocystis* spp., and prokaryotic microbial communities in the intestinal microbiome observing that Individuals with intestinal microbiota dominated by *Bacteroides* were much less prone to having *Blastocystis*-positive stool than individuals with *Ruminococcus*- and Prevotella-driven enterotypes. Otherwise, different studies focused on the study of the cohabitation of microbiota in the intestine and the interactions among bacteria, helminth parasites, fungi, and viruses. Their results showed that helminth colonization is associated with

increased diversity of the gut microbiota observing that the presence of commensal bacteria within the host is essential for the successful establishment of parasites such as *Trichuris* infection (Hayes et al. 2010; Lee et al. 2014; Reynolds 2015). Hayes et al. (2010) observed that the commensal spp. and Fam. Hymenolepididae—*Hymenolepis* spp. microbiota of the gut induced successful hatching of embryonated eggs of parasites, and removal of bacterial or yeast cells from cultures prevented hatching, and thus, revealing a close relationship between a metazoan parasite and the gut microbiota.

2 Acquisition, Development and Role of GIT Microbiota in the Human Body

2.1 GIT Microbiota in Infants

The gut microbiota develops immediately after birth influenced by delivery mode, early nutrition and host genotype (Lozupone et al. 2012). It was generally believed that the development of gut microbiota starts at birth, being considered the GIT of the fetus sterile. However, recently it has been acknowledged that microbial colonization may occur in the fetus (Romano-Keeler and Weitkamp 2015). During birth, microorganisms from the mother, including vaginal, fecal, skin, breast and environment microbiota, quickly colonize the infant GIT. Colonization varies depending on the gestational age, the mode of birth (Neu and Rushing 2011) and thus, cesarean birth was observed to alter the pattern of gut microbiota development relative to that of vaginally born infants (Francino 2014). After birth, the composition of the intestinal microbiota is affected by different factors such as the mode of delivery, breast or bottle-feeding. Thus, the microbiota of breastfed infants is mainly dominated by *Bifidobacteria* but also contains bacteria from the skin of the mother and from the breast milk. Breast milk, traditionally believed to be sterile, was found to have 10^3–10^4 cfu/mL including hundreds of species including Gram-positive bacteria such as lactic acid bacteria and Gram-negative bacteria such as *Serratia* and *Pseudomonas* (Jeurink et al. 2013; Francino 2014). The oligosaccharides of human milk are only partially digested in the small intestine and mostly reach the colon, where they are fermented, mainly by *Bifidobacterium*, to produce short-chain fatty acids (SCFA) leading to acidification. Besides colonizing the gut, the bacteria acquired at birth and by breast milk may stimulate the immune system of the infant and the immune-protective function of the gut mucosa (Francino 2014). During the first year, the GIT microbiota of the infant evolves and becomes dominated by the phyla Bacteroidetes and Firmicutes, mainly represented by the genera *Bacteroides*, *Faecalibacterium*, *Clostridium* and *Ruminococcus* and by the age of three, microbiota becomes stable and similar to that of adults (Yatsunenko et al. 2012; Francino 2014). Further, infant nutrition may have life-long consequences through microbial modulation of the immune system (Lopez-Legarrea et al. 2014).

2.2 GIT Microbiota in Adults

About 60 % of the composition of the adult GIT microbiota is considered overall stable. However, several factors can modulate the adult GIT microbiota including age, antibiotics and drugs, diet, or may alter the physiological state of the host like in pregnancy, inflammatory bowel disease, obesity, diabetes, metabolic syndrome, autoimmune illness, atherosclerosis, non-alcoholic fatty liver disease and liver cirrhosis (Brahe et al. 2015). Namely, the diet has a major role in shaping the composition and activity of the human gut microbiota, particularly macronutrients including carbohydrates, fats and protein, shifts in intake of animal—versus plant-based diets can influence the microbial profiles (David et al. 2014; Conlon and Bird 2014). The role of diet in gut microbiota modulation is strengthened by the recent metagenome-wide association studies. Thus, in type 2 diabetic (T2D) patients, with a diet-associated insulin resistance; they have lower butyrate-producing bacteria, an increase in various opportunistic pathogens (Qin et al. 2012). Gut microbiota play a part in vitamin synthesis and in absorption of calcium, magnesium, and iron. Absorption of these ions in the cecum is improved by carbohydrate fermentation and productions of SCFAs, especially acetate, propionate and butyrate (Martin et al. 2008). On the last decades, all microbiological/clinical research have helped to understand how food components, macro and micronutrients, probiotics, prebiotics, synbiotics, organic acids, exogenous enzymes, polyunsaturated fatty acids (PUFAs), phytobiotics, antibiotics and drugs and xenobiotics can modulate the intestinal bacteria quali-/quantitative pattern as well as the microbial-host relationships (Mai and Dragonov 2009; Quigley 2010). Understanding the role and the patterns of modulation of the GIT microbiota may help to normalize the GIT microbial ecosystem and immune system as well as the prevention and/or treatment of enteric infections. Turnbaugh et al. (2009) found that mice on a western diet (increase in fat intake) experienced an increase in the abundance of bacteria of the phylum Firmicutes and a decrease in the abundance of those of the phylum Bacteroidetes. Changing from a diet low in fat and rich in polysaccharides to a diet rich in fat and sugar and low in plant polysaccharides (western diet) changed their microbiota in just 1 day. Gut microbiota supply the human body with energy from dietary plant polysaccharides producing enzymes, glycoside hydrolases, polysaccharide lyases and carbohydrate esterases, which are absent in the human genome. Gut microbiota may adjust to metabolize and get energy from dietary components. Such is the case of Japanese people that can digest seaweeds (*Porphyra* spp. or nori is a part of their daily diet) thanks to specific enzymes, porphyranases, that their microbiota has acquired from marine bacteria (Hehemann et al. 2010).

2.3 *Microbiota in Aging People*

The balance of the GIT microbiota may be affected during the aging process and, consequently, the elderly have substantially different microbiota compared to younger adults (Claesson et al. 2012; Yatsunenko et al. 2012). Consequently, GIT microbiota may be used as an indicator of the health status in elderly. Since they are more susceptible to infections, increased inflammation and the food intake is less varied. This can also modulate the changes in microbiota composition and activity (Claesson et al. 2012). In fact, diet is a major, controllable modulator of the GIT microbiome, with the high-fat, sugar-rich Western diet contributing to a Bacteroides-dominant microbiome and high-fiber diet to one dominated by *Firmicutes* (Wu et al. 2011).

The changes on the composition of the GIT microbiota during aging can greatly affect and be affected by human health. An increase in the proportion of *Ruminococcus*, *Atopobium*, and Enterobacteriaceae and a significant reduction in the proportion of *Lactobacillales*, *Bacteroides/Prevotella* and *F. prausnitzii* were seen in frailty individuals and the number of *Enterobacteriacea* increases greatly in very frail individuals (Van Tongeren et al. 2005). This change may also affect to the fermentation of undigested carbohydrates as well as other nutrients, and affect some treatments in elderly, such as anticoagulants. In fact, Enterobacteria were identified as vitamin K producers, concretely, *Enterobacter agglomerans*, *Serratia marcescens* and *Enterococcus faecium* produce various forms of menaquinone, the naturally occurring form of vitamin K identified in bacteria (Cooke et al. 2006). Van Tongeren et al. (2005) also observed that decreased microbial diversity correlated with increased frailty, decreased diet diversity and health parameters and with increased levels of inflammatory markers. Lifestyle also modulates GIT microbiota, and thus, individuals living in a community had more diverse microbiota and were healthier than those in short- or long-term residential care services.

3 Role of the Gut Microbiota in the Human Body

As mentioned above, the human microbiota participates in the digestion of complex carbohydrates in the gut, in the protection of the host from pathogen invasion, in modulating proper immune function, and in the maintenance of epithelial homeostasis (Neish 2009). In most examples of eukaryotic–prokaryotic symbioses, the microbe benefits by acquisition of a stable nutrient supply and immediate environment, while the eukaryotic host gains extended metabolic/digestive ability and competitive exclusion of less-benign microbes, an arrangement that clearly applies to the intestine-microbiota system (Neish 2009; Gibson et al. 2014) (Fig. 2). Thus, some authors discovered symbiosis factors from symbionts that facilitate the peaceful coexistence of the microbiota and the host immune system (Rupa and Mine 2012). These communities can live under extreme conditions, at pH level,

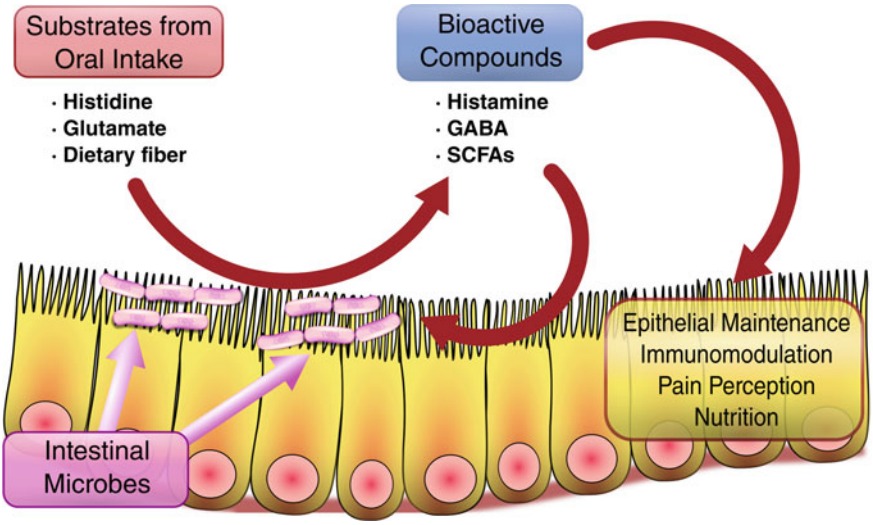

Fig. 2 Example of the extended metabolic/digestive ability produced in the intestinal lumen. Nutrients such as vitamins, amino acids or dietary fiber consumed by the host can be assimilated and converted into other metabolites by intestinal microbes. GABA, gamma-aminobutyric acid; SCFAs, short-chain fatty acids. Figure modified from Hemarajata and Versalovic (2013)

pressure, and temperatures, in which no other organism can survive, being adapted through numerous strategies developed to survive. Their genomes contain a vast array of biochemical transformations, and the microbial cells have accumulated DNA changes over a period of billion years of environmental change and evolution. In the past years, many authors demonstrated that gut intestinal microbiota has a protective effect of against pathogen infection. Indeed, gut microbiota play a crucial role in host resistance against invading or overgrown pathogens within the intestine (Kamada et al. 2013). The mechanisms suggested preventing pathogen colonization of the gut fall into two categories: (1) Direct interactions between commensals and pathogens, such as competition for shared nutrients and niches and (2) Commensal-mediated enhancement of host defense mechanisms.

3.1 GIT Microbiota in Healthy Humans

Most GIT microbiota are mutalists (Backhed et al. 2005) adapted to the adverse conditions of the intestinal environment, such as high pH, low oxygen levels, nutrient limitation and competition for nutrients, and elevated osmolarity (Sears 2005). In order to acquire nutrients, they have developed a complex relationship with other microorganisms as well as with the host. Otherwise, while the general composition of the intestinal microbiota is similar in most healthy people, one third

of the composition of the microbiota is highly personalized like a fingerprint. It is determined by different factors like aging, diet, drugs, environment and the relationship host-gut microbiota. The microbiota benefits of the products of the metabolism of the surrounding microbial species, even from the lysis of the microbiota, but human body also benefits from some of these bio products. Thus, bacterial species that lack the ability to synthesize certain amino acids may acquire them from the intestinal environment. Intestinal bacteria also play a role in synthesizing vitamin B and vitamin K (Cooke et al. 2006). In patients treated with antibiotics, a decreased number of gut microbionts was observed, leading to reduced vitamin K2 (menaquinone) concentrations, and thus as a vitamin K deficiency (Alkhalil and Tate 2010).

Gut microbiota are able to ferment undigested carbohydrates producing SCFA such as butyrate, acetate or propionate, metabolized in the colonic epithelium (Den Besten et al. 2013). Human gut microbiota metabolize undigested carbohydrates producing enzymes that human cells lack for breaking down certain polysaccharides of the human diet including certain starches, fiber, oligosaccharides, and disaccharides as lactose, sugar alcohols like mannitol, providing up to 15 % of total caloric intake (Hooper et al. 2002). The end-products of the fermentation include SCFAs (Fig. 2 in Chap. 13) such as acetic acid, propionic acid, and butyric acid that can be used by host cells as a source of energy and nutrients (Sears 2005). SCFAs can be modified by the amount of fiber in the diet: this in turn, affects the composition of the microbiota. Some GIT microbiota have specific enzymes, glycoside hydrolases and polysaccharide lyases, to break down plant polysaccharides that cannot be digested by humans alone because it lacks the genes which encode the enzymes needed to cleave the glycosidic bonds. The microbiota also plays a role modulating the immune responses of the host (Hooper et al. 2012). Thus, SCFAs exert significant effects on host immune system. Butyrate can modify the cytokine production profile of helper T cells promoting intestinal epithelial barrier integrity, which in turn can help limit exposure of the mucosal immune system to luminal microbes and prevent aberrant inflammatory responses. Acetate promotes the resolution of intestinal inflammation via the G protein-coupled receptor, Gpr43. Else, acetate proved to prevent infection with the enteropathogen, *Escherichia coli* 0157: H7. Bifidobacteria can protect from enteropathogenic infection increasing the production of acetate and inhibiting the translocation of the *E. coli* O157:H7 Shiga toxin from the gut lumen to the blood (Fukuda et al. 2011). This effect was linked to its ability to maintain gut epithelial barrier function (Kau et al. 2015).

Studies on the molecular mechanisms of degradation of complex polysaccharides by the GIT microbiota were performed on the anaerobe Gram-negative *Bacteroides thetaiotaomicron* on germ-free mice. The results showed that the bacteria modify the intestinal cellular regulation of glycolytic and pentose phosphate pathways, increasing the expression of glycoside hydrolases involved in retrieving carbohydrates from mucus glycans. This results in a benefit to both host and the bacteria (Hooper et al. 2002). In a like manner, other studies give evidence of this symbiotic relationship of GIT microbiota and the host analyzing the impact of bacterial metabolism of dietary fiber on epigenetics. In general, dietetic fiber

increases stool bulk decreasing transit time, and thus modulating the composition of gut microbiota. Phytochemicals of high-fiber foods are metabolized by the gut microbiome to SCFAs, isothiocyanates, and microbial metabolites of polyphenols that interact with human gut epithelial cells and may modify epigenetic control of gene expression (Hullar and Fu 2014).

3.2 GIT Microbiota in a Distressed Body

GIT microbiota harmony can be disrupted by different body situations like the presence of antibiotics, drugs, junk food or due to different illnesses such as gastrointestinal disorders like IBD.

Systematic antibiotic (AB) therapy represents a major public health problem because gut microbiota may be transformed into a reservoir of antibiotic resistance genes. This can promote the appearance of harmful resistant strains, while suppresses some protective members of the resident microbiota. This will promote the overgrowth of opportunistic pathogens such as *Clostridium difficile* (Pérez-Cobas et al. 2013). Antibiotics change the composition and the relationships microbiota-microbiota-host. These changes can allow pathogenic bacteria growth, thus affecting human physiology, such as carbohydrate metabolism or immunity. Furthermore, suppression of species able to ferment undigested carbohydrates lead to two situations: firstly, these carbohydrates may absorb water causing diarrhea, or secondly, decrease the amount of SCFAs produced by GIT microbiota affecting and immune homeostasis as well as the health status of the body (Pérez-Cobas et al. 2013). In the past years, a new concept was introduced, the human gut resistome being studied by metagenomic sequencing and functional metagenomics (Dethlefsen et al. 2008; Hu et al. 2013; Forslund et al. 2014; van Schaik 2015; Card et al. 2014). To avoid all these obstacles, new treatment protocols for some difficult GIT infections involve fecal microbiota transplantation of donor feces.

As mentioned above, the intraluminal pH ranges from highly acidic in the stomach to about pH 6 in the duodenum, gradually increasing in the small intestine from pH 6 to about pH 7.4 in the terminal ileum. Then, the pH drops to 5.7 in the caecum, increasing again in the rectum reaching pH 6.7. Altering these values affects the balance of the GUT microbiota, absorption of vitamins and electrolytes and on the activity of digestive enzymes. Thus, GIT pH may be used as a biomarker of the health status of the GIT (Farmer et al. 2014). Due to the acidic conditions, the stomach only supports the growth of acid-tolerant microorganisms. *Helicobacter pylori* is a bacterium that infects the gastric epithelium of the human stomach (its only known natural habitat). It has been described as a major bacterial pathogen that causes different gastroduodenal diseases (Khosravi et al. 2014). The interaction between the preexisting gastric microbiota and *H. pylori* infection might influence an individual's risk of gastric disease, including gastric cancer. Additionally, *Bifidobacteriaceae* (*Bifidobacterium dentium*, *Scardovia inopinata*, and *Parascardovia denticolens*) were found in the stomach of hypochlorhydria patients

(Mattarelli et al. 2014). Inappropriate immune responses to the normal commensal gut microbiota lead to high inflammatory conditions such as IBD (Macfarlane et al. 2008).

4 Control of Pathogens by the Gut Microbiota

Gut microbiota coevolved with the host, being essential for many host physiological processes that include modulating the intestinal epithelial barrier, development of the immune system and supplying the host with nutrients obtained as end-products of their metabolism (Hooper et al. 2012). A major function of the microbiota is protection against colonization by pathogens and overgrowth of potentially harmful indigenous microorganisms that can result from the disruption in the harmony in the microbial community. The mechanisms that regulate the ability of the microbiota to restrain pathogen growth are complex and include competitive metabolic interactions, localization to intestinal niches and induction of host immune responses. Pathogens, in turn, have evolved strategies to escape from commensal-mediated resistance to colonization (Kamada et al. 2013; Lawley et al. 2012). Intestinal colonization resistance is defined as the resistance to colonization by ingested bacteria or inhibition of overgrowth of resident bacteria normally present at low levels within the GIT (Lawley and Walker 2013). For a successful colonization of the GIT, bacteria must survive the low pH of the stomach, locate to a suitable niche within the intestine competing with the resident microbiota for nutrients to begin replication to reach sufficient numbers to resist peristalsis and resist washout from the intestine. In some pathogenic bacteria such as *Staphylococcus* adherence is meiated by surface protein adhesins or microbial surface components recognizing adhesive matrix molecules (MSCRAMMs). Other pathogens are characterized by horizontal gene transfer mediated by bacteriophages, one the most important phenomenon occurring in the group A streptococcus (GAS) such as *Streptococcus pyogenes*, where they have a major impact on pathogenicity as well as bacterial genome diversity and evolution. Phage-associated GAS virulence factors include SpeA, SpeC, SpeG, SpeH, SpeI, SpeJ, SpeK, SpeL, SpeM, MF2, MF3, MF4, Sla, Ssa, Sda, Sdn, as well as HylP. Enteric bacterial pathogens, usually transmitted through foods, are well adapted to vertebrate hosts and generally colonize the gut. Out of them, some have humans as their principal host, while many others and affect humans only incidentally (Lynch et al. 2009).

A number of pathogenic bacteria such as *Salmonella* spp., *Escherichia*, *Listeria*, *Staphylococcus*, *Streptococcus*, use virulence factors to colonize and directly manipulate host cells, leading to colonic inflammation (Lawley and Walker 2013). Virulence factors refer to the properties (i.e., gene products) that enable a microorganism to establish itself on or within a host of a particular species and enhance its potential to cause disease. Virulence factors include bacterial toxins, cell surface proteins that mediate bacterial attachment, cell surface carbohydrates and proteins that protect a bacterium and hydrolytic enzymes that may contribute to

the pathogenicity of the bacterium. The virulence factor database (VFDB) (http://www.mgc.ac.cn/VFs/main.htm) is an integrated and comprehensive online resource for curating information about virulence factors of significant bacterial pathogens. Their aim is to provide a deep understanding of the structure features, functions and mechanisms of the virulence factors used by these pathogens to allow them to conquer new niches and to circumvent host defense mechanisms, and cause disease.

Nevertheless, some pathogens coexist in harmony with other microbiota. Some authors studied the case of *C. difficile* finding that 50–60 % of newborns are colonized with this intestinal pathogen, although disease is rarely observed (Jangi and Lamont 2010). Half of the adult population, which never had *C. difficile* disease, harbor serum antibodies specific for *C. difficile* toxins associated with resistance to severe, recurrent disease. Since the initial colonizing microbiota shapes the immune system, the presence of toxin-specific antibodies in adults is hypothesized to be linked to *C. difficile* exposure early in life (Jangi and Lamont 2010; Lawley and Walker 2013).

5 Effects of Pro and Prebiotics on the Modulation of Gut Microbiota Composition

Although it can adapt to change, a loss of balance in GIT microbiota may arise in some specific situations leading to dysbiosis (Fig. 3). Dysbiosis may be linked to health problems such as functional bowel disorders, inflammatory bowel disease, allergies, obesity and diabetes among others. Accordingly, when the GIT microbiota is unbalanced, several strategies can be used to restore the ecosystem back to its normal state. Although GIT microbiota is able to recover naturally, strategies like reseeding the GIT with a few well-defined good microbiota (probiotics), adding compounds that promote the growth and mutualism or symbiosis of the normal microbiota (prebiotics) or even transplanting the microbiota from a healthy stool sample (fecal bacteriotherapy) (Lozupone et al. 2012).

5.1 Role of Probiotics on the Balance of GIT Microbiota

Probiotics are defined as viable microorganisms which, when administered in adequate amounts to reach the intestine in an active state, confer a health benefit on the host. Most probiotics belong to lactic acid bacteria, a clade of Gram-positive, low-GC, acid-tolerant, generally non-sporulating, either rod or cocci-shaped bacteria that produce lactic acid as the major metabolic end-product of carbohydrate fermentation. Numerous probiotic microorganisms are used in probiotic food, particularly fermented milk products, e.g., *Lactobacillus rhamnosus* GG, *Lactobacillus reuteri*, *Bifidobacterium* spp., and certain strains of *Lactobacillus*

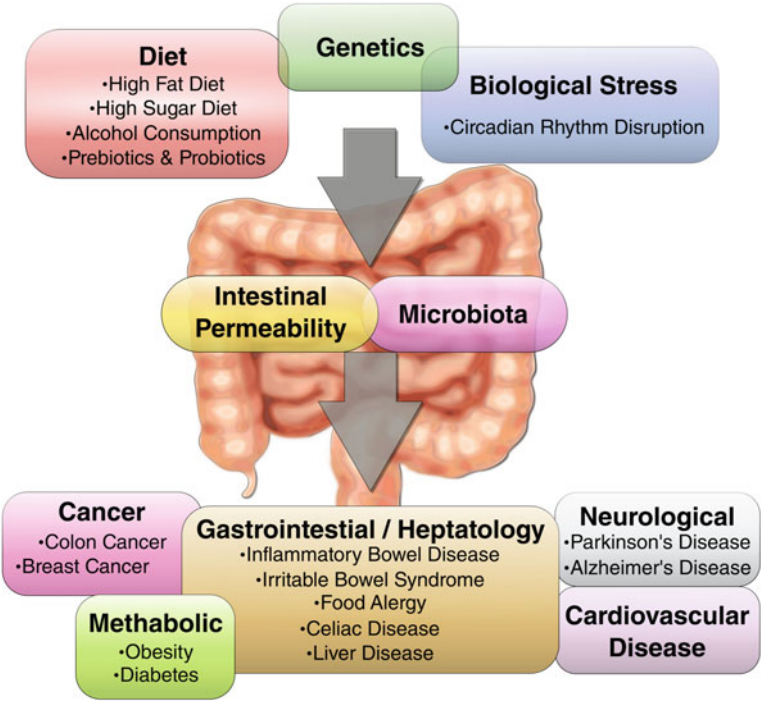

Fig. 3 Factors affecting to the disruption of the intestinal microbiota homeostasis (dysbiosis) and diseases associated. Figure modified from Engen et al. (2015)

casei or the *L. acidophilus*-group (de Vrese and Schrezenmeir 2008). Others have been investigated for their medicinal use, e.g., *E. coli* strain Nissle 1917, *Enterococcus faecium* SF68, the sporoformer *Bacillus coagulans* GBI-30, 6086, the probiotic yeast *Saccharomyces boulardii*, among others. Thanks to technological progress, the range of microbiota living in the GIT as well as their functions, their inter- and intra-relationships, and their interactions with the host are becoming better understood. Thus, new methodologies, including tools derived from molecular biology, proteomics (Fig. 4), metabolomics and transcriptomics, are available.

Probiotics are being applied for treating diarrhea, including antibiotic-associated one. Many of these bacterial species produce exopolysaccharides that are being suggested to act as prebiotics since either homopolysaccharides or heteropolysaccharides, synthesized by lactic acid bacteria and bifidobacteria can be fermentable substrates for the GIT microbiota (Salazar et al. 2015). Additionally, some probiotics produce compounds with antibacterial activity such as bacteriocins, some of them isolated from food. Bacteriocins are antimicrobial peptides produced by lactic acid bacteria, such as *Lactobacillus* spp., *Pediococcus acidilactici*, *L. lactis* and *E. faecium* (Calo-Mata et al. 2008; Hosseini et al. 2009), with a varying activity spectrum among species. They inhibit or kill other related or unrelated

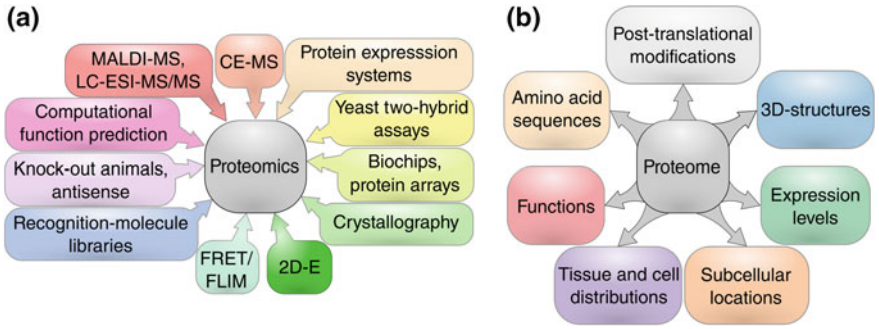

Fig. 4 **a** Technologies available for the development of proteomics. ESI, electron electrospray ionization; FLIM, fluorescence lifetime imaging microscopy. **b** Some of the biological information that proteomics can provide. Figure modified from Hebestreit (2001)

Gram-positive and Gram-negative bacteria, like *Listeria* spp., *Bacillus* spp., *Staphylococcus aureus*, *E. coli*, *Salmonella*, *C. perfringens*, *Klebsiella* sp., *Proteus* sp. Some of the requirements to use bacteriocines as therapeutics include being generally recognized as safe (GRAS), being able to survive to pH 3.0 and to the presence of bile salts, pancreatin and pepsin, not exhibiting bile salt hydrolase or hemolytic activity, showing adhesion to gut epithelial tissue, and also exhibiting sensitivity to clinically relevant antimicrobial agents (Hosseini et al. 2009). The huge amount of patents on this subject reveals the general interest on these antibacterial compounds (Benmechernene et al. 2013).

Probiotics have the ability to increase immune response playing an important role in the intestinal defense responses against pathogenic bacteria. Specific probiotics regulate some immune components modulating the intestinal epithelial cell–mediated defense responses, the intestinal barrier function, innate and adaptive mucosal immune responses like secretion of mucins, defensins, immunoglobulin A (IgA), Toll–like receptors (TLRs), cytokines as well as signaling (Wan et al. 2015). *Bacillus coagulans*, a spore-producing bacterial probiotic has been found to be effective in diverse gut related health situations such as antibiotic-associated diarrhea, gastric ulcers, irritable bowel (Horosheva et al. 2014). Baron (2009) evaluated the effects of the patented GanedenBC30 probiotic (*B. coagulans* GBI-30) on the immune system when exposed to adenovirus and influenza A (H3N2 Texas strain) (viral respiratory tract infections) in healthy adults finding an increase of the immune response of the host like increased T-cell production of TNF-alpha. The fungi *S. boulardii* has been included in numerous randomized controlled trials and strong clinical evidence exists for the use of *S. boulardii* for the prevention of antibiotic-associated diarrhea, traveler's diarrhea, and acute infectious diarrheas (Kelesidis and Pothoulakis 2012). In addition, *S. boulardii* has shown a positive impact on disease outcome in clinical studies of IBD such as Crohn's disease and ulcerative colitis, indicating an ability of *S. boulardii* to influence human immune responses underlying intestinal inflammation. Well-established probiotic effects are:

(1) Prevention and/or reduction of duration and complaints of rotavirus-induced or antibiotic-associated diarrhea. (2) Reduction of microbial metabolites in the gut such as cancer-promoting molecules. (3) Prevention and alleviation of unspecific and irregular complaints of the GIT. (4) Prevention or alleviation of allergies and atopic diseases in infants. (5) Prevention of respiratory tract infections (common cold, influenza) and other infectious diseases as well as treatment of urogenital infections (de Vrese and Schrezenmeir 2008).

5.2 *Role of Prebiotics on the Establishment and Maintenance of GIT Microbiota*

A prebiotic is a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and (or) activity of one or a limited number of bacteria in the colon, and thus improves host well being and health (Gibson and Roberfroid 1995). Prebiotics are a kind of dietary fiber that remains intact throughout the digestive process. Beneficial members of the microbiota in the large intestine and colon, especially bifidobacteria and lactobacilli, selectively use this fiber. This promotes their growth and rebalancing the microbiota on the gut, improving the health of the host (Macfarlane et al. 2008). Oligosaccharides from human milk play a key role in development of the GIT microbiota on the infant gut and a stimulating factor for the postnatal development of the immune system. Some nondigestible oligosaccharides include inulin, fructooligosaccharides (FOS), and galactooligosaccharides (GOS), isomalto-oligosaccharides (IOS), mannan-oligosaccharides (MOS) and ß-glucans. They are dietary fibers with a well-established positive impact on the intestinal microbiota as well as prevention of diarrhea. Other effects like modulation of the metabolism of the GIT microbiota, cancer prevention, positive effects on lipid metabolism and immunomodulatory properties are indirect, i.e. mediated by the intestinal microbiota (de Vrese and Schrezenmeir 2008). They are also reported to alter the uptake of various minerals from the intestine, promoting bone health and maintenance in animal models (Scholz-Ahrens et al. 2007a, b; Weaver 2005). Some dietary prebiotics such as inulin, FOS, GOS or ß-glucans occur naturally in a wide range of foods; however, it would take a large quantity of these foods for their active oligosaccharides to exert a useful prebiotic effect.

MOS like glucomannan are mannose-based polysaccharides found in many plants, like coffee and certain beans, and in yeast cell walls (Moreira and Filho 2008). MOS has been shown in vivo animal studies to inhibit bacteria such as opportunistic *E. coli*, *Salmonella* or *Cl. perfringens* from adhering to the intestines. Mammals lack the enzymes necessary to cleave the β-1,4 linked sugar residues of the mannan backbone, they get the colon undigested being metabolized by bacteria, particularly by bifidobacteria (Asano et al. 2004; Chen et al. 2008).

6 Conclusions and Future Perspectives

GIT microbiota, particularly certain organisms have adapted to the GIT niche, over the course of evolution, having different functions like nourishment of the host or training the immune system. With a better understanding of the microorganisms in the GIT, the manipulation of the GIT microbiota might prove to be a future targeted therapy for a number of conditions. Although probiotics and prebiotics are being applied and some treatments are well established, a lot of effort is still required in order to a better understanding and characterization of the microbiota and the microbe-microbe-host relationships to achieve an effective therapy.

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Fecal Matter Implantation as a Way to Fight Diarrhea-Causing Microorganisms

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Abstract This chapter summarizes the current knowledge about fecal microbiota transplantation (FMT) as a way to treat persistent diarrhea. Although this is an old concept, originated in China at least a millennium ago, it also represents a novel approach and an alternative to the use of antibiotics. This approach is a major departure from Paul Ehrlich´s old paradigm 'the magic bullet,' or a compound that supplied in a 'dossa sterilisa magna' would eliminate the parasite protozoan or bacterium, and represents a more ecological perspective in the fight against pathogenic microorganisms. This chapter includes additional topics, such as the effect of probiotics and ADP-ribosylation, as well as cAMP, cGMP, and phosphatidylinositol signaling. We also deal here with the problems associated with fecal transplant therapies when using human stool donors that, although asymptomatic, are in fact carriers of bacterial or parasite diseases.

1 Introduction

Humans have co-evolved with a high number of commensal bacteria, fungi, and viruses that colonize the intestinal tract, mouth, eyes, and skin; this normal microbial flora proliferates to such an extent that the microorganisms outnumber the human cells by a factor of ten (Hansen et al. 2010). As Savage concluded in 1977: "*the normal human organism can be said to be composed of over 10^{14} cells, of which only about 10 % are animal cells.*" In 1960 Dubos and Schaedler published an article on the effect of the intestinal flora on the growth rate of mice, and the rodent's susceptibility to experimental infections. Three years later, the same scientific group

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(Dubos et al. 1963) described that variations in the intestinal microbiota of mice had pleiotropic effects on the animal's health. Dubos et al. (1965), as well as Schaedler et al. (1965), attempted to rationalize the study of the mammal gut microbiota, as the gastrointestinal (GI) tract represents one of the most challenging study environments for a microbiologist. Intestinal bacteria are separated from intestinal cells by the mucosa, a layer that mediates the mucosal adaptive immune responses. The plasma cells that reside at the intestinal mucosa produce secretory IgA, one of the main factors controlling gut microbial population diversity, by limiting both adhesion and entry of the microorganism into the GI epithelium, and this may lead to microbial immune exclusion (Macpherson and Uhr 2004). As pointed out by Hansen et al. (2010), IgA antibodies also help to maintain mucosal homeostasis by controlling the composition of the commensal microbial community.

The mammalian gastrointestinal tract consists of distinct areas, each possessing its own associated microbiota. The anatomic parts of the digestive system include the esophagus, stomach, small intestine, cecum, and large intestine; which are subdivided into additional regions, depending on the animal. In 1977 Savage reported that the normal microbial populations found in the feces of nonruminant animals include *Lactobacillus, Streptococcus, Peptostreptococcus, Peptococcus, Bifidobacterium, Clostridium, Eubacterium, Propionibacterium, Ruminococcus, Gemminger, Coprococcus, Catenabacterium, Escherichia coli, Bacteroides, Fusobacterium,* and *Veillonella*, in addition to other bacteria and yeasts. Currently, it is estimated that the whole human gut microbiome includes approximately 1150 bacterial species (Flanagan et al. 2011), and that each person, on average, has 160 different microbial species (Qin et al. 2010). Gut colonization starts at the moment of birth, with the first dose of microbes coming from the mother. Microbial colonization of the neonatal gastrointestinal tract is completed in the first 2 weeks of life and, curiously, the microbiota remains quite stable for long periods of time. Major changes in the composition of the intestinal microbiota result from dysbiosis, such as the microbial imbalances caused by inflammatory bowel diseases (including ulcerative colitis), Crohn's disease, and pseudomembranous colitis. These pathologies are associated with changes in the stool microbiota and, in particular, in the microbial population associated with the intestinal mucosa. Savage reported, in 1977, that a variety of changes in the animal's digestive system, such as variations in temperature, pH, stasis, oxygen, oxidation/reduction potential, vitamin B_{12}, pancreatic enzymes, bile acids, epithelial turn-over, urea, mucin, as well as variations in diet and the effect of drugs, antibodies and phagocytic cells (Paneth cells in the epithelium of small intestine have been identified as functional phagocytes), can considerably influence the composition of the indigenous microbiota. Microbiologists are painfully aware that only ≈30 % of the human fecal microbiome can be grown in the laboratory and that, unfortunately, the remaining 70 % can only be hinted at by the use of techniques such as real-time PCR. Additionally, it is also clear that the human healthy gastrointestinal microbiota can vary considerably in people suffering dysbiosis, as mentioned above (Seksik et al. 2003; Flanagan et al. 2011). The abnormal gut microbiota, originated from those diseases, can be replaced with normal microbial GI populations by the procedure currently known as 'stool or fecal matter transplantation.'

Mankind has used fecal matter transplantation for at least a millennium and, while this was obviously done without the scientific knowledge that we currently possess on the procedure, people probably realized that eating fecal matter could speed the recovery from harsh diarrheas. Chinese ancient remedies used what was called 'yellow soup' (fresh or fermented fecal products) to cure chronic diarrhea. This treatment could have started by mimicking animal behavior, as many animal species, such as chimpanzees, gorillas, pandas, koalas, and hippopotamus, practice coprophagia.

Fecal matter transplantation is a simple technique in which feces from a healthy individual are introduced (normally by means of an enema or a nasogastric tube) into the patient's GI track (Bakken et al. 2011). Since the emergence of hypervirulent strains of *Clostridium difficile,* the causative agent of pseudomembranous colitis, fecal microbiota transplantation has been successfully used in several extreme cases that otherwise could have been fatal. In fact, this bacterium has been reported to cause over 100,000 deaths per year, in the USA alone. Although there is not much epidemiological data on *Clostridium difficile* infections in developing countries, we can infer that, in those countries, bacterial strains are less hyper virulent than the antibiotic-resistant bacterial strains that usually arise in developed countries from antibiotic misuse.

Eiseman et al. (1958) reported one of the first successful uses of fecal microbiota transplantation for the treatment of patients suffering from pseudomembranous colitis. Bowden et al. (1981), as well as many others, continued this pioneering work, some of which is referred to in the present chapter. Fecal matter transplantation and the use of enzybiotics are two of the techniques that currently offer great promise in the fight against pathogenic microorganisms. This is of particular importance, as they come at a time when antibiotics are increasingly becoming less effective and the number of resistant microbial strains is constantly increasing; this is aggravated by the fact that the search for new antibiotics has practically ceased, which means that society is running out of effective weapons to combat microbial diseases.

The hypothesis behind the success of fecal matter transplantation therapies rests on the concept of bacterial interference (using harmless bacteria to replace pathogenic organisms), and possibly bacteriophage interference, both of which take place in the GI tract. This is what several authors have referred to as genetic 'crosstalk' in the gut, as intestinal homeostasis depends on the crosstalk between the innate immune system and the intestinal microbiota. This is a multistage process that involves, among others, intestinal cells, pathogenic microorganisms, bacteriocin-producing bacteria, induction of lytic bacteriophages, direct transformation by naked DNA, and soluble plant fibers as 'contrabiotics' (Flanagan et al. 2011), to prevent bacterial adherence to the GI epithelium and to block microbial translocation across M cells and Peyer's patches (for a mini review, see Uzzau and Fasano 2000).

Microbiologists have long recognized that bacterial pathogens have closely evolved with their hosts and have been involved in horizontal gene exchange with them, in order to evade the host's immunological defenses or to adapt to their hosts environment. Cossart et al. (1996) coined the term 'Cellular Microbiology' to describe the

close cooperation and mutual dependency between microbiology and cellular biology, which is needed for the study of the interactions between host and microorganisms. Perhaps, the archetype is the case of *Neisseria gonorrhoeae* and *Homo sapiens*, two organisms that have shared an intimate relationship for thousands of years, to such an extent that 11 % of *N. gonorrhoeae* strains harbor a 685-bp sequence that is almost identical to the human long interspersed nuclear element L1 (Anderson and Seifert 2011; Shafer and Ohnecka 2011). From the evolutionary point of view, these findings are very important, since they help us understand the behavior/evolution of other neisserial species, such as *N. meningitides,* and contribute to the design of appropriate treatments and/or vaccines to combat these bacteria (because gonococci split from meningococci and commensal *Neisseria* spp. only recently).

Another, well-documented, example of bacterial-host adaptation can be seen by changes in bacterial surface carbohydrates (Zarrella et al. 2011). In prokaryotes, polysaccharide capsules are characteristically composed of repeating oligosaccharide units, on the outer membrane of Gram-negative bacteria and, as they are highly hydrated, the capsules serve many functions, which include adherence to either fomites or living cells, evasion of the host's immunological surveillance, and molecule and ion access to the bacterial cytoplasmic membrane. This means that these polysaccharide capsules are of cardinal importance in the pathogenesis of infections of animals, plants, and insects (for a review, see Moxon and Kroll 1990). A less well-known case of genetic crosstalk concerns the mechanism by which pathogens can evade the host's immunological surveillance and are able to grow intracellularly, either inside macrophages or other host cells. In this regard, much remains to be learnt from studying the transient association between amoebae and certain pathogenic bacteria, such as *Legionella pneumophila* (Kwaik 1996), M*ycobacterium* spp (Kusnetsov et al. 2001), or *Francisella tularensis* (Abd et al. 2003). Barker and Brown had, already in 1994, suggested that amoebae represent the bacterial environmental reservoirs, acting as 'Trojan horses' of the microbial world to help them reach their hosts. For a review on the impact of the interaction between bacteria and amoebae on the evolution of human intracellular bacterial pathogens, see Molmeret et al. (2005).

Probiotics. The term probiotic, 'for life,' refers to bacteria associated with beneficial effects for humans and animals, and hence encompasses the subject of this chapter. It was Élie Metchnikoff who, at the start of the twentieth century, suggested the possibility of using beneficial microbiota to colonize/re-colonize the gut as a way of helping the organism recover from disease (Metchnikoff 1907). *Lactobacillus acidophilus* constitutes one of the early probiotics, as has long been considered to possess health-promoting properties, such as anticarcinogenic and hypocholesterolemic, in addition to its strong antagonistic action against intestinal pathogens, since it can survive in the human gut for long periods of time (Mital and Garg 1995). Other *Lactobacillus* species (*L. gasseri* and *L. reuteri*) produce cyclic bacteriocins and also have the potential to control intestinal bacteria (Kawai et al. 2004). In 2001, a joint commission of the Food and Agricultural Organization and the World Health Organization (FAO/WHO) defined probiotics as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host."

The beneficial effects must be produced within four areas of human application: (i) metabolism, ii) chronic intestinal inflammatory and functional disorders, (iii) infections, and (iv) allergy. Lactic acid bacteria are the most commonly used probiotics, but there are other microorganisms, such as the actinomycetales or even yeasts, that also deliver these beneficial properties. However, Lewis et al. (1998) showed that *Saccharomyces boulardii* (a member of the *Saccharomycetaceae* family) could not prevent antibiotic-related diarrhea in elderly patients. All potential probiotic candidates must fulfill several criteria, which include that the probiotic must be alive when administered (otherwise it is not effective) and, most importantly, that the probiotic must be a taxonomically defined microbe or combination of microbes (the genus, species, and strain must be know). Since it is generally accepted that the probiotic activity is strain specific, strain identification becomes particularly important (Rijkers et al. 2010). A number of beneficial applications for probiotics have been reported, such as their use to prevent gastrointestinal disorders in children (Vanderhoof and Young 1998), to potentiate immunological reactions (Isolauri et al. 2001), and to alleviate Parkinson's symptoms (Ananthaswamy 2011). Other probiotic uses include *L. acidophilus* to control bacterial vaginosis; fecal flora for vitamin K production (Cooke et al. 2006); *Bifidobacterium* spp for folic acid production (Strozzi and Mogna 2008); lactic acid bacteria to regulate cytokine function and hence reduce inflammation (Reid et al. 2003); different lactic acid bacteria to reduce blood pressure (Sanders 2000); *L. acidophilus* to treat antibiotic-associated diarrhea (Arvola et al. 1999; D'Souza et al. 2002; Beausoleil et al. 2007); *Lactobacillus* GG to help recover from antibiotic-associated gastrointestinal side effects during *Helicobacter pylori* eradication therapy (Armuzzi et al. 2001); *Bifidobacterium infantis* to ameliorate the symptoms of irritable bowel syndrome (Whorwell et al. 2006); *Bifidobacterium breve, Lactobacillus casei*, and galacto-oligosaccharides as perioperative symbiotic treatment to prevent infections in patients undergoing liver transplantation (Eguchi et al. 2011).

Bacteriocin production has long been recognized as a tool in bacterial classification, and it is even useful in strain typing with epidemiologic purposes (Hall 1971); in particular, Senior described in 1977 the typing of *Proteus* strains by proticin production and sensitivity. Salle and Jann, in 1945, published an article describing subtilin production by *Bacillus subtilis*, and this represents one of the first reports on bacteriocin production. This was followed by Foster and Woodruff's publication, in 1946, describing 'bacillin' as a new antibiotic substance from a soil isolate of *B. subtilis*. In fact, that year saw a number of publications concerning the use of these new substances (now renamed bacteriocins) for controlling fungi or pathogenic bacteria, such as the use of bacteriocins to treat guinea pigs infected with *B. anthracis* (Salle and Jann 1946). Nisin, the first bacteriocin (lantibiotic) ever approved for use in food production, was used by Berridge, as early as 1949, in the treatment of bovine mastitis caused by *Streptococcus uberis* and *Staphylococcus aureus* (Hulse and Lancaster 1951). Frédéricq (1957) published an early review on colicins and, it was already clear at that stage, that bacteriocin production was strain specific and that even human pathogens (such as *Vibrio cholerae*) and animal pathogens (such as the salmon pathogen *Myxococcus columnaris*) can have bacteriocin-producing strains

(Datta and Prescott 1969; Anacker and Ordal 1959, respectively). George Ivanovics published another review on these compounds, in 1962, which describes the classical properties of bacteriocins. Basically, bacteriocins are proteinaceous toxins produced by a particular strain of bacteria to inhibit the growth of similar, or closely related, bacterial strains (Jacob et al. 1953). As a general rule, producing strains are immune to their own bacteriocin, and some toxins are only active against strains belonging to the same bacterial species as the producer strain. In addition, bacteriocin activity requires the presence of specific receptors. Ivanovics (1962) classifies bacteriocins into four groups: colicins, pyocins, megacins, and other bacteriocin-like substances. The latter includes pesticin (produced by *Yersinia pestis* and with a very narrow antibacterial spectrum), a molecule that Ivanovic described as "*A heat-labile and dialyzable substance sensitive to protein denaturants was detected in some atypical strains of Mycobacterium tuberculosis.*" This author also describes another 'bacteriocin-like' substance, produced by certain strains of *Corynebacterium diphtheriae* (gravis type), but this toxin is not sensitive to proteolytic enzymes. Reeves published an interesting review, in 1972, that includes additional bacteriocins, such as colicins (produced by 20–25 % of *E. coli* strains, *Shigella* and, less frequently, by *Salmonella* and *Aerobacter*), alveicins (produced by *Hafnia alvei* and described by Hamon and Péron in 1963a), caratovoricins (produced by *Erwinia carotovora*; Hamon and Péron 1961), arizonacins (produced by *Paracolobactrum arizonae*, currently known as *Salmonella arizonae*; Hamon and Péron 1963a), cloacins (from *Enterobacter cloacae*; Hamon and Péron 1963a), marcescins (isolated from *Serratia marcescens*; Hamon and Péron 1961), pneumocins (produced by *Klebsiella pneumonia*), aerocins (synthesized by *Aerobacter aerogenes*), pyocins (from *Pseudomonas pyocyanea*; Hamon 1956), fluocins (synthesized by *P. fluorescens*; Hamon et al. 1961) pesticins (synthesized by strains of *Y. pestis*; Ben-Gurion and Hertman 1958), megacins (produced by *B.acillus megaterium*; Ivanovics and Nagy 1958), monocins (synthesized by certain strains of *Listeria monocytogenes*; Hamon and Péron 1962), cerecins (produced by *B. cereus*; Hamon and Péron 1963b), enterococcins (produced by *Streptococcus* species; Brock et al. 1963), and staphylococcins (produced by *Staphylococcus* strains; Hamon and Péron 1963b). An interesting, and well-known, characteristic of bacteriocinogenic strains is that, although they are capable of producing bacteriocins, they only do so under certain metabolic conditions (Reeves 1965). In addition to the above mentioned, there are other types of bacteriocins, such as meningococins, produced by highly virulent *Neisseria meningitidis* strains (Kingsbury 1966), and boticins, synthesized by non-toxigenic strains of *Clostridium botulinum* (Ellison and Kautter 1970).

From the time of their discovery, bacteriocins were considered as potential tools for the control of human and animal bacterial infections, in particular those occurring in the oral cavity and in the GI tract, as well as important in ecological studies concerning mixed microbial populations. Unfortunately, the studies carried out thus far do not support this early optimism, as the results obtained were often contradictory. Kelstrup and Gibbons (1969) used gnotobiotic mice harboring single *E. coli* strain (either colicinogenic, colicin-sensitive, or colicin-resistent) and found that the bacteriocins were inactivated by resident proteases, both in the intestinal tract and

oral cavity. Recently, a new application for bacteriocins has emerged: the use of bacteriogenic strains in fecal microbiota transplantation. The reasoning behind this idea is that, as the half-life of bacteriocins in the intestinal track is low, the presence of bacteriocin-producing bacteria in the GI tract would ensure continuous production of these compounds. The most suitable bacteriocins appear to be those produced by *Bifidobacterium* strains (Collado et al. 2005), as they have a broad spectrum and are active not only against Gram-positive, but also against, Gram-negative bacteria. These bacteriocins are active at pH values between 3 and 10 and are stable even when heated at 100 °C for 10 min. Bacteriocins are also useful in the identification of bacterial strains, and the bacteriocins present in human stools have been used for bacterial strain typing (Mahony and Swantee 1978); they are also helpful as epidemiological markers to assist in the identification of non-agglutinable (serogroup O:1-negative) *V. cholerae* (Israil et al. 1983). Additional roles for these compounds include to assess the presence of *Morganella morganii* in the bowel (Senior 1987) and for identification of *C. difficile* in hospitalized newborns affected with persistent, and potentially fatal, diarrhea (Bacon et al. 1988; Camorlinga et al. 1991). More recently (Al Kassaa et al. 2014), it has been suggested that probiotics and their bacteriocins can act as antivirals. The bacteria found in healthy human GI tracts probably produce substances yet to be identified (including antibiotics and bacteriocins) that control the growth of pathogenic bacteria. This is supported by Dalhoff who, in 1982, demonstrated that *E. coli* strains found in healthy human GI tracts could control the growth of *S. faecalis*, both in mixed cultures and in experimental animal infections. Additionally, Toshima et al. (2007) showed that normal and prevalent human enteric bacteria could effectively inhibit the growth of enterohemorrhagic *E. coli* O157. In 2008, Millette et al. published an interesting study describing that bacteriocin-producing lactic acid bacteria can reduce intestinal colonization by vancomycin-resistant enterococci. Anaerobic bacteria from human fecal matter, such as some *Bacteroides* species, can also produce bacteriocins, and many of these compounds have been described as having 'hetero-antagonism' or otherwise broad spectrum (Miranda et al. 1993).

2 ADP-Ribosylation

ADP-ribosylation is the addition of one or more ADP-ribose moieties to a protein (Ziegler 2000); this action triggers a cell signaling pathway that can control many cell processes, including DNA repair and apoptosis (Berger et al. 2004; Corda and Di Girolamo 2003). The process of ADP-ribosylation was described by Goor and Maxwell, as early as 1969, as a mechanism of ADP-ribosylation of aminoacyl transferase II, mediated by the diphtheria toxin from toxigenic *C. diphtheriae* strains (i.e., lysogenized by β bacteriophage). Sometime later, Maxwell et al. (1975) demonstrated that the eukaryotic translation elongation factor 2 is indeed the primary target for diphtheria toxin-mediated ADP-ribosylation. The mechanism of this, apparently unusual, reaction was unraveled by Chung and Collier (1977a).

However, it was soon realized that ADP-ribosylation-mediated processes are more common that originally thought. Some examples are *E. coli* DNA-dependent RNA polymerase modification/alteration for specific transcriptions of T_4 promoters during lytic infection (Rohrer et al. 1975), and the *P. aeruginosa* exotoxin-derived fragment (26 kDa), which has a similar mode of action as the diphtheria toxin (Chung and Collier 1977b). In conclusion, some bacterial toxins, including cholera, pertussis, and diphtheria toxins, are ADP-ribosyltransferases that modify target human proteins. One such case is the cholera toxin, a heat-labile enterotoxin that activates adenylate cyclase and ADP-ribosylates G proteins, which causes massive fluid secretion (up to 20 l per day) from the lining of the small intestine, resulting in life-threatening diarrhea (De Haan and Hirst 2004). All of this, taken together, suggests a broader role for NAD than that originally thought. Some of the processes NAD involved in are (i) participation in biological oxidations; (ii) interaction with mitochondrial proteins; (iii) activity on epimerases; (iv) involvement in gene expression; (v) interaction with histones and endonucleases, or nuclear proteins in general, thus affecting DNA synthesis; (vi) interaction with polynucleotide ligases, hence affecting ligation of nicked DNA; and (vii) activation of eukaryotic adenylate cyclase (Suhadolnik et al. 1977).

In fecal matter transplantation, cholera toxin-related molecules, such as the ones mentioned in this chapter, are more likely to reach the afflicted intestine than diphtheria toxins; but this could result in the alteration of the intestinal function, by stimulation of adenylyl cyclase [ATP pyrophosphate-lyase (cyclizing) or adenylate cyclase, EC 4.6.1.1], as there areadenylyl cyclase regulatory subunits in the intestinal brush border (Domínguez et al. 1987). Indeed, van den Berghe et al. (1991) demonstrated that G proteins (α and β subunits of guanosine-nucleotide-binding proteins) are asymmetrically distributed along both the apical and basolateral membranes of rat enterocytes. Diarrheal disease is a major cause of morbidity and mortality in children worldwide, and all our current knowledge indicates that the interaction of immature human enterocytes with bacteria and/or their enterotoxins may be responsible for the increased susceptibility of neonates to diarrheal diseases, all of which must be taken into account when considering fecal microbiota transplantation (Lu et al. 2009). Another matter to consider is the existence of shiga toxin-producing *E. coli* (STEC) strains, and Osek et al. (2000) demonstrated that calves are reservoirs for these microbes, which harbor pathogenic genes and are dangerous to humans.

In addition to the above, *C. difficile* often produces a binary toxin (belonging to the ADP-ribosylating family of toxins; Gerding et al. 2014), formed by two components: i) component A, that harbors the ADP-ribosyltransferase activity and modifies actin (thus inducing cytoskeletal depolymerization); and ii) component B, which binds to a lipoprotein receptor and translocates the enzyme through the plasma membrane. Component A is 463 amino acids long and has a molecular mass of *ca* 53 kDa (Perelle et al. 1997). After processing, the mature protein has a mass of *ca* 48 kDa and is 84 % homologous to *C. perfringens* iota toxin (Ib), as well as 82 % homologous to *C. spiroforme* toxin (CSTb) (Gerding et al. 2014). Component A ADP-ribosylates act in at position arginine-177 (Gülke et al. 2001), the same amino

acid modified by binary actin ADP-ribosylating toxins (Vandekerckhove et al. 1987). Component B, on the other hand, consists of 876 amino acids, exhibits a molecular mass of 98.8 kDa, and is nearly 80 % homologous to its counterpart from *C. spiroforme*. These binary toxins increase the bacterial adhesion to the affected cells, by inducing the redistribution of microtubules and formation of long tentacle-like extensions at the surface of intestinal epithelial cells, which helps the pathogenic bacterium remain in the gut and increases mortality. The rationale behind using fecal matter transplantation to treat the severe diarrhea caused by *C. difficile* relies on the control the ADP-ribosyltransferase secreted by the bacterium. Unfortunately, up to date and to the best of our knowledge, this application for fecal matter transplantation remains untested.

3 cAMP Signaling

Cyclic adenosine monophosphate (cAMP, 3'-5'-cyclic adenosine monophosphate) is synthesized from ATP by the enzyme adenylyl cyclase [Systematic name: ATP diphosphate-lyase (cyclizing; 3′,5′-cyclic AMP forming), EC 4.6.1.1; CAS registry number: 9012-42-4]. This compound acts as a second messenger and, as such, is involved in a multitude of processes (Neer and Murad 1979; Paunescu et al. 2010; Rahman et al. 2013). The cellular cAMP concentration is regulated by phosphodiesterases, a family of enzymes (EC 3.1.4) with different isoforms depending on the organism, or even the tissue, in the case of higher eukaryotes (Uzunov and Weiss 1972). Both cAMP and cGMP play crucial roles in intestinal epithelial cell homeostasis and the two signaling pathways form a complicated web of interactions in these cells, which probably involves crosstalk between the pathways. In eukaryotes, cAMP works by activating protein kinase A, which is normally inactive as a tetrameric holoenzyme, causing dissociation of the enzyme into two catalytic and two regulatory subunits. Once free, the catalytic subunits are activated and can phosphorylate proteins, by transferring phosphate from ATP to specific serine or threonine residues. The phosphorylated proteins can now activate ion channels on the intestinal epithelial cells, originating a variety of effects that include the typical symptoms caused by bacterial diseases, such as cholera, shigellosis, and pseudomembranous enterocolitis. Protein kinase can also cause increased expression of certain genes, by specific phosphorylation of promoter-binding proteins. In higher eukaryotes, there is an additional family of cAMP-interacting proteins that act as guanine nucleotide exchange factors (GEF); these are termed 'Exchange proteins activated by cAMP (Epac)' and include Epac1 and Epac2 (de Rooij et al. 1998).

Cyclic AMP has a different mechanism of action in bacteria, requiring a regulatory protein known as cAMP receptor protein (CRP) or catabolite activator protein (CAP). CRP binds cAMP, which causes a conformational change, which allows CRP to bind tightly to specific DNA sequences in the promoters of the genes it controls (see Fig. 1). CRP then activates transcription through direct interaction with bacterial RNA polymerase (Inada et al. 1996). For the purpose of the present

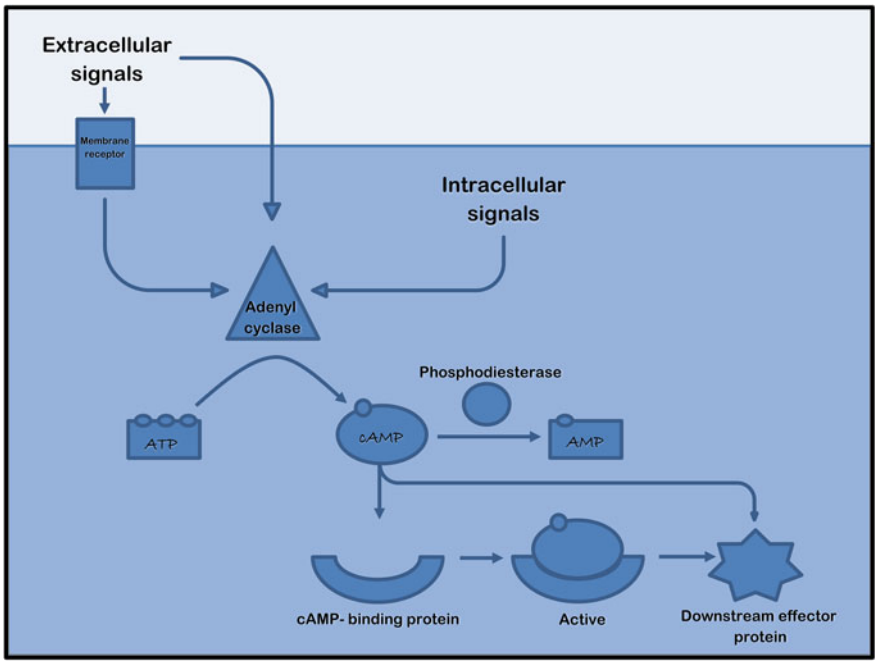

Fig. 1 Indirect activation of adenylyl cyclase (AC) pathways often involves membrane receptors that transmit extracellular signals to the AC by a phosphorylation event, although other cytoplasmic factors might also be required. cAMP then relays the signal to downstream effector proteins either directly, by interacting with the downstream effectors, or indirectly, by allosterically activating one or more cAMP-binding proteins (modified from McDonough and Rodriguez 2011)

chapter, cAMP is important because a change in its concentration can alter the normal function of the human gut. It has been known for some time that compounds such as methylxanthines (caffeine) produce intracellular accumulation of cyclic 3′ 5′-AMP, by inhibiting phosphodiesterase, and that may play a role in the symptoms experienced by some patients with functional diarrhea (Wald et al. 1976); but these symptoms must not be mistaken with similar syndromes caused by some of the microbial inhabitants of the human gut.

Diarrhea can be defined, according to Ewe and Wanitschke (1977), as "*increased frequency of bowel movements (greater than 3 per day) plus decreased consistency of stools (volume greater than 200 ml per defecation).*" It can be caused by either secretion of electrolytes, and hence water, as a consequence of the increased level in the mucosal cells (usually caused by bacterial toxins or by vasoactive intestinal peptide) or by certain substances, such as dihydroxylated bile acids, diphenolic laxatives, or fatty acids, which act on the tight junctions between mucosal cells and cause electrolyte and water leakage into the intestinal lumen. Quantification of cAMP in patients with diarrheal syndrome confirmed that most typical diarrhea-causing microorganisms (e.g., *V. cholerae* that produces cholera toxin, or *E. coli* that generates LT toxin) indeed increase the amount of this cyclic

nucleotide in stool samples (Chen et al. 1971; Molla et al. 1985). Additionally, the stool's cAMP concentration could help diagnose the pathogenic microorganism involved, and could even be used in the future as an index of intestinal dysbiosis that could indicate the need for fecal matter transplantation. It is well known that cholera has spread, from the Indian subcontinent to most of the world, at least seven times during the past 185 years (Sack et al. 2004) and, in recent years, *V. cholerae* (serogroup O1, biotype El Tor) has spread from Asia to Africa and South America. In these pandemics, cAMP quantification is a must, and perhaps, in the not so far future, stool transplantation may be used to complement the classic treatment of fluid and electrolyte replacement and antibiotic treatment. In addition, somatostatin, a molecule present throughout the intestinal tract, is known to have an important regulatory role for water and electrolyte absorption and secretion, by affecting epithelial transport and intestinal motility. This suggests that stools containing high amounts of somatostatin could ameliorate the diarrheal symptoms in a variety of diseases (Dharmsathaphorn 1985).

Intestinal fluid secretion is a well-regulated process, driven by active transport of Cl^- ions across the epithelium (Barrett and Keely 2000), and disruption of this process often results in secretory diarrheas. Cl^- ions accumulate intracellularly, above electrochemical equilibrium, at the basolateral membrane of intestinal epithelial cells, due to the action of the Na^+–K^+–$2Cl^-$ cotransporter. The ion gradient that drives the Cl^- influx is maintained by the Na^+–K^+ pump (Flores et al. 2007), and the K^+ channels are involved in both K^+ secretion and recycling of the proton by the Na^+–K^+–$2Cl^-$cotransporter (NKCC1) and the Na^+–K^+ pump. The most important intracellular mediators of intestinal Cl^- secretion are cAMP and cGMP as well as, of course, intracellular Ca^{++} (Kunzelmann and Mall 2002). Indeed, Cl^- secretion in the adult colon relies on the luminal Cystic Fibrosis Transmembrane Conductance Regulator (CFTR), which is a cAMP-regulated Cl^- channel. Most acquired diarrheas are the result of pathogenic microorganisms, such as *E. coli, Shigella flexneri, Salmonella typhimurium,* and *V. cholerae,* growing in the intestinal tract (Hyams 2000). These microorganisms alter the intestinal ion transport and activate an inflammatory response (Lencer 2001), which, in turn, increases the concentration of second messengers, such as cAMP, leading to overstimulation of the secretory pathway by activating luminal CFTR (Kunzelmann and Mall 2002). It must also be noted that nonselective cationic channels are also present in colonic epithelial cells (Champigny et al. 1991) and they may play a role in volume regulation (Koch and Korbmacher 1999), although their actual role is yet unclear, which is surprising as so much is known about the basolateral Na^+–$2Cl^-$–K + cotransport and K^+ channels. Wuensch et al. (2013) demonstrated, in both mice and humans, that the peptide transporter PEPT1 (expressed in distal colon) contributes to water absorption, which represents yet another matter to take into account when considering fecal donors for stool transplantation.

It has now become clear that coordinated regulation of accessory genetic elements, involving cyclic di-nucleotides (particularly cyclic di-GMP), can alter *V. cholerae* virulence (Davies et al. 2012). Considering the myriad of roles played by cAMP (and cyclic di-AMP) in microbial pathogenesis, we feel compelled to include

here a review on the roles of this cyclic nucleotide, which is a universal messenger in bacteria, fungi, and protozoa, as well as contributing to their pathogenic status. These roles include the coordination of intracellular and extracellular processes and the manipulation of host immunity by increasing its cAMP levels (see review by McDonough and Rodríguez 2011).

4 CGMP Signaling

cGMP is a cyclic nucleotide that acts as a second messenger and is generated from GTP by the action of the enzyme guanylate cyclase (EC 4.6.1.2). This enzyme is part of G protein signaling cascade (Sakurai et al. 2011) and is regulated by intracellular calcium concentration (low concentrations activate the enzyme). Guanylate cyclase contains a heme molecule, capable of binding nitric oxide, which also results in enzymatic activation (Derbyshire and Marletta 2009). Nitric oxide can act as a non-adrenergic, non-cholinergic neurotransmitter released from enteric inhibitory nerves in the gastrointestinal tract (Ward et al. 1992). When the enzyme is stimulated, cGMP increases and this produces relaxation, and hence this process is coupled to homeostatic mechanisms such as intestinal peristalsis (de Jonge 1981, 1984). cGMP is degraded by phosphodiesterases and these enzymes are regulated differently, depending on the tissue where they reside.

High luminal bile salts concentration (>1 mM) increases tight junction permeability; hence, the salts can reach the epithelial basolateral membrane, causing an increase in free cytosolic Ca^{++}, as well as transcellular Cl^{-} secretion (Dharmsathaphorn et al. 1989). Toskulkao and Rao, in 1990, described that cGMP and Ca^{++}can inhibit salt absorption. They characterized a 50 kDa phosphoprotein, regulated by Ca^{++}, cAMP, and cGMP, and present both in the cytosol (50 kDa-C) and particulate fractions (50 kDa-P).

Particular care must be taken to eliminate, from putative fecal matter donors, asymptomatic carriers of enterotoxigenic *E. coli* strains, such as those harboring the heat-stable enterotoxin STa. In these donors, the intestinal protein kinase C can be up to 5 times more active than in normal donors, and can cause the phosphorylation of specific membrane proteins (of 37, 100, and 140 kDa, respectively) that alter normal ion translocation across GI membranes (Chaudhuri et al. 1993). Identifying the specific functions mediated by cGMP-dependent protein kinases is a key to understand the biological role of the nitric oxide/cGMP-signaling pathway (Ruth 1999). cGMP concentration can be regulated by the hormone uroguanylin, through activation of the enzyme guanylate cyclase (Silos-Santiago et al. 2013).

Cyclic GMP can also operate as a second messenger, in the form of cyclic di-GMP (c-di-GMP) [bis(3′,5′)-cyclic diguanylic acid], originally identified as an allosteric activator of cellulose synthase activity in *Gluconacetobacter xylinus* (Ross et al. 1987). Subsequently, this molecule was found to play a role in a variety of processes (Novak et al. 2014), such as virulence of plant and animal pathogens (Cotter and Stibitz 2007), heavy metal resistance (Brown et al. 1986), and phage

resistance (Chae and Yoo 1986). The discovery of new signaling molecules in both archaea and bacteria, mediated by other di cyclic nucleotides, such as cyclic di-AMP (Corrigan and Gründling 2013), has brought insights into their signaling mechanisms and those of their host eukaryotic cells. Patently, these new pathways must be taken into consideration when screening putative fecal matter donors. For instance, Koestler and Waters (2014) recently found that bile acids regulate intracellular cyclic di-GMP in bacteria such as *V. cholerae*. These authors proposed that the bacterium senses microenvironments within the small intestine, using bile acids and bicarbonate as chemical cues, and responds by modulating the amount of intracellular c-di-GMP, which can cause a variety of side effects. In addition, contact with the host cell induces expression of virulence factors and cyclic di-GMP phosphodiesterase, an enzyme involved in the regulation of c-di-GMP concentration in *V. cholerae*. We must assume that this process is not unique to *V. cholerae,* but that it can happen in other human intestinal pathogens (Dey et al. 2013). In fact, interaction of enterotoxigenic *E. coli* strains (ETEC), which can colonize asymptomatic human carriers, with human intestinal cells results in the modulation of a variety of bacterial genes, such as those coding for motility, adhesion, toxin production, cAMP, cyclic receptor protein (CRP), and c-di-GMP, as well as the sequential deployment of multiple virulence molecules (Kansal et al. 2013). Indeed, McKee et al. (2013) studied *C. difficile* toxin production and concluded that c-di-GMP can inhibit both motility and toxin production, via the signaling molecule SigD, a key virulence gene regulator for this intestinal bacterium. Also, in 2013, Fatima et al. used a chicken experimental model, where the birds were challenged with *C. difficile* and treated with c-di-GMP plus penicillin, and found that the nucleotide can effectively restore the normal host's GI microflora, in fact reducing the colonization by *C. perfringens*. Cyclic di-GMP positively regulates intestinal epithelial cell and tissue colonization by *E. coli* O157: H7, as well as bacterial adhesion (Hu et al. 2013). The case of *V. cholerae*, a well-documented example of the effect of cyclic di-GMP on virulence, merits being included here. Krasteva et al. (2010) were able to demonstrate coordination between cyclic di-GMP and transcription in this Gram-negative bacterium. They found that the binding of *V. cholerae* to the transcriptional regulator Vps resulted in the up-regulation of a variety of genes, including the virulence regulator AphA (Srivastava et al. 2011). Additionally, Ahmad et al. (2011) reported that, in pathogenic *Salmonella*, c-di-GMP signaling was important, not only in the regulation of bacterial-host interaction, but also in the regulation of biofilm formation in external environments.

Another important factor to take into account is the amount of IgA present in the stool of putative donors, as it may protect against diarrhea. Accordingly, Amarasinghe et al. reported in 2013 that Sal4 (a monoclonal polymeric IgA antibody that targets *S. typhimurium* O antigen, and induces stress on its external membrane) renders the bacterium avirulent, by triggering a c-di-GMP-dependent signaling pathway. This pathway involves YeaJ (a membranal diguanylate cyclase) and leads to the suppression of bacterial motility, as well as stimulation of exopolysaccharide production (Amarasinghe et al. 2013).

5 Phosphatidylinositol Signaling

The intestinal epithelium is one of the most rapidly proliferating tissues in the body (Sheng et al. 2003); hence, it is important to take into account the biochemical and physiological condition of this tissue in the fecal matter transplant recipient. The intestinal epithelial cells constantly and constitutively proliferate to compensate for the continual cell loss they suffer (Podolsky 1993), and the epidermal growth factor (EGF) family of proteins and their receptors (EGFR) play a critical role in this process. This pathway starts with the binding of the ligand to EGFR, followed by activation of the appropriate tyrosine kinase and phosphorylation of tyrosine residues in the EGFR, and finally results in activation of pathways such as the phosphoinositide 3-kinase (PI3 K)/Akt, involved in cell growth, migration, and apoptosis (Jones et al. 1999). Phosphoinositide 3-kinases, also known as phosphatidylinositol-4,5-bisphosphate 3-kinases and PI 3-kinases (the human genome organization official stem symbol for the gene family is PI3K), are a family of enzymes involved in cellular functions such as cell growth, motility, survival, and intracellular trafficking. They are signal transducer enzymes with the ability to phosphorylate the 3-position hydroxyl group of the inositol ring of phosphatidylinositol (Whitman et al. 1985; Whitman et al. 1988; Auger et al. 1989). In 1994, Cantley´s group published a strategy to determine the sequence specificity of phosphopeptide-binding domains, and later on the same peptide library approach was used to identify their substrate specificity (Songyang et al. 1994) and to characterize the specificity of Ser/Thr kinases and phospho-Ser/Thr (Yaffe et al. 1997).

There are three classes of PI 3-kinases: Class I that produces phosphatidylinositol 3-phosphate, phosphatidylinositol (3,4)-bisphosphate, and phosphatidylinositol (3,4,5)-trisphosphate and is activated by either tyrosine kinase receptors or G protein-coupled receptors; Class II contains three isoforms, but it does not contain regulatory subunits, with the characteristic of containing a C-terminal C2 domain that lacks the critical Asp (this suggests that Class II PI-3 kinases bind lipids in a Ca^{++} -independent manner); finally, Class III is similar to Class I, as it includes heterodimers composed of a catalytic and a regulatory subunits and mainly involved in cellular trafficking.

The protein ghrelin is another factor that must be taken into account when considering fecal matter transplantation. Ghrelin is a short peptide of 28 aminoacids produced by a variety of GI organs, including the lower gastrointestinal tract, which can stimulate the growth hormone-releasing hormone that triggers the release of growth hormone from the pituitary (Yu et al. 2013). Ghrelin also plays a role on appetite regulation and gastric acid secretion (Yu et al. 2013), and stimulates small intestine motility (Tack et al. 2006). Recent evidence suggests that ghrelin plays a role on cell proliferation of both normal and neoplastic cells: it stimulates the proliferation of cardiomyocytes (Baldanzi et al. 2002) and pancreatic β-cells (Granata et al. 2006), while inhibiting the proliferation of lung (Ghè et al. 2002) and prostate carcinomas (Cassoni et al. 2004). Ghrelin plays additional roles, such as enhancing intestinal growth by suppressing apoptosis in the small intestine (Park et al. 2008).

The parasitic protozoan *Giardia intestinal*, a major cause of diarrheal disease, is another factor that needs to be considered in fecal matter transplants. Cox et al. established in 2006 that disease transmission depends on the ability of the parasite to differentiate, back and forth, between an intestine-colonizing trophozoite and an environmentally resistant infective cyst. These authors used phylogenetic analysis to identify two genes in this protozoan that encode putative Class I and Class III, but not Class II, PI3 K isoforms. This strongly suggests the involvement of both classes of PI 3 kinases in the parasitic activity of *Giardia intestinalis*, with Class I playing a role in the initiation of parasitic infestation, and Class III involved in intracellular vesicle trafficking. The gastrointestinal pathogen *Campylobacter jejuni* should also be included in this section, since this bacterium, when alive, induces strong anti-inflammatory response in human intestinal epithelial cells. This effect is carried out by activation of the phosphatidylinositol 3-kinaseAkt pathway (Li et al. 2011), and this process would clearly interfere with fecal matter implantation therapy.

The presence of viruses, belonging to the *enteroviridae* family, in fecal matter must also be considered, since they can induce the production of interferon beta (Chi et al. 2013). Burke et al. (2014) described a role for IFN-β in metabolism regulation, mediated by the PI3 K-Akt pathway (resulting in glucose uptake and ATP production), to meet the cellular energy requirements for a strong antiviral response.

6 Asymptomatic Human carriers and Stool Transplantation

The subject of asymptomatic human carriers immediately brings to mind the article written by the microbiologist George A. Soper in 1907: "The work of a chronic typhoid germ distributor." This author describes how Mary Mallon (aka. 'Mary typhoid'), an asymptomatic carrier of *Salmonella typhi*, the typhoid fever etiological agent, was responsible for several outbreaks of this disease in the New York area at the turn of the twentieth century. Although, it was never determined how many people were infected by Mary, and probably will never be, since she refused to cooperate with the health authorities. Kayser, in 1906, described another well-documented case of an asymptomatic typhoid carrier; he recounts the case of a female baker in Strasburg who was responsible, in 1906, for a number of typhoid cases, resulting in two deaths. Cummins (1910) classified people who pass typhoid bacilli in their excreta as (i) *Precocious carriers* or persons within the incubation period but without the disease; (ii) *Paradoxical carriers* or persons who have contracted the microorganism without concomitant illness and who continue to pass them in their excreta; (iii) *Typhoid cases* both recognized, unrecognized or convalescent; and finally (iv) *Chronic carriers* or persons who continue to and constantly pass the bacterium in their excreta. In a lecture read before the Ottawa Medico-Chirurgical Society, Dr W.T. Connell, as early as 1911, pointed out

"*In certain infections it has long been recognized that the body tissues may, at least temporarily, gain the upper hand, check the propagation of the infecting microbe, and cause health to be restored. The microbes, however, are not always entirely destroyed, but some may lie latent and, later on, again find favorable conditions for propagation and a rekindling of infection.*" And later on he said "*In certain other diseases, after the tissues have gained the upper hand and health has been restored, the causal microorganisms may still remain within the body, and may even multiply largely. Such lodgement may be but temporary, or may continue for long periods, the microbes being excreted either continuously or intermittently, and the subject thus becoming a possible distributor of infection. It is not necessary that the individual should suffer an actual infection of his tissues for him to become a carrier of the microbe, as this may acquire a habitat and yet the tissues be able to withstand actual infection. It is particularly in connection with these apparently healthy individuals, who, after contact or convalescence, continue to harbor and excrete the causal microbes, that we apply the term 'carriers'.*" This means that, even at the beginning of the twentieth century, it was generally accepted the importance of the asymptomatic humans (or carriers) as a way of spreading sickness, mainly through their stools. This is the case for *S. typhi*, a bacterium that causes an estimated 22 million typhoid fever cases (and *ca* 220,000 deaths) worldwide every year. This means that *S. typhi* asymptomatic carriers, as well as carriers of any other intestinal pathogens that can be transmitted by asymptomatic humans, must be excluded from being stool donors. These bacterial pathogens include *Pseudomonas aeruginosa*, a microorganism that caused nursery outbreaks of severe diarrhea in Brazil in 1970, transmitted by asymptomatic nurses (Falcão et al. 1972), as well as the organisms responsible for cholera and related pathologies.

Cholera, in particular, has currently re-emerged as a major infectious disease, even in areas that had been free from those illnesses for decades, or even centuries. One such example is Haiti, where *Vibrio cholerae* made a come back in the aftermath of the 2011 earthquake (Sigman and Luchette 2012). As microbiologists well know, *V. cholerae* is a Gram-negative, facultative anaerobe, non-spore forming curved bacillus that belongs to the *Vibrionaceae* family and, as such, is oxidase positive; this microbe has a single polar-sheathed flagellum. Pacini (1854) and Koch (1884) were the first to describe this bacterium, but it was not until 1959 that De (1959) reported the presence of a toxigenic substance, in the culture broths of *V. cholerae*, which elicited great accumulation of "rice-water" fluids in ligated ileal rabbit loops (Bharati and Ganguly 2011). Two *V. cholerae* serogroups, O1 (classical and El Tor biotypes) and O139, are primarily responsible for cholera outbreaks worldwide. In endemic areas, 75 % of the cases are asymptomatic, 20 % are mild to moderate, and only 2–5 % are severe (Crump et al. 2003; Bronze and Greenfield 2005). The high percentage of asymptomatic carries justifies the inclusion of cholera in the present chapter. In the past 200 years, there have been eight main cholera pandemics, with the disease affecting mainly tropical and subtropical areas (Ryan and Ray 2004) mostly in the Indian subcontinent and Africa. *V. cholerae* has the added complication that 'dormant' forms of this pathogenic bacterium can undergo 'resuscitation' after passage through a mammalian intestine. Unfortunately, the

search for resuscitation factors has not been very successful, although Bari et al. (2013) reported that the molecular signals that activate dormant *V. cholerae* could be related to structural analogs of 'quorum-sensing' auto inducers, either natural or man-made, which are present in water supplies. Both the 'classical' and 'El-Tor' biotypes can cause cholera; they both belong to the serogroup O1 and contain Ogawa, Inaba, and Hikojima serotypes. El Tor was the biotype responsible for the seventh pandemia (Barua and Cvjetanovic 1972) and it got its name from the place where E. Gotschlich identified it in 1905 (El Tor, Egypt). This author found the biotype in six Mecca pilgrims, although postmortem analyses did not reveal any signs of cholera, i.e., they were typical cholera carriers. Asymptomatic carriers can be responsible for fecal contamination of large quantities of water, either directly or through the use of sewerage facilities. Toxigenic *V. cholerae* strains produce cholera toxin; this toxin shares many similarities with *E. coli* heat-labile enterotoxin, as they are both oligomeric in nature and formed by six subunits (one A and five B). The A subunit (28 kDa) is, in turn, formed by two segments: A_1 and A_2. The A_1 segment ADP-ribosylates G proteins thus activates eukaryotic adenylate cyclase (cAMP can undergo a 100-fold increase in concentration, as compared to normal conditions) and, in turn, activates protein kinase A (involved in efflux of Cl^-, Na^+, K^+, HCO_3^- ions) resulting in the secretion of large amounts of water into the intestinal lumen (Galloway and van Henyningen 1987). The A_2 segments are involved in the internalization of A_1. The 5 B subunits, with a molecular weight of *ca.* 12 kDa, recognize the membrane receptor GM1 ganglioside (for a review, see De Haan and Hirst 2004; Fig. 2). Cholera toxins are encoded by *V. cholerae* structural genes, horizontally transferred by the temperate filamentous phage CTXφ, related to the M143 coliphage (Waldor and Mekalanos 1996). Prophage induction, to produce progeny virions, can aggravate water contamination with choleragenic *V. cholera*, since the bacteriophage may infect non-toxigenic strains of this bacterium, thus rendering them toxigenic. Zhang et al., in 1995, published the three-dimensional crystal structure of cholera toxin.

Purging the patients with compounds such as magnesium sulfate may facilitate detection of cholera infection in asymptomatic human carriers (Pollitzer 1959; Gangarosa et al. 1966), as this treatment induces bacterium discharge in convalescent cholera patients, even in people not previously shedding *V. cholerae* in their stools. Purging also results in bacterial stool shedding in other asymptomatic carriers, such as in people infected with the typhoid bacilli (Huckstep 1962), and also inpatients suffering from amoebiasis (Castellani and Chalmers 1919). This topic was re-evaluated by Pierce et al., in 1970, who found that the carrier state of infected people, aged over 50 and not receiving effective antibacterial treatment, could last up to 331 days. These authors also confirmed that purging with magnesium sulfate was a good method to detect cholera carriers. These asymptomatic human carriers tend to generate rough, avirulent, *V. cholerae* bacterial forms that undergo antigenic changes; but these avirulent bacteria can revert to virulent smooth forms, when placed in an appropriate environment (i.e., when released through contaminated stools) (Miller et al. 1972). Paraphrasing Shandera et al. (1983): "*toxigenic V. cholerae 01 can multiply and persist for years in some*

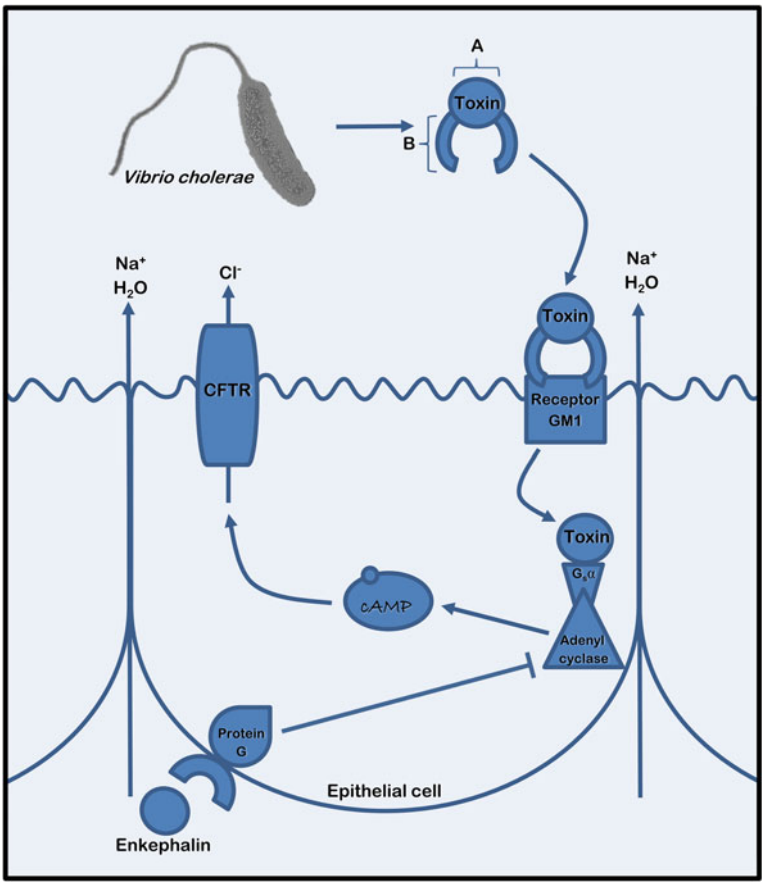

Fig. 2 Mechanism of action of cholera toxin on intestinal epithelial cells. *A* and *B* Cholera toxin subunits; *GM_1* Ganglioside receptor; *Gsα* G protein; *ACy* Adenylate cyclase; *Gi* G protein; *cAMP* Cyclic AMP; *CFTR* Cystic fibrosis transmembrane conductance regulator. Modified from Thiagarajah and Verkman (2005)

environments, making eradication of cholera a formidable task." Szostak et al. (1997) tried vaccination with bacteria ghosts in an attempt to control this and other pathogens (even in the case of potential carriers). Bacterial ghosts are empty, nonliving bacterial envelopes of Gram-negative bacteria with high immunogenic potential, produced by controlled expression of the cloned φX174 gene E, which represent an alternative to heat or chemically inactivated bacteria. They have been produced from a variety of bacteria including *E. coli, S. typhimurium, S. enteritidis, V. cholerae, K. pneumoniae, Actinobacillus pleuropneumoniae, Haemophilus influenzae, Pasteurella haemolytica, P. multocida, and H. pylori* (Szostak et al. 1997). We currently know that the asymptomatic state of *V. cholerae* in humans could be due to a variety of reasons, which range from immunological to

antagonism against this pathogen by diffusible substances produced by bacterial components of the human fecal microbiota (Silva et al. 2001). These authors reported that *Lactobacillus* and *Peptostreptococcus* species produce a compound antagonistic, in vitro, to *V. cholerae,* in such a way that, when bi-associated with germ-free mice, those bacterial strains readily eliminated *V. cholerae* from their intestinal ecosystem in only 5 days. The work of Halpern et al. (2007) must be mentioned in relation to the dispersion of *V. cholera* in nature, as it could explain the simultaneous cholera outbreaks in two different regions. These authors found a high diversity of culturable bacteria from chironomid egg masses collected from two freshwater habitats. Apart from *V. cholerae,* they identified representatives of the following genera: *Acinetobacter, Aeromonas, Klebsiella, Shewanella, Pseudomonas, Paracoccus,* and *Exiguobacterium*, including three important human pathogens, *Aeromonas veronii*, *A. caviae*, and *A. hydrophila*. These results suggest that chironomid egg masses could be a natural reservoir for these bacterial pathogens. What is clear is that rapid diagnosis of human carriers is important to isolate the disease outbreak at an early phase, and this can be accomplished by following the LAMP (Loop-mediated isothermal amplification) method, without need for pre-cultivation of the bacteria from intestinal samples of putative carriers (Okada et al. 2010).

Regarding humans as Shiga´s bacillus carriers, it is currently well established that the genus *Shigella* contains the species *S. dysenteriae* (group A), *S. flexneri* (group B), *S. boydii* (group C), and *S. sonnei* (group D). Those bacteria are non-motile Gram-negative rods and, except for *S. sonnei*, they are all unable to ferment lactose and lack the ability to produce H_2S in triple sugar iron agar. All those species can produce 'shigelosis' (also known as bacillary dysentery, Marlow syndrome, or non-amoebic dysentery), and hence produce several toxins, including shiga toxins (Stx1 and Stx2; see Fig. 3). The genes encoding these toxins are thought to originate from lambdoid prophages (Friedman and Court 2001), which may be also present in shigatoxigenic *E. coli* strains (STEC, including serotypes O157:H7, O104:H4, and other enterohemorrhagic *E. coli*, EHEC) (Beutin 2006). Even 117 years after the discovery of the Shiga toxin (Shiga 1898), the disease still kills hundreds of thousands of people every year. The victims are primarily children in developing countries (Trofa et al. 1999), military units under field conditions (Grant et al. 1969), and Kitchen personnel or food suppliers (Tjoa et al. 1977). *Shigella* carriers were already described in the 1960s (i.e., Nakamura 1967; Kerekes 1968) as apparently healthy humans, who can shed the dysentery bacillus (usually resistant to several antibiotics) at concentrations ranging from 10^4 to 10^7 cells per gram of fecal matter (Levine and Hornick 1975). Oral lactulose can be used, with moderate success, to treat *Shigella* carriers (Levine and Hornick 1975), but oxolinic acid is the treatment of choice, as it causes immediate cessation of the carrier state (Ribner and Freimer 1978); more recently, the antibiotic pivmecillinam has also been found effective (Katzung 2007). Asymptomatic carriers have been incriminated in the maintenance and spread of shigellosis in the community, in countries such as Bangladesh, where dysentery is endemic (Hossain et al. 1994).

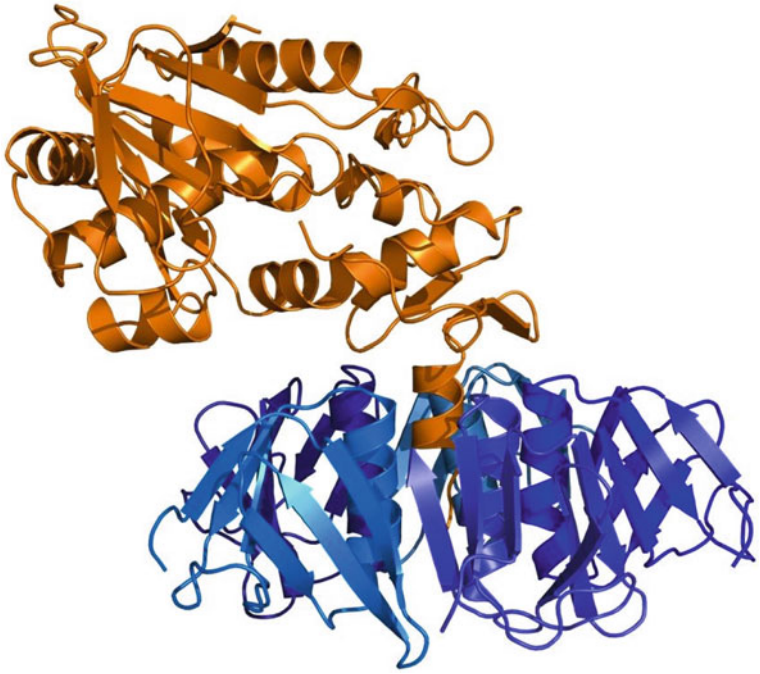

Fig. 3 Diagram of shiga toxin (Stx) from *Shigella dysenteriae* (Olsnes et al. 1981). A subunit is shown in *orange*, whereas the B-subunits (forming the B-pentamer) are shown in shades of *blue*

Other bacteria that also use human carriers as reservoirs, although not as well documented as those mentioned above, are worth to mention here. One of those is *H. pylori*, the causative agent of peptic ulcers, a discovery that earned Barry J. Marshall and J. Robin Warren the 2005 Medicine Nobel Prize. In some individuals, this bacterium (that is found only in the human stomach) can infect the corpus region of the stomach, thus predisposing the patient, not only to a stomach ulcer, but also to stomach cancer (Caruso and Fucci 1990; Loffeld et al. 1990) Also, and interestingly, carrying this bacterium appears to offer protection against allergic and chronic inflammatory GI disorders (Arnold et al. 2012). Additionally, people infected with *H. pylori* (particularly those with an A blood type) tend to suffer from serious iron deficiency anemia, due to the bacterium close adhesion to host erythrocytes (Wang et al. 2012). The work of Schiutze et al. (1995) provides evidence that people can be re-infected with the same strain of *H. pylori* and that this occurs in people sharing a close relationship (such as husbands and wives). This conclusion warrants screening (not only the putative donors, but also their close family) for *H. pylori* IgG antibodies, before a candidate is accepted as stool transplantation donor. Fortunately, and thanks to the pioneering work of Ameglio et al. (1991), it is currently easy to detect *H. pylori* carriers by urea and pH analyses in gastric juices. The prevalence of this bacterium in a fairly large sample of apparently healthy people (*ca* 1050) was high (15-17 %;

Blecker et al. 1994) and accentuates the importance of eliminating these carriers as stool donors. *Gastrospirillum hominis* is a spiral-shaped bacterium that, like *H. pylori*, is found in the human stomach; this microorganism has been suggested as the cause of certain chronic gastritis (Mazzucchelli et al. 1993). Both *H. pylori* and *G. hominis* must be tested for, or at least their specific circulating antibodies, before a person can be considered as a fecal matter donor.

Mycobacterium species are rarely present in human stools, but they need to be taken into account, since human carriers can transmit them. Already in 1963, Virtanen recognized the occurrence of atypical acid-fast bacilli in otherwise healthy human feces. Additionally, Allen discovered in 1989 that 90 % of the patients suffering from any phase of tuberculosis could disseminate the bacterium in their stools. The transmission rate is even higher for dairy products, or muscle tissue, contaminated with *M. paratuberculosis* (*M. avium* subspecies *paratuberculosis,* a bacterium that commonly infects dairy cattle and can cause Johne's disease), when consumed by humans (Collins 1997; Alonso-Hearn et al. 2009). This makes it mandatory that people are tested for mycobacteria before being considered as stool donors, especially as diagnosis can be easily carried, by highly reliable real-time PCR analyses, directly from fecal matter. Wolf et al. (2008) successfully analyzed pediatric stools for the presence of Koch´s bacillus, while Imirzalioglu et al., in 2011, used the same technique to detect *M. avium* subsp. *paratuberculosis.*

In addition to pathogenic bacteria, there are a variety of viruses that can be transmitted by fecal matter and, hence, must be addressed here. This is the case for the HB6-Ag virus, since human carriers shed viral particles that can be detected in their body fluids, such as urine, feces, saliva, tears, semen, vaginal discharge, menstrual flow, and breast milk, as well as in joints, ascitic, and cerebrospinal fluids (Steigmann and Dourdourekas 1976). Other viruses, such as rotavirus, can be also present in the stools of asymptomatic adults. This was shown by Horst and Kohlhase (1986), who reported that rotaviruses can be present in the stool of 1.6 % of healthy adults, and that virus shedding is evenly distributed throughout the year, and not limited to the winter season. Nicand et al. (2001) found that stools from asymptomatic carriers can transmit acute hepatitis, caused by the hepatitis E virus (HEV). We must again stress that the possibility being an enterovirus carrier must be fully explored and eliminated before a person is cleared to become a fecal matter donor. In poliovirus, infection occurs via the fecal–oral route and viral replication occurs in the alimentary tract (Bodian and Horstmann 1965); therefore virions are released in the feces of non-vaccinated infected individuals, who can be entirely asymptomatic (paralytic poliomyelitis occurs in less than 1 % of poliovirus infections). The advantage with this viral group is, however, that there are no long-term human carriers and that the viruses are rapidly neutralized, mainly in people vaccinated with IgA, both on the intestinal mucosa and the tonsils.

Stool samples from putative donors must also be investigated for the presence of the fungus *Candida albicans*, as well as for *Cryptosporidium* oocysts. Holt and Newman, in 1967, reported the presence of *C. albicans* in human stools, in particular after antibiotic or steroid treatments. In addition, Nucci and Anaissie (2001) found that opportunistic fungal pathogens, such as *Candida*, could colonize the

human GI; this is significant because it is believed that these organisms were originally commensals. Although this yeast is generally benign, recent studies indicate that *Candida* colonization, in fact, delays the healing of inflammatory lesions and that inflammation, in turn, promotes further yeast colonization and inflammation (Kumamoto 2011), as indicated by the high levels of the pro-inflammatory cytokine IL-17 detected. This is of the highest importance, since the human (and indeed the mammal) GI tract provides an important reservoir for this yeast (Soll et al. 1991), and *C. albicans* can rapidly adapt to changing GI conditions, such as alterations in pH, oxygen, and nutrient levels. In particular, this fungus quickly senses decreasing microbial diversity in the gut, in what Rosenbach and coworkers have called: "*Adaptations of Candida albicans for growth in the mammalian intestinal tract*" (Rosenbach et al. 2010). Another adaptation of this yeast is its ability to respond to GI tract secretions, such as bile. All these findings confirm that *C. albicans* is adapted to efficiently colonize the human GI tract.

7 Bacteriotherapy

As indicated above, the publication by Eiseman et al. (1958) was either the first or one of the first, to proposed fecal enema as an important aid in the treatment of pseudomembranous enterocolitis. After that, there were no more publications on the subject until the second part of the twentieth century. As pointed out by Savage, in 1977, the gut microbiota is a complex an important ecological niche for microorganisms, with an estimated 10^{14} microbial cells residing in the human GI tract. The microbiotas of multipartite gastrointestinal tracts, such as those of ruminants, and those of nonruminant mammals, such as man, have striking similarities and all provide microhabitats exploited by particular microbial species (Bryant 1974). Tolkacheva et al. (1989) used bacteriotherapy to treat intestinal dysbacteriosis, in patients suffering from acute leukemia; the treatment involved using dried and milk bifidumbacterin, but the results were ambiguous. Balli et al., in 1992, explored the use of orally delivered, high-dose fecal lactobacilli, for the treatment of chronic nonspecific diarrhea in 40 infants, with the conclusion that the therapy helped resolve the disease (Balli et al. 1992). In addition to the treatment of diarrheas, the therapy may also benefit patients suffering from other dysbiosis. This is the case for patients suffering from cancers, such as carcinoma of the uterine cervix or endometrium, who undergo postoperative radiation therapy that produces intestinal dysbiosis; these people may benefit from the intake of oral live bacteria, such as *B. subtilis,* at the beginning of their radiation exposure (Cuzzolin et al. 1992). Mazza et al., in 1994, also studied the antidiarrheal effect of *B. subtilis* spores on long, persistent diarrheas, caused by rotavirus infections of infants. These diarrheas are biphasic, starting with an osmotic diarrhea, followed by a second stage associated with urease-producing bacteria (Isolauri et al. 1994). These authors demonstrated that treatment with oral lactobacilli shortened the duration of the diarrhea. The rational application of intestinal bacteriotherapy could have interesting additional

beneficial effects, such as the establishment of a healthy microbial system in the gut that could prevent the offset of food allergies (Kirjavainen and Gibson 1999). Indeed, these authors conclude "*the essential role of the gut microflora in the development of the gut immune system indicates that a close relationship between allergic sensitization and the development of the intestinal microflora may occur in infancy. Intestinal micro-organisms could down-regulate the allergic inflammation by counterbalancing type 2 T-helper cell responses and by enhancing antigen exclusion through an immunoglobulin A response.*" These results, however, must be taken with caution, as Helin et al. (2002) found that oral treatment with *L. rhamnosus* did not improve birch-pollen allergy. "Flora Power," a term coined by Borody in 2000, refers to the treatment of recurrent *Clostridium difficile*-associated diarrhea by administration of stool, from a healthy donor, transplanted via colonoscopy. This procedure represented a breakthrough therapy in the treatment of persistent diarrheas (Borody 2000). An important factor to consider in bacteriotherapy is the fact that the GI mucous membrane, or at least that of the large intestine, can be resistant to bacterial re-colonization, and that this re-colonization can be assisted by infusion of 'healthy' bacteria, such as bifidobacteria, lactobacilli, Bacteroidetes, *Escherichia,* and enterococci, all of which could be delivered by the appropriate probiotic. Gionchetti et al. (2000) used oral bacteriotherapy (involving the use of three lactobacilli strains and three bifidobacteria, as well as *S. salivarius* subsp. *thermophiles* to successfully treat patients with chronic pouchitis (complication after ileal pouch–anal anastomosis for ulcerative colitis). Bazzocchi et al. (2002) investigated the effects of the intestinal microflora and oral bacteriotherapy on patients afflicted by irritable bowel syndrome. Their results suggest that probiotic administration may ameliorate the symptoms from irritable bowel syndrome, a disease characterized by sudden changes from diarrhea to constipation in short periods of time. Famularo et al. reported similar results in 2003. Borody et al. (2003) were also successful in using fecal bacteriotherapy for the treatment of ulcerative colitis; they used healthy donors that where cleared from parasites and bacterial pathogens. As reported by these authors, the patients were prepared by treatment with antibiotics and oral polyethylene glycol lavage and the fecal matter was administered, as retention enemas, within 10 min of preparation. The treatment resulted in complete reversal of symptoms, in all patients, 4 months after the fecal probiotic infusions (Borody et al. 2003, 2004). The query is how durable is this treatment for the patient? Grehan et al. answered it in a publication in 2010, which reported that, 24 weeks after stool transplant, the patient´s microbiota still resembled that of the healthy donor.

Ranganathan et al. published, in 2006, one of the first reports on the use of intra-intestinal bacteriotherapy in chronic kidney disease. As pointed out by these authors, this illness can progress to end-stage renal disease, requiring either dialysis or kidney transplantation. Since there are not currently any useful therapies to slow down renal failure, bacteriotherapy represents a promising approach to mitigate uremic intoxication. This therapy uses the probiotic effects of certain bacteria that, upon ingestion, lower the uremic solutes in the gut. The authors evaluated the use of the nonpathogenic soil-borne alkalophilic urease-positive bacterium *Sporosarcina pasteurii* as a filter to eliminate what they called 'enteric dialysis' uremic solutes.

This experiment was successful, with the bacterium not only surviving, but also showing beneficial effects, both in vivo and in vitro. Additionally, the use of synbiotics (combination of probiotics and prebiotics) is starting to gain application, not only in gastrointestinal infections, but also as an effective way of preventing infections after major surgery (Nomoto 2008). Khoruts et al., in 2010, detected changes in the composition of the human fecal microbiome after bacteriotherapy for recurrent *Clostridium difficile*-associated diarrhea, and reported: "*striking similarity of the recipient's and donor's intestinal microbiota following after bacteriotherapy,*" which suggests that the donor's bacteria quickly occupied the intestinal niches in the patients. Infection by *Clostridium difficile* is a potentially serious disease, particularly in children in developed countries, where the number of cases has increased dramatically in the last 20 years. Accordingly, Russell et al. (2010) proposed a protocol, based on bacteriotherapy, for the treatment of relapsing *C. difficile* infection in children. The authors demonstrated, for the first time, that fecal transplantation was a practical and effective way for treating *C. difficile* infections, and recommended that this strategy be reserved for those *C. difficile* infections that did not respond to conventional therapy. Figure 4 outlines the procedure for fecal matter transplantation (bacteriotherapy).

Even now, the mechanism of action of fecal bacteriotherapy is not totally clear; as suggested by Russell et al. (2010) the success of this procedure could be due to multiple reasons, such as the diverse and more robust microbiota from the donor, elements such as bacteriocins present in the transplanted stool, modulation of the

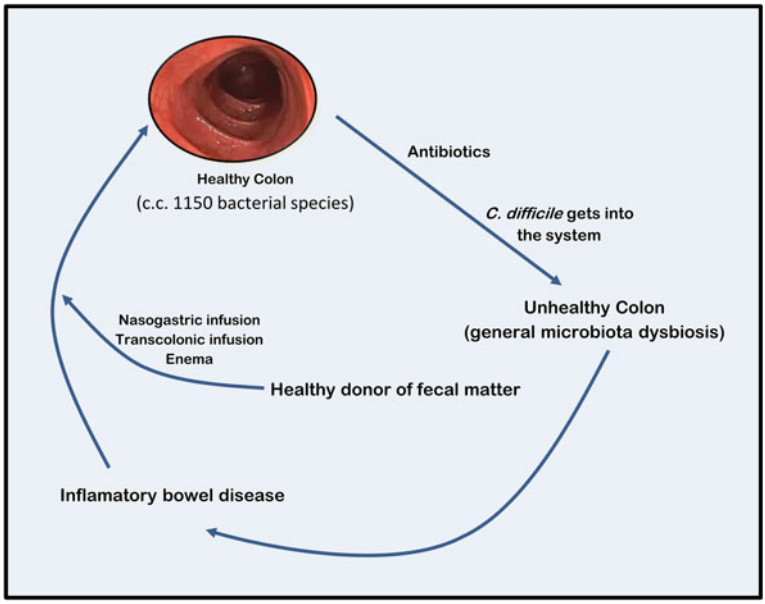

Fig. 4 Schematic protocol for fecal matter transplantation (bacteriotherapy)

recipient's immunity by the new microbiota (Kelly and LaMont 2008), up-regulation of anti-inflammatory factors, suppression of pro-inflammatory factors, or activation of phagocytes, natural killer cells, and regulatory T cells.

Anderson et al. conducted, in 2012, a systematic review on the effect of fecal microbiota transplantation in the management and treatment of inflammatory bowel disease, with the conclusion that this technique has the potential to be an effective and safe treatment for these syndromes (including pseudomembranous colitis caused by *C. difficile)*. The authors concluded that "*Well-designed randomised, controlled trials are necessary to confirm the positive findings from case reports, to evaluate safety and to develop optimal protocols for the use of fecal matter transplantation in inflammatory bowel disease, prior to this becoming a standard part of clinical therapy.*" Shahinas et al. reported, in 2012, statistical analyses based on 16S rRNA deep-sequencing data from healthy people and patients affected by pseudomembranous colitis. They found that the healthy gut microbiota contained mainly the phyla Bacteroidetes, whereas the patients' intestines contained an over abundance of Proteobacteria, accompanied by a markedly decreased microbiotal richness and diversity.

Also in the same year, Petrof et al. (2013; see also Adamu and Lawley 2013 and Mitchell et al. 2013) described the use of a stool substitute preparation. This preparation included 33 intestinal bacteria isolates from healthy donors, and was used to successfully treat two patients infected with resistant *C. difficile* infections, and the treatment produced major changes in the patients' stool microbiota. These authors concluded that "*a stool substitute mixture comprising a multi-species community of bacteria is capable of curing antibiotic-resistant* C. difficile *colitis.*" The bacterial species used as stool substitute in this study were *Acidaminococcus intestinalis, Bacteroides ovatus, B. adolescentis, B. longum, Blautia product, C. cochleatum, Collinsella aerofaciens, Dorea longicatena, E. coli, Eubacterium desmolans, E. eligens, E. limosum, E. rectale, E. ventriosum, Faecalibacterium prausnitzii, Lachnospira pectinoshiza, L. casei/paracasei, L. casei, Parabacteroides distasonis, Raoultella* sp.*, Roseburia faecalis, Ruminococcus torques, R. obeum, and S. mitis. B. longum* has been reported to possess an intriguing property that could render the microbe ineffective for use in stool transplantation. This bacterium undergoes a targeted loss of gene clusters, encoding traits pertinent to the human intestinal environment, when grown axenically in vitro. This loss of traits that confer competitive abilities in the gut may render the microorganism unable to inhibit the growth of other microorganisms (Lee et al. 2008).

To the best of our knowledge, there have only been 55 fecal matter transplantation treatments worldwide (ClinicalTrials.gov). These include 13 in Europe, 4 in Israel, 5 in China, 3 in Australia, 27 in the USA, and 3 in Canada. Although the number is still small, their success is very encouraging, as the patients recovered from their different pathologies, and this success will encourage new research in the subject. Regarding the use of fecal matter implantation for treatment of Crohn's disease and ulcerative colitis, we must remark that this research is still in its infancy, and that it should treated with great care and only performed as part of controlled clinical trials. Despite the increasing interest on the use of fecal matter

transplantation to treat different gut disorders, many questions still remain unanswered; these include those concerning the methodology to be used for actual fecal matter transfer, the optimal route of administration, the long-term effect, and, outstandingly, what makes a good and a bad donor (Borody et al. 2013). Some of these issues have been addressed above.

Another subject that warrants inclusion here concerns familial diarrhea syndromes, which could represent one of the primary subjects for multiple fecal microbiota transplantations. Familial diarrhea disorders are, in most cases, severe and caused by recessive mutations (Fiskerstrand et al. 2012). These authors described the case of a novel dominant disease in 32 members of a Norwegian family, generated by a heterozygous missense mutation (c.2519G → T) in the guanylyl cyclase 2C receptor (GUCY2C, a transmembrane receptor with an extracellular domain that can be activated by either the endogenous ligand or by an external ligand, such as *E. coli* heat-stable enterotoxin ST). Exposure of the mutant receptor to its ligands resulted in markedly increased production of cyclic guanosine monophosphate and, consequently, increase in the cystic fibrosis transmembrane regulator (CTFR); this, in turn, resulted in higher chloride and water secretion from the enterocytes, resulting in diarrhea. CFTR mutations, modulated by yet unidentified modifier genes in humans, could play a role in this type of dysbacteriosis. Accordingly, Romi et al. (2012) showed that in two unrelated consanguineous Bedouin kindreds, an autosomal-recessive mutation in GUCY2C led to a dramatic reduction, or even total elimination, of the enzymatic activity of the encoded guanylyl cyclase 2C.

Fecal matter transplantation from asymptomatic donors, carrying *E. coli* strains that produce heat-stable enterotoxin, can have deleterious effect on recipients but, surprisingly, it may also have positive outcomes in patients with intestinal neoplastic processes. In these cases, activation of guanylyl cyclase increases the intracellular cGMP concentration, which, in addition to modulating intestinal fluid–ion homeostasis, it also causes reduction in carcinogen-induced aberrant crypt foci formation, at least in a mouse model (Basu et al. 2014). Fecal microbiota transplants containing bacteriocins with a very narrow action spectrum is another area of interest that would benefit from further research, as they could specifically target intestinal pathogens. Rea et al., reported in 2010 that thuricin CD, a post translationally modified bacteriocin produced by *B. thuringiensis*, displays a narrow spectrum of activity against *C. difficile.*

In conclusion, the old, and yet new, bacteriotherapy technique is a very promising approach for the treatment of long lasting and life-threatening diarrheas, but it must be implemented with great care. To be successful, it requires the genetic/biochemical background for both the recipient and stool donors to be thoroughly investigated, but as indicated by Willing et al., in 2011, "*the transfer of resistance through microbial transplantation (bacteriotherapy) provides additional mechanisms to alter 'host' resistance, and a novel means to alter enteric infection and to study host-pathogen interactions.*"

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Human Mutations Affecting Antibiotics

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Abstract Ever since the introduction of antibiotics in human therapy to treat infectious diseases, it was known that some individuals responded better than others and that some were altogether resistant. In addition, some antibiotics were able to produce side effects of different strength that were in the majority of the cases transmissible to the progeny, while others seemed to ameliorate certain syndromes. These and other aspects shall be addressed in the present chapter.

1 Introduction

Throughout history, humans have suffered from many diseases (microbial and nonmicrobial) being then the cause that life span, until quite recently, had been of only 40 years. Anderson and May recorded in their article of 1991, that in the Paleolithic, the life expectancy was of only 25 years, being infection diseases the main cause of premature death. The increase in life span to more than 80 years in many developed countries has been due to the massive introduction of antibiotics in the middle of the twentieth century, preparation of vaccines against many infectious diseases, understanding and treatment development of the cancerous disease, and so on. Some infectious diseases are thought to be eradicated from earth, while others such as tuberculosis are increasing again, as society runs out of effective antibiotics and antimicrobial compounds that are useful to control such infections. Underlying all these known aspects, there are others such as mutations, always present when genetic systems are involved, that constantly drawback the increasing life span of the human. Some of these mutations affect mitochondria that are cytoplasmically

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and maternally inherited in higher eukaryotes, while others affect nuclear genes. The first type, occurring in a haploid genome has more chances to generate a phenotype, while the second, taking place in a diploid genome may or may not produce a phenotype. Normally these mutations are recessive, but when expressed do generate an unwanted disease (many related to the appearance of infectious diseases and only a handful of them correcting the symptoms of a previous syndrome).

Nearly one half of the current antibiotics target the ribosome of bacteria to inhibit the translation, hence its duplication, since mitochondrial ribosomes are thus far considered as bacterial-type ribosome. If antibiotics could penetrate or be transported into mitochondria, the mitochodrial ribosomes would be, therefore, inhibited (Franceschi 2007). Some acts on the 30S ribosomal subunit and include the aminoglycosides (i.e., gentamicin, tobramycin, or streptomycin) and the tetracyclines (i.e., doxycycline or minocycline), while others affect the 50S subunit and include the macrolide group (i.e, erythromycin, clarithromycin, or azithromycin), the ketolides (i.e, telithromycin), phenylpropanoids (chloramphenicol), lincosamide (clindamycin), the oxazolidinones (i.e., linezolid, posizolid, and cycloserine), or the Streptogramins Quinupristin/dalfopristin. In particular, the tetracycline antibiotics, that are commonly used to treat skin infections, respiratory systemic infections, chlamydia, Lyme disease, brucellosis, Rocky Mountain spotted Fever, anthrax as well as plague, have a wide margin of safety of use, and although they may cause dysfunction in human mitochondria, do not seem to have a particular negative impact in human health (McKendrick 1968).

By the time Jacobs produced his paper in 1997 entitled "Mitochondrial deafness" up to 100 nuclear genes had been described as involved in the disorder although only around 30 had been mapped. As we will see later in this chapter, there are many mitochondrial mutations involved in syndromic and nonsyndromic loss of hearing ability. In addition to genetic factors there are environmental ones, such as aminoglycoside antibiotics (i.e, streptomycin) that when used in long periods, such as in tuberculosis treatment, also lead to deafness. As suggested in Jacobs paper and taking everything together (including the mechanistic studies at the molecular level) "*the pathogenic process involves the accumulation of abnormal translation products inside mitochondria, in sensitive cells of the auditory system. This leads to a prediction of the involvement of a novel class of nuclear genes in hearing impairment, namely those with roles in 'mitochondrial protein quality control*". Hearing loss is perhaps the most prevalent sensorial deficit in the general population (de Moraes et al. 2009), and it is generally admitted that congenital deafness occurs with a frequency of 10^{-3}. Nonsyndromic deafness can be caused by mutations in both nuclear and mitochondrial genes, and as a matter of fact mutations in the mitochondrial DNA have been associated with aminoglycoside-induced such deafness and nonsyndromic deafness. The influence of the nuclear genetic background (mutations) may be quite relevant, although as yet scarce, but could explain the penetrance of mutations in a given population.

Many of these mutations affect genes (nuclear genes) involved in immunity development, thus causing immunodeficiencies (Casanova and Abel 2004) that

ultimately are the cause of deaths (antibiotics or antimicrobials only buy time for the immunity system to get rid of the infectious agent). There is a considerable host variation as far as immunodeficiencies are concerned, and the first evidence supporting such variability and that immunodeficiencies were in fact hereditary (mono or polygenicaly inherited), came from observations of familial aggregations considering a variety of common or rare diseases, including cancer, or from the studies concerning monozygotic or dizygotic twins.

Primary immunodeficiencies were only hinted during the first half of the twentieth century and in fact the first, Bruton's agammaglobulinemia, was reported as linked to the X chromosome as late as 1954 (Notarangelo et al. 2004). According to these authors, "*at least 200 primary immunodeficiencies are known, most of which have a prevalence of less than 1 per 50,000 births*" and they appear at childhood as sensitivity to a huge variety of infectious agents, reticular dysgenesia (total lack of granulocytes and lymphocytes) being the most severe form of primary immunodeficiency known. According to Abel and Demenais the first identified predisposing gene to an infectious disease was the one controlling susceptibility to leprosy (Abel and Demenais 1988). There are also nonconventional immunodeficiencies (dominant or recessive) including predisposition to viral infections that also have a genetic background. Other predispositions based on genetic grounds include the syndromes known as *Mendelian susceptibility to mycobacterial diseases (MSMD)* when mutations affect the interleukin-12–interferon-γ cytokine system (Newport et al. 1996; Casanova and Abel 2002).

Some antibiotics such as tunicamycin may induce endoplasmic reticulum stress due to the over accumulation of unfolded proteins that may lead to resistance to antibiotics and apoptosis originated by the microtubule-targeting chemotherapeutic drugs, such as docetaxel and vincristine (Jiang et al. 2009). The cells, however tend to adapt to this stress by mechanisms largely unknown, but today it is known that some eukaryotic organisms such as yeasts may overcome in part this situation by expressing the HOG (high-osmolarity glycerol) pathway (Torres-Quiroz et al. 2010).

2 Deleterious Effects of Human Mitochondrial Mutations Related to Antibiotic Effectiveness

Following the endosymbiont theory, mitochondria has a bacterial origin (Gray 1998; Andersson et al. 1998), and accordingly their expression systems are altogether quite similar (Singh et al. 2014). As the mutational rate of the mitochondrial rRNA is much higher as compared to that of nuclear rRNA, the mitochondrial functions may be compromised thus generating a variety of side effects upon antibiotic treatment. Point or deletion mutations in the mammalian mitochondrial DNA (Table 1) originate a variety of syndromes (including aging) that are difficult to treat because of the "high copy number effect" and the mitochondrial double membrane (Zullo et al. 2005). Mitochondria in eukaryotic systems, besides being

Table 1 Some mitochondrial human mutations related to antibiotics responsiveness

Locus	Mitochondrial mutations	Response
Mitochondrial genes mutations		
MT-RNR1 (mitochondrially encoded 12S RNA)	A1555G	Most common mutation involved in streptomycin-based deafness
	C1494T	Maternally transmitted aminoglycoside-induced and nonsyndromic hearing loss (Wei et al. 2013)
	T1095C	The mutation increases gentamicin-mediated apoptosis (Muyderman et al. 2012)
	A827G	
	961delT	
	T1291C	
	A1491G	Transitional mutation affecting the 12S rRNA. The resulting associated deafness may be modulated by TRMU (see Table 3)
MT-RNR2 (mitochondrially encoded 16S rRNA)	2706A	Mutation in the MT-RNR2 gene encoding the 16S rRNA. Haplogroup H (see Table 2)
	3010A	Mutation in the MT-RNR2 gene encoding the 16S rRNA. Subhaplogroup H1 (see Table 2)
MT-CO2 (complex IV: mitochondrially encoded cytochrome c oxidase II)	G7598A	Mutation that may have a modifying role in the phenotypic manifestation of aminoglycoside antibiotic-induced deafness associated with 12S rRNA A1555G mutation (Chen et al. 2013)
MT-CO1 (complex IV: mitochondrially encoded cytochrome c oxidase I)	G7444A	Mutation in the mitochondrial CO1/tRNASer(UCN). Co-segregates with A1555G
MT-ATP6 (complex V: mitochondrially encoded ATP synthase 6)	T8993C and T8993G	Transitional and transversional point mutations, respectively, in the ATP synthase 6 gene thus originating low amounts of ATP and contributing to causing Leigh's syndrome or NARP (neurogenic weakness, ataxia, retinitis pigmentosum). Expected to generate oligomycin-resistant phenotypes
	T9176C	Transitional mutation. The mitochondrial ATPase is less efficiently inhibited by oligomycin

(continued)

Table 1 (continued)

Locus	Mitochondrial mutations	Response
MT-ND5 (mitochondrially encoded NADH dehydrogenase 5)	T12338C	All these variants may have a modifying role in the phenotypic manifestation of deafness-associated 12S rRNA A1555G mutation (Chen et al. 2008)
MT-TC (mitochondrially encoded tRNA cysteine)	T5802C	
MT-TT (mitochondrially encoded tRNA threonine)	G15927A	
MT-TS1 (mitochondrial tRNA for serine (UCN))	T7505C	Mutation in the tRNASer(UCN) associated to maternally transmitted hearing loss. Deafness may be modified by the highly conserved tRNA (Ala) T5587C variant, according to Tang et al. (2010)
	T7510C	
	T7445C	
MT-TL1 (mitochondrially encoded tRNA leucine(UUR) (R = A or G))	G3243A (A > G)	Causes a variety of diseases including deafness upon treatment with aminoglycosides as well as diabetes mellitus
MT-TI (mitochondrially encoded tRNA isoleucine)	T4290C	
	4269	Mutation at that particular nucleotide results in reduced stability tRNA (Ile) both in vitro and in vivo, thus affecting mitochondrial function
MT-TV (mitochondrially encoded tRNA valine)	C1624T	Mutation that causes rapid degradation of deacylated form of the abnormal mt-tRNA(Val)
	G1644A	Transitional mutation G1644A affecting the tRNA(Val) contributing to development of myopathy, encephalopathy, lactic acidosis, and stroke-like episodes in MELAS syndrome

(continued)

Table 1 (continued)

Locus	Mitochondrial mutations	Response
MT-TK (mitochondrially encoded tRNA lysine)	G8342A	Transitional mutation in the mitochondrial tRNA(Lys) gene. Associated with progressive external ophthalmoplegia and myoclonus
	A8344G	Transitional mutation within a conserved region of the mitochondrial tRNA(Lys) gene and highly prevalent in patients with MERRF
	G8363A	Transitional mutation in the mitochondrial tRNA(Lys) that originates cytochrome c oxidase deficiency
Nuclear genes mutations		
Strain ERY2301 (an erythromycin-resistant mutant)		Point mutation in that nucleotide HeLa cells mitochondrial DNA that originates eritromycin-resistance phenotype
ATPIF1		Mutations in this gene ameliorate symptoms associated to dysfunctions in the mitochondrial respiratory chain in mammals
TYMP	Thr151Pro and Leu270Pro mutations in mitochondrial thymidine phosphorylase gene (TYMP)	These mutations in mitochondrial thymidine phosphorylase gen originate neurogastrointestinal encephalomyopathy (MNGIE), a rare autosomal recessive multisystemic disorder (Suh et al. 2013)

involved in energy generation, have two other critical functions or side effects that we should consider here, and these are: (i) generation of free radicals and (ii) involvement in cellular apoptosis (Cohen and Saneto 2012). According to these authors, there are over 200 mutations affecting the mitochondrial DNA and many more in 150 nuclear genes that cause human diseases (many of these mutations are involved in microbial resistance to antibiotics or tumor cells resistant to anticancer drugs). Among other diseases, these are associated to mutations in the mitochondrial DNA in humans: dementias, ataxias, myopathies, otologic disorders, optic atrophy, pigmentary retinopathy, sideroblastic anemia, gastrointestinal dysmotility including pseudoobstruction including cyclic vomiting, several hepatopathies, lipodystrophy (for further information see the review by Cohen and Saneto in 2012). In higher eukaryotes the mitochondrial DNA is maternally inherited (Wallace 2008), while paternal DNA is mysteriously degraded in the oocyte soon after fertilization (Al Rawi et al. 2011).

The knowledge on mitochondrial gene expression in higher eukaryotes experienced an important advance after the works of Aloni and Attardi in 1971, Attardi and Attardi in 1971 and finally, Attardi and Attardi in 1972. Mitochondrial DNA in mammals is a double-stranded circular molecule that codes for 22 tRNAs, 2 rRNAs and 13 out of the 87 proteins forming part of the oxidative/phosphorylation system (Moreno-Loshuertos et al. 2011), so, mutations in mitochondrial DNA may be the cause of the aforementioned diseases (Jacobs 2003) based on an abnormal working condition of the oxidative/phosphorylation system and approximately 250 of such mutations are located in the tRNA genes (Fig. 1) (Ruiz-Pesini et al. 2007) (http://www.mitomap.org). Of these tRNA genes affected, MT-TL1 (Mitochondrially encoded tRNA leucine 1 (UUA/G) exhibited 23 mutations followed by MT-TK (Mitochondrially encoded tRNA lysine) and MT-TI (Mitochondrially encoded tRNA isoleucine) with 15 and 14 mutations, respectively (Moreno-Loshuertos et al. 2011). Accordingly the cells harboring these mutations have defficient respiration coefficient as well as reduced growth rates in medium with galactose and the homoplasmic animals (all the copies of the mtDNA are mutant) tend to suffer from different syndromes.

In 1998 Guan et al. found that mutation 7445 in the mitochondrial DNA thus affecting tRNASer (MT-TS1) precursor processing, had long-range effects on the NADH dehydrogenase subunit ND6 gene expression, this playing a determinant role in the deafness-associated respiratory phenotype of the mutated cell lines characterized by very significant decrease in glutamate- or malate-dependent O_2 consumption (Guan et al. 1998).

Loss of chloramphenicol effectiveness was reported as soon as 1974 by Bunn et al. who also signaled that in higher eukaryotes (mice) such a property could be cytoplasmatically inherited; the same was reported one year later by Wallace et al. for human cells and corroborated by Mitcbell and Attardi in 1978 and Munro et al. the same year (1978). Another example of antibiotic mitochondrial resistance is the one showed by mouse cell lines against rutamycin that is accompanied by reduced activity of mitochondrial ATPase (Lichtor and Getz 1978). Chemically-induced mitochondrial mutations in Chinese hamster cells (V79 strain) that resulted in

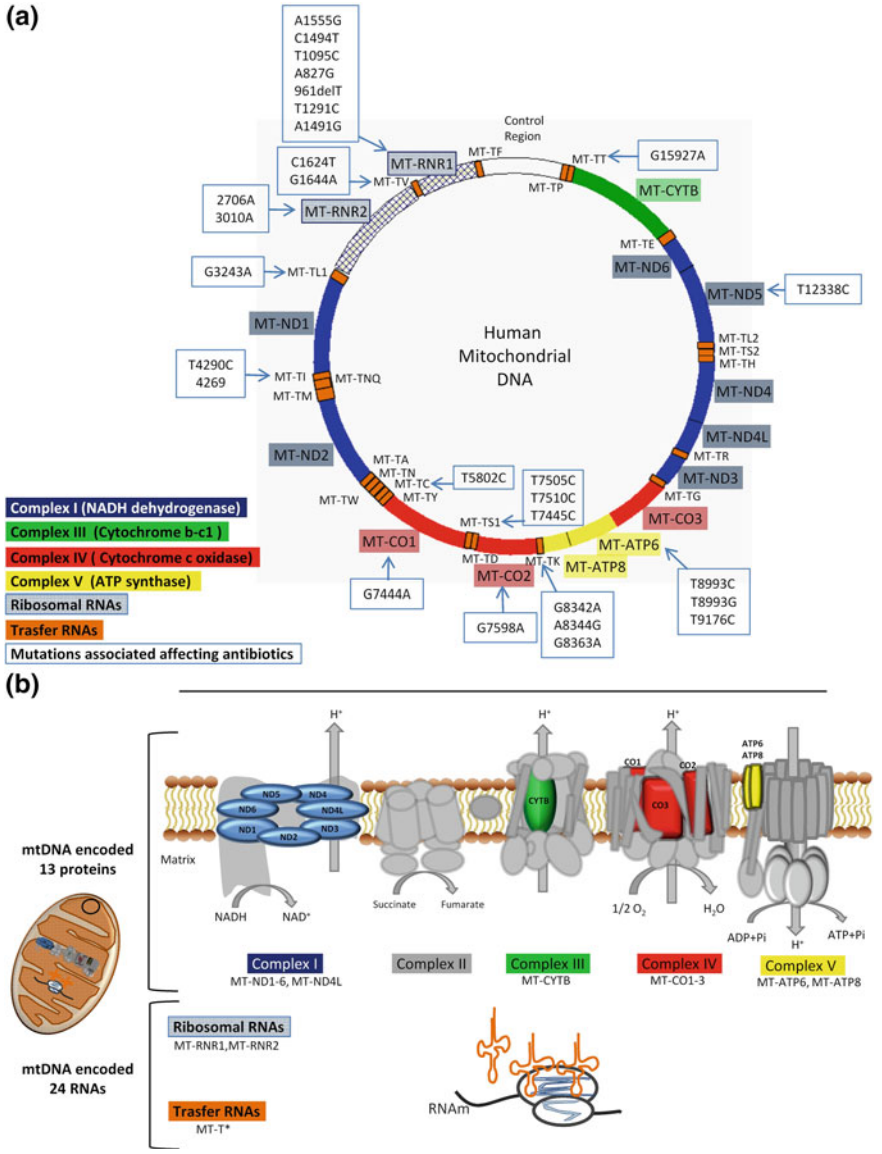
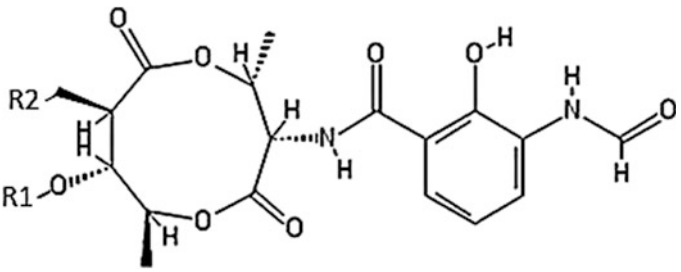


Fig. 1 **a** Map of human mitochondrial DNA (mtDNA) and mutations related to effect of antibiotics. The mtDNA encodes 37 genes: 13 polypeptides, 22 transfer RNAs, and 2 ribosomal RNAs. The polypeptides belong to mitochondrial respiratory chain included: 7subunits of complex I (MT-ND1, MT-ND2,MT-ND3, MT-ND4, MT-ND4L, MT-ND5, MT-ND6) are presented in *blue*; 1 subunit of complex III (MT-CYTB) is presented in *green*; 3 subunits of complex IV (MT-CO1,MT-CO2 and MT-CO3) are presented in *red*; 2 subunits of complex V (MT-ATP6, MT-ATP8) are presented in *yellow*. The genes for 22 tranfer RNAs (MT-T*, * letter amino acid codes; one for each of 18 amino acids two each for Serine and Leucine) are presented in *orange* and the genes for ribosomal RNAs (MT-RNR1 (12S rRNA), MT-RNR2 (16S rRNA)) are presented in *blue* frame. Mutations are presented in *boxes*. **b** Creative drawing of proteins and RNAs encoded for mtDNA genes (represented with the same colors as the genes that encode)

resistance to antimycin (CAS number 642-15-9; Table 2) were described by Harris in 1978; this resistance is of particular interest since the agent is a well-known inhibitor of electron transport between cytochrome *b* and cytochrome c_1, and has been used in a preclinical evaluation as a potential antilung cancer stem cell agent (Yeh et al. 2013). Resistance to this antibiotic also takes place in plant choloroplasts as recently demonstrated by Sugimoto et al. (2013). In humans, mitochondria are affected by antimycin causing respiratory chain disorder and loss of electron transport chain, but there are mutations (i.e, mutations in the ATPIF1 nuclear gene) that protect against antimycin-induced dysfunction I and is even essential for the viability of human ρ° cells that lack mitochondrial DNA. It follows then that controlling ATPIF1 function may be used to fight back intoxications associated to antimycin or related antibiotics (Chen et al. 2014).

Soon after unraveling the chemical structure of antimycins, resistant mutants started to be described in a variety of organisms such as the yeast *Torulopsis utilis* (Butow and Zeydel 1968) *Neurospora sitophila* (Sherald and Sisler 1970), *Ustilago maidis* (Georgopoulos and Sisler 1970) or *Candida utilis* (Grimmelikhuijzen and Slater 1973). Antimycin was first identified by Leben and Keitt (1956) as a potent fungicide and synthesized by species of the bacterial genus *Streptomyces* (Slater 1973), exhibiting a powerful inhibitory role on the mitochondrial respiratory chain at the level of the ubiquinol-cytochrome c oxidoreductase (cytochrome bc1) enzyme complex (Ahmad et al. 1950; Lang et al. 1975). The genetic determination of the antimycin-resistance phenotype was soon demonstrated to be extrakaryotically inherited in the yeasts *Schizosaccharomyces pombe* (Wolf et al. 1976) and

Table 2 Antimycins

Chemical Structure: Antimycins	
	
The groups at positions R1 and R2 vary according to the type of actinomycin	
CAS number	642-15-9 (Antimycin A_1)
Molecular formula	$C_{28}H_{40}N_2O_9$ (for Antimycin A_1; R1 = $COCH(CH_3)CH_2CH_3$ and R2 = $(CH_2)_4CH_3$)
Source	*Streptomyces* sp.
Mechanism of action	It inhibits mitochondrial electron transport specifically between cytochromes b and c1, resulting in inhibition of ATP synthesis and in toxic free radical superoxide formation
Uses	Used as a piscicide in the catfish farming industry and also has potent killing activity against insects, nematodes, and fungi

Saccharomyces cerevisiae (Michaelis 1976), and this property was also demonstrated two years later for higher eukaryotic cells (Harris 1978). As expected, nuclear-dependent mutant resistant strains were soon found in different yeasts (Lucchini et al. 1979), thus turning out as in the majority of antibiotic exhibiting mitochondria or chloroplasts as primary targets, that resistance phenotypes could be either chromosomically or cytoplasmically inherited.

Human mutation A1555G affecting the locus MT-RNR1 which encodes mitochondrial 12S RNA is perhaps the most common one involved in the production of deafness due to streptomycin-based therapy, although the nuclear background (human GTPBP3 gene, which encodes a GTP-binding protein that is localized in the mitochondria and may play a role in mitochondrial tRNA modification) and homolog to the yeast allele MSS1), indeed determines the biochemical phenotype in the associated deafness (Guan et al. 2001; Li and Guan 2002). Streptomycin is still extensively used in some countries such as China, India, or some African countries, as a way to treat the resistant forms of tuberculosis (Sande and Mandell 1990; Chamber and Sande 1996). The ototoxicity shows a pattern of familial aggregation suggesting autosomal dominant inheritance (Viljoen et al. 1983) and may even cause deafness in the unborn (Donald and Sellars 1981). Human et al. in 2009 genotyped a South African family and found that variants in the some mitochondrial proteins originated by the genic variants tRNA(Ser(UCN)), A10S in TRMU (responsible for the 2-thiolation of the wobble U in tRNALys, tRNAGlu and tRNAGln) and 35delG in GJB2 genes (encoding conexin 26) did not act as genetic modifiers at least in this family, although all had been initially reported as being involved in streptomycin-based deafness (Yan et al. 2006); the work by Abe et al. in 2001, concerning also GJB2 mutation in relation to A1555G mutation, merits also to be cited here. It is well known that ototoxicity is perhaps the main side effect exhibited by aminoglycosides, this leading to permanent deafness in some individuals particularly sensitive to streptomycin (Prezant et al. 1993; Guan et al. 2000) due to mitochondrial mutations in the MT-RNR1 gene which was the first reported mutation causing this phenotype, although today others such as C1494T (Zhao et al. 2004), [T1095C (Wang et al. 2005) A827G (Xing et al. 2006), 961delT (Yoshida et al. 2002)] and T1291C (Ballana et al. 2006) have been reported. Other mutations maternally inherited nonsyndromic hearing loss, involving mitochodrial tRNAs (T7510C en el locus MT-TS1) has been reported by del Castillo et al. in 2002 and others such as G7444A mutation in the mitochondrial CO1/tRNASer(UCN) genes cosegregates with A1555G mutation (Yuan et al. 2005). The G7444A mutation is of special interest because it results in a read-through of the stop condon AGA of the COI message, this adding three amino acids (Lys–Gln–Lys) to the C-terminal of the polypeptide; in addition, this mutation is adjacent to the site of 3′ end endonucleolytic processing of L-strand RNA precursor, spanning tRNASer(UCN) and ND6 mRNA. The deafness associated to the A1555G mutation, however, may vary accordingly depending on the different variants in the mitochondrial tRNA(Glu), tRNA(Arg), and tRNA(Thr) (Young et al. 2006). Chen et al. reported in 2008 that variants in mitochondrial MT-ND5 T12338C, tRNA(Cys) T5802C, and tRNA(Thr) G15927A may have a significant

modifying role in the phenotypic manifestation of deafness-associated 12S rRNA A1555G mutation and more recently (Chen et al. 2013) have reported that mitochondrial MT-CO2 G7598A mutation may have also a modifying role in the phenotypic manifestation of aminoglycoside antibiotic-induced deafness associated with 12S rRNA A1555G mutation. In fact there are many hints strongly suggesting that such a mutation by itself is insufficient to produce the various deafness phenotypes, and that there must be other over-accumulated mutations as the one described above, as well as nuclear modifier genes and mitochondrial variants/haplotypes, responsible for the penetrance of hearing loss associated with the A1555G mutation (Hutchin et al. 1993; Bykhovskaya et al. 2000).

Tang et al. reported in 2010 after a deep study on mutations in mtDNA on a novel maternally inherited homoplasmic tRNA(Ser(UCN)) T7505C mutation plus 37 variants of haplogroup F1. 65 % of mutation-harboring individuals showed significant reduction in the level of tRNA(Ser(UCN)) in the lymphoblastoid cells and seven out of nine matrilineal relatives exhibited a variable hearing loss (Tang et al. 2010). The T7505C mutation was found located at a highly conserved base-pairing (10A-20U) of tRNA(Ser(UCN)).

Additional mutations produce several defects that include reduction in the half-life of tRNAs (Yasukawa et al. 2000), impaired aminoacylation (Chomyn et al. 2000), or decrease in the steady-state levels of tRNA (Guan et al. 1998; Moreno-Loshuertos et al. 2011) which results in heavy organular protein synthesis deficiency. Homoplasmic mutations affecting tRNA(Ile) have been also described to produce a familial progressive necrotising encephalopathy with different degree of penetrance (Limongelli et al. 2004), or multiple neonatal deaths due to severely mutation-affected mitochondrial DNA (McFarl et al. 2002). Other mutations affect mitochondrial tRNAs and it has been estimated (Park et al. 2008a) that more than 130 pathogenic mutations affect mtDNA-encoded tRNA genes, including the 24 in the tRNALeu(UUR) gene (MTTL1) and among these the A 3243G was the first and most commonly identified (with a prevalence in humans range from 1 to 236 per 100,000 individuals; Majamaa et al. 1998) as being the cause of mitochondrial myopathy, lactic acidosis, and stroke-like episodes (MELAS) syndrome (Goto et al. 1990). These authors found that mitochondrial DNA with a point mutation in the tRNA(Ile) gene (MT-TI) at nucleotide position 4269 and with a point mutation in the tRNA(Arg) gene (MT-TR) at 10410 could get transferred cytoplasmically to rho zero HeLa cells (i.e., cells lacking mtDNA) in order to determine if the mutations were in fact responsible for the defects in mitochondrial respiration function; the results being that 4269 mutation (even in the absence of any defects in the nuclear genes) produced an altered phenotype, while mutation 10410 did not produce phenotypic changes whatsoever.

Tiranti et al. described in 1999 the G8342A transition in the mitochondrial tRNA (Lys) gene (MT-TK) associated with progressive external impaired ocular motility and myoclonus (sudden muscle contractions or brief lapses of contraction, including hiccups) that resulted in high percentage of cytochrome c oxidase negative fibers but not ragged fibers; according to Shtilbans one year later (Shtilbans et al. 2000), this mutation should also be associated to maternally inherited Leigh

syndrome. Association of mutations in tRNs (Lys) had been already decribed by Silvestri et al. in 1992 as associated with myoclonic epilepsy and ragged red fibers although in this case affected a A > G transition at position 8344 of the same tRNAS. Yet, an additional mutational event, (8363A > G), for the same tRNA has been described by Ozawa et al. in 1997 in two Japanese families and originating cytochrome c oxidase deficiency.

As indicated before, the A3243G transitional mutation is associated with numerous other clinical outcomes including cardiomyopathy, chronic progressive external ophthalmoplegia, diabetes mellitus (Wang et al. 2013), and diabetes with deafness (Finsterer 2007). Estimates of the population prevalence of the A3243G mutation in humans range from 1 to 236 per 100,000 individuals (Majamaa et al. 1998; Chinnery et al. 2000; Manwaring et al. 2007; Uusimaa et al. 2007; Schaefer et al. 2008). Such mutation may be phenotypically corrected by overexpressed mitochondrial leucyl-tRNA synthetase (Park et al. 2008a) and as a matter of fact this procedure may be used to overcome undesirable effects caused by tRNA gene mutations. Although there are not yet curative treatments for mitochondrial disorders, rapid advances are being made in for treating human disorders by manipulating the nuclear genome in order to produce extra copies of genes encoding proteins that interact with tRNAs (De Luca et al. 2006).

Wilson et al. described in 2004 a cluster of metabolic defects associated to mutated mitochondrial DNA. The authors described a syndrome which included hypertension, hypercholesterolemia, and hypomagnesemia in such a way that each phenotype is transmitted on the maternal lineage which suggested mitochondrial inheritance and the analysis did reveal a homoplasmic mutation C > U immediately 5′ to the mitochondrial transfer RNA Ile anticodon.

Pathogenic mutations in mitochondrial DNA and hence the associated phenotypic diversity observed in patients has been postulated by an unequal segregation of the mutated and wild-type genomes, but there are some mutations, such as C1624T, in the locus MT-TV (tRNAVal) that provokes the Leigh Syndrome and that causes serious metabolic disorders with extremely low respiratoty index and quite low levels of tRNAVal resulting in neonatal deaths. The phenotype can be partially corrected, at least in vitro by overexpression of the human mitochondrial valyl tRNA (Rorbach et al. 2008). To the best of our knowledge no data have been published on the use of antibiotics to correct this phenotype. Other mitochondrial mutations, such as the G1644A transition described by Menotti et al. in 2004 and that affects the tRNA for valine was identified in the proband and maternal relatives. The mutation have been described as a pathological mutation because the G at the 1644 position is a highly conserved base (Menotti et al. 2004), being thus involved in severe hypertrophic cardiomyopathy development as well as deafness.

Some neurological human disorders have been reported as dependent (at least in part) on mitochondrial DNA mutations that involve the synthesis of ATP. One such syndromes involve the transversion T9176C that changes a highly conserved leucine residue into proline at position 217 of the mitochondrially encoded Atp6p

(locus MT-ATP6), subunit of the F1FO-ATP synthase (Kucharczyk et al. 2010), resulting that the yeast cells are less sensitive to the antibiotic oligomycin.

According to the foregoing it is clear that sensitivity to antibiotics is a multifactorial trait depending to a great deal on the genetic variability of the sensitive individuals as far as the side effects of the drugs are concerned; in turn, this may be an important factor explaining the diverse degree of reactions in a given population. As we know, the protein synthesis in mitochondria resembles that of bacteria and hence mitochondrial ribosomes are frequently affected by these antibiotics (Pacheu-Grau et al. 2013). Such adverse reactions may be so strong at times that in fact are estimated to be the 4th–6th leading cause of death at least in the USA. The genetic basis explaining this drug-related toxicity of the different antibiotics affecting the mitoribosomes is not well known yet but as Pacheu-Grau et al. recall "this in fact may represent the Achilles heel of ribosomal antibiotics" (Bottger 2007). Naturally, the antibiotic-sensitive ribosomal pockets in mitochondria and bacteria are slightly different, in that the Ki for the second is lower than for the first; this being the reason why a given antibiotic eliminates the pathogenic bacterium without or only with minor side effects.

The above-mentioned classical human mutation (A1555G; Bottger 2010), as reported by Pacheu-Grau et al. (2013), has been found also completely fixed in Bornean and Sumatran orangutan species (Pacheu-Grau et al. 2011) and negatively affecting the oxidative phosphorylation function and the amount and activity of respiratory complex IV (CIV). SNPs in human mitochondrial DNA (Table 3) define haplogroups and according to Pacheu-Grau et al., almost half of the western European population belongs to haplogroup H, including mutation 2706A in the MT-RNR2 gene that encodes the 16S rRNA. Within this haplogroup, the subhaplogroup H1 (accounting for *ca* 50 % of H individuals) harbors mutation 3010A in this rRNA. The 2706 and 3010 mutations being close to the ribosomal peptidyl transferase center (Ruiz-Pesini and Wallace 2006) would provoke that when antibiotherapy (i.e., linezolid or chloramphenicol) is used on these individuals chances are that they develop side effects such as lactic acidosis (Carson et al. 2007) or neuropathy (Gould 2011) as the oxidative phosphorylation function is affected.

Resistance to chloramphenicol in lower eukaryotes has been described since early seventies of last century (Tait 1972; Roberts and Orias 1973), and so the protozoon *Paramecium* was soon shown to be sensitive to bacterial antibiotics such as chloramphenicol or erythromycin, but as in bacteria, mutant strains appear with frecuency only possible by assuming that it was encoded by haploid genomes, and indeed the resistance in these mutants is inherited cytoplasmically (Beale 1969) and

Table 3 Association between combinations of 16S rRNA (MT-RNR2) polymorphisms and mtDNA haplogroups

Haplogroup	Mutation 2706	Mutation 3010
H non-H1	A	G
H1	A	A
J1	G	G
T	G	G
Uk	G	G

transferred by the mitochondrial DNA (Beale et al. 1972). Also, similar cytoplasmically inherited mutants have been reported in yeasts (Wilkie et al. 1967),also involving mitochondrial DNA.

Resistance to chloramphenicol has been also reported in mice-derived cellular lines (Bunn et al. 1974) and in human-derived ones (Wallace et al. 1975). In this second case, the resistance was demonstrated by enucleation of HeLa cells followed by cell fusion with appropriate HeLa sensitive cells (lacking nuclear thymidine kinase). The cybrids able to grow in bromodeoxyuridine (Kit et al. 1966, 1972) were enucleated again and fused to hypoxanthine phosphoribosyl transferase-deficient HeLa cells and the cybrids selected in thioguanine. Following this strategy of obtaining different cybrids, the authors concluded that chloramphenicol resistance is coded in the cytoplasm and not in the nucleus and that this resistance could be transferred to cells of different origin. According to these authors these studies represent the first genetic evidence of cytoplasmic inheritance in human cells.

Another type of mutations concern the oligomycin-resistant variant in human HT1080 fibrosarcoma (Fig. 2 See next section) that has been assigned to chromosome 10 (gene ATP5 J, encoding for the oligomycin-interacting F6 component of the F0 subunit of mitochondrial ATPase). Mutations in such a gene originate oligomycin-resistant phenotypes and behave as codominants (Webster et al. 1982;

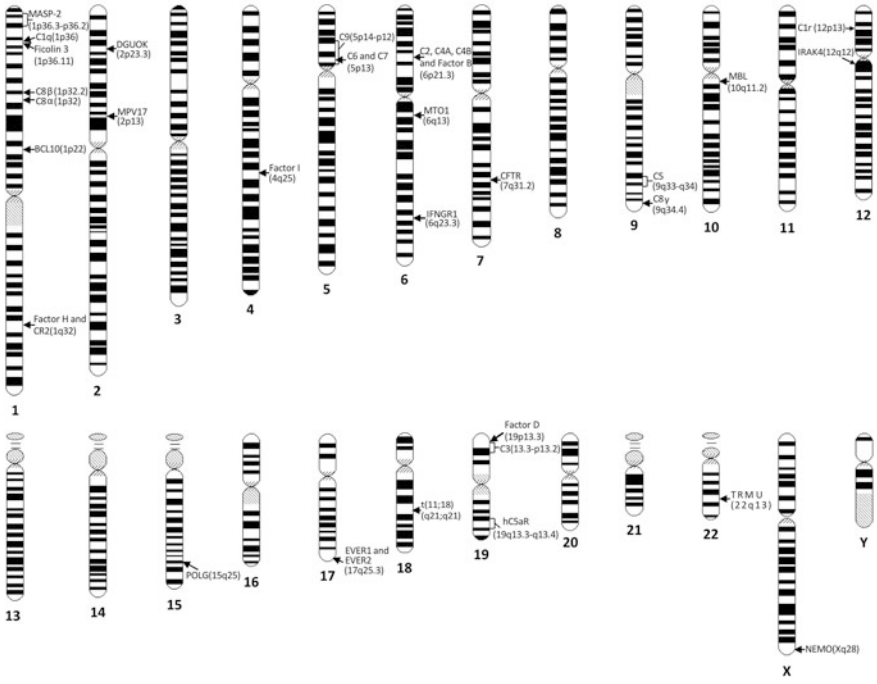


Fig. 2 Chromosome position of some nuclear human mutations related to antibiotics responsiveness

Higuti et al. 1991). One of the most common disease-associated point mutations in such a gene is the thymine to guanine transversion at nucleotide position 8993 (T8993G) or the thymine to cytosine transition at nucleotide 8993 (T8993C), this leading to the substitution of a highly conserved leucine residue by arginine at amino acid position 156 or by proline, respectively, in the ATPase 6 protein (Morava et al. 2006). Symptoms include **n**eurogenic muscle weakness, **a**taxia, and **r**etinitis **p**igmentosa, otherwise a syndrome termed NARP (Holt et al. 1990; Carelli et al. 2002; Zullo et al. 2005). High load of the first mutation is lethally associated to NARP syndrome, whereas high load of the second originates a less severe syndrome (de Vries et al. 1993).

Recently, Mihaylova et al. in 2013 reported on a mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) as rare autosomal recessive multisystemic disorder caused by a novel homozygous TYMP mutation (p.Leu347Pro) as the leading cause for possible toxicity of tuberculostatic agents in a Bulgarian patient showing signs of cachexia, hearing loss, ptosis, ophthalmoparesis, polyneuropathy, and cognitive impairment among others, suggesting mitochondrial toxicity of these agents.

3 Nuclear Mutations

We do tend to separate the genes involved susceptibility/resistance to certain diseases as main or primary and minor or secondary genes, but this may be an altogether erroneous classification since the so-called minor genes may turn out to be as important as the primary ones, this being particularly true for heterozygous traits, such as the classical case of resistance of heterozygous carriers for the sickle cell anemia to *Plasmodium falciparum,* than the homozygous for the wild-type hemoglobin allele (Allison 1954, 2002).

Humans tend to suffer from a variety of diseases which have been the cause of death throughout the history of mankind (Anderson and May 1991). Being the burden of infectious diseases high in the different civilizations, life expectancies was as low as 25 years in the Paleolithic or 40 years in Europe by the nineteenth century. After introduction of antibiotics and vaccination, life expectancy increased rapidly to an average of 80 years in developed countries. Therefore, the increase in life expectancy is recent, is the cause of the ongoing population explosion, but can drop again to low numbers if antimicrobial compounds lose their effectiveness.

In 1982 Doersen and Stanbridge reported on the characterization of two new erythromycin-resistant mutants of HeLa cells (ERY2305 and ERY2309) that expressed the phenotype even when no mitochondrial protein synthesis occurred and that it could not be transferred by cybridization that indeed represent a new class of erythromycin-resistant mutants in human cells altogether different from the cytoplasmically inherited mutation in strain ERY2301 already known at that time (Doersen and Stanbridge 1979).

Anderson and May (1991) indicate a quite complex interplay of factors such as the microorganisms themselves and the immunity of humans to fight back microbial colonization and hence disease. Development of resistance to antimicrobials by the microorganisms but also failures of the immunity system due to mutational events do determine the result of the clinical outcome. This is particularly relevant since treatment with antimicrobials only buys time to the sick and it must be the immune system the one that gets rid of the pathogen (Casanova and Abel 2004). Also, we must keep in mind that by artificially increasing the life expectancy with antimicrobials we have introduced a negative factor in genetic selection thus favoring the maintenance of deleterious or mutated alleles in the population. Such mutations, when occurring in the nucleus may be inherited in the so-called Mendelian fashion (either monogenic or not, that is, multigenic and sometimes referred as non-Mendelian) and is classical that the predisposition to a given microbial infection is circumscribed to a family or particular ethnics. Mutations in the mitochondrion (see previous section) are inherited cytoplasmically with an altogether different segregation from the Mendelian one.

The first primary immunodeficiency was linked to chromosome X as soon as 1954 and characterized as a recessive agammaglobulinemia (Notarangelo et al. 2004); for a recent review on immunodeficiencies see: Geha et al. (2003). Although rare, there are over 200 primary leukocyte-affecting immunodeficiencies (including reticular dysgenesia in which patients lack granulocytes and lymphocytes) with a prevalence of near 2 in every 100.000 and characterized because the ill in the childhood are quite sensitive to a variety of microbial pathogens and the patients must be constantly subjected to different antibiotics.

There are also nonconventional immunodeficiencies (dominant or recessive) twelve of which are associated to diverse infection phenotypes. Mutations in EVER1 and EVER2 may be a good example of the foregoing being the cause of epidermodysplasia verruciformis. Also, there are some predispositions in humans to certain pathogenic bacteria, this including the Mendelian Susceptibility to mycobacterial Diseases (also known as MSMD). These patients exhibit mutations in the interleukin-12–interferon-γ cytokine circuit and suffer from recurrent infections caused by otherwise low virulent mycobacteria (Newport et al. 1996; Casanova and Abel 2002). All humans are in constant contact primarily with air-born environmental mycobacteria, *ca*. 80 % of the population are or has been intradermically inoculated with the Bacillus of Calmette and Guerin (BCG) and yet many people are also exposed to infection caused by full mycobacterial pathogens such as *Mycobacterium tuberculosis* or *M. leprae*.

In fact, segregation studies revealed that human genetic predisposition to leprosy is associated to PARK2 and PACRG (Mira et al. 2004; candidate region on 6q25). The system involves an E3-ubiquitin ligase, an unexpected but with utility as aid to leprosy diagnosis.

In animals there may be regular exposure to environmental mycobacteria but they are rare among humans, except in those harboring the mutations reported here.

Classical genetic disorders such as Papillon–Lefèvre syndrome (PLS), characterized by palmoplantar keratodermosis and periodontitis is an autosomal genetic

disorder recessive (chromosome 11q14.1–q14.3) due to deficiency in cathepsin C (Ahuja et al. 2005; Cagli et al. 2005). In the homozygous state, this mutation (G415A) is associated with an almost complete loss of activity of cathepsins as well as elastase, this giving rise to periodontal state (a group of diseases characterized by progressive destruction of the structures that support the teeth) that results, despite the use of antibiotics in the complete loss of primary teeth. In fact the great number of polymicrobial and polygenic infections that we term as "periodontitis" are caused by a number of environmental and host-modifying conditions being therefore multifactotial (Armitage 2002; Hart and Atkinson 2007) and the actual evidence suggests a significant role of genetic factors as a main cause of periodontitis susceptibility. This conclusion is based on studies of the role of the disease in family trees on the one hand, and on studies of segregation analysis and linkage studies in the disease (Marazita et al. 1994; Hart and Kornman 1997; Li et al. 2004a). So, when autosomal-dominant traits are concerned, mutation of one of the two alleles may cause the disease, whereas in autosomal-recessive situations, as it is known both alleles must be mutated for the disease to occur. In the first case, the resulting severe congenital neutropenia is due, as indicated, by a mutation in the neutrophil elastase gene (ELA2). Such an enzyme is a chymotryptic serine protease localized in neutrophils and monocytes and is the point of inhibition caused by the α-serpin-antitrypsin. The other types of autosomal-dominant neutropenias such as that caused by mutations in the growth factor independent 1 gene (GFI1) whose gene product plays an essential role in hematopoiesis and in neutrophil differentiation. In addition there are other severe congenital neutropenias such as Kostmann syndrome that are of recessive trait and that involves HAX1 gene whose product is a mitochondrial protein that works in signal transduction and also controls neutrophil apoptosis. The associated periodontitis and the failure of antibiotics as tool of treatment in all these forms of neutropenic conditions is constant.

Interesting mutations are IRAK4 (acromym for **i**nterleukin-1 **r**eceptor-**a**ssociated Kinase and characterized by impaired cellular responses to interleukin-1) and NEMO (acronym, standing for **N**F-Kappa-B **e**ssential **mo**dulator affects tumor necrosis factor receptor stimulation, impairment of NF-kappaB activation, and also impaired cellular response to IL-1R, TLR) that occur in otherwise healthy children but that suffer from recurrent invasive pneumococcal disease (Casanova and Abel 2005; Ku et al. 2007). The recurrent episodes take place in at least 2 % of patients that include infants or adults suffering from HIV infection, organ failure, or cancer (Eskola et al. 1992); the situation in the case of infant population may include sickle-cell disease, congenital, or acquired cerebrospinal fluid leaks (Wong et al. 1992), plus primary immunodeficiencies that include defects in the classic complement activation pathway (Figueroa and Densen 1991) or congenital asplenia. All these aspects start a process that leads to the phagocytic interruption of opsonized bacteria by splenic macrophages. NEMO and IRAK4 mutations do aggravate the situation by involving impaired mucosal and systemic inflammation responses. These mutations although initially thought to be very rare there are almost 200 cases worldwide and all of them are quite sensitive to be affected by pyogenic Gram-positive microorganisms (Picard et al. 2011). Patients affected with this

mutation exhibit low-level or delayed fever, as well as slow rise of the inflammatory markers and antibodies against estreptococal carbohydrates (Enders et al. 2004). Particularly, in a NEMO situation, the degree of responsiveness varies from anything to a variety of pathways being affected (Puel et al. 2004), although generally NEMO patients suffer from lack of IL-10 production (Puel et al. 2004; Hanson et al. 2008) and impaired antibody response to glycans, including to pneumococcal capsules. Devora et al. reported in 2010 the T437G missense mutation that originates a V146G substitution that resulted in infectious susceptibility to methicillin-resistant *S. aureus* thus broadening the clinical spectrum associated with the disease. Also, and in 2005, it was reported (Chapel et al. 2005) the first case of systemic shigellosis caused by *Shigella sonnei.* These authors concluded that immunity mediated by IRAK-4 must be crucial for the inflammatory response to this bacterium in the intestinal mucosa and that IRAK-4 deficiency and related disorders should be considered exhibiting these pathologies. Patients with this mutation may show specific responsiveness to pyogenic estreptococci but also, to other microorganisms (Picard et al. 2003), and so, patients IRAK-4 mutation, according to Picard et al. (2011) must be immunized with *S. pneumoniae* conjugated and nonconjugated vaccines, *Haemophilus influenzae* conjugated vaccine, and *Neisseria meningitidis* conjugated and nonconjugated vaccines. Besides a life-lasting preventive antibiotic treatment is suggested (cotrimoxazole plus penicillin V), and even an empirical intravenous or subcutaneous IgG injections until the patient is at least 10 years old, is suggested (Picard et al. 2011). As indicated by these authors, the aforementioned mutations confer predisposition to invasive infections caused by *S. pneumoniae*, *S. aureus*, and *P. aeruginosa*, as indicated, particularly in children and a dramatic improvement with age due to the development of adaptive antigen-specific T- and B-lymphocyte responses.

The pathogenic and tumor-causing *Helicobacter pylori* if appropriately treated with antibiotics is completely eradicated in *ca* 75 % of patients but Dong et al. reported in 2008 have reported that BCL10 nuclear expression and t(11;18)(q21; q21) indicate nonresponsiveness to *H. pylori* eradication of Chinese primary gastric MALT lymphoma.

Some nuclear mutations (i.e, resistance to the aminoglycoside paromomycin) occurring in certain transformed human cell lines such as HeLa, are of striking interest since, unlike previously reported ribosomal mutations that originate general resistance to amynoglycosides, seems to derive from differences in the intracellular metabolism of paromomycin (Bunn et al. 1986). This broad spectrum antibiotic, also known as monomycin, was first isolated from the actinomycete *Streptomyces krestomuceticus* (Davidson et al. 2008) that acts by inhibiting protein synthesis by binding to the 16S rRNA at the A-site, affecting translational fidelity by stabilizing two adenines (A1492 and A1493; Romanowska et al. 2011) in the flipped-out state and thus disrupting translocation of tRNA during the translation process (Vicens and Westhof 2001)and soon introduced in humans to treat cryptosporidiosis or leishmaniasis (Sundar et al. 2007). The antibiotic is also effective against both gram-positive and gram-negative bacteria. The resistance against paromomycin must not be mistaken with the “streptomycin –dependent phenotype” (today known

as "streptomycin dependent" mutants) caused by mutations that originate depending on chemically unrelated drugs that by binding to the ribosome induce misreading in vitro and suppression in vivo (Gorini et al. 1967). In bacteria, ribosomes containing 16S rRNA with mutations at positions 1408, 1407 + 1494, or 1495 had reduced affinity for the antibiotic (Recht et al. 1999) and Romanowska et al. (2011) found that most pronounced changes as far as resistance to this antibiotic, were observed in U1406C:U1495A mutants, where important hydrogen bonds between the RNA and paromomycin were disrupted.

TRMU nuclear gene encodes for the enzyme 5-methylaminomethyl-2-thiouridylate-methyltransferase, a mitochondrial-specific tRNA-modifying enzyme. Mutations in this gene may result in fatal liver failure (Zeharia et al. 2009), particularly in infant population, so mutations in this gene may lead to cirrhosis as the primary manifestation (Schara et al. 2011). The human TRMU gene containing 11 exons encodes for a 421 residue protein showing high homology with bacterial TRMU-like proteins of bacteria. Mitochondria of tissues with high respiratory or metabolic rate (i.e., heart and liver) are heavily loaded with this enzyme. Individuals exhibiting the deafness-associated 12S rRNA A1491G mutation may have phenotypically modulated the hearing loss with the TRMU protein that is ubiquitously expressed in various tissues, but abundantly in tissues with high metabolic rates including heart, liver, kidney, and brain. Immunofluorescence analysis of human 143B cells expressing TRMU-GFP fusion protein demonstrated that the human TRMU localizes and functions in mitochondrion. Furthermore, we show that in families with the deafness-associated 12S rRNA A1491G mutation there is highly suggestive linkage and linkage disequilibrium between microsatellite markers adjacent to TRMU and the presence of deafness. These observations suggest that human TRMU may modulate the phenotypic manifestation of the deafness-associated mitochondrial 12S rRNA mutation (Yan et al. 2006; Guan et al. 2006). Other nuclear mutations affecting mitochondrial DNA depletions, thus causing combined respiratory chain defect and liver failure may take place at genes POLG, DGUOK, and MPV17 (Gaignard et al. 2013).

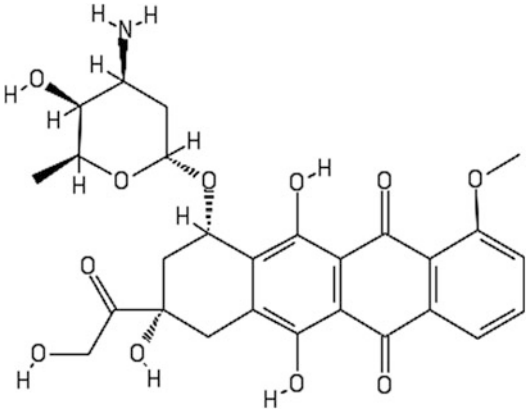
MTO1 is a nuclear gene located in chromosome 6 of humans (mitochondrial translation optimization) encoding for a protein that catalyzes the 5-carboxymethylaminomethylation of the wobble uridine base in three mitochondrial tRNAs (Ghezzi et al. 2012). It is involved in accuracy and efficiency of mtDNA translation, thus affecting the three mitochondrial RNAs. Mutations affecting this gene (i.e., G1282A (p.Ala428Thr)), result in hearing loss pathologies and hypertrophic cardiomyopathies.

Adriamycin (name standing for Adriatic sea), also known as doxorubicin (because its reddish color), and daunorubicin known as daunomycin (indeed, prototype compounds for the anthracycline group), originally described as produced by *Streptomyces peucetius* var. *caesius* (Arcamone et al. 1969) that acts as intercalating agent in the DNA (Momparler et al. 1976; Fornari et al. 1994) and used in cancer chemotherapy with a variety of collateral effects (hair loss, myelosuppression, diarrhea, anaphylaxis, and most importantly heart damage and liver dysfunction; Rossi 2013), in the treatment of AIDS (Johansson et al. 2006), or as antimalarial

drug (Friedman and Caflisch 2009). Today there are well over 2000 analogs of doxorubicin (Table 4) and by 1991, 553 of them had been already evaluated as anticancer drugs (particularly for solid tumors); unfortunately all maintained the above-mentioned cardiotoxicity (Di Marco et al. 1969) and the syndrome characterized by cell vacuolization and mitochondrial degeneration accompanied by myofibrillar breakdown, lymphocytic infiltration, and myocardial cell destruction appeared 2–3 months after the end of treatment with total drug dose of 500–600 mg/m^2 (Blum and Carter 1974; Fioretti et al. 1976). This antibiotic is not restricted to activity on tumor cells, but also shows activity on a vast variety of microorganisms as reported by Pittillo and Woolley in 1971.

Resistance to adriamycin was soon reported by DanØ in 1972 in a case of in vivo treatment of an Ehrlich ascites tumor. The acquisition of resistance against adriamycin (as well as other DNA-binding antibiotics) is a multistep process including drug uptake, ion transport, and energy production as well as regulation of macromolecular synthesis (Di Marco 1971) indeed the results obtained by Nishimura et al. in 1979 suggested that the adriamycin resistance could be due to alteration of the cytoplasmic membrane that resulted in diminished uptake and concomitantly a possible accelerated excretion of the antibiotic. The second premise was demonstrated in the same year (1979) by Inaba et al. while studying the active

Table 4 Doxorubicin

Chemical Structure: Doxorubicin (adriamycin)	
	
CAS number	23214-92-8 (doxorubicin free base) 25316-40-9 (doxorubicin hydrochloride)
Molecular formula	$C_{27}H_{29}NO_{11}$
Source	*Streptomyces peucetius* var. *caesius*
Mechanism of action	It acts as intercalating agent in the DNA
Uses	Cancer chemotherapy, in the treatment of AIDS or as antimalarial drug

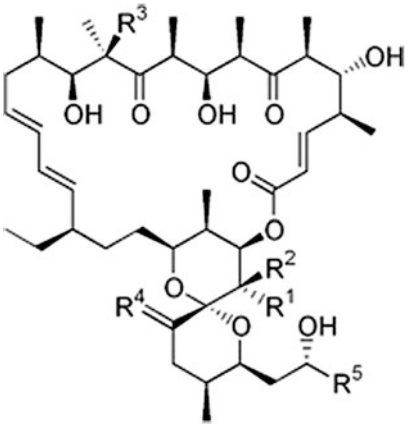
outward transport mechanism for anthracyclines in P388 leukemia cells in sensitive or resistant cells. Resistance of mammalian cells to this antibiotic might be easily recognizable by their higher resistance to X-ray denaturation (Belli and Harris 1979). Much of the resistance mechanisms have been learnt by studying the adriamycin-producing *Streptomyces peucetius,* since producing strains contain two genes, *drrA* and *drrB,* both of which required for daunorubicin and doxorubicin resistance in other *Streptomyces* species such as *S. lividans*. The product of the first gene belongs to a large ABC family with one nucleotide (ATP) binding domain (to which P-glycoprote of cancer cells belongs; Gottesman and Pastan 1993), and homologous to the *mdr* (standing for multiple drug resistance)-encoded protein in mammals (Guilfoile and Hutchinson 1991), that also confers resistance to daunorubicin and doxorubicin, while protein *DrrB*, being hydrophobic is integrated in the bacterial membrane acting as efflux pump protein showing eight membrane-spanning domains with both the N-terminus and the C-terminus in the cytoplasm (Gandlur et al. 2004); in fact membrane lipid composition is involved in resistance adriamycin-type (Le Moyec et al. 1996). Both genes were cloned in *Escherichia coli* (Kaur 1997) that resulted to be resistant to doxorubicin and other structurally unrelated drugs. There is a strong dependence of both proteins on each other for their expression as well as for functional role, and in fact they behave as domains of a larger protein (Kaur and Russell 1998); The interlink between both subunits takes place through cysteine residues (Kaur et al. 2005). Similar genes have been also detected in *Mycobacterium tuberculosis* and in turn are related with exhibiting multiple resistances to unrelated antimicrobial agents; in addition the same phenotype is originated when functional overexpression of these genes takes place in nonpathogenic mycobacteria such as *M. smegmatis* (Choudhuri et al. 2002). In 2014 Li et al. produced a fine paper that demonstrated after a first time in-depth study of the *S. peucetius* drug resistance system, based on the efflux system DrrAB, as being responsible for multiple drug resistances. A horizontal dispersion of this gene cluster would originate resistance to this group of antitumoral drugs.

Resistance to Adriamycin (doxorubicin) may be however be overcome by simultaneously using oligomycin, in which case the fatal apoptosis is triggered (cells tend to accumulate higher amounts of doxorubicin), thus controlling the outgrowth of cancerous cells (Li et al. 2004b).

Resistance to oligomycin (Table 5) is one of those examples involving mutations both in the nuclear genome and in the mitochondria. It has been assigned to chromosome number 10 in humans by Webster et al. in 1982 while working with the human fibrosarcoma HT1080, concluding that the determinant gene as nuclear and codominant. The mutation resulted in that mitochondrial ATPase exhibited less susceptible to oligomycin than the wild type allele. Manfredi et al. dag into this aspect and reported in 1999 that human fibroblasts exhibiting the mutation T8993G (leucine instead of arginine at position 156 of ATPase 6) showed that when selected on galactose, the antibiotic caused a significant increase in the fraction of wild-type molecules (from 16 to 28 %).

Table 5 Oligomycins

Chemical Structure: Oligomycins



The groups at positions R1–R5 vary according to the type of oligomycin.

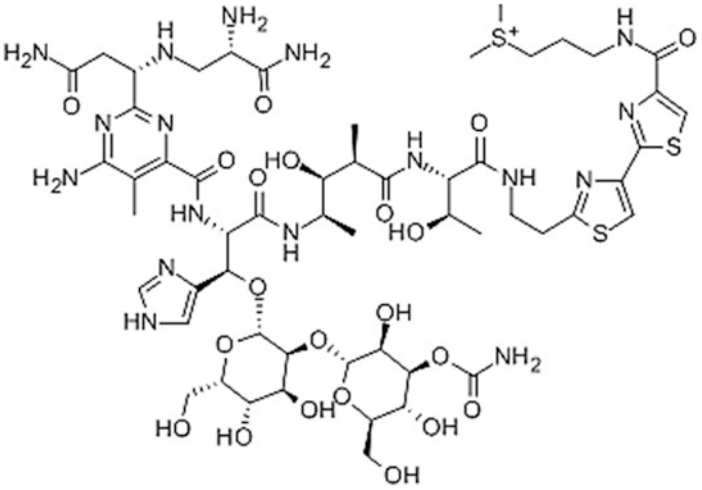
CAS number	1404-19-9 (mixture of A, B, and C isomers)
Molecular formula	$C_{45}H_{74}O_{11}$ (for Oligomycin A)
Source	*Streptomyces* sp.
Mechanism of action	It inhibits ATP synthase by blocking its proton channel (F0 subunit)
Uses	It is used in scientific research to modulate ATP synthesis in studies of cell or organ function. It is not used therapeutically

Oligomycin is a macrolide antibiotic that was described as being produced by *Streptomyces* sp by Smith et al. in 1954 as a new antifungal antibiotic which targets mitochondrial ATPase. The biological properties were described four years later (Marty and McCoy 1959). The inhibition of ATP synthesis (Lardy et al. 1958) caused by this antibiotic significantly reduces the electron flow through the electron transport chain, although not completely, thus occurring a proton leak into the mitochondrial matrix mediated by uncoupling proteins such as thermogenin (this protein is exclusively localized in brown fat mitochondria; Cannon et al. 1982). The amino acid residues that form the oligomycin-binding in ATPase site are highly conserved in eukaryotic organisms, including man, and are clearly different from that of bacteria and this explains their different sensitivity. Despite more than 50 years of studies on oligomycin and ATPases has elapsed, only recently Symersky et al. (2012) have found the primary binding subunit of oligomycin, resulting to be c subunit of F0 (subunit c is an integral membrane protein consisting of two helices). As a consequence of oligomycin effects, higher eukaryotic cells may develop serious syndromes (including neurological disorders) that may degenerate into life-threatening diseases.

Bleomycin is a glycopeptide (nonribosomal synthesis) and belongs to the group of cationic antibiotics. It is produced by *Streptomyces verticillus* (Table 6) and first discovered in 1966 by Dr. Umezawa who reported on its anticancer activity. The antibiotic is also active on bacteria and is totally dependent on RNA synthesis (Cohen and Josephine 1976; Holmes et al. 1993). Today this antibiotic is important in the treatment of Hodgkin's lymphoma, squamous cell carcinomas, testicular cancer, or plantar warts (Lewis and Nydorf 2006). This antitumor activity has been reported to be representatively increased by a small (25 kDa) protein produced by *Bacillus thuringiensis* subsp. *israelensis* toxin (Yokoyama et al. 1991). The mechanism of action involves breaking DNA (Takimoto and Calvo 2008), near or at the replication forks (Yamamoto and Hutchinson 1979), thus causing a variety of lethal mutations making the cells unable to grow. Additionaly, bleomycin has antiviral properties against drug-resistant HIV strains and indeed is currently used as antiviral agent (Georgiou et al. 2006). The drug has however serious collateral effects including pulmonary fibrosis and possibly sensitivity to oxygen toxicity through Cytokine IL-18 and IL-1 produced in bleomycin-induced lung Injury

Table 6 Bleomycin A2

Chemical Structure: Bleomycin A2*



* Bleomycin is a mixture of glycopeptide antibiotics containing primarily Bleomycin A2 and B2

CAS number	11116-31-7 (Bleomycin A2) 9060-10-0 (Bleomycin B2)
Molecular formula	$C_{55}H_{84}N_{17}O_{21}S_3$ (Bleomycin A2) $C_{55}H_{84}N_{20}O_{21}S_2$ (Bleomycin B2)
Source	*Streptomyces verticillus*
Mechanism of action	It inhibits DNA metabolism
Uses	Antitumor antibiotic, it is used to treat Hodgkin's lymphoma, squamous cell carcinomas, testicular cancer or plantar warts

(Hoshino et al. 2009); pulmonary fibrosis may be however prevented in mice, if an adenovirus harboring the bleomycin-resistance gene is supplied together with the antitumoral (Tran et al. 1997). Soon after discovery, the antibiotic showed activity on mycobacteria (Schröder and Hensel 1972) and was even able to induce prophages of lysogenized bacteria (Fujimura et al. 1972). Resistance to bleomycin has been studied in bacteria as being due to a protein coded by a bleomycin-resistance gene (*ble*) present in bleomycin-producing strains. The protein binds strongly to the antibiotic making it totally unable to exert its action on bacterial DNA (Gatignol et al. 1988). *Ble* gene is contained within transposon Tn5 making it thus amenable for researchers to study the intimate mechanisms of resistance to bleomycin in bacteria (Blot et al. 1994). In humans, a single nucleotide polymorphism (SNP A1450G) in the bleomycin hydrolase gene encoding a specific neutral cysteine protease able to metabolise bleomycin has been proposed as a plausible candidate to the observed variation in bleomycin sensitivity (Maffei et al. 2008).

The complement system is a multicomponent (C_1 trough C_9) and sophisticated mechanisms present in warm-blooded vertebrates that play a vital role in the innate host defense against microbes (particularly Gram-negative bacteria) producing finally bacterial lysis. Alterations in one or more of its components may cause apparent resistance (phenotypic resistance) to antibiotics and higher doses must be supplied to patient to eliminate the pathogen, being this the origin of recurrent episodes in short periods of time. Human C8 forms part of the membrane attack complex (MAC) which generates pores in the microbial membrane and hence lysis (Ross and Densen 1984; Figueroa and Densen 1991; Arnold et al. 2009). C8 is an oligomeric protein composed of three subunits (α, 64 kDa; β, 64 kDa; γ, 22 kDa; Arnold et al. 2009) encoded by C8A, C8B, and C8G genes, respectively. C8A and C8B are linked closely on chromosome 1p32, whereas C8G is located on 9q22.3–q32 close to a cluster of lipocalin genes (that encode proteins involved in the transport of small hydrophobic molecules) (Kaufma et al. 1989; Arnold et al. 2009). Mutations in the C8B gene (normally in exon 9 C > T transitional mutation; Saucedo et al. 1995) originates a considerable decrease (up to 85 %) in the amount of serum C8β protein. In addition, Arnold et al. reported in 2009 two more mutations: a novel duplication mutation, c.1047_1053 dupGGCTGTG in exon 7 that introduced a frame shift, resulting in the addition of seven novel amino acid residues and a premature stop codon, and a previously reported mutation, c.271C > T in exon 3.

Other mutations may affect complement factor I, as the potential cause of neuroinflammation in acute hemorrhagic leukoencephalitis (Broderick et al. 2013). This is a rare disorder, fatal if untreated, characterized as indicated by these authors by rapid neurologic decline, peripheral and cerebrospinal neutrophilia and focal demyelination observed on brain biopsy or postmortem examination (Payne et al. 2007). The etiology of the disease has been associated with upper respiratory infections such as mumps, and colonization by *Mycoplasma pneumoniae*. Complement fragment C5a is produced following the activation of the complement cascade, it is a potent chemoattractant for leukocytes and involved in the pathogenesis of several inflammatory disorders as well as myocardial infarction

(Gallin et al. 1992). The effects of C5a are mediated by G protein-coupled receptors expressed on the surface of the cells. Receptors such as human C5a Receptor have seven transmembrane domains linked by alternating intra- and extracellular loops (Boulay et al. 1991). Mutation of Lys68, Leu72, or Arg74 at the C-terminus (intracellular domain) of hC5a receptor, substantially inhibits binding ligand and receptor. Studies with antibodies against the hC5a receptor (Oppermann et al. 1993) as well as receptor mutations (DeMartino et al. 1994) have highlighted the role of the receptor N-terminus, containing a number of aspartate residues, in ligand binding. Mutations in both C-terminus or N-terminus (including that of glutamate 199; Monk et al. 1995) result in defective complement activation, thus interrupting the transducing signal to the C8 subunit and difficulty in eradicating microbial pathogens.

In general, deficiencies in the complement cascade comprise between 1 and 10 % of all primary immunodeficiencies (Grumach and Kirschfink 2014) and still considered rare syndromes among clinicians but the genetic deficiency of any early component of the classical pathway (C1q, C1r/s, C2, C4) is mostly associated with autoimmune diseases, whereas individuals, deficient of properdin or of the terminal pathway components (C5–C9), are highly susceptible to meningococcal disease (Grumach and Kirschfink 2014). As many genes are involved (Table 7) in the complement cascade, the probability of encountering syndromes (included those caused by bacteria) associated to total lack of functional genes or disfuntional alleles, is high. This is the case for a deficiency of C1 Inhibitor or the dysfunctional corresponding alleles concerning factors H, MBL, or MASPs.

4 Beneficial Effects of Antibiotics on Mutation-Caused Human Syndromes

Contrariwise to what one could expect from the mutation world, there are many, that instead of being neutral or exhibiting a variety of deleterious effects to the subject, are amenable to be phenotypically corrected by some antibiotics. Particularly, this is the case of certain hepatobiliary disorders observed in cystic fibrosis (the most common lethal autosomal recessive disease among Caucasians) and aminoglycosides (Wilschanski et al. 2000; Zsembery et al. 2002). The disease that is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene that contains a premature termination signal so, little or no CFTR chloride channels are produced, depending on the mutation (Howard et al. 1996; Bedwell et al. 1997). Gentamicin topically applied in the nasal epithelium (0.3 %, 3 mg/ml three times daily for 14 days) also had effect in producing functional CTFR channels (Wilschanski et al. 2000) and significant repolarization of the nasal epithelium occurred. Zsembery et al. studied in 2002 the role of CFTR and of Ca^{++} and Cl^{-} channels in promoting HCO_3^{-} secretion. The studies were performed by isolation of cholangiocytes from one patient with cystic fibrosis and exhibiting

Table 7 Some nuclear human mutations related to antibiotics responsiveness

Gene/mutation	Response
CFTR/delta F508	CFTR improper folding and degradation in endoplasmic reticulum
CFTR/G542X	Stop codon and no CTFR is produced; the mutation is phenotypically corrected by aminoglycosides
CFTR/W1282X	Nonsene mutation. Truncated CTFR gene product
CFTR/3849 + 10 kb C > T	Aberrantly spliced CFTR transcripts containing an additional cryptic exon
TRMU (also known as MTO2)	Mutations in this gene may result in fatal liver failure, but individuals showing the deafness-associated 12S rRNA A1491G mutation, may have their deafness modulated
MTO1/G1282A	Mutations in this gene (i.e., G1282A (p.Ala428Thr)) cause relevant decrease in the accuracy and efficiency of mtDNA translation affecting the three mitochondrial RNAs and generating hypertrophic cardiomyopathies and involved in the pathogenesis of deafness-associated mitochondrial 12S rRNA A1555G mutation. Phenotype is hardly worsened in the presence of a paromomycin-resistant (P(R)) mitochondrial rRNA mutation
NEMO/T437G	Missense mutation that originates a V146G substitution in the encoded polypeptides of affected people that results in infectious susceptibility to methicillin-resistant *S. aureus*
POLG, DGUOK, and MPV17	Mutations of these nuclear genes are the major causes of combined respiratory chain defects in early infancy (Gaignard et al. 2013)
IRAK4 and NEMO	Mutations in IRAK4 and NEMO originate recurrent infection by *Streptococcus penumoniae*
G415A	Transitional mutation in chromosome 11 in humans, resulting in loss of antibiotic efficiency and periodontal disease with complete primary teeth loss: Papillon–Lefèvre syndrome.
BCL10 nuclear expression and t(11;18)(q21;q21)	Nonresponsiveness to *Helicobacter pylori*
ERY2305 and ERY2309	Mutations originating eritromycin phenotype in HeLa cells
Exon 9 C > T transition in C8beta gene Exon 7 1047_1053 dupGGCTGTG Exon 3 C271T transition	These mutations affect complement C8 resulting in low amounts or unterminated polypeptides that originate difficulties in

(continued)

Table 7 (continued)

Gene/mutation	Response
Exon 3 C388T transition Exon 3, C298T transition Exon 6, C847T transition	eliminating pathogens and the appearance of recurrent forms of diseases such as meningococcal meningitis (Saucedo et al. 1995; Arnold et al. 2009)
Mutations in aminoacids Lys68, Leu72, or Arg74 of hC5aR	Failure to completely or partially completing the complement activation pathway to the C8 level, thus expressing difficulties in eradicating microbial pathogens
EVER1 and EVER2	Mutations in two genes of unknown function that are normally expressed in keratinocytes and the cause of epidermodysplasia verruciformis (EV)
Mutation at 395nt (6q-mapping) interferon-gamma receptor 1 gene	Mutation causing sensitivity to weakly virulent mycobacteria
Mutations affecting C1q gene: location in1p36	General infection sensibility and systelic lupus erytematosus
Mutations affecting C1r/s genes: location 12p13	
Mutations affecting C2 gene: Location 6p21	
Mutations affecting C4 (C4A, C4B) gene: location 6p21	Although often clinically nonapparent, the mutations explain infections in general and systemic erythematosus-like syndromes
Mutations affecting C3 gene: location 19p13	Pyogenic infections
Mutations affecting C5 gene: location 9q33-34	Susceptibility to Neisserial infections
Mutations affecting C6 gene: location 5p13	Susceptibility to Neisserial infections
Mutations affecting C7 gene: location 5p13	Susceptibility to Neisserial infections
Mutations affecting C8α/β genes: location 1p32	Susceptibility to Neisserial infections
Mutations affecting C8γ gene: location 9q34	Susceptibility to Neisserial infections
Mutations affecting C9 gene: location 5p14-p12	Asymptomatic Neisserial infections
Mutations affecting Factor B gene of the alternative complement fixation pathway: location 6p21	Susceptibility to Neisserial infections
Mutations affecting Factor D gene of the alternative complement fixation pathway: location: 19p13	Susceptibility to Neisserial infections
Mutations affecting Factor H gene of the alternative complement fixation pathway: location 1q32	Susceptibility to general infections or autoimmune diseases due to either overactive or underactive polypeptides
Mutations affecting Factor I (also known as C3b/C4b inactivator) gene: location 4q25	Deficiency of factor I is associated with recurrent bacterial infections in children, and involved in the haemolytic uremic syndrome (Saunders et al. 2007)

(continued)

Table 7 (continued)

Gene/mutation	Response
Mutations affecting Complement receptor type 2 gene (CR2; also known as complement C3d receptor, Epstein-Barr virus receptor orCD21: Location 1q32	Infections associated to common variable immunodeficiency (CVID), which is the most prevalent cause of primary immunodeficiency (Park et al. 2008b)
Mutations affecting Mannan-binding lectin gene(MBL, also called mannose-binding protein) : location 10q11	MBL is a protein belonging to the collectin family and produced in the liver and able to initiate the complement cascade by binding to pathogen surfaces. Underproduction may lead to recurrent bacterial infections
Mutations affecting Ficolin 3 gene: location 1p36	The protein can activate the complement pathway through the activation of the lectin pathway. Underproduction leads to respiratory infections and necrotizing enterocolitis
Mutations affecting MASP-2 gene: location 1p36	MASP-2 is similar to the C1s molecule, so it is involved in the classical complement fixation pathway. Underproduction may result in respiratory infections

TRMU: TRMU nuclear gene (22q13) encoding for the human mitochondrial enzyme 5-methylaminomethyl-2-thiouridylate-methyltransferase, a mitochondrial-specific tRNA-modifying enzyme

POLG: nuclear gene (15q25) encoding for human mitochondrial DNA polymerase γ (Zullo et al. 1997)

DGUOK: nuclear gene (2p13) encoding for human deoxyguanosine kinase (Johansson et al. 1996)

MPV17: nuclear gene encoding for a mitochondrial inner membrane protein involved in the metabolism of reactive oxygen species. Mutations in this gene have been associated with hepatocerebral diseases (Karasawa et al. 1994)

MTO1: nuclear gene (chromosome 6, mitochondrial translation optimization) encoding for a protein that catalyzes the 5-carboxymethylaminomethylation of the wobble uridine base in three mitochondrial tRNAs (Ghezzi et al. 2012)

EVER1 and EVER2: epidermodysplasia verruciformis (EV) is a rare disorder characterized by widespread human papillomavirus infection and malignant transformation. EV may be caused by mutations of the genes EVER1 or EVER2, which are located on the EV1 locus, 17q25

CTSC: Nuclear gene (11q14.2) encoding for cathepsin C

the Delta F508/G542X mutations. The results were consistent with other reports in that premature stop codon mutations such as G542X could be overpassed by treatment with aminoglycoside antibiotics (that increase the frequency of erroneous insertion of nonsense codons and hence allowing the translation of CFTR alleles to the end of the gene; Wilschanski et al. 2000) and that gentamycin restored activation by cAMP of Cl^- current and HCO_3^- secretion. Indeed, aminoglycosides have been quite useful to understand the synthesis of protein in microorganisms since its addition (streptomycin) was able to suppress a number of early stop-generating codons mutations in *E. coli*, *Saccharomyces cerevisiae,* or *Tetrahymena thermophila* (amber mutation TAG) due to a general decrease in the fidelity of the proteins synthesis process, so instead of stop signal an additional

amino acid is incorporated into the polypeptide and translations continues to the end of the gene (Palmer and Wilhelm 1978; Singh et al. 1979; Burke and Mogg 1985).

Cystic fibrosis is one of the most common genetic disorder among Caucasians, that according to Haenisch et al. (2010) occurs once among 2500 live births in the USA and with a frequency as high as one in every twenty, in some populations (Kerem et al. 1989; Mateu et al. 2002). Infectious bacteria most of the times colonize the lungs and *ca* 80 % of patients are infected with *Pseudomonas aeruginosa* (Lyczak et al. 2002). Strains belonging to this species and as a matter of fact, almost all species of the genus (including the more recent one *Burkholderia*) are able to grow aerobically almost on anything due to their enormous genetic plasticity and ability to induce a large variety of operons depending on the ecological niche they colonize; no wonder why *P. aeruginosa* tends to colonize the lungs of patients affected by this disease. The fact, however, that normal people are resistant to such colonization points toward the existence of mutations in humans sensitive to the disease. Pier et al. reported in 1996 that CFTR could act as a receptor to bind and help to phagocytize *P. aeruginosa*, and in fact antibiotic protection-based bacterial invasion assays showed a CFTR-dependent internalization of this pathogen. The defects in CFTR induce abnormal chloride and sodium transport and besides the lungs, it may also affect pancreas, intestine, as well as the male genital system (Davis et al. 1996). In the case of lungs the associated suppurative bronchiectasis leads in severe cases to early death of patients.

According to Wilschanski et al. in 2000 more than 800 mutations affecting the CFTR gene have been identified, these missense, deletions, insertions, frameshift, or nonsense mutations (Tsui 1992), premature stop mutations accounting for ca 5 % of the total mutant alleles in CF patients (Kristidis et al. 1992). Certain subpopulations, however may have drastically increased this percentage (more than 60 % in the Ashkenazi Jewish population in Israel according to Shoshani et al. in 1992). Other mutations such as 3849 + 10 kb C > T mutation described by Chiba-Falek et al. in 1998 produce aberrantly spliced CFTR transcripts containing an additional cryptic exon, or at least only aberrantly spliced CFTR RNA could be detected in the lung of a patient before undergoing lung transplantation. Indeed, the severity of the lung disease did correlate with the insufficienct amount of normal CFTR RNA in the cells.

Renal function may be affected in cystic fibrosis as well, so Stanton (1997) plus others researchers have accumulated considerable evidence supporting a role for CFTR in mediating Cl^- secretion by the distal tubule, the cortical collecting duct and the inner medullary collecting duct. CFTR, besides working as a 3′,5′-cAMP-activated Cl^- channel, it may play a role in intracellular protein trafficking except in the most common mutation in humans (delta F508) that causes improper folding of CFTR and finally degraded in the endoplasmic reticulum where it is degraded without exerting its biological action (Stanton 1997). CFTR has also been involved in phagosome trafficking and acidification in pulmonary cells (Di et al. 2006).

Other times some DNA-damaging antibiotics such as mitomycin may be used as an aid in the diagnosis of certain tumors (i.e., Wilms'-tumour; Imray et al. 1984).

The response to gentamicin in preterm infants is another aspect worth of being treated in this chapter, because its use in the neonatal intensive care units has resulted in dramatic increase in the rates of survival (Lorenz et al. 1998), although the prevalence of deafness is ca 1–2 % also including the low birth weight babies, and is up to ten times more prevalent than in term babies (Marlow et al. 2000; Bitner-Glindzicz et al. 2014). The cause of such acquired deafness has been ascribed to a variety of reasons that include medication (Vohr et al. 2000) and infection (Yoshikawa et al. 2004) which commonly occurs in the neonatal period and aminoglycosides such as gentamicin are profusely used for stopping potential life threatening bacterial sepsis. A genetic predisposition (1 in 520 children; Bitner-Glindzicz and Rahman 2007) renders some of them far more susceptible to deafness even at therapeutic range of gentamycin or aminoglycosides in general to such an extent that even a single dose of the aminoglycoside may cause permanent hearing loss. Such predisposing mutation is the mitochondrial A1555G transition that is maternally inherited (Bitner-Glindzicz et al. 2014). Early reports showed that deafness could be close to 100 % in patients harboring this mutation and treated with aminoglycosides; however some identical twins are separated from this generalization in that one of the them was insensitive to aminoglycoside-caused deafness, whereas the other developed hearing loss. These findings suggest that other factors in the case of gentamicin-caused deafness in new borns with mutation A1555G, must be involved (Bitner-Glindzicz et al. 2014) but perhaps alternative to amynoglycosides as first line antibiotics in preterm babies should be taken into account.

In the light of the reported in the present chapter it would be interesting, particularly in the individuals belonging to risk groups, to investigate their genotypes or mutated alleles in order to prescribe accordingly and avoid the generation of antibiotic-associated syndromes such as the described here.

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Different Approaches for Searching New Microbial Compounds with Anti-infective Activity

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Abstract New antibiotics are continuously required to combat antibiotic-resistant bacteria. This resistance increasingly limits the effectiveness of current antimicrobial drugs. Besides the problem of antibiotic resistance, new families of antibiotics are needed to enter the marketplace at regular intervals to face new diseases caused by evolving pathogens. Antibiotics toxicity is another reason for developing new antibiotics. Microorganisms are amazing producers of a great diversity of natural products including secondary metabolites. These metabolites constitute half of the pharmaceuticals on the market today and provide agriculture and livestock with many essential products. This chapter centers on the different approaches for searching new antibiotic-producing microorganisms with special emphasis in novel potential sources, bioprospecting, and new molecular tactics to look for novel compounds.

1 Introduction

Naturally occurring antibiotics can be produced by fermentation, an old technique that can be traced back almost 8000 years, initially for beverages and food production (Demain and Sanchez 2015). Thanks to technical improvements in screening programs, and the advance in separation and isolation techniques, the number of natural compounds discovered exceeds the half a million (Bérdy 2015). The discovery of penicillin by Alexander Fleming back in 1928 triggers a scientific revolution on searching novel compounds with antimicrobial activities. The mold, identified as *Penicillium notatum*, produced an active compound (penicillin) against *Staphylococcus aureus*. Later, other naturally occurring substances, such as streptomycin and tetracyclines, were isolated and used to fight microbial infections.

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As a consequence, life expectancy increased, at the same time as deaths caused by bacterial infection decreased. Unfortunately, after a while some microorganisms generate resistance to conventional compounds used for treatments and therefore, promoted evaluation of a broad number of new substances for anti-infective effects (Demain and Sanchez 2015). The continuous search for new molecules with antibiotic activities has undergone changes over the time. Hence, the procedures, techniques, targets, selection criteria, etc., have evolved in function of the appearance of new diseases, as well as new technologies that decrease the time from the first laboratory evaluation, until the product is placed on the market (Park and Thomas 2015). Due to the richness of compounds and the easy way to get them, microbial natural products have been one of the most important sources of new compounds with antibiotic activity (Bérdy 2015). From the 38,000 biologically active compounds that have been obtained from microbes, 75 % corresponded to antibiotics, which are mainly produced by Actinomycetes (51 %), followed by fungi (36 %) and eubacteria (13 %) (Bérdy 2015). Some decades ago, pharma industry people led the most important discovery programs of natural products. They expended a lot of resources in high-throughput screening (HTS) for searching novel molecules. Nowadays, major pharmaceutical companies have abandoned the antibiotic field, leaving much of the discovery efforts to small companies, biotechnology industries and universities (Butler et al. 2013). Traditionally, these natural compounds produced by bacteria and fungi generally have been the dominant source of clinically useful antibiotics, whereas the completely synthetic chemical scaffolds are in minority. Microbial antibiotic research has largely relied on bioactivity detection by means of two strategies, bioactivity-guided fractionation and chemical screening. Genomic age and molecular biology tools opened new doors for finding new secondary metabolite structures (Martin and Liras 2015). New "omics" approaches to search for new biosynthetic genes for antibiotic production included genome mining, single cell genome amplification, metabolomics and proteomining, among others.

The purpose of this chapter is to review different approaches for searching new antibiotic-producing microorganisms with special emphasis in novel potential sources, bioprospecting, and new molecular strategies to find out novel compounds.

2 Antibiotics

2.1 Importance and Definition

Since their introduction to therapy, antibiotics have been the primary option for treating bacterial infections and literally saved millions of lives (WHO 2011; Butler et al. 2013). Nevertheless, due to bacterial resistance, antibiotics tend to loss effectiveness over time, situation that continuously demands introduction of new antibiotic therapies.

However, it is necessary to clarify what an antibiotic is, because this term is surrounded by controversy, and its initial definition has been refined over time, even Selman A. Waksman who in 1947 defined an antibiotic as "a chemical substance produced by microorganisms, which has the capacity to inhibit the growth of and even destroy bacteria and other microorganisms," later added to his definition that "antibiotics should act in low concentrations." Subsequently, during the antibiotic golden age, due to the fact that most of the isolated antibiotics were effective only against bacteria, the term antibiotic was often used as synonym of antibacterial. Hence, this definition excluded antifungal, antiviral, or antiparasitic compounds (Bennett 2015). Even the World Health Organization preserves this term, defining antibiotic as "synonym for antibacterial used to treat bacterial infections in both people and animals" (WHO 2011).

However, according to Bennett (2015), most of the antibiotic definitions do not mention "the selective toxicity," a desirable property of antibiotics. Therefore, in contrast to a poison, an antibiotic can be able to kill bacteria without causing damage to human cells (Bennett 2015).

On the above basis, in this chapter the term "Antibiotic" will be referred as a chemical agent from natural, synthetic, or semisynthetic source, that at low concentrations exhibit antibacterial activity and presents selective toxicity; implying little or no damage to animal cells.

3 Microbial Diversity

Natural products have a huge beneficial potential for mankind. They are source of the majority of drugs in clinical use today, mainly secondary metabolites, which is especially true in the case of anti-infective agents (Taylor 2013). According to most views, 50–65 % of our pharmaceutical drugs are direct natural products, or biologically or chemically modified natural products (semisynthetic derivatives), or compounds obtained by chemical synthesis on the basis of natural product structures mimics, and analogs (Butler et al. 2013).

Traditionally, these natural compounds produced by bacteria and fungi have been the dominant source of clinically useful antibiotics, whereas the completely synthetic chemical scaffolds are in the minority (Wright 2014). Although exploitation of genomics, in silico drug design and target-based high-throughput screening of combinatorial compound libraries was believed that would improve the repertoire of new antimicrobials, unfortunately these methods remain less effective despite massive investment (Singh and Palaez 2008; Sams-Dodd 2005; Payne et al. 2007).

Of all microbial antibiotics, approximately 40 % are obtained from fungal strains, 40 % from actinomycetes, and 20 % from unicellular bacteria (Bérdy 2012). Unfortunately, only 1.5 % of the existing actinobacteria and about 5 % of the known fungi are cultivable by current methodologies, limiting the possibilities of isolating new species. However, microbes are always amazing, as one single

species may contain between 10 and 80 unexpressed biosynthetic clusters, just as reported for *Streptomyces coelicolor*, where already 25–30 independent clusters producing secondary metabolites have been identified (Ochi and Hosaka 2013).

The absolute number of discovered microbial secondary metabolites is continuously increasing. In the past, most of these compounds were isolated from *Streptomyces* species. However, today the share of isolated fungal derivatives has increased to 62–65 %, and the portion of actinomycete products decreased almost to 30 %. Among the underexplored bacterial taxa, myxobacteria certainly deserve the spotlight. Recent studies of myxobacteria have produced several new bioactive molecules (Bérdy 2015; Wenzel and Müller 2009).

Widely speaking, the increase in cyanobacterial secondary metabolites discovery is more significant, since from only 15 products initially reported, today we have over 1500 bioactive metabolites and, as in the case of marine-derived bioactive compounds, approximately 7000 antibiotics have been identified (Bérdy 2015). Many marine-derived compounds are similar to metabolites synthesized by terrestrial microbes, such as microsclerodermins, polytheonamides, nodularins, swinholides, etc., (Radjasa et al. 2011).

Most of the known taxa (80–90 %) are not antibiotic producers or only very poor producers, but there are privileged types of microorganisms that are the main factories of bioactive natural products principally the actinobacterial, some specific fungal, and other non-actinobacterial eubacterial strains (Table 1), Currently, almost 35,000 antibiotics have been isolated from microorganisms.

Depending on the isolated microorganisms, there is a variation on the microbial molecules distribution. For example, cyanobacteria and eubacteria produce more bioactive peptide type (62 and 53 %, respectively) molecules than any other bacteria, while fungi generate more terpenoids (18 %), and rare actinobacteria produce

Table 1 Number of microbial natural antibiotics

Microorganisms	Number	Proportion (%)
Actinobacteria	~14,500	
Streptomyces sp.		75.80
Other actinobacteria		24.20
Eubacterias	~4000	
Bacillus sp.		35.00
Pseudomonas sp.		23.75
Myxobacteriales		11.25
Cyanobacteria		10.00
Other bacteria including proteobacteria		20.00
Fungi	~15,860	
Microscopic		56.80
Aspergillus		13.80
Penicillium		10.40
Basidiomycetes		18.30
Other fungi including yeasts, slime molds, etc.		0.70

Table 2 Type of microbial antibiotics

	Terpenoids type (%)	Peptide type (%)	Macrocyclic lactone type (%)
Streptomyces	3.0	18	10.0
Rare actinobacteria	1.5	17	27.0
Fungal	18.0	10	5.5
Eubacteria	1.3	53	2.5–3.0
Cyanobacteria	3.0	62	–

a lot of macrocyclic lactone compounds (27 %) (Table 2). But there are some compounds, which are exclusive from certain microorganisms, like the tetracycline-type compounds, echinomycin-type, quinoxaline peptides, and polyene macrolides, which are derived from actinobacteria, mainly from *Streptomyces* species. In addition, brefeldin and cytochalasin type, peptaibol antibiotics and gliotoxin-like diketopiperazines, are frequently found among fungal metabolites (Bérdy 2015).

Of all the bioactive molecules isolated from microbial sources, 67 % of the actinobacterial products and 65 % of other eubacterial products exhibit antimicrobial activities, whereas only 36 % of fungal and 14 % of cyanobacterial compounds are antimicrobials. From these, 16,000 exhibit antibacterial (against Gram-positive, Gram-negative, and other bacteria, e.g., mycobacteria) activities, 6800 show antifungal (against microscopic fungi, yeasts, etc.) and about 700 have antiprotozoal activities (Bérdy 2015).

Microbial natural products have high steric complexity with many chiral centers, less nitrogen and more oxygen content, as well as less aromatic and more ether linkages (Bérdy 2012); their MW are between 400 and 600 Da. Generally, actinobacterial products ($\sim$550 Da) have more complicated structures than most fungal metabolites ($\sim$380 Da) (Clardy et al. 2006; Sheridan 2006).

Likely microbial metabolites have not evolved to act as drugs, but rather to fulfill normal functions in cell physiology and ecology (Wright 2014). At high concentrations, they may be toxic, but at low (subinhibitory) concentrations they may act as modulators or signal regulators.

Thanks to the new focuses and screening methods, all the "inactive" compounds already isolated, sooner or later will become bioactive, discovering their still unknown role in the regulation of life processes (Bérdy 2015).

4 Biosynthetic Pathways

Although natural antibiotics of microbial origin have a huge chemical architecture diversity, a wide range of them can be included in four general groups of secondary metabolites: polyketides; nonribosomal peptides; terpenoids; and lantibiotic bacteriocins, among others. The structure and functional diversity of these natural

metabolites are generated by specific enzymes, forming biosynthetic pathways frequently encoded in gene clusters with coordinated regulation (Brakhage 2013; Liu et al. 2013). Through understanding of the chemical logic and protein machinery in the producer microbes, it has been possible to discover novel natural antibiotics and explore their structural diversification to create semisynthetic antibiotics (Scheffler et al. 2013; Pidot et al. 2014a).

4.1 Polyketides and Nonribosomal Peptides

Polyketides (PKS), nonribosomal peptides (NRPS), and PKS-NRPS hybrid products, compose one of the major families of natural products with important clinical applications including antibiotics. The structural and functional diversity in PKS and NRPS products arises from a consecutive condensation and template-directed elongation of a set of monomer units catalyzed by enzymatic complexes known as polyketide synthases (PKSs) and nonribosomal peptide synthases (NRPSs), respectively (Fischbach and Walsh 2006). In the PKS assembly lines, the monomers are acyl-CoA substrates such as malonyl-CoA, methylmalonyl-CoA, and ethylmalonyl-CoA (Banskota et al. 2006), while the monomer units for NRPS assembly can be proteinogenic and nonproteinogenic amino acids and heterocyclic rings (Hur et al. 2012). The essential unit for PKSs and NRPSs consists of three domains responsible for (i) selection and/or activation of each monomer: acyltransferase domain (AT) for PKSs and adenylation domain (A) for NRPSs; (ii) propagation of the growing PKS and NRPS chain: acyl carrier (ACP) domain and peptidyl carrier protein (PCP) domain, respectively; and (iii) condensation and elongation of the growing polyketide or peptide chain: ketosyntase (KS) domain from PKSs and condensation (C) domain from NRPSs (Strieker et al. 2010; Tibrewal and Tang 2014). In summary, the AT/A domains select and/or activate each monomer unit as acyl-CoA or aminoacyl-AMP, and load them onto the ACP/PCP domain. The growing polyketide or peptide chain, are then loaded into the KS/C domain, which catalyzes C–C/C–N bond formation. After each extension, the fate of the resulting β-keto and peptidyl intermediaries can undergo further chemical transformations that depend on the presence of optional processing domains. In the case of PKSs, ketoreductase (KR), dehydratase (DH), and enoyl reductase (ER) domains are frequently found. NRPSs contain epimerization (E), oxidation (Ox), *N*-methylation (MT), amongst others (Fischbach and Walsh 2006). Finally, the TE domain is located in the termination module and catalyzes polyketide and peptide release either by hydrolysis or macrocyclization (Fischbach and Walsh 2006).

According to the architecture and mode of action of the enzymatic assembly lines, PKSs can be classified as type I, type II, and type III (Hertweck 2009). The modular bacterial type I PKSs, usually consist of archetypical large multidomain enzymes, organized into modules. Each module is responsible for only a single elongation cycle, and the number of modules, frequently correlate with the number

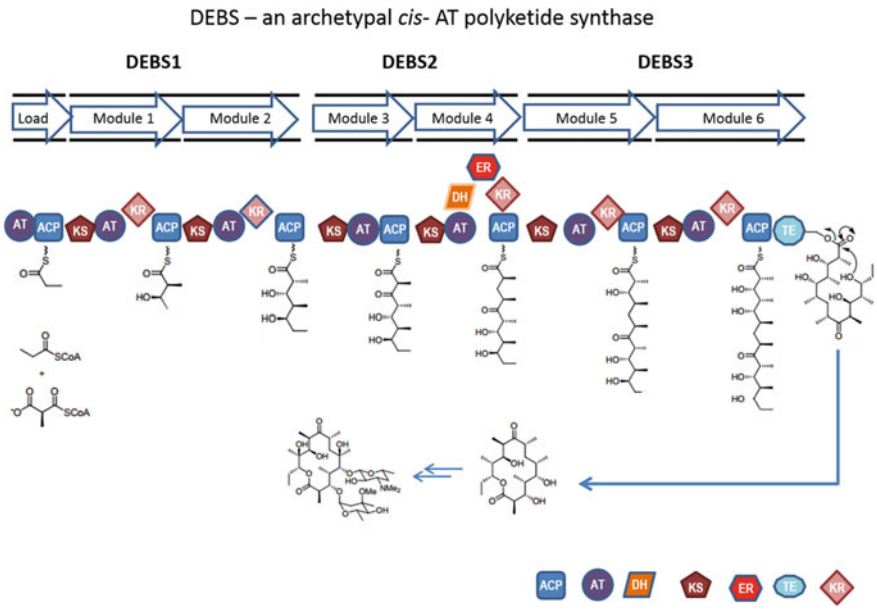

Fig. 1 Organization of the type I PKS 6-deoxyerythronolide B synthase (DEBS)

of extension cycles. A prototypical example of this PKS subfamily is the 6-Deoxyerythronolide B synthase (DEBS), involved in erythromycin biosynthesis (Khosla et al. 2007). The biosynthesis of 6-Deoxyerythronolide requires 28 active sites that are precisely arranged along three large polypeptides, denoted as DEBS1, −2 and −3, respectively (Fig. 1). As shown in this figure, each polypeptide has two unique modules responsible for one round of the polyketide chain extension and subsequent processing of its backbone. From the knowledge of this assembly line logic, combinatorial biosynthesis has been an attractive target for biosynthetic engineering to make 'unnatural' products. Specifically, combinatorial biosynthesis can be achieved by manipulating enzymes responsible for primer unit incorporation, chain elongation, and chain termination (McDaniel et al. 1999; Wong and Khosla 2012). The type II PKS synthases are monofunctional enzymes operating as a "minimal PKS synthase complex" (KR-ACP-KS) (Hertweck et al. 2007), which is characterized by an iterative mechanism, where a heterodimeric KS (KSα and KSβ) catalyzes more than one round of chain elongation (Szu et al. 2012). Polycyclic aromatic polyketides, such as the tetracyclines and anthracyclines, are synthesized by these kinds of PKSs. Recently, a novel type II PKS antibiotic from anaerobic bacterium was discovered in *Clostridium beijerinckii* (Pidot et al. 2014b). Although no special secondary metabolites had been reported from this organism, the authors noted that the genome of *Clostridium* species harbor cryptic polyketide synthase genes (Behnken and Hertweck 2012). This clue led the authors to isolate a purple pentacyclic polyphenol named clostrubin from fermentation products of

C. beijerinckii. Clostrubin was a potent antibiotic with pronounced activity against various pathogenic bacteria, such as methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant enterocci (VRE) bacteria, and the multidrug-resistant tuberculosis (MDR-TB) mycobacteria (Pidot et al. 2014b). The genetic basis for the production of this compound are not yet identified. Structural data suggest a unique biosynthetic route not previously observed in type II PKS (Pidot et al. 2014b). Today, innovative bioinformatic tools have emerged to predict the type II PKS gene clusters that would be useful for research in the discovery of novel bacterial type II polyketides (Kim and Yi 2012).

Microbial type III PKSs from bacteria and filamentous fungi, consist only of a single homodimeric protein that catalyzes the priming, extension, and cyclization reactions that iteratively form polyketide products, without requiring an ACP domain to deliver the extender units (Yu et al. 2012). A novel type III polyketide synthase (PKS) known as Cpz6 is used in the biosynthesis of caprazamycins (CPZs), a liponucleoside antibiotic with potent activity against *Mycobacterium tuberculosis*. The enzyme introduces a sulfate group at the 2″-hydroxy of the CPZs aminoribosyl moiety. Meanwhile, nonribosomal peptide synthetases follow the general assembly line mechanism of type I PKS chemical logic (Gene et al. 2012). Thus, the biosynthetic machinery is comprised of multimodular enzymatic assembly lines that contain one module for each amino acid monomer incorporated (Strieker et al. 2010). An example is ramoplanin A2, a new NRPS compound in clinical development (Montecalvo 2003). This is a 17-residue nonribosomally produced lipoglycodepsipeptide antibiotic, obtained from *Actinoplanes* sp. ATCC 33076. The 33 kb biosynthetic gene cluster of this microorganism reveals architecture of fatty acid and nonribosomal peptide synthase biosynthetic genes (NRPSs) (Hoertz et al. 2012). Structurally, ramoplanin consists of 17 amino acid residues, among which there are a considerable number of non-proteinogenic amino acids, including 4-hydroxyphenylglycine (Hpg) (Chen et al. 2013). Ramoplanin A2, exhibits activity against Gram-positive bacteria including vancomycin-resistant *Enterococcus* sp. (VRE), methicillin-resistant *S. aureus* (MRSA), and the vancomycin-intermediate resistant *Clostridium difficile* (Cudic et al. 2002).

Furthermore, a wide range of semisynthetic antibiotics currently in clinical trials (http://www.pewtrusts.org/) have been created based on the chemical architecture of NRPS and PKS (Table 3). Moreover, antibiotics like ramoplanin, dalbavancin and oritavancin, with great potential to treat serious bacterial infections have been developed from novel microbial natural NRPS (from *Actinoplanes* and *Actinomadura*, respectively) (Billeter et al. 2008; Hoertz et al. 2012).

4.2 Terpenoids

Terpenoids or isoprenoids are natural bioactive products which may have antibiotic, antitumor, vitamin and hormone effects (Brahmkshatriya and Brahmkshatriya 2013). The biosynthesis of terpenoids start with condensation of isopentenyl

Table 3 Examples of antibiotics in current clinical development based on the chemical architecture of NRPS and PKS

Antibiotic name	Company	Chemical base architecture	Target	Reference
Ceftaroline + avibactam	Astra Zeneca/ Forest Labs.	Cephalosporin + novel β-lactamase inhibitor (NRPS)	*Enterobacteriaceae*	Castanheira et al. (2012)
			Streptococcus aureus (MRSA)	
			Pseudomonas aeruginosa	
			Streptococcus β-hemolytic	
Ceftazidime + avibactam	Astra Zeneca/Forest Labs.	Cephalosporin + β-lactamase inhibitor (NRPS)	*P. aeruginosa*	Zhanel et al. (2013)
EDP-788	Enanta Pharm.	Bicyclolide macrolide derivative (PKS I)	Gram-positive (MRSA)	de Souza Mendes and de Souza Antunes (2013)
Eravacycline	TetraphasePharm.	Tetracycline (PKS II)	*Enterobacteriaceae*	Sutcliffe et al. (2013)
			Streptococcus pneumoniae	
Omadacycline	Paratek Pharm.	Tetracycline (PKS II)	*S. pneumoniae*	Draper et al. (2014)
			Haemophilus influenzae	
			Enterococcus faecium (VBR)	
Plazomicin	Achaogen, Inc./Isis Pharm. Inc.	Aminoglycoside (NRPS)	*S. aureus* (MRSA)	Walkty et al. (2014)
			Gram-negative multidrug resistant (MDR), Enterobacteriaceae (CRE)	
Ramoplanin	Nanotherapeutics Inc.	Lipoglycopeptide (NRPS)	*S. aureus* (MRSA)	Hoertz et al. (2012)
			Clostridium difficile	
Solithromycin	Cempra Inc. Pharm.	Macrolide (PKS I)	*Legionella pneumophila*	Oldach et al. 2013
			Haemophilus influenza	
			Neisseria gonorrhea	
TD-1792	Theravance Inc.	Glycopeptide-cephalosporium heterodimer (NRPS)	*Staphylococcus epidermidis*	Long et al. (2008), Hedge et al. (2012)
			S. pneumoniae	
			Streptococcus pyogenes	
Teixobactin	Novobiotic Pharm.	Depsipeptide (NRPS)	*S. aureus* (MRSA)	Ling et al. (2015)
			Mycobacterium tuberculosis	

Avibactam is a new non-β-lactam, β-lactamase inhibitor

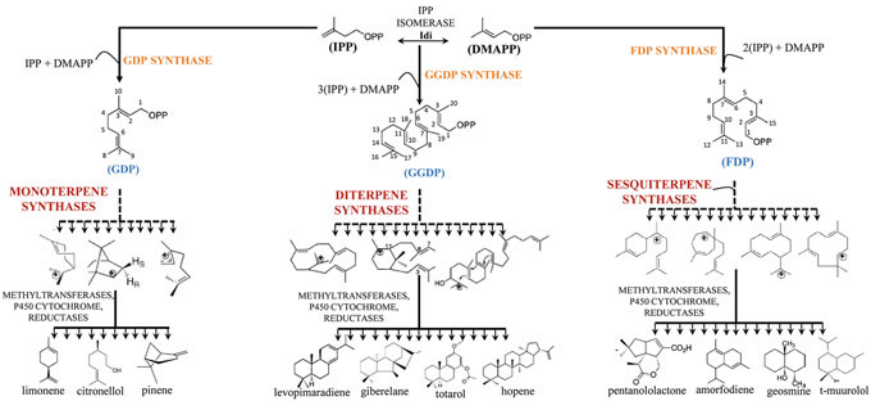

Fig. 2 Biosynthesis of terpenoids

diphosphate (IPP) with dimethylallyl diphosphate (DMAPP) to form the precursors geranyl diphosphate (GDP), geranyl-geranyl diphosphate (GGDP) or farnesyl diphosphate (FDP) (Fig. 2). This step is catalyzed by prenyltranferases called GDF, GGDP, or FDP synthases, respectively. In the second part of the terpenoids biosynthesis, the diphosphate precursors undergo a wide range of cyclizations and rearrangements by terpene synthases to produce the parent carbon skeletons of a terpeneoid. GDP is converted to the monoterpenes (C_{10}), FDP is converted to the sesquiterpenes (C_{15}), and GGDP is converted to the diterpenes (C_{20}). Finally, a variety of oxidations, reductions, isomerizations, conjugations, and other transformation reactions take place, to convert the parent skeletons of each terpene to thousands of distinct terpene metabolites (Ashour et al. 2010). Terpene synthases possess two highly conserved functional motifs: (i) a rich DDXX aspartic (D/E); and (ii) a NSE/DTE (N/D) DXX (S/T) XX (K/R) (D/E) triad motif (Cane and Ikeda 2012). Both, the rich aspartic and the NSE triad are responsible for the cooperative binding of three divalent cations (Mg^{2+}) to produce a carbocation and the binding of a pyrophosphate group, respectively (Christianson 2006).

Generally, terpenoids have been studied in plants and fungi (Ashour et al. 2010). However, in the last years, extensive bacterial genome sequencing and bioinformatics analysis have revealed more than a hundred distinct predicted terpene synthase genes in bacteria, many of them present in Actinomycetes and filamentous fungus (Dairi 2005; Ebel 2010; Cane and Ikeda 2012). Sesquiterpene synthases from *Streptomyces* have been shown to produce pentalenene and epi-isozizaene, precursors to the pentalenolactone and albaflavenone antibiotics, respectively (Tetzlaff et al. 2006; Zhao et al. 2008). Pentalenolactone has been shown to be active against both Gram-positive and Gram-negative bacteria, as well as a variety of fungi and protozoa through the selective inhibition of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (You et al. 2007). The pentalenene and epi-isozizaene synthases are encoded in the terpene biosynthetic cluster of the *Streptomyces avermitilis* and *S. coelicolor* chromosomes, respectively

(Tetzlaff et al. 2006; Zhao et al. 2008). These sesquiterpene synthases catalyze cyclization of FDP to tricyclic hydrocarbon, pentalenene and epi-isozizaene, respectively.

4.3 Lantibiotics Bacteriocins

Bacteriocins are antimicrobial low molecular weight peptides, ribosomally synthetized by Gram-positive and Gram-negative bacteria (Karpiński and Szkaradkiewicz 2013). Lantibiotics are a class of complex polycyclic modified peptides of Gram-positive bacteriocins (Willey and van der Donk 2007). Lantibiotics biosynthesis involves posttranslational modification of peptides with molecular weights below 19–38 amino acids. Basically, lantibiotics formation involves two steps generated by two independent enzymes (type A lantibiotic synthases LanB and LanC) or by a single enzyme (type B lantibiotic synthases LanM) (Goto et al. 2010). These enzymes, catalyze dehydration of specific serine and threonine residues in a prepeptide (LanA), and the stereospecific intramolecular Michael addition of Cys thiols to the resulting dehydro amino acids to form lanthionine and methyllanthionine bridges, respectively (Karpiński and Szkaradkiewicz 2013). Finally, an ABC transport system (LanT) exports the mature peptide out from the cell. Additionally, participation of regulatory proteins (LanRK), peptidases (LanP) and transport/immunity (LanFEG), have also been reported (Chatterjee et al. 2005). Examples of type B antimicrobial lantibiotics in current clinical development are NVB302 (aminoheptylamido-deoxyactagardine B) by Novacta Biosystems Ltd. Hertforshire UK, and MU1140 (mutacin) by Oragenics, Tampa FL, USA. NVB302 is a semisynthetic type B lantibiotic derivative of deoxyactagardine B isolated from *Actinoplanes liguriae* (Boakes et al. 2010). The biosynthetic gene cluster, encoding deoxyactagardine B in *A. liguriae* (DAB) (Fig. 3), consists of a prepeptide *ligA* (45 amino acids), a dehydratase, a cyclase (*ligM*), a transporter (*ligHT* and *ORF14*), and regulatory (*ligR-R1*) genes, as well as a monooxygenase, responsible for the oxidation of the sulfur atom to the sulfoxide (*ligO*) (Boakes et al. 2010). NVB3202 is a novel type B lantibiotic that inhibits cell wall biosynthesis in the Gram-positive *C. difficile* by binding lipid II at an alternative binding site to that of vancomycin (Crowther et al. 2013). MU1140 is a 21-amino acid type B lantibiotic produced by *Streptococcus mutans* (Hillman et al. 1998). The molecular structure of mutacin contains four macrocyclic rings, each of which contains a meso-lanthionine or 3-methyllanthionine residues. The mutacin biosynthetic gene cluster consists of seven genes (*mutRAMTFEG*): a regulator (*mutR*), the prepromutacin structural gene (*mutA*), a modifying protein (*mutM*), an ABC transporter (*mutT*), and an immunity cluster (*mutFEG*) (Fig. 3) (Chen et al. 1999). Mutacin has a unique mechanism of action that involves inhibition of peptidoglycan synthesis by binding to and abducting lipid II from its site of action at points of peptidoglycan synthesis (Hasper et al. 2006).

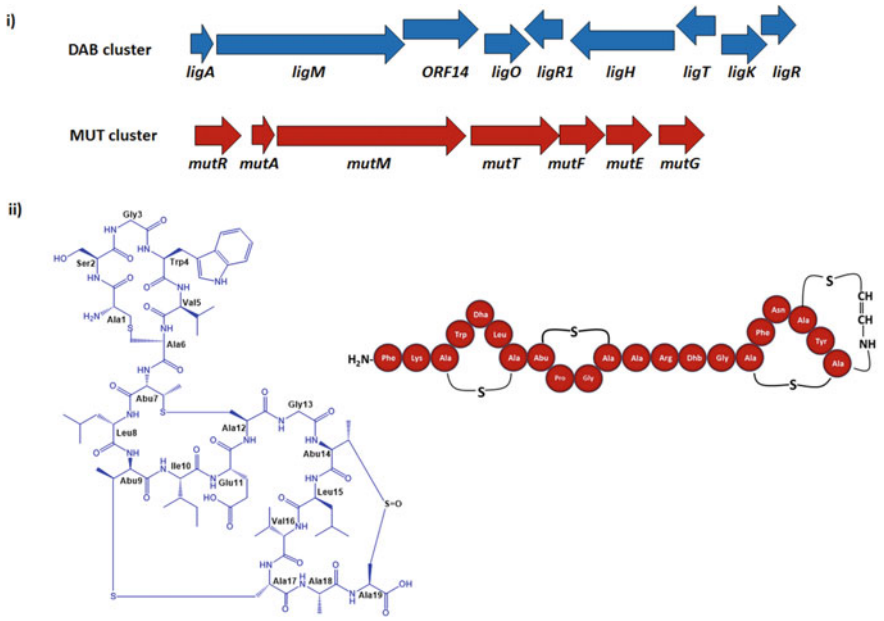

Fig. 3 Gene cluster organization (**a**) and structure of the peptide sequence (**b**) of deoxyactagardine B (*blue*) in *Actinoplanes liguriae* (DAB) and mutacin (*red*) in *Streptococcus mutans* (MUT). The order and direction of transcription of all the open reading frames are shown. Abu, 2-aminobutyric acid, Dha 2,3-didehydroalanine, Dhb 2,3-didehydrobutyrine (Chen et al. 1999; Smith et al. 2008; Boakes et al. 2010)

5 Searching for Novel Antibiotics

All types of antibiotics are susceptible to elicit microbial resistance. However, it is common to observe cross-resistance between antibiotics of the same class (Genilloud 2014), e.g., the beta-lactams and aminoglycosides are susceptible of enzyme inactivation, the macrolides and glycopeptides became unable to bind to their targets due to target modification, quinolones, tetracyclines and macrolides are susceptible to be exported out of the cells by efflux pumps (Lynn et al. 2003).

Therefore, a huge interest in discovering new antibiotic agents does exist, mainly against Gram-negative bacteria, associated to high lethality and because the existing options to treat these bacterial infections are limited (Genilloud 2014). In particular, novel antibiotics are needed to treat antibiotic-resistant strains of Gram-negative bacilli (GNB) (Fabbretti et al. 2011). This is due to problems of the current antibiotics to cross the bacterial outer membrane.

Antibiotic Discovery Has Declined Since 1970s and Was not Until the Year 2000, When a New Class of Systemic Antibiotic Appeared

From the year 2000 to 2014, only 8 new classes resulted from the 26 new antibiotics introduced to the market (Table 4). Three out of eight first-in-class antibiotics, are synthetic: linezolid (oxazolidinone, approved in 2000), bedaquiline (diarylquinoline, approved in 2012), and tedizolid (oxazolidinone, approved in 2014), while the other five are from natural sources (fungi or actinomycetes): daptomycin (approved in 2003), tygecicline (approved in 2005), retapamulin (for topical use, approved in 2007), fidaxomicin I (approved in 2010), and dalvavancin (approved in 2014) (Bassetti et al. 2013; Butler et al. 2013).

From 2011 to date, only 10 new antibiotics, developed on the chemical architecture of NRPS or derived from them, are in clinical trials (Table 3).

Natural products represent the main source of antibiotics, including a large list of launched antibiotics still useful to control microbial infections (Genilloud 2014). They have provided most of the known chemical classes of antibiotics, and their repertory is the result of a large evolution process (Fabbretti et al. 2011).

Novel antibiotic classes are expected to have new action mechanisms and at the same time, less likely to be susceptible to any resistance mechanism. Then, development of new class of antibiotics is a mandatory medical need (Genilloud 2014).

Microbial natural products have been one of the most important sources of new compounds with antibiotic activities. According to Newman and Gragg (2012), between 1981 and 2010 from 118 new antibacterial compounds, 92 were from natural products. This is due to the richness of compounds produced by

Table 4 New classes of antibiotics launched since 2000

Year	Antibiotic name	Chemical class	Origin	Marketed by
2000	Linezolid	Oxazolidinone	Synthetic	Pfizer Inc.
2003	Daptomycin	Lipopeptide	Natural (*Streptomyces roseosporus*)	Cubist Pharm.
2005	Tigecycline	Glycylcyclines	Semisynthetic (*Streptomyces aurefaciens*)	Wyeth
2007	Retapamulin	Pleuromutilin	Semisynthetic (*Pleurotus mutilus*)	GlaxoSmithKline
2011	Fidaxomicin	Macrocyclic	Natural (*Actinoplanes decanensis*)	Cubist Pharm.
2012	Bedaquiline	Diarylquinoline	Synthetic	Johnson and Johnson
2014	Dalbavancin	Lypoglycopeptide	Semisynthetic (*Nonomuria* spp.)	Pfizer Inc.
2014	Tedizolid	Oxazolidinone	Synthetic	Cubist Pharm.

microorganisms and the easy way to obtain them. Bacteria and fungi represent the organisms with greatest biosynthetic potential for antibiotic synthesis specially actinomycetes and endophytic fungi (Genilloud 2014).

Discovering new antibiotics involves the use of multidisciplinary tools in the culture of wild strains, screening and strain selection, genetic improvement programs, early de-replication to avoid rediscover of known antibiotics, expression of silenced biosynthetic pathways by stimulation and exploration of its biosynthetic potential by genome mining or metagenome and genetic engineering (Genilloud 2014). The research strategy has varied depending on the budget, the ability of the multidisciplinary groups in the field, as well as the particular goals in each laboratory.

5.1 Source Consideration

Microorganisms can be found virtually in every corner of the earth (soil, plants, rivers and oceans, caves, geysers, etc.) and these microbes are potential factories of a huge amount of bioactive natural products, some of them are highly valuable to mankind like antibiotics.

Soil represents a very rich source of microbial biodiversity. The number of prokaryotic cells in soils is estimated to be 4×10^7 per gram in forest soil and 2×10^9 per gram in cultivated soils and grasslands (Daniel 2004), many of which make chemical structures of pharmaceutical interest (Taylor 2013).

From these bacteria, about 70 % of secondary metabolites are produced by *Streptomyces* (Zengler et al. 2005), the rest being distributed across other families, mainly *Micromonosporaceae* and *Pseudonocardiaceae*. Likewise, there are fungi such as *Penicillium, Aspergillus, Trichoderma* or *Fusarium*, which are much more productive than any other fungal genera (Pelaez and Genilloud 2003).

It makes sense to predict that unexplored microbial strains represent an excellent approach to discover metabolites with novel chemical structures. However, not all the microbes have the same ability to produce specialized metabolites. Therefore, some microbial taxa could be more promising than others (Donadio et al. 2007).

The main new places to start searching for new antimicrobial producing microorganisms are oceans, and endophytic organisms from plants or similar living things. There is a relatively new trend as well, on the investigation of some 40 million insect species and their products (Ramadhar et al. 2014; Piel 2009, 2011), which may result in discovery of new chemical types.

Endophytes are a relatively new field for searching new compounds with antimicrobial activity, since they can be found in practically all plants on earth (Guzman-Trampe et al. 2015). Each of the higher plants, from mosses and mangroves to green trees, are the hosts of possible producers of new microbial metabolites. It has been estimated that at least 1 million species of fungi alone have been isolated as endophytic microorganisms (Dreyfuss and Chapela 1994). Some

fungal endophytes and their host plants are capable to produce the same or similar compounds. From these microorganisms, diverse structural classes of compounds with antibiotic activity have been isolated. Alkaloids, peptides, steroids, terpenoids, phenols, quinones, flavonoids, and others are representative of these compounds (Zhao et al. 2010; Tan and Zou 2001; Gunatilaka 2006; Schulz et al. 2002; Schulz and Boyle 2005; Kusari et al. 2012).

Microbes that occupy sites characterized by extreme temperatures, pressure and light availability, like the polar sea ice (Junge et al. 2004) and ice cores (Price and Bay 2012), hydrothermal vents (Beatty et al. 2005; Petersen et al. 2011), sub-seafloor sites (Forschner-Dancause et al. 2012) and caves (Barton 2006; Bastian et al. 2010), are also rich sources of novel compounds, probably due to their notorious specialized metabolic adaptations.

A high thermostable peptide antibiotic that strongly inhibits the growth of Gram-positive bacteria was isolated from *Bacillus licheniformis*, strain 189. This strain was isolated from a hot spring environment in Azores, Portugal (Mendo et al. 2004).

Cervimycin A–D is a novel polyketide glycoside with significant activity against multidrug-resistant staphylococci and vancomycin-resistant enterococci. It is produced by *Streptomyces tendae*, isolated from a cave located at Grotta dei Cervi, Porto Badisco, Italy (Herold et al. 2005).

The oceans seem to be the richest source to find new microbes, better than soil samples. One milliliter of seawater contains almost 1 million free-living cells and microbes, and they are generally of different types, including many new species previously isolated from terrestrial sources (Imhoff et al. 2011). Rare actinomycetales are relatively more frequently isolated from marine habitats.

From marine microorganisms, there have been found metabolites with antibiotic and antitumor properties, such as salinosporamides (Williams et al. 2005), sporolides (Buchanan et al. 2005), the terpenoid chlorodihydroquinones (Soria-Mercado et al. 2005), marinomycins (Kwon et al. 2006) and others (Jensen and Fenical 2005), as well as known metabolites such as rifamycins (Kim et al. 2006). Examples of novel or rare structural scaffolds discovered from marine strains include abyssomicins, antibacterial compounds that interfere with folate metabolism produced by a *Verrucosispora* sp. (Bister et al. 2004); salinosporamide A, a potent proteasome inhibitor isolated from *Salinispora tropica* (Gulder and Moore 2010); and the enediyne-derived cyanosporaside from *Salinispora pacifica* (Oh et al. 2006).

Walsh and Wencewitz (2014) pointed out that the pressing need for new antibiotic frameworks and new scaffolds may be reached by culturing of the so-called “unculturable” species, and by precursor-directed biosynthesis, in order to discover the hidden biosynthetic capacity of the microbes, which give us a new outlook on this inexhaustible microbial world.

It should be noted that natural product screening has changed. Currently, it is mostly a business of academia and small biotech companies, with a few notable exceptions of large companies still active in the field; contrary to how it was done a few years ago (Monciardini et al. 2014).

5.2 *Bioprospecting*

Bioprospecting represents the main strategy in the search for new antimicrobial compounds. According to the United Nations Environment Programme (UNEP), bioprospecting is the exploration of biodiversity for commercially valuable genetic and biochemical resources. It can be defined as the process of gathering information from the biosphere on the molecular composition of genetic resources for the development of new commercial products. Genetic resources can yield either small organic molecules called secondary metabolites, gene-encoding proteins such as enzymes, or metabolic pathways linking enzymatic reactions, e.g., in a process known as microbial fermentation (UNEP 2000).

Bioprospecting has great importance in the pharmaceutical industry due to the need for new drugs to treat diseases affecting a large number of worldwide populations (AIDS Alzheimer, cancer, diabetes, hypertension, infectious diseases caused by virus, bacteria, fungi or parasites). However, it is also widely used by several industries like health, beauty, biotechnology, agricultural and food security, among others.

Bioprospecting uses empirical evidence and traditional knowledge in the search and use of natural resources. This strategy allows the sustainable use of endemic natural resources for exploitation, conditioned to respect and conserve the biological diversity. It allows the sustainable use of its components, and commits the fair and equitable sharing of benefits arising from the use of genetic resources, according to The Convention on Biological Diversity http://www.cbd.int/secretariat/ (which came into force on December 29, 1993).

In regard to the use of microorganisms as natural source for anti-infective compounds bioprospecting, it begins with sample collection, chosen by some specific criteria. In some cases, microorganisms are isolated and identified to know the metabolic potential previously reported, followed by growing those microorganisms in some selected cultured media to improve production of the active compounds. Then, it comes the process of extraction with diverse polarity solvents and subsequent fractionation, which allows a bio-directed search “hits” and thus the chemical characterization of the structure by spectroscopic methods. This will eventually allow selection of “leads” and make product optimization.

In recent decades, automation and miniaturization technologies enabled the application of complicated and expensive methodologies to improve, with higher sensitivity, the protocols previously used. Within this framework, there are different techniques that have allowed the search for new molecules with different activities between them; this technique was named by the pharma industries as HTS (Koehn 2008).

The discovery of streptomycin, gentamicin, tetracycline and other antibiotics, spurred industry to develop large research and development programs around natural product discovery, particularly microbial fermentation based technologies (Baker et al. 2007).

Sample collection. In order to select a potential microorganism, it is important to consider the research objectives, the necessity of the whole microorganism or its DNA, as in metagenomics (Nováková and Farkašovský 2013; Genilloud 2014). The parameters to be evaluated and the locations where the microorganisms or its DNA are going to be sampled are important too (plants, extreme or unexplored environments). A lot of examples exist in the literature about different metabolites from microorganisms found in rare places. For instance, abyssomicin, an antibacterial compound produced by a marine *Verrucosispora* sp. that interferes with folate metabolism (Bister et al. 2004) or "ergosterol" with antibiotic activity, found in the fungus *Gliomastix murorum*, who inhabited in the medicinal plant *Paris polyphylla* var. *yunnanensis* (Zhao et al. 2012).

Antibiotic susceptibility testing (AST). There are different methodologies for antibiotic activity evaluation. They allow determination of the antibiosis pattern of different compounds, with special interest in searching for microbial resistance to one or more antibiotics. They are used for diagnosis of antimicrobial treatment of different microorganisms, supporting decision-making processes, sample analysis, and collaboration to help microbiologists to identify compounds with antibiotic activity.

These methods can be organized into three major groups as described below:

1. Quantitative methods: these methods give a viability measure of the test microorganisms, in some cases by determination of the minimum inhibitory concentration (MIC), dilution tests (macrobroth and microbroth) or by he Agar dilution method. There are also colorimetric methods that quantify cell viability. For example, with the use of the redox indicator Alamar Blue as an indicator of cell viability (Collins and Franzblau 1997).
2. Qualitative methods: these methodologies categorized the level of sensitivity of the isolated bacteria against antibiotic concentrations. The antibiotic susceptibility test, using standardized single disks (Bauer et al. 1966) has been widely used for antibiotic activities. Some variations of this methodology have emerged as stokes method, spiral gradient endpoint method and E-test (used to monitor resistance to antibiotics), supporting their principle in the diffusion and dilution methods (Hill 1991). They are performed on solid culture and there are certain advantages to testing anaerobic bacteria on solid media by agar dilution rather than microbroth dilution.

   Spiral gradient, allows evaluation of different antibiotic concentrations on a plate. For this purpose, the antimicrobial is radially distributed through the plate, thus allowing the selective growth of the test microorganism, according to their ability to grow at different concentrations of the tested compound. This methodology seems to be more efficient than the standard agar dilution method and it is a reproducible alternative for the traditional one (Hill 1991).
3. Automated techniques: these techniques are based on detection of genes coding for resistance to one or several drugs by techniques such as PCR and DNA hybridization.

Susceptibility tests have been automated, with computer-assisted methods, as the VITEK®, BD Phoenix^{MT}, autoSCAN®-4 system and the ALADIN (automated instrument that uses video imaging for determination of biochemical and antimicrobial susceptibility test reactions) (D'Amato et al. 1988).

The Clinical Laboratory Standards Institute (CLSI) is a nonprofit organization, established by the mid-twentieth century, that develops clinical and laboratory consensus practices and promotes their use in worldwide organizations. As directed by the Congress, the FDA has legal responsibility for determining safety and efficacy of new anti-infective agents. When a new drug is released for sale in the U. S., the FDA-approved package insert, includes specific guidelines for interpretation of in vitro tests and quality control limits for tests performed according to CLSI procedures (Barry 2007).

Several methodologies have been previously named highly standardized and are part of the CLSI for determination of antibiotic activities (http://www.crcnetbase.com/doi/pdf/10.1201/9781420014495.ch1).

Microbial isolates characterization and review of their metabolic potential. In addition to the morphology traits, for bacterial genus determination it is common to use the 16S DNA ribosomal sequence information. In the case of fungi, the corresponding 5.8S and the ITS regions are commonly used. This information, allows better decisions about selection of the specimen to be used, based on bibliographic information and data libraries of its possible metabolic potential.

Examples of search libraries and their successful for finding microbial compounds with antibiotic activity have been chronologically exposed (Monciardini et al. 2014) using the Naicons technology (information containing metabolites produced by the actinomycetes strains and the filamentous fungi).

In the case of a metagenome study, functional and sequence-driven screens are the major approaches employed to mine libraries, in order to find interesting substances, followed by the heterologous expression of DNA, as exemplified for Nováková and Farkašovský (2013).

Different studies have uncovered this methodology, finding metabolites with antibiotic activity. For example, fluostatins were obtained using an homology screening of cDNA mega-libraries for clones containing natural product biosynthetic genes, coupled with transformation-assisted recombination (TAR) in yeast (Feng et al. 2010), Another example is the identification and heterologous expression of the type II polyketide synthase cluster found in a soil environmental DNA (eDNA) sample. In *Streptomyces albus* it was revealed the presence of diverse metabolites belonging to well known, rare, and previously uncharacterized structural families (Feng et al. 2011). Finally, Chang and Brady (2013) used homology-guided metagenomic library screening, cloning, and heterologous expression for discovery of the indolotryptoline-based borregomycin A with CaMKIIδ kinase inhibitory activity.

Microorganism cultivation. Metabolites may be obtained from microorganisms by different ways. When the whole microorganism is used, a lot of techniques could be applied to improve the production of the desired substances. These include modification of media components, nutrient depletions, incubation conditions

affecting O_2 and CO_2 concentrations, temperature and pH variations, as well as changes in osmolarity, iron homeostasis, or redox stress (Genilloud 2014).

New strategies to explore novel uncultivable species include synthetic biology approaches like genome mining of microbial strains and obtention of cryptic biosynthetic pathways followed by their heterologous expression. A combination of these methodologies with high-throughput sequencing platforms, integrated bioinformatic analysis, and on-site analytical detection and de-replication tools for novel compounds are very used nowadays (Genilloud 2014; Zotchev et al. 2012).

Extract preparation. This step involves the selective separation of compounds with different polarities produced by the selected microorganism. They can be obtained from the culture medium or from the cell after fermentation. Organic solvents are used to eliminate those compounds that can provoke some interference in the subsequent study (Weiss and Eisner 1998).

Fractionation. Having microbial crude extracts, it is possible to generate a chemical library with some particular characteristics. Some libraries are composed by crude extracts, by fractionated extracts or by pure components, and depending on the library used is the complexity of the subsequent procedures like bioassay guided isolation, structure elucidation and subsequent scale-up. As purest the library is, as easier are the next steps in bioprospecting (Koehn 2008).

With all the information reported in libraries of crude extracts, semi or fractionated and pure compounds, it is possible to find those which are novel structures and those which correspond to compounds previously reported, avoiding de-replication and finding potential metabolites for future studies (Ito and Masubuchi 2014).

Screening of potential uses. This step is necessary to verify the biological activity of the compounds found in the fractions obtained previously. It is important to mention that those metabolites that present high probability to de-replication, not necessarily have to be excluded from the screening because they may already be reported but have not been tested against the same biological activity.

In these tests, various concentrations of the fractions are tested on different possible biological assays. Typical screen tests could be the inhibition of a specific enzyme reaction, specific antibiotic activity tests against some microorganisms or else attaches itself to the receptor molecule that serves as a proxy for the organ or organism the researcher wishes to target (Weiss and Eisner 1998).

HTS is an experimental method used for the rapid identification of active compounds, it has gained great importance in drug discovery, being used in processes of detection activities in natural products, synthetic or semi synthetic drugs, either based or not based on natural products (Koehn 2008). According to Weiss and Eisner (1998) in HTS the results of these bioassays eliminate the vast majority of samples, allowing researchers to focus on the tiny minority that have real prospects of commercial success. The flip side of this efficiency is the disadvantage that these bioassays will eliminate a sample that is active in the particular application but that works on a principle different from the one embodied in the bioassay.

A clear example of the use of this methodology is the discovery of platensimycin by the Merck Gropup, a selective FabF inhibitor with potent antibiotic properties. It was made by a systematic screening of 250,000 natural product extracts (83,000

strains in three growth conditions), with the use of a combination of target-based whole-cell and biochemical assays, which led identification of a potent and selective small molecule from a strain of *Streptomyces platensis*, recovered from a soil sample collected in South Africa (Wang et al. 2006). This technique could also be employed to evaluate some physicochemical characteristics of certain compounds like absorption or permeation, which is one of the latest phases in drug discovery (Kansy et al. 1998).

Product purification and characterization. The already obtained and tested biologically active fractions generate "hits-to-lead" that are those who had a desired biological activity, and depending on the research group one hit has to fulfill certain characteristics. For example, in Schering AG, the hit has to have two positive results against different targets (Steinmeyer 2006). If the evaluated library has crude extracts or fractionated extracts, the next step is to purify the active compound by different methods. Chromatographic separation (either gas or liquid) can be used, although other methodologies such as capillary electrophoresis could also be applied. Finally, spectroscopic methods must be used to determine each one of the compounds of interest, being the most important NMR and mass spectroscopy.

Product development. Knowing the chemical identity of the compounds of interest, it is necessary to perform chemical modifications that allow the "lead" to have better features for use, such as increase in activity and decrease in their toxicity and side effects, improved pharmacokinetic properties, etc. At this point, the redox activity assays of molecules with in vitro and in vivo assay variations are important (Steinmeyer 2006).

An additional highlight to consider is the use of chemical synthesis of the selected compounds, or in some cases only a semisynthesis from the obtained natural products. Improving fermentation conditions, strain optimization, and scaling up the fermentation are the next steps.

6 Molecular Approaches in the Search of New Biosynthetic Genes for Antibiotic Production

Since its beginning, microbial antibiotic research has largely relied on bioactivity detection by means of two strategies, bioactivity-guided fractionation and chemical screening. The first one employs crude fermentation broth extracts, bioactivity screening, fractionation, and final structure elucidation through HPLC, MS, and NMR spectroscopy techniques (Bachmann et al. 2014). The second one involves isolation of TLC-resolvable spots, followed by structure elucidation. Both techniques were suitable in the past, since they conducted the discovery of a plethora of molecules with antibiotic activity. Nonetheless, these techniques reached the peak at the end of the 80s with the continual re-isolation of already known compounds and the subsequent lost of interest from companies due to the blind chance to find new bioactive molecules and the high cost of these procedures (Demain and Sanchez 2015).

When the idea of disclosing new natural products seemed to have reached the end, the genomic age and molecular biology tools opened new doors for finding new secondary metabolite structures. As discussed earlier in this chapter, almost all kinds of antibiotics can be synthesized by one or more genes, which are grouped in clusters (Winter et al. 2011). Microbial DNA molecules encode basic information about assembly and sometimes the structure of these molecules. In this regard, earlier findings help us to understand cluster organization, linearity, and continuity of these genes inside the genome and how these three areas are closely related with the final antibiotic structure. One of the first and most beautiful examples belongs to the modular type I PKS, encoding for the erythromycin aglycone (Caffrey et al. 1992). Breaking points were papers describing the complete genome sequence from two Gram-positive bacteria, *S. coelicolor* (Bentley et al. 2002) and *S. avermitilis* (Omura et al. 2001; Ikeda et al. 2003), which help us to realize that secondary metabolite production had been long underestimated.

We stand now into a new stage on antibiotic research supported by the "omics" evolution that during the last 20 years has conducted isolation to a whole different level. Some strategies enclose genome mining, single cell genome amplification, metabolomics and proteomining, among others (Fig. 4). In the following paragraphs we briefly discuss some of these techniques.

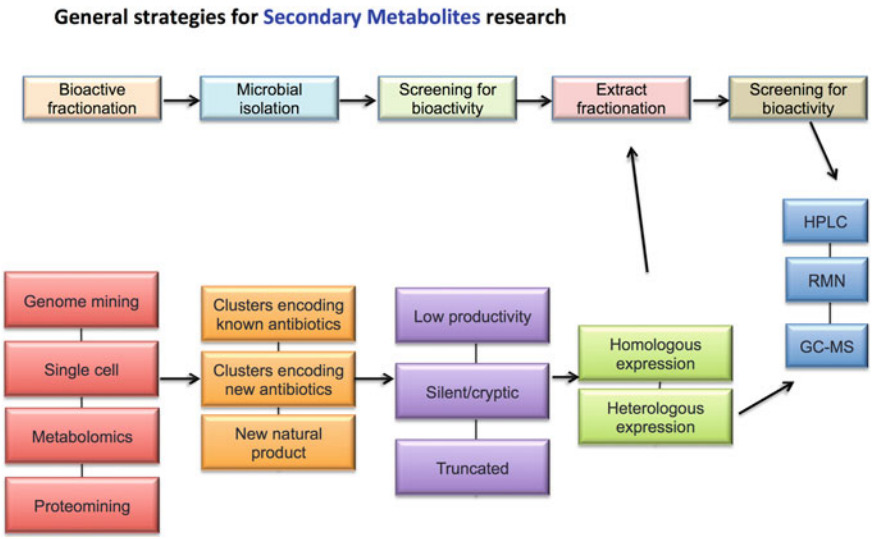

Fig. 4 Strategies for antibiotic discovery. Bioactive fractionation involves standard techniques including microbial selection and many steps of screening bioactivity. Microbial isolation includes all "omics" strategies associated, in which the isolation from a microorganism is not necessarily required. First and second steps include discovery of a new antibiotic molecule or cluster and isolation of the chemical entity or DNA sequence. Some troubles regarding antibiotic concentration, cluster low or miss-expression, and incomplete DNA sequence can be solved through homologous or heterologous expression of clusters. Both strategies converge into the finals steps, since screening for bioactivity and chemical structure elucidation are always needed

6.1 Genome Mining

For the chapter purposes, we define genome mining as a process in which DNA is translated into coding sequences (CDS) in order to deduce the production of antibiotics or other bioactive metabolites. Since the completion of *Haemophilus influenzae* Rd sequence in 1995, more than 3200 genomes have been deposited in the NCBI database (Fleischmann et al. 1995). The genome of many streptomycete strains and other bacteria genus such as *Pseudomonas* and *Bacillus* have been obtained in the last 10 years. This genomic era has conducted to the astonishing increase of DNA sequence data available in public databases. Until now, more than 3214 complete genome sequences are available in one or more scaffolds and only 10 of these belong to fungi. Just 17 genomes were completed before 2000 and more than 50 % have been released after 2010 (NCBI 2014).

The general strategy for genome mining is described in Fig. 5. The fist step is to obtain the genome sequence from the microbial strain. Different HTS platforms such as Illumina (Solexa) sequencing (Luo et al. 2012), 454 pyrosequencing (Balzer et al. 2010), SOLiD sequencing (Mardis 2008), Ion Torrent Sequencing, Single Molecule Real Time (SMRT) sequencing (Quail et al. 2012) and nanopore DNA sequencing are the most widely used technologies. Next step could be considered as the key point and

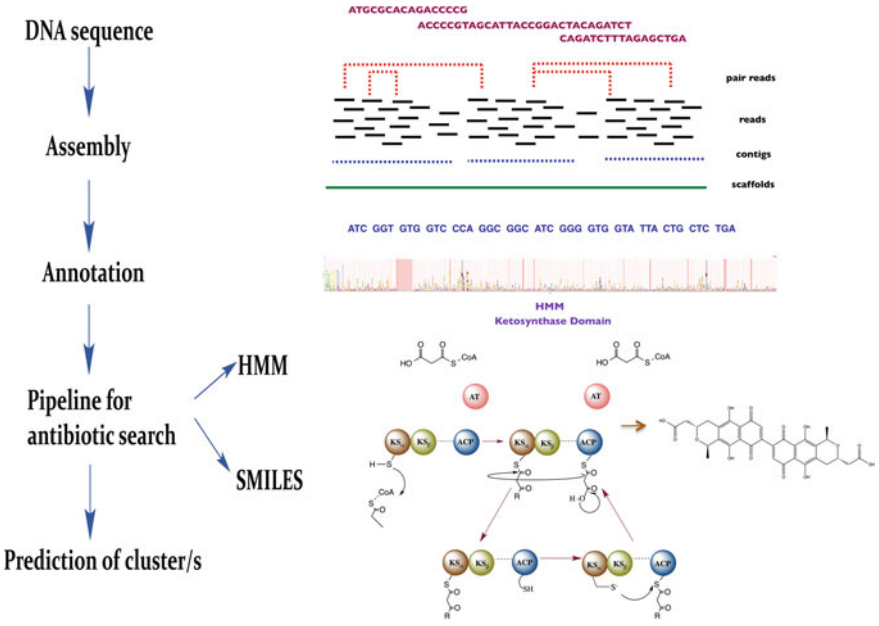

Fig. 5 General strategy for genome mining. Pair read assembly into large contigs and scaffolds, sequence alignments and enzyme motives identified and final cluster expression or isolation, and chemical structure elucidation are described. Type II PKS actinorhodin is the example molecule assembled

takes advance from bioinformatic tools to align and merge million of short reads into long contigs and finally realign these contigs in an ordered fashion to obtain scaffolds. Ideally, complete genomes assembled into less than five scaffolds implicate high quality DNA molecules. Third generation sequencing platforms such as SMRT and nanopore have the power to sequence the whole DNA molecule in real time, so very long sequence fragments can be obtained thus eliminating the library construction step. However, if just one SMRT cell is acquired, the probability to obtain low-quality data increases mainly because polymerase enzyme errors. A complete compilation about HTS and assembly algorithms was prepared by Magi et al. (2010). Once you get a high-quality assembly, the third step becomes easy. Most commonly, FASTA sequences are employed to feed different databases such as RAST (Aziz et al. 2008) or pipelines like MGA (Noguchi et al. 2008), Prodigal (Hyatt et al. 2010), and Prokka to predict CDS and generate the annotation file. This means that each coding sequence will be screened against different nonredundant databases, naming each CDS or gene sequence. The fourth step uses conserved motifs or sequences regarding each enzyme involved in key points from antibiotic assembly. Consensus sequences obtained through alignments from the enzyme domains are used to construct Hidden Markov Models (HMM) or the chemical structure to predict SMILES (Fig. 5). To fulfill this goal, many online and stand-alone sources are freely available, some of these include antiSMASH 2.0 (Blin et al. 2013), which detects 24 types of secondary metabolites. CLUSEAN, NaPDoS, NP.search, Bagel2 are used for lantipeptides, while PKS/NRPS Analysis, SBSPKS (Anand et al. 2010) and SMURF pipeline were developed for fungi systems. More complete tools such as RAST offer a complete analysis of metabolic pathways supported by your target sequence (Aziz et al. 2008). AntiSMASH is one of the most popular softwares since it is friendly and easy going. Basically works by searching HMM profiles for each key enzyme of specific secondary metabolites (KS, C, A, ACP, PCP, terpene cyclases, prepeptides, DH). Once a candidate has been found, clusters of orthologous groups (smCOGs) are assigned, different alignments conducted and detection of secondary metabolites clusters is supported by PFAM. Despite accurate results, beware of false positives with data obtained through antiSMASH. In all the above-mentioned algorithms, the final result will be the prediction of the presence of a gene or gene clusters encoding for antibiotics and other secondary metabolites production. De-replication of molecules is allowed when you compare your cluster or query sequence against those of compounds already available.

Before genome mining, four secondary metabolites had been studied in *S. coelicolor* including the calcium dependent antibiotic CDA (Kempter et al. 1997), and the anthelmintic avermectin produced by *S. avermitilis* (Ikeda and Omura 1997). After the availability of genome sequences around 30 and 37 clusters were discovered, respectively. Additionally 27, 36, 9, 14 and 31 clusters have been described in *Saccharopolyspora erythraea* NRRL2338 (Oliynyk et al. 2007), *Streptomyces griseus* IFO 13350 (Ohnishi et al. 2008), *Pseudomonas fluorescens* Pf5 (Paulsen et al. 2005), *Rhodococcus* sp. RHA1 (McLeod et al. 2006) and *S. tropica*, respectively (Udwary et al. 2007). NRPS coelichelin, isolated from *S. coelicolor* was the first siderophore whose complete structure was decrypted by genome mining

(Lautru et al. 2005). In 2003, genome mining derived benzodiazepinone ECO4601 became the pioneer compound in clinical trials (Gourdeau et al. 2008). Germicidin, filipins, grixazones, enedynes, and rhizoxin are some examples of bioactive compounds discovered through genome mining (Nett et al. 2009).

An outstanding work published last year describes the use of computational analysis to detect biosynthetic gene clusters from genomes of 2430 gut-living bacteria. Donia and collaborators evaluated the potential for secondary metabolites production toward ClusterFinder followed by antiSMASH and the final proteins alignment was conducted applying mblastx algorithm. Surprisingly, they found an outstanding arsenal of natural products machinery including NRPS, ribosomal peptides, carbohydrates derivatives, PKS and terpenes among others, being the first three the most widely represented. The authors describe how these types of secondary metabolites are precisely located into human body, like PKS obtained from actinobacteria isolated from oral cavities. Meanwhile NRPS, saccharides and ribosomal compound were produced mainly by intestinal bacteria. This work is conducted for the isolation of the thiopeptide lactocillin, which is a ribosomal antibiotic, isolated from the vaginal microbiota. Interestingly, they also describe how the production of some of these ribosomal antibiotics is conserved between other animals besides human (Donia et al. 2014).

6.2 Single Cell Genome Amplification

Genome mining has proven a very powerful resource to untapped real potential for antibiotic production. But, what happens when no single colonies can be obtained? Or there is not enough information available for bioinformatic proceedings? Single cell amplification (SCA) or single cell genomics (SCG) has come to untangle these problems. SCA refers to a novel procedure, which employs a non-PCR-based technique to obtain a template DNA from a single isolated cell (Wang and Bodovitz 2010). SCA steps are summarized and briefly discussed afterwards:

a. Collection of microbial samples
b. Single cell isolation: flow cytometer (FACS), piel pooling strategy, dilution
c. Cell lysis: freeze-thaw process, alkaline, heat
d. Amplification of genome through multiple displacement amplification (MDA)
e. Genome sequencing
f. De novo assembly
g. Genome annotation

Single cell isolation is a crucial step, in order to avoid or reduce contamination. Cells must be hooked and deposited into single wells often containing TE buffer. Individual cells are stained with fluorescent labels, so they can be selected by flow cytometry. Ideally cell lysis should be clean, no reaction chemicals or contaminants

must remain after this step. Alkaline lysis is the most extensively used, it will allow not damage to the DNA and after neutralization and washing steps, just around 50 % lysis rate will be succeeded and according to Stepanauskas (2012), further improvements are still required.

Amplification achieved by MDA has been an innovative tool, is a non-PCR dependent technique, which allow you up to 90 % coverage of genome in long overlapping amplicons. Amplification is carried out via random primers (usually 3′-PO modified), which will be amplified using a special polymerase, Φ29. Bacteriophage Φ29 polymerase can produce fragments as large as 10 kb with a very low error rate. It is considered a high fidelity enzyme and it has 3′–5′ proofreading activity. MDA goal is to increase the DNA concentration in order to allow further sequencing. Genome sequencing is conducted by the HTS platforms mentioned earlier. Regularly Illumina pair-end and 454 pyrosequencing are the most suitable methods. Assembly of reads could be completed through bioinformatic sources such as MALBAC, EULER-SR, or Newbler. Sequencing the whole consortium of cells to complete or improve DNA sequence might cover common bias obtained by MDA sequencing.

Standard annotation tools such as RAST or homemade pipelines could be useful for genome annotation (Stepanauskas 2012).

At the same time, metabolomic or proteomic analysis (described later in this chapter) can be useful tools in order to allow antibiotic identification. Analytical methods like MALDI, MALDI-TOF, HPLC-MS, MS-CPA, LAESI-MS are also useful to corroborate or predict specific compound properties, and some times structure elucidation. Assays may be performed with culture broth extracts or directly over lysed single cells. Discovery of Apratoxin A (Fig. 6) a hybrid PKS/NRPS + butyl group isolated from an Indo-Pacific species of cyanobacteria was accelerated, thanks to this strategy (Grindberg et al. 2011).

At this moment the Joint Genome Institute is revealing interactions between rhizosphere microorganisms isolated from the plant *Arabidopsis thaliana* throughout SCA technique (JGI web page 2014 http://genome.jgi.doe.gov/). There is a chance that new antibiotics will be revealed inside these fellows after sequence is completed and analyzed.

6.3 Metabolomics and Proteomining

Although DNA sequence gave us an inside look into the complete potential of antibiotic productivity in one organism, different approaches have been displayed to survey cluster expression troubles or silent clusters. Metabolomics refers to the analysis of the complete profile of metabolites (primary or secondary) produced by one or more organisms in a given set of conditions (Baker 2011). Somehow, metabolome completes genome approach since encloses phenotype, which means unravel expression and biological function of gene cluster encoding for antibiotic biosynthesis. It has the advantage that analyses could be directed on cells, extracts,

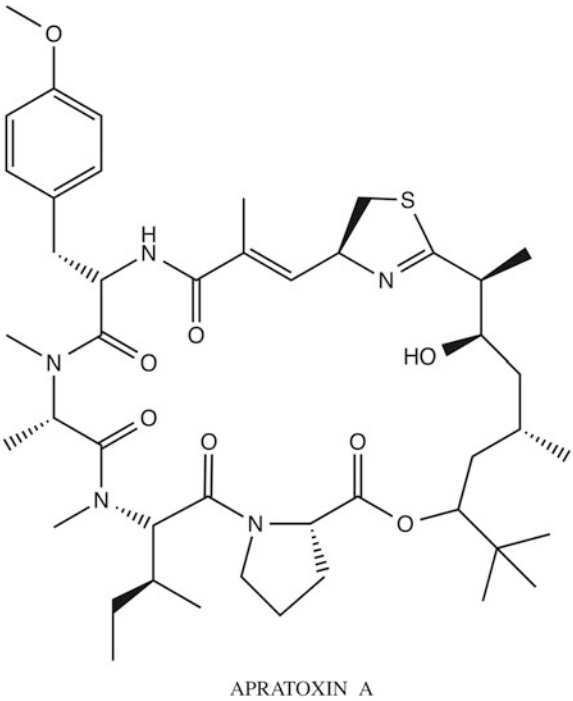

Fig. 6 Hybrid PKS/NRPS Apratoxin a compound, isolated from the cyanobacterium *Lyngbya majuscule*

or even growth medium (liquid or agar) in which interesting metabolic interactions, such as competition cell to cell, can be measured. Analyses through this technology could be separated into three steps: detection of antibiotics profile peaks by analytical methods, de-replication of molecules and structure elucidation (Krug and Müller 2014).

Analysis of metabolic profiles. In order to achieve this goal, metabolomics takes hand of high-resolution techniques in which mass spectrometers are combined with separation devices such as liquid chromatography (LC-HRMS), high-performance chromatography (UPLC-MS), ion mobility–mass spectrometry (IM-MS), nano-DESI, different types of MALDI analyses and one of the most used, tandem mass spectrometry (MS-MS or MS^n) (Krug and Müller 2014; Macintyre et al. 2014). Basically, mass spectrometry platforms have their basis in the measurement of mass-to-charge ratios (m/z) of ions. So, after separate gas phase ions, targeted analysis divided in two or three steps searching ions of specific m/ratios could be directed. Statistical analysis will be required in order to eliminate the background noise.

De-replication. This step may be conducted comparing structurally similar compounds using direct matching of peak positions, estimation of molecular formula, isotope pattern or/and compare spectral data against available metabolome databases such as METLIN, and MassBank (Krug and Müller 2014; Macintyre et al. 2014).

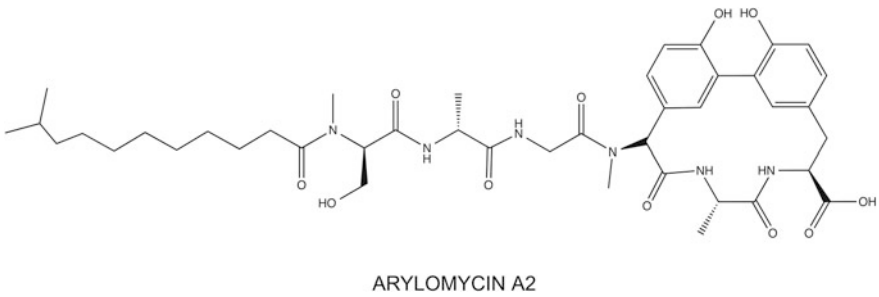

Fig. 7 Structure of the peptidic compound Arylomycin

Structure elucidation. The final step could be completed with the support of NMR and genome mining data. Until now, there are many examples of secondary metabolites identified by this means. One example is the peptidic compound arylomycin (Fig. 7), which was identified trough the merging of three strategies metabolomics, peptidogenomics, and genome mining (Meier and Burkart 2011). Employing MALDI-IMS and short sequence tags (SST) it was possible to discover new peptidic and NRPS molecules. Cocultivating *Streptomyces roseosporus* with two pathogens, allowed further detection of the peptidic compounds responsible for the inhibition zone into the Agar medium and correlation of SST, achieved their goal with genome sequence in order to identify the antibiotic cluster. The second example is the mutaxanthenes (Fig. 8), a new type II PKS-like antimicrobials isolated from the actinomycete *Nocardiopsis* sp. FU40 (Derewacz et al. 2013). Rifampicin and streptomycin resistant mutants were analyzed through UPLC coupled with ion mobility and mass spectrometry. The authors found 311 unidentified peaks, three of which were isolated and NMR elucidated conducting to a new family of xanthenes (Derewacz et al. 2013). This work also probed how drug resistance or gene mutations can display broad changes in metabolic phenotypes.

In conclusion, "omics" approaches have provided researchers with the opportunity to mine different microorganisms, which means more compound diversity and new antibiotics. To our understanding, each of the past reviewed techniques should be complementary to each other, in order to accelerate discovery of new antibiotics with reduced toxicity. So, metabolomics or proteomics may succeed in scenarios where genome mining or bioinformatic approaches have failed and vice versa.

7 Conclusions and Perspectives

Antibiotics have been and continuous to be the primary option for treating bacterial infections saving annually millions of lives. However, after being released to the market, they must face different factors in order to survive. Among these factors,

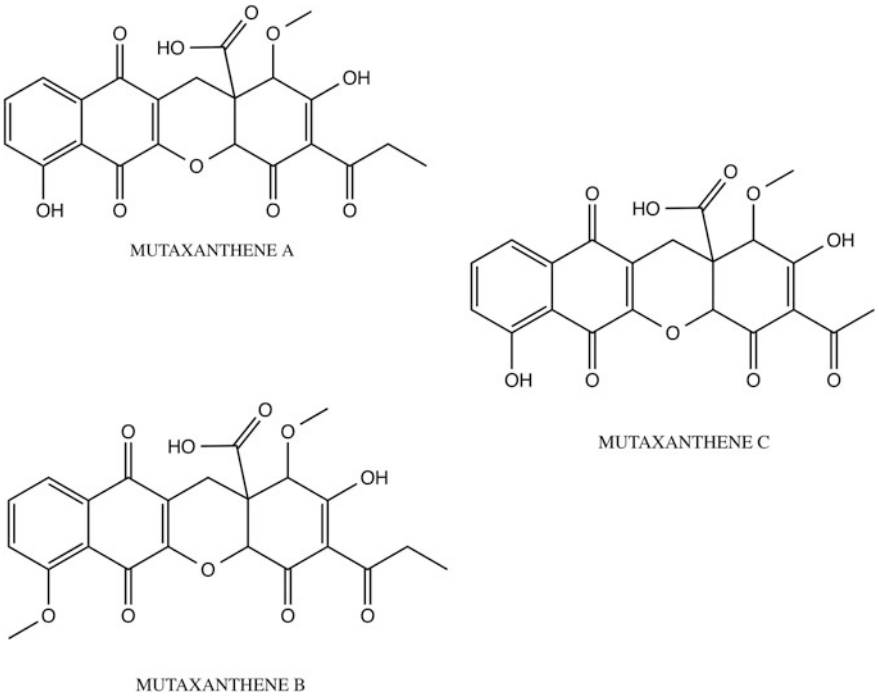

Fig. 8 Structure of type II PKS mutaxanthenes A, B, and C

antibiotic resistance by pathogenic bacteria is probably the main issue affecting their clinical period of useful time. Because of this, and considering the increased costs and time to get an antibiotic into the market, pharmaceutical companies have decreased their anti-infective research programs from 35 in the 1950s to less than ten in 2013 (Torrice 2013). However, antibiotics are still a big business for industry. In 2009 the antibiotics market was US$42 billion, which represented 46 % of the global anti-infective sales (Hamad 2010), and there are many new opportunities for the isolation of new antibiotics for treatment of resistant GNB. Furthermore, as part of the Food and Drug Administration Safety and Innovation Act, on 2012, the USA President signed into law the Generating Antibiotic Incentives Now (GAIN), that grants an additional 5 years the exclusivity period during which certain antibacterial and antifungal drugs can be sold without generic competition. In October 2013, at least 16 antibiotics have been designated as qualified infectious disease products under GAIN (Gingery 2013). In addition, the Infectious Diseases Society of America (IDSA) supported a program, called "the '10 × 20' initiative" (Boucher et al. 2013), aimed to develop ten new systemic antibacterial drugs within 2020. The aim targets of this program are the so-called ESKAPE pathogens (*Enterococcus faecium*, *S. aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa*, and enterobacteriaceae).

Natural products continued to be the main source of antibiotics in clinical use today. Traditionally, bacterial and fungal natural compounds have been the dominant source of clinically useful antibiotics, whereas the completely synthetic chemical scaffolds are in the minority. Microbes are always amazing, as one single species, may contain between 10 and 50 unexpressed biosynthetic clusters, just as reported for *S. coelicolor*, where already 25–30 independent clusters producing secondary metabolites have been identified (Ochi and Hosaka 2013). Perspectives in the field are quite promising as new microorganisms from unconventional sources are being isolated. These include caves, hot springs, plant endophytes and marine microbes among others. In addition, novel targets for antibacterial compounds like inhibitors of peptide deformylase and fatty acid biosynthesis are already in clinical trials. In addition, application of modern technologies of molecular biology and genomics to look for new compounds is being also applied. Among them, genome mining, combinatorial biosynthesis, and metagenomics deserve special mention. These approaches will undoubtedly contribute to develop a robust platform for discovery of many unknown complex structures. Additionally, using these methodologies the novel structures will be produced in sufficient quantities that will allow their testing for antibacterial activity and chemical characterization. Although discovery of new antibiotics is not an easy task, industry and universities together or in different venues should continue to challenge their selves and take actions to find innovative and improved antibiotics.

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Therapeutic Potential of Antimicrobial Peptides

Francesc Rabanal and Yolanda Cajal

Abstract The emergence of pathogens which are resistant or multi-drug resistant to most of the currently available antibiotics is posing an immense burden to the healthcare systems throughout the world. The development of new classes of antibiotics has also suffered a decline since many pharmaceutical companies have gradually abandoned the field. Fortunately, several public–private initiatives to spur the development of new antibiotics have been recently launched. Antimicrobial peptides are thus attracting a renewed interest as potential therapeutic antibiotic candidates. In fact, some of the oldest available antibiotics in the market are cyclic antimicrobial peptides, such as polymyxin B, colistin, gramicidin or bacitracin. However, pharmacological and toxicological problems associated with the systemic use of antimicrobial peptides are slowing their development and drug approval. An overview of the advantages and drawbacks of antimicrobial peptides as antibiotic drugs and a report of compounds that are in development are described.

1 Introduction

Antibiotics have contributed to save millions of lives since their discovery. In spite of that, infectious diseases remain as one of the major causes of morbidity and mortality in much of the world. One of the culprits lies in the fact that bacteria adapt, evolve, and acquire antibiotic resistance. In fact, resistance to antibiotics emerged as early as the late 1930s, not long after the introduction of the first antibiotics, namely, sulfonamides (Davies and Davies 2010).

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Nowadays, the burden caused by the resistance to antibiotics is immense. Around 2 million patients get hospital-acquired infections every year in the EU, of which 175,000 die. The economic burden caused by multidrug-resistant bacterial infections is estimated to be €1.5 billion annually in the EU. In the US, the Centre of Disease Control of Atlanta estimates that more than 2 million patients get antibiotic-resistant infections every year of which at least 23,000 die (CDC 2014). The cost to the US health system has been estimated in some 30–45 billion a year, according to the WHO and the CDC. Numbers reflect, for instance, that more people in the US die of methicillin-resistant *Staphylococcus aureus*, than because of AIDS (Klevens et al. 2007).

In spite of these daunting figures, only seven new antibiotics have been approved by the FDA from 2003 to 2012 and only two new classes since 1998, most of them against Gram-positive bacteria (Butler et al. 2013; Boucher et al. 2013; Walsh and Wencewicz 2014; CDC 2014; WHO 2014). The high cost of drug development and the low return on investment of antibiotics has compelled many pharmaceuticals companies to abandon the field and move to other more profitable (i.e., chronic diseases, cancer).

Multi-drug resistance is observed in both Gram-positive and Gram-negative bacteria. However, two thirds of deaths are due to infection by Gram-negative bacteria. Hence, there is an urgent need to develop new antibiotics, in particular against Gram-negative bacteria, such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Enterobacteriaceae* (i.e., *Escherichia coli* or *Klebsiella pneumoniae*), that are particularly prone to acquire resistance to current antibiotics. In this respect, the Infectious Disease Society of America (Gilbert 2010) has issued a proposal to develop new antibacterial drugs by 2020, particularly against "ESKAPE" bacteria (the former ones in addition to Gram-positive *Enterococcus faecium* and *S. aureus*).

Altogether it is evident that there is a clear unmet medical need in the field of infectious diseases and the development of new, active, and safer antibiotics is urgently required, particularly to fight infections caused by resistant and multi-drug-resistant bacteria.

2 Initiatives to Address Antibiotic Scarcity

In recent years, several important initiatives to fuel the discovery and development of new antibiotics have been established. For instance, the Innovative Medicines Initiative (IMI, co-funded by the European Union and EFPIA pharmaceutical companies) launched in 2012 a €223 million New Drugs 4 Bad Bugs (ND4BB) program to spur the development of new antibiotics (Rex 2014). Three projects are active so far within the ND4BB, namely the COMBACTE, TRANSLOCATION and ENABLE. Initiated in early 2014, the European Gram-negative Antibacterial Engine (ENABLE) aims at identifying new chemical entities that are active against resistant Gram-negative bacteria and to take at least one new compound through

Phase I clinical studies. The project involves 32 partners in 13 countries from academia, small–medium enterprises (SMEs) and the pharmaceutical industry (EFPIA). It has €85 million in available funding for 6 years (2014–2020). Similarly, the Joint Programming Initiative on Antimicrobial Resistance (JPIAMR) aims at coordinating research between European countries and to create a common European research agenda to fight antimicrobial resistance (JPIAMR webpage).

In the US, the NIAID (National Institutes of Allergy and Infectious Diseases) is also leading an Antibacterial Resistance Program that offers an extensive range of preclinical and clinical services. The Biomedical Advanced Research and Development Authority (BARDA) is also supporting the development of new antibiotics. Finally, the GAIN Act (Generate Antibiotic Incentives Now) provides an economic incentive to developers providing five additional years of marketing exclusivity for Qualified Infectious Diseases Products (QIDP) with the purpose of encouraging pharmaceutical companies to develop new antimicrobial drugs to treat serious and life-threatening infections.

3 Antimicrobial Peptides: Mechanism of Action

Antimicrobial peptides (AMPs) are considered to have a great potential to become a new class of antibiotics to treat resistant bacterial infections including septic-shock (Fjell et al. 2012; Pasupuleti et al. 2012; Yount and Yeaman 2003, 2012; Brogden 2005; Brodgen and Brodgen 2011; Afacan et al. 2012; Finlay and Hancock 2004; Hancock and Scott 2000; Vaara 2013; Rabanal et al. 2015; Viñas et al. 2014; Grau-Campistany et al. 2015, 2016). They are a unique and diverse group of molecules produced by a variety of invertebrate, plant and animal species, and despite the diversity in structure, most of them share common trends, such as an amphipathic character and several charged residues. Recently, the use of antibacterial agents that disrupt the bacterial membrane bilayer have been claimed as an alternative and promising approach to regular antibiotics since they have the additional advantage of being active in dormant bacteria (with slow growth or even no growth). These metabolically inactive organisms can survive high concentrations of classical antibiotics, and extended treatment is therefore required for drug efficacy (Hurdle et al. 2011).

The mode of action of antimicrobial peptides involves disrupting the integrity of the bacterial membrane in different ways, although new evidence points to intracellular targets, including DNA and protein synthesis, protein folding, enzymatic activity, and cell wall synthesis. In any case, membrane interactions are important even for intracellular-targeting peptides, because they must translocate the lipid membrane. Diverse studies have indicated that the interaction of AMPs with the bacterial membranes is specifically based on their structural properties. It is thus their sequence, size, cationic nature, hydrophobicity and amphipathicity that govern their interaction with target cells (Yeaman and Yount 2003; Nguyen et al. 2011).

Despite the diversity of their sequences, common features are shared by many AMPs. They are typically 10–40 amino acids in length, and are usually amphipathic

molecules with hydrophobic and hydrophilic moieties segregating into distinct patches on the molecular surface. In addition, they usually possess a net positive charge at physiological pH, although a few negatively charged AMPs have also been found (Toke 2005). Topologically, they can be divided into two groups: linear peptides and cysteine-bridged peptides. Many experiments using model membranes, such as liposomes and monolayers, have shown that most AMPs disturb the anionic lipid membrane and enhance ion permeability (Zhao et al. 2001; Sun et al. 2014; Hallock et al. 2003; Domingues et al. 2013).

3.1 Peptide Binding and Disruption of the Cytoplasmic Membrane

Bacterial membranes are negatively charged, with lipids bearing anionic phospholipid headgroups such as phosphatidylglycerol, cardiolipin, or phosphatidylserine (Epand et al. 2008). In addition, Gram-negative bacteria have an outer membrane composed of anionic lipopolysaccharide (LPS). This particular lipid composition explains the selectivity of AMPs for bacterial over eukaryotic membranes. The last ones are rich in zwitterionic phospholipids such as phosphatidylcholine, especially in the outer leaflet, and thus are overall neutral. In addition, bacterial membranes lack cholesterol, and are less rigid and more susceptible to peptide-induced permeabilization. Cationic AMPs directly interact with bacterial anionic membrane lipids. Anionic antimicrobial peptides act by an analogous mechanism; however, in this case, they are absolutely dependent on the presence of calcium ions. Most likely, the divalent Ca^{2+} ions serve to confer a pseudo positive charge to the anionic peptides, thus promoting the interaction with the anionic bacterial membrane. This is the case of daptomycin, an acidic cyclic lipopeptide that is highly active against Gram-positive bacteria, including methicillin-resistant *S. aureus* (MRSA). The proposed mechanism of action includes interaction with the anionic lipids of the cytoplasmic membrane in a calcium-dependent manner (Straus and Hancock 2006; Jung et al. 2008). Once in the membrane, Ca^{+2} locks daptomycin in a membrane-active conformation, forming oligomers that span the bilayer and induce potassium leakage, thus causing cell death (Silverman et al. 2003). In a revised mechanism, calcium binds to daptomycin and induces oligomerization and change in conformation previously to insertion into the membrane (Straus and Hancock 2006).

The absence of specific chiral receptors or enzymes for antimicrobial peptides is demonstrated with synthetic D-enantiomers, showing activities that are comparable to their natural L-counterparts (Wade et al. 1990). The lack of specific receptors will also make it difficult for bacteria to develop resistance to the peptides, since bacteria would have to alter the properties of their membrane as a whole, rather than specific receptors, and this is highly costly. In fact, acquisition of resistance by a sensitive microbial strain against antimicrobial peptides is surprisingly improbable, and even

in this case most of the resistance mechanisms described have a moderate increase on the minimal inhibitory concentration (Hancock 2001). The first consequence of lipid binding is the structuration of the peptides that are mostly unstructured in solution, folding into amphiphilic structures (Saberwal and Nagaraj 1994; Laverty et al. 2011). Details on what happens next at the molecular level have been dissected successfully by biophysical techniques using model membranes, such as monolayers and lipid vesicles, and later corroborated on microbial cells predominantly utilizing membrane potential sensitive dyes and fluorescently labeled peptides (Cajal and Jain 1997; Clausell et al. 2006; Bocchinfuso et al. 2011). Studies with unilamellar vesicles that reproduce the bacterial cytoplasmic membrane have shown that antimicrobial peptides at low peptide-to-lipid ratio remain adsorbed in the headgroup region of the outer leaflet, lying parallel to the membrane surface causing a thinning effect but without any destabilization of the membrane function (Yang et al. 2001). As the peptide/lipid ratio increases, peptide molecules rearrange and insert into the bilayer, forming transmembrane pores that result in permeabilization and cell death. There are three different models proposed to explain membrane permeabilization by cationic peptides: the toroidal pore, barrel stave, and carpet models (Brodgen 2005). In addition, the membrane–membrane contact formation and lipid exchange is the mechanism of action of certain AMPs on Gram-negative bacteria (Oh et al. 1998a, b, 2000) (Fig. 1). In the toroidal pore model, peptide molecules aggregate and insert perpendicularly into the lipid bilayer, with electrostatic interactions between the hydrophilic domain of the peptide and the anionic phospholipid head groups. Hydrophobic regions of the peptide bend the lipid monolayers forming a central water core lined by lipid head groups and inserted peptides. A toroidal pore is thus formed by positive curvature, allowing entry of further antimicrobial peptide. This has been demonstrated for magainins and melittin (Yang et al. 2001; Hallock et al. 2003). The barrel-stave pore has been demonstrated for alamethicin, an α-helical peptide antibiotic that aggregates and spans de membrane with the hydrophobic regions aligned parallel with the lipid core of the bilayer, thus forming a pore that is hydrophilic in the interior (Spaar et al. 2004). Finally, the carpet model refers to peptides that bind parallel to the bilayer surface; once a threshold concentration in the membrane is reached, clusters of high peptide density are formed, causing significant curvature strain and ultimately resulting in membrane disruption (Oren and Shai 1998). In fact, this mechanism is considered to be an extension of the toroidal pore model that will take place at high peptide concentrations, such as those used in the experiments with model membranes (Nguyen et al. 2011).

There does not appear to be one model that fits all peptides, with various factors contributing to the process of pore formation. The actual antimicrobial activity in order to kill bacteria resides in part in the disruption of the bacterial cell membrane, which can occur in a number of ways. These include membrane depolarization, permeabilization or creation of pores which could cause cellular contents to leak out, degradation of cell walls, and alteration of the lipid composition in the membrane bilayer which could lead to the disturbance of membrane functions (Straus et al. 2006).

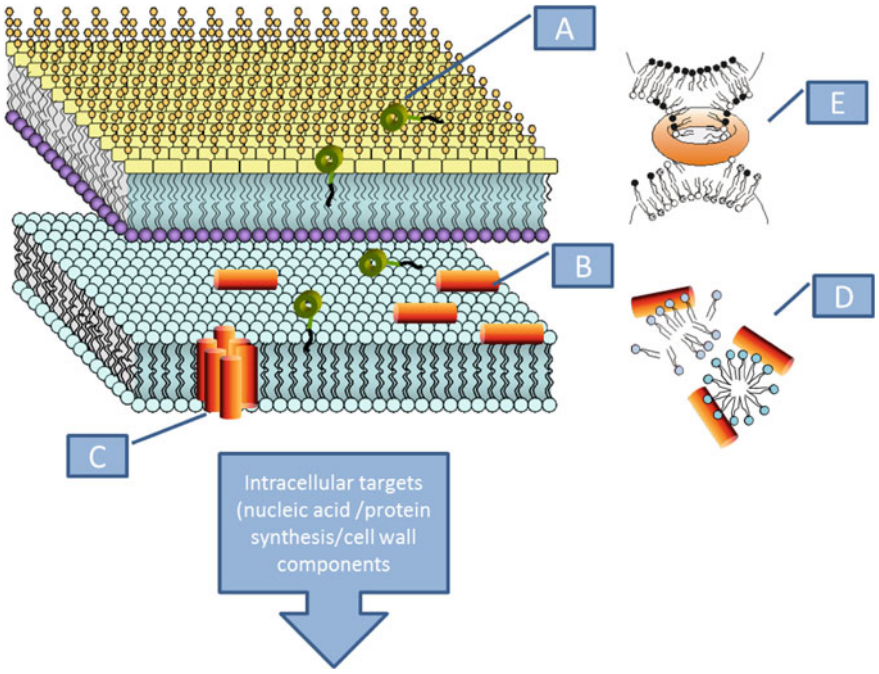

Fig. 1 Model for the mechanism of action of antimicrobial peptides. In Gram-negative bacteria, peptides must bind LPS in the outer membrane in order to enter into the periplasmic space (**A**). Subsequently, peptides interact with the anionic cytoplasmic membrane, adopting an amphiphatic secondary structure (**B**). This structure is able to integrate into the outer leaflet, causing a thinning effect that is followed by channel formation, thus inducing depolarization and/or leakage of intracellular contents (**C**), or destruction of the bilayer structure by formation of micelles (**D**). In some cases, peptides induce contacts between outer and inner membranes, allowing mixing of lipids and therefore changing the membrane composition (**E**), thus resulting in osmotic imbalance. Finally, peptides may translocate across the cytoplasmic membrane and attack intracellular targets

3.2 Peptide Binding to LPS and Self-promoted Uptake

Gram-negative bacteria has a highly complex membrane, with an additional outer lipid membrane that is formed by lipopolysaccharides in the outer leaflet, and phospholipids in the inner leaflet. In order to reach the cytoplasmic membrane, antimicrobial peptides must first cross the outer membrane, a process known as self-promoted uptake (Hancock 1997; Sawyer et al. 1988). The peptides possess greater affinity for the negatively charged LPS than native divalent cations, such as Ca^{2+} and Mg^{2+}, and as a result, the divalent cations are displaced. Both magnesium and calcium ions are required for cell surface stability via the cross-linking of carboxylated and phosphorylated head groups of lipids (Chen and Cooper 2002). Removal of these divalent cations leads to distortion of the outer membrane, thus enabling the translocation of the peptides. In addition, this alteration, which in itself

is not enough to kill the bacteria, can allow other small molecules (such as conventional antibiotics) to cross the outer membrane, thus explaining why in Gram-negative bacteria many cationic antimicrobial peptides act in synergy with conventional antibiotics (Giacometti et al. 1999).

Once the antimicrobial peptides access the periplasmic space, there are two possible mechanisms. One possibility is that they associate with the phospholipids of the cytoplasmic membrane and disrupt its function by any of the models described above. Another mechanism of action has been described that involves contact formation between the outer and inner membranes of Gram-negative bacteria. According to this model, once in the periplasmic space the peptides form contacts between the two enclosed phospholipid interfaces and promote a fast and selective exchange of certain phospholipids. The resulting changes in the membrane lipid composition trigger an osmotic imbalance that leads to bacterial stasis and cell death (Velkov et al. 2010; Yu et al. 2015). This phenomenon has been proposed as the mechanism of antibacterial action of polymyxins (Cajal et al. 1996a, b; Oh et al. 1998a) and other antibiotic peptides, including cecropins (Oh et al. 1998b, 2000). Biophysical studies using model membranes have demonstrated that at the concentrations around the MIC, polymyxin B (PxB) induces the apposition of anionic vesicles and the formation of functional vesicle–vesicle contacts that support a fast and selective exchange of phospholipids exclusively between the outer monolayers of the vesicles in contact and maintaining intact the inner monolayers and the aqueous contents (Cajal et al.1996a, b). For example, monoanionic phospholipids such as phosphatidylglicerol are transferred through the contacts, whereas zwitterionic phosphatidylcholine or dianionic phosphatidic acid are excluded, independent of the composition of the fatty acid chains. Sublethal concentrations of PxB in growing *E. coli* induce a highly selective cellular stress, with transcription of the *osmY* gene without leakage of solutes and protons (Oh et al. 2000). Since *osmY* expression is also induced by hyperosmotic stress, the interpretation is that PxB forms functional contacts in the periplasmic space between the anionic phospholipid-containing outer surface of the cytoplasmic membrane and the inner surface of the outer membrane.

3.3 Intracellular Targets of Peptide Antimicrobials

There is increasing evidence that although membrane interaction must occur, it is unlikely that this is the only mechanism of bacterial killing in all cases (Hale and Hancock 2007; Afacan et al. 2012). Many AMPs are able to translocate across the cytoplasmic membrane to act on various cytoplasmic targets, generally anionic molecules, as both alternative and synergistic pathways to membrane rupture and cell lysis. Some of the proposed targets are DNA/RNA synthesis, condensation of intracellular DNA, protein synthesis/folding, cell wall synthesis/integrity, and cell division (Hale and Hancock 2007; Park et al. 1998).

In some cases, antimicrobial peptides can translocate the cytoplasmic membrane without any alteration in membrane potential and function, as is the case of buforin II, a peptide derivative from histones of the Asian toad Bufo *Bufo gargarizans* (Park et al. 1998). Once in the cytoplasm, buforin binds and inhibits DNA and RNA, exerting in this way its antimicrobial activity (Lan et al. 2010). Other AMPs that bind nucleic acids are tachylepsin from horseshoe crabs (Brodgen 2005), and bovine lactoferricin, although both of them are also membrane-active.

Cathelicidin is a family of host defense peptides that exert their antimicrobial action through interactions with cell membranes and pore formation, but other killing mechanisms based on interactions with internal microbial targets have also been reported. For instance, some cathelicidin peptides interfere with DNA/RNA/protein synthesis (for review see Brodgen 2005). Indolicidin, a 13-amino acid antimicrobial peptide from the cathelicin family, is present in the cytoplasmic granules of bovine neutrophils. Indolicin disrupts the cytoplasmic membrane by channel formation (Rokitskaya et al. 2011), but it has also the ability to penetrate the cytoplasm and bind DNA, interfering with DNA-binding enzymes (Marchand et al. 2006) and protein synthesis (Falla et al. 1996). Alternative intracellular mechanisms include inhibiting the formation of structural components of the cell wall, such as HBD-3 and plectasin, a fungal defensin that acts by binding to the bacterial cell wall precursor Lipid II to inhibit cell wall biosynthesis (Nguyen et al. 2011; Brotz et al. 1998; Brotz and Sahl 2000). Recently, an intracellular mechanism of action has been described for polymyxin B, where bacterial death will occur through the accumulation of hydroxyl radical (·OH). This hypothesis is based on the oxidative stress due to polymyxin induced formation of reactive oxygen species (ROS) in Gram-negative bacterial cells (Imlay 2013).

4 Antimicrobial Peptides: Market and Pipeline

The first antimicrobial peptides available in the market were cyclic peptides, such as polymyxin B, colistin (polymyxin E), tyrocidin, gramicidin, bactracin, and daptomycin (Fig. 2).

The case of polymyxin is of particular interest as it is currently being reintroduced as a last resort antibiotic. Polymyxins were discovered in 1947 and entered into clinical use in the 60s. They are highly active against Gram-negative bacteria, particularly most of the ESKAPE bacteria: *A. baumannii, P. aeruginosa, K. pneumoniae, and E. coli.* Polymyxins have been mostly used for topical application, superficial eye infections and, for selective decontamination of the digestive tract. Systemic use was abandoned in the 1970s because of reports on their toxicity (nephrotoxicity, neuromuscular blockage and neurotoxicity).

However, due to the scarcity of antibiotics to fight infections caused by multi-drug-resistant Gram-negative bacteria, polymyxins have been reintroduced mainly as a treatment of last resort for patients who have serious infections caused by such pathogens for which no other therapeutic options exist. In addition, recent

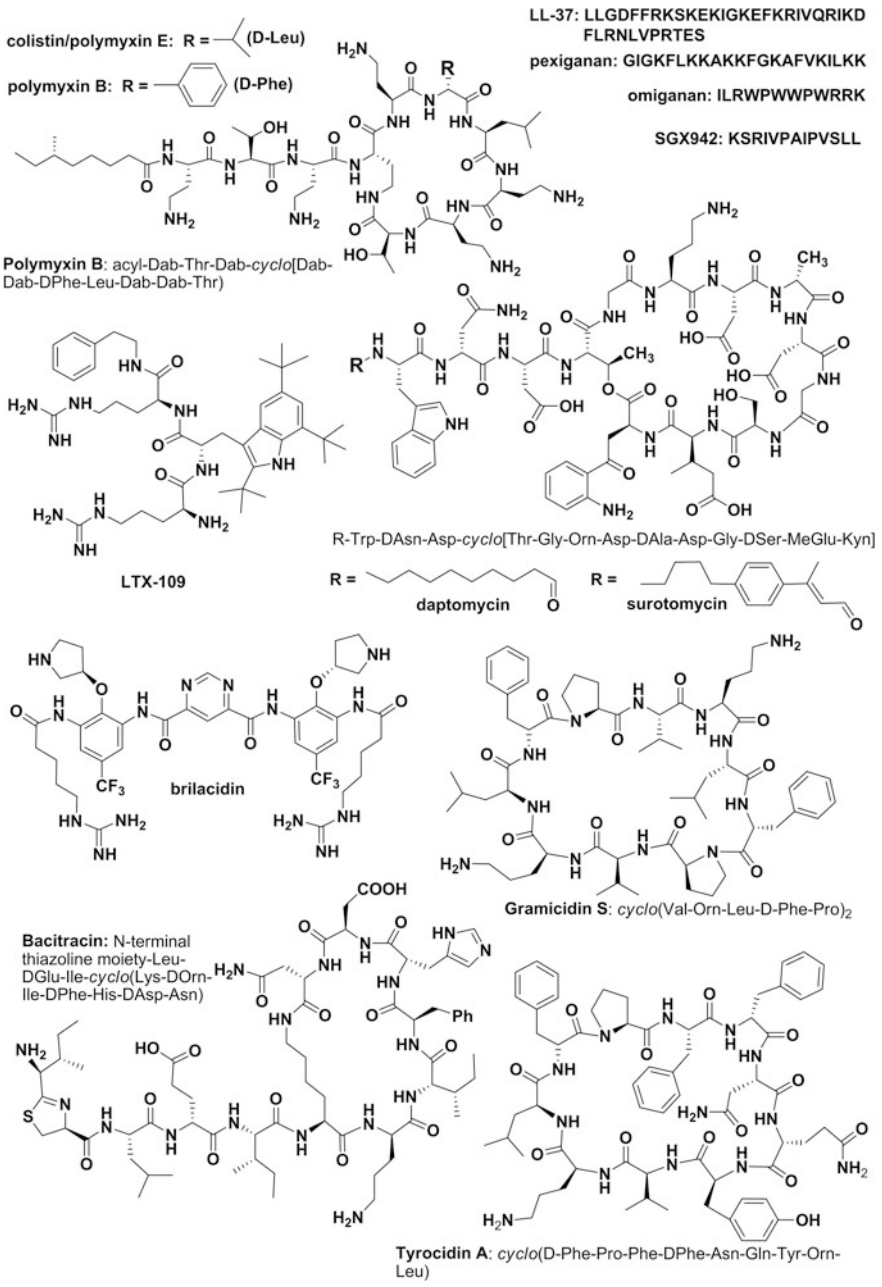

Fig. 2 Available structures of some of the antimicrobial peptides that are described in the text

clinical research on polymyxins has led to better dosing strategies and found that polymyxins show a better therapeutic index than originally reported. Furthermore, a great deal of effort is being invested in developing new polymyxin analogs with reduced toxicity (Vaara 2013; Rabanal et al. 2015; Velkov et al. 2014; Magee et al. 2013; Clausell et al. 2007).

Bacitracin is also a cyclic peptide antibiotic that is commonly used in combination with polymyxin and neomycin (an aminoglycoside) as triple antibiotic ointment (Neosporin™) for the topical treatment of skin and eye infections. Parenteral (intramuscular) administration of bacitracin is highly restricted due to its nephrotoxicity. It is only used as an intramuscular injection for infants with pneumonia and emphysema caused by staphylococci (Stevenson 2009).

Gramicidin is a heterogeneous mixture of three antibiotic linear pentadecapeptides named, gramicidins A, B, and C. They are obtained from the soil bacterial species *Bacillus brevis* and known with the global name of gramicidin D. Gramicidin S is a cyclic decapeptide. Gramicidins are particularly effective against Gram-positive bacteria. They cannot be used systemically as they are highly hemolytic. They also show toxicity to liver, kidneys, meninges, and the olfactory system. Hence, they are only administered topically. Their primary use is in the treatment of infected surface wounds, and in ocular, nasal and throat infections. They are also commercialized as an ophthalmic solution in combination with two other antibiotics (neomycin and polymixin B) (Stevenson 2009).

Tyrocidine is also produced by *B. brevis*. It is a mixture of cyclic peptides (tyrocidines A–D, containing ten amino acids) and is the major constituent of tyrothricin (80 %). Discovered in 1939 by René Dubos, tyrothricin, the active substance of *B. brevis*, contains also gramicidin (20 %). Tyrocidine attacks the membranes of both Gram-positive and Gram-negative bacteria, whereas gramicidin is mostly a bacteriostatic agent that selectively inhibits the growth of Gram-positive bacteria. The discovery of tyrothricin and gramicidin is considered to have launched the era of antibiotics, together with prontosil, a sulfamide discovered also back then, and helped revive the stalled interest in penicillin, shelved for almost a decade after its discovery by Fleming in 1929. Tyrothricin is used only as a topical antimicrobial agent since it has been found to be highly toxic when administered parenterally (Van Epps 2006). An example of the current use of tyrothricin, usually formulated with benzocaine, could be the throat lozenges, to treat throat irritation and infection.

Daptomycin (cubicin®), a cyclic anionic lipopeptide antibiotic, was approved by the FDA in 2003. Daptomycin is indicated for the treatment of complicated skin and skin structure infections (cSSSI) caused by susceptible isolates of Gram-positive bacteria, mainly *S. aureus* (including methicillin-resistant isolates) and *S. aureus* bloodstream infections (bacteremia), including patients with right-sided infective endocarditis (Cubicin webpage). Daptomycin is considered a blockbuster drug as 2014 sales added upto *ca* $1000 million.

Other AMPs are at different stages of development (Fox 2013; Afacan et al. 2012; The Pew Trust webpage). Pexiganan, a 22-amino acid magainin analog, is now in clinical trials (Phase III) for the treatment of bacterial infections associated with diabetic foot ulcers (Locilex, pexiganan cream 0.8 %). Pexiganan shows

activity against a broad spectrum of Gram-positive, Gram-negative, aerobic, and anerobic bacteria, as well as fungi. In addition, it is also active against a variety of resistant bacteria, including methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant *Enterococcus* (VRE), extended-spectrum beta-lactamases (ESBL) and multidrug resistant bacteria (Dipexium pharmaceuticals webpage).

Omiganan (CLS001, topical gel), a cationic peptide derived from indolicin, is currently in phase II clinical trial to find the best dose when treating the papules and pustules of rosacea. Rosacea is a chronic skin disease that affects over 14 million North Americans and 45 million people globally. The US rosacea prescription drug market is estimated to be greater than US$200 million annually (Cutanea Life Sciences webpage).

LTX-109 (Lytixar) is a short cationic peptide developed for the treatment of skin infections. It is in phase II trial for the treatment of impetigo and uncomplicated skin and skin structure infections (uSSSi, abscesses, impetiginous lesions, furuncles, and cellulitis) and phase I/II trial for nasal decolonization of *S. aureus* including methillicin- and vancomycin-resistant strains (Lytix Biopharma webpage; Butler et al. 2013).

Pergamum AB, which is a portfolio company of Karolinska Development AB (Karolinska Institutet, Sweden), has developed three clinical stage peptides, namely LL-37, PXL01 and DPK-060, for the topical administration and treatment of chronic wounds, prevention of scars and adhesions and, as an anti-infective. LL-37 (hCAP-18, C-terminal fragment of human cathelidicin) has broad-spectrum antibacterial activity. Its enantiomer, D-LL-37, shows a similar activity, what suggests a nonspecific mode of action (Karolinska development webpage).

POL7080 is a macrocyclic protegrin I analog highly active and selective against *P. aeruginosa*. It acts by a novel mechanism of action since it targets protein LptD, which functions in outer membrane biogenesis. In preclinical studies, POL7080 showed high activity on a broad panel of clinical isolates including multidrug resistant *Pseudomonas* with outstanding in vivo efficacy in thigh, lung, and septicemia infection models. In a phase I clinical trial with healthy volunteers, it was well-tolerated showing no serious side effects (Polyphor webpage; Srinivas et al. 2010).

SGX942 is a fully synthetic, 5-amino acid peptide derived from indolicin. SGX942 demonstrated safety and tolerability in a healthy volunteer phase I clinical trial. A phase II clinical study to treat oral mucositis is currently ongoing. SGX942 is also an innate defense regulator. Preclinical data indicate that SGX94 is active in models of a wide range of therapeutic indications including severe side effects of chemo- and/or radiation-therapy and inflammation (Soligenix webpage).

Brilacidin, an arylamide foldamer peptidomimetic of human defensin, shows high bactericidal activity against both Gram-positive and Gram-negative bacteria. A clinical Phase IIb study comparing brilacidin to daptomycin at three dosing regimens for the treatment of Acute Bacterial Skin and Skin Structure Infections (ABSSSI) yielded top-line positive results. Brilacidin has received QIDP designation, which means that it is eligible for additional FDA incentives in the approval

and marketing path, including Fast Track designation and Priority Review for development and a five year extension of market exclusivity (Cellceutix webpage).

NP213 is a cyclic arginine-based heptamer based mostly on α- and β-defensins. It shows fungicidal activity and it has been formulated as a brush-on-treatment for onychomycosis (fungal nail infection). NP213 is the active pharmaceutical ingredient of Novexatin®. Phase I and IIa clinical studies have demonstrated that NP213 is safe, well-tolerated and effective in resolving toenail infections. This toenail condition is a clear unmet medical need (it affects *ca* 12 % of the population). By 2022, the global market for this disease has been forecasted to be greater than $6 billion (Novabiotics webpage).

Arenicin-3 was isolated from the marine lugworm *Arenicola marina*. It is a 21-residue amphipathic β-hairpin peptide that contains two disulfide bridges. It exhibits a potent bactericidal effect against multidrug-resistant Gram-negative bacteria. It has been shown to be of particular interest in the fight against urinary tract infections caused by *E. coli* strains and for the treatment of pneumonia (HAP, hospital-acquired pneumonia, and VAP, ventilation-associated pneumonia). It is still in preclinical development stage (Adenium biotech webpage).

Another family of peptides in development as potential antimicrobials includes the lantibiotics. Lantibiotics are a naturally occurring class of antibiotic peptide compounds found in bacteria. Their name comes from the lanthionine amino acid present in their sequence. Perhaps the most known member of the family is nisin, which is widely used as food preservative (E324 food additive number). Other lantibiotics are in development to fight infections. Compound MU1140, which is in the preclinical stage, has shown promising activity against *S. aureus* (including MRSA), *Enterococci* (including VRE), *Clostridium difficile*, *Mycobacterium tuberculosis* (the causative agent of tuberculosis), and *Bacillus anthracis* (the causative agent of anthrax). However, the main drawback with lantibiotics is the production in large quantities, as standard fermentation methods, such as those used to make a variety of other antibiotics, typically result in production of only minute amounts (Oragenics webpage). Similarly, lantibiotic NVB302 has completed a phase I clinical trial for orally administered treatment for *C. difficile* infection. NVB302 proved to be safe and well-tolerated. In addition, it is not absorbed in the gastrointestinal tract, thus resulting in optimal concentrations there. It is also highly selective against *C. difficile* over other members of the normal gut flora which may result in additional therapeutic benefits (Novactabio and Cantab webpages).

Finally, surotomycin (also CB-315, CB-183315) is an antibacterial drug candidate derived via semi-synthesis from daptomycin. It is in phase III clinical trials for the treatment of *C. difficile*-associated diarrhea. It is a potent, oral, cidal, cyclic depsipeptide. In 2012, Qualified Infectious Disease Product Designation was assigned in the U.S. under the FDA GAIN Act for the treatment of *C. difficile*-associated diarrhea. This means that priority review, fast-track status, and five year exclusivity after license are applicable (Knight-Connoni et al. 2016).

5 Development of Antimicrobial Peptides as Drugs

The discovery and development of drugs is a long and expensive process (some 10–12 years and several hundred millions of euros, taking into account the cost of opportunity) with many regulatory hurdles and a low success rate (approximately, 1 compound out of 5–10 thousand reaches the market) (Walsh and Wencewicz 2014; Herper 2013; Tufts CSDD 2014). Despite these generic pitfalls, more than a hundred peptide-based drugs with different therapeutic applications have reached the market. The global peptide drug market will move from $14.1 billion in 2011 (*ca* 1.5 % of global drug sales) to an estimated $25.4 billion in 2018 (Fosgerau and Hoffman 2015).

Therapeutic peptides show advantages and drawback compared to small molecule drugs. On the one hand, peptides are potent and selective for their target in vivo and tend to be less toxic than small molecules. On the other hand, they usually show poor systemic stability, membrane permeability, oral biovailability, and high clearance levels. In addition, their production costs are generally high (Uhlig et al. 2014).

The development of peptides as drugs is thus a challenging area. Due to their physicochemical properties and size, compared to small molecule drugs, peptides show poor oral biovailablity and are not likely to pass through the gastrointestinal tract. Hence, the majority of peptide drugs are administered parenterally (75 % are injectables), such as the case of antimicrobial peptides polymyxin B, colistin or daptomycin in the treatment of systemic infections. In spite of that, alternative administration forms are under development, such as mucosal (intranasal sprays, pulmonary delivery), transdermal (patches), or oral delivery routes (Uhlig et al. 2014; Goodwin et al. 2012). Great efforts are invested in increasing orally biovailable peptide forms mostly for the convenience of patients. Oral delivery implies that peptide drug antibiotics not only should be stable to acids in the stomach and to proteolytic degradation by enzymes but they should also be capable of crossing the intestinal mucosa to reach the circulatory system. Alternatively, pulmonary intake has the advantage of showing a highly absorptive and permeable surface. Hence, several antibiotics including polymyxins have been assayed for inhaled administration to treat pulmonary infections such as ventilator-associated pneumonia or lung infections associated with cystic fibrosis (Falagas and Kasiakou 2005). Compared to systemic routes of administration (enteral and parenteral), inhalation achieves higher pulmonary concentrations of antibiotics, with 30–50 % dose reduction and the potential to ameliorate systemic toxicity.

Formulations trying to deal with the aforementioned peptide pharmacological limitations are also in constant development. Many of these medicinal preparations are chemistry- and nanotechnology-based. Chemical strategies have been devised to enhance the pharmacologic properties of peptides, such as introduction of D-amino acids, *N*-methyl amino acids, ciclyzation (some are naturally cyclic, e.g., polymyxins and daptopmycin), or stapling/cliping peptide sequences (Goodwin et al. 2012; Fosgerau and Hoffman 2015). Nanocarrier technology indeed shows the

potential of increasing peptide pharmacodistribution and biovailability. Nanocarriers include nanoparticles, liposomes and micelles which protect the peptide against degradation and provide a sustained release. Examples are nanoparticles composed of polymeric formulations such as polylactic acid, poly (lactic-co-glycolic) acid or polysaccharides (i.e., chitosan). Other strategies use binding to circulating albumin to increase the half-life of peptides, conjugation to polyethylene glycol moieties (PEGylatgion) or the use of special formulations. Finally, drug delivery approaches also include implantable devices that deliver the therapeutic dose of peptide for up to one year by means of osmotic pump mechanisms (Zhang et al. 2010; Uhlig et al. 2014; Fosgerau and Hoffman 2015).

6 Synthesis at Large Scale

Peptides, as any other potential drug, need to be produced in large amounts following good manufacturing practice (GMP) guidelines. The method for producing a peptide is mostly determined by its size and chemical structure. Several technologies exist for this purpose such as chemical synthesis, recombinant DNA technologies, and cell-free expression systems (in vitro translation) (Uhlig et al. 2014).

Chemical synthesis of peptides may be challenging and expensive, even at low scale. Therefore, recombinant biotechnology will certainly be an alternative approach in peptide manufacturing in cases where chemical complexity or certain quantity requirements are needed. Biotechnology procedures will thus be a complement to chemical peptide synthesis in the production of peptide therapeutics.

Nevertheless, cost of large-scale peptide chemical synthesis has significantly decreased over the last years, mainly due to technological evolution and chromatographic systems. Chemical synthesis is more easily scalable for manufacturing short or medium-size peptides up to ten- or hundred-kg scale. It is also less demanding in terms of process development, quality assurance and regulatory affairs. Finally, chemical approaches are far more versatile than biotechnological ones, mostly regarding flexibility in the design of analogs requiring unnatural amino acids or non-proteogenic building blocks. Nowadays, some peptides are produced up to the scale of metric tons, such as enfuvirtide (Fuzeon), which prevents the infection of CD4+ T-cells by HIV-1 and is used as salvage therapy in patients with multi-drug-resistant HIV (Bray 2003).

7 Concluding Remarks

The emergence of resistant and multi-drug-resistant pathogens has put a spotlight on the urgent need to develop new antibiotic compounds that are highly active, selective and safe. Particularly interesting would be those having novel chemical

scaffolds and acting by alternative mechanisms of action. Antimicrobial peptides potentially offer such advantages to become useful therapeutic candidates as they are unlikely to promote microbial resistance, act preferentially at the membrane level, can disrupt multiple biochemical processes in the pathogen (multimodal mechanism of action) and may activate the immune system of the host. Nevertheless, there are still some pitfalls that need to be addressed in the development of antimicrobial peptides, such as toxicity, pharmacological issues (enhance peptide stability, reduce clearance rates and so on) and cost of manufacturing. In fact, very few antimicrobial peptides have reached the market, and those that have are mainly cyclic peptides that usually contain non-coded amino acids (D-amino acids, for instance). Hence, much effort is needed from the chemical and pharmaceutical point of view, but the fact that many peptides have reached the market for different therapeutic indications (oncology, diabetes, blood pressure control, and so on) and that the regulatory approval rate for peptides is around 20 % (vs. 10 % for small molecules) augurs well for the future arrival of new antimicrobial peptides to fight the threat of infections caused by resistant and multidrug resistant bacteria.

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The Use of Nanoparticles for Antimicrobial Delivery

María Moreno-Sastre, Marta Pastor, Amaia Esquisabel and José Luis Pedraz

Abstract Many currently used antibiotics suffer from some drawbacks such as local and systemic side effects, inadequate therapeutic index, and high antimicrobial resistance to bacteria. Since the emergence of multidrug-resistant bacteria, new antibiotic approaches are required. In recent years, nanotechnology has appeared as a successful tool for the encapsulation of antibiotics into nanoparticles (NPs) aiming to treat bacterial infections and overcoming, at the same time, some of the limitations of traditional antimicrobial therapeutics. Drug delivery systems (DDS) provide several advantages over the free drug such as protection from environmental inactivation and specific target site that can lead to an improvement in the treatment of such diseases. Moreover, NPs can overcome tissue and cellular barriers, thereby can treat infections caused by intracellular microorganisms. NPs are capable of reducing drug dose and toxicity as well as dosing frequency which improve patient compliance. Many nanostructures including liposomes, nanoparticles, or dendrimers have demonstrated their ability to increase the therapeutic efficacy of antibiotics and fight against infectious diseases. In this chapter we provide an overview of the current progress of the latest nanosystems developed to delivery antibiotics to combat microbial infection.

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1 Introduction

Since the discovery of penicillin in 1928, antibiotics have made significant improvement in public health reducing the morbidity and mortality associated with infectious diseases. However, the growth of bacterial resistance over the last decades forces an enormous economic and social problem on the healthcare system worldwide (Huttner et al. 2013). The main causes of this phenomenon are the overuse and misuse of anti-infection drugs that contribute to the development of new defense mechanism of bacteria by means of innate resistance, genetic mutations, or acquisition of resistance genes. In addition, bacteria can modify the antibiotic by inactivation or enzymatic modification, alteration of the antibiotic target, or changes in cell permeability and efflux (Brooks and Brooks 2014). Figure 1 summarizes the main mechanisms bacteria have for antibiotic resistance.

The emergence of drug-resistant microorganisms, such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus*, fluoroquinolone-resistant *Escherichia coli,* and multidrug-resistant mycobacteria intensifies the needs for new antimicrobial agents with novel drug targets (Yang 2014). Besides that, another important challenge in antimicrobial therapy is the treatment of chronic infections, which are often caused by intracellular microbes or by extracellular microorganisms able to form biofilms. Intracellular infections are more difficult to treat than the extracellular ones due to the low availability of drug inside the cells; the intracelullar location of the microorganism which protects them from the host defense mechanisms and also because the drugs are unable to penetrate the cell efficiently (Ray et al. 2009).

Currently, a great number of pathologies are caused by intracellular microorganisms such as leishmaniasis, tuberculosis, legionellosis (pneumonia), or salmonellosis. The antibiotics mainly used today to treat these infections

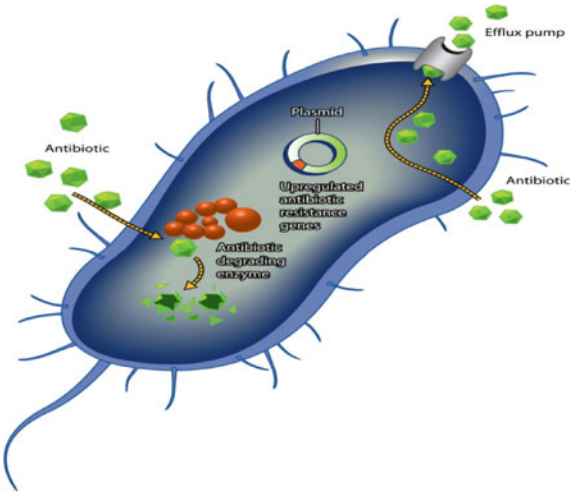

Fig. 1 Main antibiotic resistance strategies used by bacteria. Reproduced with permission Brooks and Brooks (2014)

(aminoglycosides and beta-lactams) have a limited capacity to penetrate into the cells, hindering their treatment, with only a few (quinolones, macrolides) being efficient in the treatment of phagocytic cells (Briones et al. 2008).

Taking all the above into account, the failure of current therapies of bacterial infectious diseases could be related with:

- Low bioavailability of antibiotics
- Side effects of antibiotics
- Rapid clearance of antibiotics from organs
- The environmental deactivation of the drug
- Tissue and cellular barriers (mucosal barriers of the ocular, gastrointestinal, and respiratory tissues)
- Biofilm formation (renders the bacterial less susceptible, i.e., 10 to 1000-fold to antimicrobial agents compared to their planktonic cell counterparts) (Mah and O'Toole 2001)
- Intracellular bacterial infection
- Emergence of resistant bacteria
- No specific accumulation

This chapter is aimed to raise the awareness of an urgent need for new therapies to fight against multidrug-resistant pathogens in the treatments of infectious diseases. In this regard, nanotechnology has emerged as an approach to encapsulate antibiotics in drug delivery systems (DDS) in order to improve the therapeutic indexes of antimicrobial drugs (Zhang et al. 2010). DDS including dendrimers, micelles, polymeric nanoparticles (NP), liposomes, solid lipid nanoparticles (SLNs), or nanostructured lipid carriers (NLCs) are structures in the nanometric range (Fig. 2) that have been explored for the delivery of different antimicrobial agents showing high bactericidal activity in vitro with an improvement on conventional therapies (Kalhapure et al. 2015).

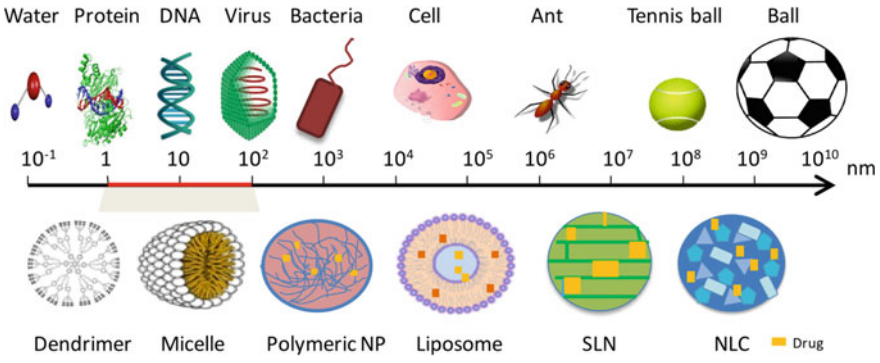

Fig. 2 Main NPs employed for antibiotic delivery

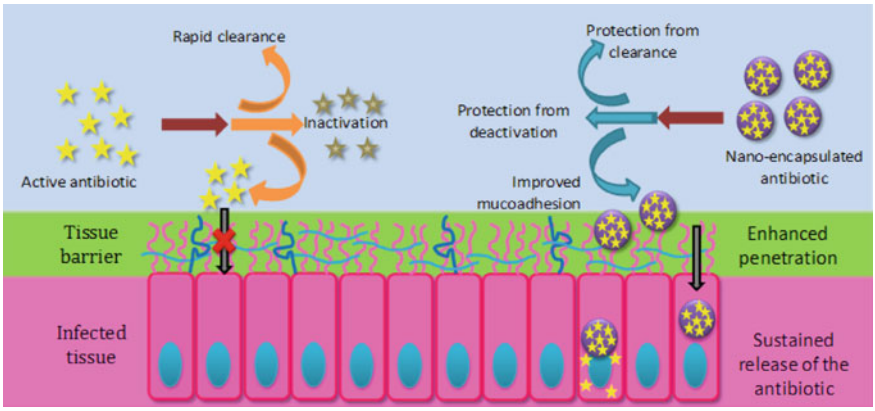

Fig. 3 Main advantages of nanoencapsulated antibiotics over free antibiotics. Adapted from Xiong et al. (2014)

Antimicrobial nano-DDS can provide several advantages over other conventional drugs that are enumerated in Fig. 3. Besides them, other advantages also worth it to mention are: high drug payload, ability to encapsulate a wide range of molecules and to increase drug solubility of hydrophobic drugs, target the drugs to specific cells, tissues or organs, reduction of side effects or toxicity of the drug as well as their ability to facilitate transport across critical and specific barriers. Moreover, NPs are also able to combat intracellular bacteria as they are small enough to be phagocytozed by host immune cells and release high concentrations of antimicrobial drugs inside the infected cell (Huh and Kwon 2011; Xiong et al. 2014).

In general, NPs are well-tolerated systems for oral, parenteral, inhalational, ocular, and dermal applications. The administration route plays an important role in their therapeutic efficiency. When NPs are administrated intravenously, they rapidly accumulate in the cells of mononuclear phagocyte system (MPS), therefore are useful in the treatment of infections involving these cells, particularly in the liver and spleen. On the other hand, oral administration is effective in treating intestinal tract infections and the pulmonary route is commonly used to the treatment of respiratory infections such as tuberculosis, cystic fibrosis, and chronic obstructive pulmonary disease (Pinto-Alphandary et al. 2000). The main applications of nanoparticles to the treatment of infectious diseases will be revised in Sect. 4 of this chapter.

Although these new drug delivery systems promises a number of benefits for the treatment of infectious diseases, there is a lack of evidence of the potential toxicity of nanoantibiotics to human health at the moment mainly related to their small size (El-Ansary and Al-Daihan 2009).

2 State of the Art

Since 2009 (report of antibiotic resistance from the Infectious Disease Society of America) only two new antibiotics (telavancin and cetaroline fosamil) have been introduced into the market (Infectious Diseases Society of America (IDSA) et al. 2011).

Nanomedicine has altered the landscape of the pharmaceutical and biotechnology industries but the marketing process of nano-DDS by the FDA is long and difficult as evidenced by the few FDA approved products in the market at the moment (Duncan and Gaspar 2011). Up to date, more than 10 nanoparticle-based products have been marketed for bacterial diagnosis, antibiotic delivery, and medical devices (Etheridge et al. 2013).

Before reaching the market, many preclinical and clinical studies should be carried out in order to confirm that the DDS are safe and viable and can provide therapeutic benefits to humans. Nanotechnology has become a promising strategy to improve the limitations of conventional formulations and to treat resistance to antibiotic drugs. This chapter aims to provide an overview of the latest DDS approaches. First, the different types of nanoparticulate systems loaded with antibiotics are described, second different approaches of antibiotic nanoencapsulation classified according to their administration route are reviewed and finally the limitations and new challenges are discussed.

3 Drug Delivery Systems (DDS) for Nanoencapsulation

Nanocarriers used in drug delivery systems are structures of sizes in the nanoscale ranging from 1 to 100 nm in at least one dimension as it is defined by National Nanotechnology Initiative (NNI). However, the literature describes nanoparticles of up to 1000 nm size.

The way of incorporating the active compound such as antibiotic drugs into the nanocarrier and the strategy for its targeting is highly important for a targeted therapy. The nanoparticulate systems (NPs) can load drugs through physical encapsulation, adsorption, or chemical conjugation and are able to deliver their payloads into host cells through different pathways, e.g., contact release, absorption, and endocytosis. These antimicrobial delivery systems may allow sufficiently high concentrations and extended release mechanism to effectively eradicate resistant microorganisms. The behavior (intracellular delivery, biodistribution, release profile, or antibacterial effect, for instance) of the NPs can be controlled by their composition or properties, e.g., targeted NPs delivery to the infection site could also be achieved by surface modification with targeting ligands or by microenvironment responsiveness. Once the nanoparticles reach the target site, the therapeutic agents are released in a controlled manner which depends on the nature of the delivery system, pH, osmotic gradient, and the surrounding environment (Wilczewska et al. 2012).

Nanoparticles used for medical applications must be biocompatible, biodegradable, and nontoxic. Undesirable effects of nanoparticles strongly depend on their hydrodynamic size, shape, amount, surface chemistry, the route of administration, reaction of the immune systems, and residence time in the bloodstream (Ai et al. 2011). For that reason, it is important to consider several parameters such as physicochemical properties of the compounds used, the drug to be loaded, particle size and polydispersity index (PDI), surface charge, stability in storage, reproducibility, and feasibility for scale-up before their utilization as drug delivery systems. Liposomes, lipid nanoparticles, polymeric nanoparticles, dendrimers, or metallic NPs are examples of nanocarriers that have been tested as drug delivery systems for antibacterial treatment and that are evaluated in the following section. Table 1 summarizes the main approaches described in the recent literature for the delivery of antibiotic drugs by means of these systems.

3.1 Lipid Nanoparticles

3.1.1 Liposomes

Liposomes are widely investigated for drug delivery since Doxil® [doxorubicin-encapsulating PEGylated liposomes (liposomes with polyethylene glycol chains in their surface)] became the first liposomal drug approved by the FDA in 1995 (Lian and Ho 2001). Liposomes are spherical vesicles comprising one or more phospholipid bilayers with an aqueous core. Those with a single bilayer are known as single-layer vesicles (SLV) and the others as multilayer vesicles (MLV). They are usually made of natural or synthetic phospholipids and cholesterol. Liposomes can encapsulate hydrophilic or hydrophobic drugs; soluble drugs such as aminoglycosides are enclosed in the aqueous phase, whereas hydrophobic molecules as penicillins are incorporated into the lipid bilayer (Samad et al. 2007).

The most commonly applied methods for liposome elaboration include sonication, reverse-phase evaporation technique, thin film hydration, or ethanol injection (Vemuri and Rhodes 1995). Sonication is one of the most extensively used methods for liposome preparation (SUV) by using a bath-type sonicator or a probe sonicator under a passive atmosphere. However, the main disadvantages of this method include a low encapsulation efficacy (EE) or possible degradation of the phospholipids or compounds to be encapsulated. When preparing liposomes by ethanol injection, a lipid solution of the ethanol is quickly injected into a huge excess of buffer. This technique, however, presents some disadvantages because the liposomes are not homogenous, they are much diluted and removal of all the ethanol is difficult to achieve. Reverse-phase evaporation is based on the formation of inverted micelles that are shaped by sonication with an aqueous phase containing water-soluble molecules (to be encapsulated inside) and an organic phase in which the amphiphilic molecules are solubilized. The slow elimination of the organic solvent leads to the formation of a complete bilayer around the residual micelles

Table 1 Summary of the main approaches used for the development of drug delivery systems containing antibiotic drugs

Drug delivery system (DDS)		Antibiotic	Bacteria tested	Main findings	References
Lipidic NPs	Liposomes	Gentamicin	*Pseudomonas aeruginosa*	MICs of liposomal gentamicin for all clinical isolates of *P. aeruginosa* were lower than the MICs of free gentamicin Liposomal gentamicin altered the susceptibilities of these clinical isolates from gentamicin resistant to either intermediate or susceptible	Mugabe et al. (2005)
			Pseudomonas aeruginosa	MIC values significantly lower than free drug against gentamicin—sensitive and resistant strains Improved killing time and prolonged antimicrobial activity	Rukholm et al. (2006)
		Polymixin B	*Pseudomonas aeruginosa*	Improve antimicrobial activity Prevent drug resistant of microbes by saturation of the efflux pumps	Alipour et al. (2008)
		Ciprofloxacin	*Pseudomonas aeruginosa*, *Haemophillus influenza*, *Streptococcus pneumoniae*	High antimicrobial activity	Chono et al. (2011)
			Pseudomonas aeruginosa, *Staphylococcus aureus*	Same antimicrobial activity as the free drug Lower MBC against *P. aeruginosa*	Ong et al. (2012)
		Vancomycin	None	Increased lung tissue concentration of vancomycin for effective treatment of pneumonia caused by MRSA with a reduction of nephrotoxicity	Muppidi et al. (2011)
		Rifampicin	*Mycobacterium bovis*	MIC values lower than the free drug Less negatively charged liposome displayed the greatest activity against intracellular growth of *M. bovis*	Changsan et al. (2009)
			MAC (*Mycobacterium avium* complex)	Able to inhibit the growth of MAC in infected macrophages (J774 cells) and to reach the lower airways in rats	Zaru et al. (2009)
		Tobramycin	*Pseudomonas aeruginosa*	The MIC of bismuth-ethanedithiol-loaded tobramycin was 16-fold lower than free drug Less CFU in the lungs of treated rats with the liposomal formulation compared to free drug and untreated animals	Alhariri et al. (2013)
		Meropenem	*Pseudomonas aeruginosa*	Enhancement of antibiotic activity None of the studied liposomal forms of meropenem exhibited bactericidal activity against drug-resistant isolates strains	Drulis-Kawa et al. (2006)
		Benzyl penicillin	*Staphylococcus aureus*	Lower drug concentrations and shorter time of exposure Enhanced the inhibition of bacterial biofilms growth	Kim and Jones (2004)
		Clarithromycin	*Pseudomonas aeruginosa*	The highly resistant strains of *P. aeruginosa* isolated from CF patients became susceptible to liposome-encapsulated clarithromycin Liposomal clarithromycin reduced the bacterial growth within the biofilm, significantly attenuated virulence factor production, and reduced bacterial twitching, swarming, and swimming motilities and were less cytotoxic than the free drug	Alhajlan et al. (2013)

(continued)

Table 1 (continued)

Drug delivery system (DDS)		Antibiotic	Bacteria tested	Main findings	References
	Niosomes	Isoniazid	None	High cellular uptake by macrophages cells (J744) capable of achieving effective treatment of tuberculosis	Singh et al. (2011)
		Pyrazinamide	*Mycobacterium tuberculosis*	Target maximum concentration of drug to the affected site (lungs) and able to exclude undesirable side effects and decrease toxicity	El-Ridy et al. (2011)
		Ciprofloxacin	*Staphylococcus aureus*	Niosomes were 2–8 times lower than MICs of free drug High intracellular antimicrobial activity	Akbari et al. (2013)
	SLN	Ciprofloxacin	None	Sustained and prolong drug release	Jain and Banerjee (2008)
		Tilmicosin	*Staphylococcus aureus*	Sustained drug release Improved antibacterial activity in vitro and in vivo	Wang et al. (2012a)
		Norfloxacin	*Escherichia coli*	Sustained drug release Improved antibacterial activity in vitro and in vivo	Wang et al. (2012b)
		Vancomycin	*Staphylococcus aureus* and MRSA strains	Lower MICs Con- encapsulation an antibiotic and a fatty acid (linoleic acid) enhance encapsulation efficiency and antibacterial activity	Kalhapure et al. (2014)
		Amikacin	*Pseudomonas aeruginosa*	Higer antimicrobial activity than the free drug taking into account the profile release over 6 days	Ghaffari et al. (2011)
		Rifampicin, isoniazid and pyrazinamide	*Mycobacterium tuberculosis*	After pulmonary administration to tuberculosis-infected guinea pig no CFU in lung and spleen No hepatotoxicity	Pandey and Khuller (2005)
	NLC	Sodium colistimethate	*Pseudomonas aeruginosa*	More effective than free drug against clinically isolated strains from CF patients	Pastor et al. (2014)
Polymeric NPs	PLGA-NP	Ciprofloxacin	*Pseudomonas aeruginosa*, *Staphylococcus aureus*	Prolonged drug release. The same activity as free drug	Dillen et al. (2004, 2006)
			Escherichia coli	Superior effectiveness to inhibit the growth of bacteria in vivo than the free drug due to the sustained release of the NPs	Jeong et al. (2008)
		Azithromycin, rifampicin	*Chlamydia trachomatis*	Enhanced effectiveness of the antibiotic in microbial burden by intracellular targeting	Toti et al. (2011)
		Levofloxacin and ciprofloxacin	*Escherichia coli*	Biofilm inhibition activity	Cheow et al. (2010a, b)

(continued)

Table 1 (continued)

Drug delivery system (DDS)		Antibiotic	Bacteria tested	Main findings	References
	PLGA, PVA, chitosan and alginate	Tobramycin	*Pseudomonas aeruginosa*	Good in vitro antibacterial activity of NP formulations according to the biphasic release profile	Ungaro et al. (2012)
	O-carboxymethyl chitosan	Tetracycline	*Staphylococcus aureus*	Sustained release, improved bioavailability and intracellar targeting	Maya et al. (2012)
	Chitosan and herparin	Amoxicillin	*Helicobacter pylori*	A multifunctional NP system for targeting *H. pylori*, clearance effect and decrease gastric inflamation	Lin et al. (2013)
Dendrimers	PANAM	Nadifloxacin and prulifloxacin	*Escherichia coli*	Improved solubility without affecting antibacterial activity	Cheng et al. (2007)
		Sulfamethoxazole	*Escherichia coli*	Increased antibacterial activity via enhanced penetration of antibiotics through the bacteria membrane	Ma et al. (2007)
		Azithromycin	*Chlamydia trachomatis*	The conjugations with the drug was efficient to treat intracellular infections	Mishra et al. (2011)
	HPO hexadentate-based dendrimeric quelator	Norfloxacin	*Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*	Synergistic bactericidal effect	Zhou et al. (2014)

MIC minimum inhibitory concentration; *MBC* minimum bactericidal concentration; *MRSA* methicillin-resistant *Staphylococcus aureus*; *CFU* colony forming units; *CF* cystic fibrosis; *NP* nanoparticle

forming the liposomes (Akbarzadeh et al. 2013). The physicochemical properties of liposomes can be modified and optimized depending on their therapeutic application. For example, cationic or anionic liposomes can be prepared by using cationic or anionic ingredients in the formulation (Immordino et al. 2006).

Interactions of liposomes with cells can be realized by: adsorption, fusion, endocytosis, and lipid transfer. Among the mechanism of action, they can release the drug incorporated in two different ways: (a) antibiotics are released inside the bacterial cells after the liposomes fuse with the microbial cell walls, (b) antibiotics are released outside the bacterial cells by the liposomes adsorbed on the cell walls, followed by the diffusion into the bacteria. Both mechanisms resulted in a high concentration of antimicrobial drug into the plasma membrane or cytoplasm (Zhang et al. 2010).

An important benefit of using liposomes for infectious diseases is that they can treat extracellular or intracellular infections. As the most popular route for liposomal administration is the parenteral one, they are rapidly uptake by the reticuloendothelial system (RES), especially liver and spleen, which may be advantageous for the treatment of intracellular infections involving this type of cells (Allen et al. 1989). However, in some cases, it is necessary to incorporate or conjugate them with other materials [e.g., polyethylene glycol (PEG) or glycolipids] in order to improve their stability and prolong their circulation time.

During the last decade there have been a surmountable number of approaches for the delivery of antibiotics by means of liposomes (Table 1). The encapsulation of the drug into liposomes has proven to be an effective method for reducing minimum inhibitory concentration (MIC), compared to the free drug, as it is the case of gentamicin. Liposomal gentamicin showed significantly lower MIC values than those of free drug (32 vs. 512 mg/L) tested in a gentamicin-resistant non-mucoid strains of *Pseudomonas aeruginosa* isolated from cystic fibrosis (CF) patients (Rukholm et al. 2006).

Another advantage of the use of liposomes is the reduction of the drug side effects. Liposomes containing polymixin B have shown to reduce its nephotoxicity, ototoxicity, and neuromuscular blockade after a systemic administration while improving its antimicrobial activity against *P. aeruginosa.* Together with this, polymixin-loaded liposomes fuse with the bacteria's membrane causing its deformation and delivering a high dosage of antimicrobial agents inside the bacteria that prevent drug resistance of microbes by saturation of the efflux pumps (Alipour et al. 2008).

In another study, Ong et al. developed ciprofloxacin-loaded liposomes for the treatment of bacterial infections in cystic fibrosis (CF) and non-CF bronchiectasis patients, achieving the same antimicrobial activity in vitro against *P. aeruginosa* and *S. aureus* as the free drug. In addition, liposomal ciprofloxacin presented lower minimum bactericidal concentration (MBC) against *P. aeruginosa* than the free drug. However, it did not provide bactericidal activity against *S. aureus* (Ong et al. 2012).

Moreover, by modifying the surface properties by PEGylation, liposomes have shown to increase the activity of ciprofloxacin and vancomycin in respiratory infections, reducing at the same time the adverse effects of the drugs incorporated (Chono et al. 2011; Muppidi et al. 2011).

Besides the previous strategies, liposomes have been widely used for the treatment of respiratory diseases, and in particular, tuberculosis. Pulmonary tuberculosis is treated by a standard short-term tuberculosis chemotherapy, which consists of a daily oral administration of isoniazid, rifampicin, ethambutol, and pyrizinamide. The co-encapsulation of isoniazid and rifampicin in liposomes for tuberculosis treatment showed a significant reduction of mycobacterias in lungs, liver, and spleen of infected mice compared with untreated animals (Labana et al. 2002).

Changsan et al. investigated the properties of liposomes containing rifampicin and different amount of cholesterol and soybean L-α-phosphatidycholine prepared by the chloroform film method. The liposomes particles were a mixture of unilamellar and multilamellar vesicles in a size range of 200–300 nm. The MICs of liposome containing rifampicin and free rifampicin were 0.2 and 0.8 μM, respectively, determined against *Mycobacterium bovis* for all formulations (Changsan et al. 2009).

In another approach, Zaru et al. (2009) carried out in vitro and in vivo studies to investigate the targeting of rifampicin-loaded liposomes to alveolar macrophages. In order to improve the stability problems associated with low transition temperature, lecithin and Phospholipon 90 with or without cholesterol were used to prepare the liposomes via the film hydration method followed freeze-drying. These authors tested different drug containing liposomal formulations, observing that the formulation with the lowest drug concentration (0.05 μg/mL) showed a complete inhibition of the growth of *Mycobacterium avium* complex (MAC) in infected alveolar macrophages (AMs) (J774 cells) whereas the free drug exhibited a 80 % reduction in bacterial growth. In the in vivo experiments, rifampicin-loaded liposomes were administered into rats by nebulization, showing that they were able to reach the lowest airways in comparison with the free drug.

Similarly, other antibiotics with successful results have been incorporated to liposomes demonstrating effective antimicrobial activity against different organisms, as they are illustrated in Table 1.

To sum up, antibiotic-loaded liposomes are effective against a wide range of microorganisms, have a potential for treating numerous diseases, reducing toxicity, and achieving a sustained drug release. However, the use of liposomes for antibiotic delivery over the last few years has decreased, probably due to the introduction of newer DDS, discussed later in this chapter, that overcome some of their drawbacks, e.g., low stability in the bloodstream and during storage, low encapsulation efficiency or the presence of residues of toxic solvents in the final preparation (Barratt 2003).

3.1.2 Niosomes

Niosomes are very similar to liposomes but they are prepared with nonionic surfactants, which give them more stability, compared to liposomes. Moreover, the surfactants used are biodegradable, biocompatible, and non-immunogenic. As is the case of liposomes, niosomes have a bilayer structure which can enclose aqueous or lipophilic drugs. There are numerous methods of preparation such as thin film hydration, ether injection, reverse-phase evaporation or active trapping techniques, as those revised in the previous section (Sankhyan and Pawar 2012).

Different drugs have been reported to be delivered using niosomes. A niosomal formulation of isoniazid showed a high cellular uptake (≈61 %) by macrophages cells, as consequence, dose and frequency could be reduced with an enhancement of patient compliance (Singh et al. 2011). Similar findings were found with pyrazinamide-loaded niosomes that were effective in killing tubercle bacilli and decrease toxicity (El-Ridy et al. 2011).

Akbari et al. prepared niosomes containing ciprofloxacin by remote loading method followed by sonication. The MIC values of ciprofloxacin-loaded niosomes were from 2 to 8 times lower than MICs of free drug against intracellular *S. aureus* infection of murine macrophage-like (J774 cells) and provide a high intracellular antimicrobial activity (Akbari et al. 2013).

Others examples of antibiotics encapsulated in niosomes are cefuroxime (Sambhakar et al. 2011) and gallidermin (Manosroi et al. 2010), although these studies did not test antimicrobial efficacy.

3.1.3 Solid Lipid Nanoparticles (SLN and NLC)

Solid lipid nanoparticles (SLNs) appeared as an alternative drug delivery system in the early 90s, thanks to their advantages such as the use of biocompatible materials, high EE of lipophilic drugs, controlled release, protection of drug against degradation, tissue tolerance, and large-scale production (Müller et al. 2000).

SLNs are made of solid lipids (e.g., stearic acid, palmitic acid, glycerol behenate, or glyceryl monostearate) stabilized by surfactants (e.g., poloxamer 188, 182, 407, 908, Tween 20 or 80, and solutol HS15) (Kovacevic et al. 2011).

The main methods for the production of SLNs are high-pressure homogenization, emulsion-solvent-evaporation, and microemulsion technique (Mehnert and Mäder 2001). The most recurrent technique of elaboration is by emulsion-solvent-evaporation where the aqueous phase containing a surfactant as stabilizer agent is added to lipid phase (drug and organic solvent). After the emulsification step the volatile solvent is removed through evaporation while magnetic stirring leading to the formation of drug-loaded nanoparticles with a lipid matrix solid at room and body temperature. Figure 4 shows a schematic representation of the method (Pardeike et al. 2009).

Many studies are reported with SLN-based antibiotic delivery systems. Jain and Banerjee (2008) encapsulated ciprofloxacin into SLNs that provided a prolong

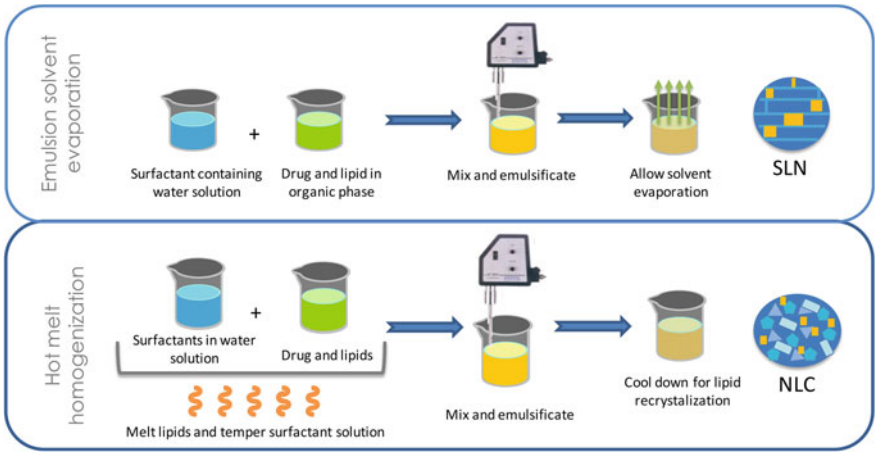

Fig. 4 Schematic representation of emulsion-solvent-evaporation and hot melt homogenization techniques

release of the drug in a controlled manner. Similarly, tilmicosin–SLNs demonstrated an improvement antibacterial activity in vitro and in vivo against *S. aureus* (Wang et al. 2012a). In a further research, norfloxacin-loaded SLNs were found to be stable 9 months at 4 °C and with a sustained release until 48 h. Also demonstrate in vitro antibacterial activity and in mice against *E. coli* (Wang et al. 2012b).

Kalhapure et al. developed a compritol-based SLN formulation of vancomycin and linoleic acid using an ion paring mechanism. The SLN formulation was active against *S. aureus* and MRSA strains (MICs of 31.25 and 15.62 μg/mL, respectively) suggesting that the co-encapsulation of a fatty acid with an antibiotic may enhance its antibacterial activity (Kalhapure et al. 2014).

Ghaffari et al. (2011) encapsulated amikacin into SLNs leading to an increasing antimicrobial activity against *P. aeruginosa* than the free drug.

For the treatment of tuberculosis, a multidrug solid lipid particle loaded with rifampicin, isoniazid, and pyrazinamide was developed by Pandey et al. The formulations were produced by the emulsion solvent diffusion technique and achieved drug incorporation efficiencies between 41 and 51 % for the three drugs. After their pulmonary administration in *Mycobacterium tuberculosis*-infected guinea pigs, no colony forming units (CFU) could be detected in the lungs and spleen after seven times one dose weekly of treatment whereas 46 daily oral doses of free drugs were required to obtain the same therapeutic effect. Moreover, those nanoparticles did not cause hepatotoxicity. Thus, nebulization of SLN-based antitubercular drugs could be a higher potential antituberculosis therapy improving drug bioavailability and patient compliance (Pandey and Khuller 2005).

Although SLNs have shown great therapeutic potential for delivering drugs they presented some disadvantages such as a low-soluble drug loading capacity, the possibility of drug expulsion after crystallization, and a relative high water content of the dispersions (Müller et al. 2002).

Nanostructured lipid carriers (NLCs) have been developed to overcome these limitations of conventional SLNs and represented the second generation of lipid nanoparticles. The main difference between them is that NLCs are produced by mixing solid lipids with liquid lipids, which leads to imperfections in the lipid matrix with an increased payload and prevented drug expulsion (Das et al. 2012). Hot melt homogenization or emulsification is one of the main methods used to elaborate NLCs that consists on heating the oil phase (drug and lipids) and the aqueous solution (surfactants) at the same temperature, high enough to able to melt the lipid compounds, mixing then afterward by sonication in order to obtain an emulsion containing the nanoparticles (Fig. 4). By using this method, recently, Pastor et al. reported a novel formulation of NLCs for the encapsulation of sodium colistimethate to reduce its side effects, more precisely, nephrotoxicity, neuromuscular blockage and ototoxicity while improving its antimicrobial activity. The nanoparticles were more effective against clinically isolated *P. aeruginosa* strains than free drug and the authors suggested that Colist-NLCs administered by the pulmonary route could be an alternative for the treatment of the infections associated to cystic fibrosis patients (Pastor et al. 2014).

3.2 Polymeric Nanoparticles

Polymeric nanoparticles are solid colloidal particles composed by polymers. Polymers can be divided into natural, e.g., albumin, chitosan, gelatin or alginate or synthetic such as poly-ε-caprolactone (PCL), poly (L-lactide) (PLA), poly-glycolide (PGA), polyvinyl alcohol (PVA), poly (lactic-co-glycolic acid) (PLGA), and polyethylene glycol (PEG) (Pinto-Alphandary et al. 2000). PLGA is widely use for NPs preparation as it is approved by FDA for therapeutic use in humans and has biodegradable and biocompatible properties (Makadia and Siegel 2011).

There are a considerable number of methods for polymeric particles elaboration reported in the literature, being the most common ones; emulsion polymerization, nanoprecipitation, emulsification-solvent diffusion, or solvent evaporation method. Drugs can be entrapped, adsorbed, or covalently attached in the polymeric matrix and may be released by desorption, diffusion, or nanoparticle erosion in the target tissue (Kumari et al. 2010).

Emulsion diffusion is a method which begins by dissolving the preformed polymer in an organic solvent that is partially miscible with water (e.g., ethyl acetate, dichloromethane, or acetone/methanol). Then, an aqueous solution containing a stabilizer is added to emulsify the solution. The mixture is then stirred while a large quantity of water is added promoting the formation of oil-in-water emulsions. Under continuous stirring, the organic solvent evaporates from the

emulsions which results in the formation of NPs. Emulsion evaporation is another method used for the Elaboration of polymeric NPs. It involves the emulsification of an organic solvent with the polymer and the compound to be encapsulated into an aqueous phase containing a stabilizer. A high shear stress is then applied to break the emulsion droplets into even smaller ones. The size of the droplet is directly related to the size of the NPs that will be formed. Finally the organic solvent is removed through evaporation causing the precipitation of the polymer and the formation of NP. Nanoprecipitation is also known as solvent diffusion or solvent displacement technique. First, the polymer must be dissolved in a water miscible solvent. Second, this mixture is added to an aqueous solution containing a stabilizer. This causes NPs to precipitate instantly due to solvent diffusion into the aqueous matrix. The NPs can be purified by removing the remaining organic solvent (Lai et al. 2014). High-pressure homogenization is a scalable method and has been commercially used for several FDA approved drugs. The drug and the stabilizer are pressurized with an intensifier pump to 100–2000 bars. The high-pressure stream then passes through a relief valve where cavitation, high shear force, and collision between the particles are induced by a sudden release of pressure which gives rise to a progressively reduction of particle size and results in a homogeneous and uniform product (Keck and Müller 2006).

Due to their polymeric composition, polymeric NPs may have greater stability than liposomes in biological fluids and under storage. Their main characteristics for antibiotic delivery include: structural stability, ability to control their physicochemical properties (size, zeta potential, and release profile) by the selection of appropriate components during preparation (polymers, surfactants, and organic solvents) and possibility to introduce functional groups onto the surface of NP by using drug moieties or targeting ligands (Zhang et al. 2010).

Since the twenty-first century, scientists have focused on the use of biodegradable and biocompatible materials, such as PLGA. Initially, Dillen et al. (2004), studied the antibacterial activity of ciprofloxacin PLGA NPs, modified or not with a cationic polymers (Eudragit RS100 or RL100) against *P. aeruginosa* and *S. aureus*. In the case of PLGA nanoparticles no differences in activity were found. However, when Eudragit was added into PLGA NPs, the drug was less active in killing *S. aureus* compared with *P. aeruginosa* (Dillen et al. 2006). Using the same antibiotic, Jeong et al. developed PLGA NPs, obtaining particle size between 100 and 300 nm and demonstrating higher antibacterial activity in vivo than the free drug although in vitro was superior. This fact was explained by the sustained release of the drug that lasted for 14 days, whereas the in vitro study was only performed for 24 h (Jeong et al. 2008). Other NPs formulated using PLGA have been shown to improve the delivery of azithromycin and rifampicin aimed to treat intracellular *Chlamydia* infections (Toti et al. 2011).

In another approach, Moghaddam and coworkers encapsulated clarithromycin into PLGA nanoparticles by the emulsification-solvent-evaporation technique using PVA as emulsifying agent. Drug release studies showed a biphasic profile lasting for 2 days. They developed a respirable formulation adding leucine to the NP formulation for the treatment of pulmonary infections (Moghaddam et al. 2013).

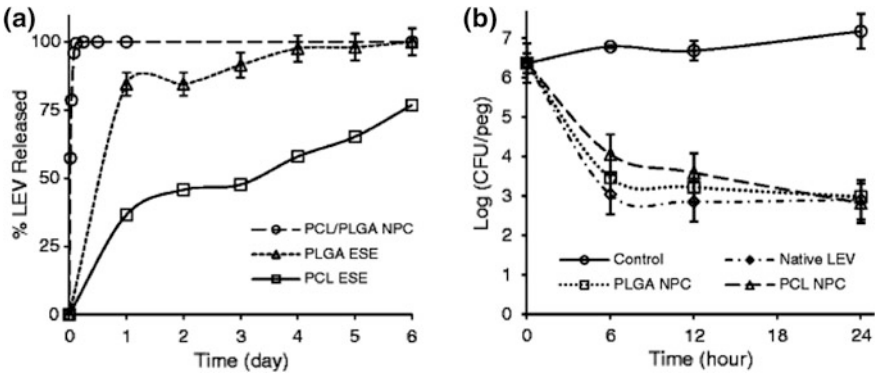

Fig. 5 **a** In vitro release profiles of the LEV-loaded nanoparticle formulations. **b** Effect of encapsulation on the LEV antibacterial activity. Reproduced with permission Cheow et al. (2010b)

Cheow et al. elaborated levofloxacin and ciprofloxacin-loaded PLGA or poly-ε-caprolactone NPs by nanoprecipitation or emulsification-solvent-evaporation methods. Their biphasic release over 6 days period permitted a high initial drug concentration at the beginning and the extended release profile above the MIC value able to inhibit the biofilm growth, both in biofilm cells and in biofilm-derived planktonic cells (Fig. 5) (Cheow et al. 2010a, b).

Tobramycin encapsulation was described by Ungaro et al. An emulsion-solvent diffusion technique was slightly modified for the preparation of PLGA nanoparticles including different types of hydrophilic polymers, such as cationic chitosan (CS) and nonionic polyvinylalcohol (PVA). When chitosan or PVA were added in an appropriate concentration, i.e., PVA/PLGA 3:5 w/w and CS/PLGA 1:20 w/w, the nanoparticles obtained displayed a 250–300 nm diameter and a positive or neutral charge, respectively. Moreover, by adding alginate in the internal phase of the emulsion, the EE increased up to 80 % and the drug release was optimal, providing a sustained drug release with a burst release followed by maintained liberation of the drug for a month. The MIC values of the PLGA formulations against *P. aeruginosa* in planktonic cells were higher than that of the free antibiotic. This fact could be due to the biphasic extended antibiotic release profiles of the nanoparticles that liberated small amounts of the drug into the media, very likely below the MIC. Moreover, the nanoparticles were able to inhibit the growth of planktonic cells even at low antibiotic loadings (i.e., <2 %, w/w) (Ungaro et al. 2012).

Apart from PLGA, chitosan alone is another interesting polymer for NPs preparation because of its antimicrobial and antifungal inherent activities. It is considered that the positive charge of chitosan bind with the negative charge of the bacteria cell walls destabilizing the cell and altering the permeability, subsequently it might get attached to DNA and inhibit the replication (Kong et al. 2010). Successful examples using this polymer are tetracycline encapsulated

O-carboxymethyl chitosan NPs for the eradication on intracellular *S. aureus* infections (Maya et al. 2012) and amoxicillin-entrapped chitosan/heparin NPs to eradicate *Helicobacter pylori* (Lin et al. 2013).

3.3 Other DDS

3.3.1 Dendrimers

Dendrimers are polymers with a well-defined monodispersed structure: a core, dendrons, and surface active groups (which determinate the biocompatibility and physicochemical or biological properties). Both hydrophobic and hydrophilic drugs may be entrapped in the internal structure of dendrimers or it can be chemically attached or physically adsorbed on their surface (Menjoge et al. 2010). Functionalization of the surface with specific antibodies may enhance potential targeting. The main mechanism of antimicrobial action is directly by destroying the cell membrane of bacterials or by disrupting multivalent binding interactions between the microorganism and host cell (Chen and Cooper 2002).

Dendrimers are normally synthesized from a central polyfunctional core by repetitive addition of monomers. The core is characterized by a number of functional groups. Addition of monomers to each functional group results into next dendrimer generation as well as expression of end groups for further reaction. The size of dendrimers increases as the generation number increases. Divergent and convergent methods are the most frequently used for dendrimer synthesis (Kesharwani et al. 2014).

The first family of dendrimers and the most frequently used in biomedical application is poly (amido amine) known as PANAM. They have gained much attention for drug delivery because of their nanosize, globular shape, multivalency, tunable inner cavities, and physicochemical properties (Kalhapure et al. 2013). PANAM dendrimers grow through generations from G1 to G10 and their sizes increases from 1.1 to 12.4 nm (Tomalia 2005).

Many researchers have used dendrimers to enhance the properties of antibiotics. These drug-loaded dendrimeric nanostructrures have been explored for improving drug solubility, antibacterial activity and achieving a sustained release in vitro. In an attempt to increase the poor aqueous solubility of quinolones, Cheng et al. (2007) investigated the encapsulation of nadifloxaxin and prulifloxacin into PANAM dendrimers, resulting in an improvement in their aqueous solubility and similar bacterial activity as the free drugs. In a further study they prepared sulfamethoxazole dendrimers, finding a higher activity against *E. coli* than the free drug (Ma et al. 2007).

Mishra et al. (2011) carried out a study with a macrolide antibiotic, azitromycin, with a G4-PAMAM dendrimer for the treatment of *Chlamydia trachomatis* infections using them as intracellular drug delivery vehicles and showing that the

conjugate was significantly superior to the free drugs in the prevention of productive infections.

Recently, Zhou et al. reported a synergistic effect of norfloxacin in combination with 3-hydroxypyridin-4-one (HPO) hexadentate-based dendrimeric chelator against Gram positive (*B. subtilis* and *S. aureus*) and Gram negative (*E. coli and P. aeruginosa*) bacteria. It was postulated that the large molecular weight of the complex penetrate slowly through the membranes and with lower toxicity than the smaller ones (Zhou et al. 2014).

Little publish data are available on the toxicity of this class of particles but it is known that the size and charge of higher generation (G4–G8) PANAM dendrimers affects their citotoxicity (Shah et al. 2011) probably due to their high cationic charge density as proved when tested in Caco-2 cells (Kitchens et al. 2006).

3.3.2 Metallic Nanoparticles

Apart from microbiological agents, many organic and inorganic nanomaterials (silver, tellurium, bismuth, copper, zinc, titanium, etc.) have also demonstrated to possess potent antimicrobial properties making them attractive candidates to treat infectious diseases (Huh and Kwon 2011). The most used are silver (Ag) nanoparticles that are able to kill Gram-positive and Gram-negative bacteria and are effective against many drug-resistant microbes as the metals nanoparticles have multiple mechanisms of action. Moreover, Ag NPs have shown to inhibit biofilm growth (Knetsch and Koole 2011).

Recent efforts have attempted to couple gold (Au) nanoparticles with a variety of antibiotics (e.g., ciprofloxacin, vancomycin, gentamicin, etc.) against all types of microorganism (Burygin et al. 2009). For example, chitosan-capped Au coupled with ampicillin displayed twofold increase in antimicrobial activity compared to free drug. The antimicrobial activity of Au NPs seems to be mediated by strong electrostatic attractions to the negatively charge bilayer of the cell membrane (Chamundeeswari et al. 2010).

An important advantage of using these types of metal nanoparticles as antimicrobial agents is that they have anti-biofilm activity as the same time it is difficult for microbes to develop resistant to them, however, their limited applications is partially due to safety concerns (Zhu et al. 2014).

4 Approaches of Antibiotic Nanoencapsulation Classified According to Their Administration Route

In this section of the chapter, different nanoparticle approaches will be reviewed classified by the administration route intended for its use. More precisely, the ocular, topical, pulmonary, oral and systemic routes will be addressed.

Once administered, antibiotics should overcome the barriers that some tissues represent. These barriers might be the ocular barrier, the gastrointestinal, or the respiratory tissue, for instance. This issue could be addressed by antibiotic encapsulation that in one hand could enhance barriers trespassing and on the other hand protects the antibiotic from different inactivation processes. In addition, nanoparticles might also improve the mucoadhesion and enhance the penetration across the extra cellular matrix that in turns might allow the penetration of the particle into the infected tissue and permit a sustained drug release in the target site (Alonso 2004; Xiong et al. 2014).

4.1 Ocular Administration

The first administration route that will be addressed herein is the ocular administration. Drug delivery to the eye is still nowadays a great challenge. The main advantage of this administration route is the feasibility of achieving a local effect avoiding systemic exposure to the drug. However, drugs display low bioavailability after ocular administration due to rapid drain out by blinking, tear turnover, liquid drainage, tear evaporation and systemic absorption that in turns results in less than 5 % of the drug penetrating the ocular parenchyma (Hon-Leung Lee and Robinson 1979; Xiong et al. 2014).

In order to overcome such limitations tailored nanoparticles represent an alternative drug delivery system. In this regard, the utility of tobramycin-loaded SLNs for ocular application was firstly reported by Cavalli and colleagues. The nanoparticles elaborated displayed a 100 nm size and 2.5 % drug load of tobramycin as an ion-pair complex with hexadecyl phosphate. Firstly, the eye disposition was studied using fluorescently labeled nanoparticles without drug and compared to fluorescent dye solution. From this study it could be confirmed that the SLNs presented a prolonged residence time on the corneal surface and in the conjunctival sac. In a further experiment, tobramycin-loaded SLN were administered to rabbits and the drug level in aqueous humor was quantified for 6 h. It was verified that SLNs were able to improve the bioavailability of tobramycin (Cavalli et al. 2002).

Another example of SLNs for ocular administration was reported by Abul Kalam et al. In this research article the authors developed gatifloxacin-loaded SLNs following an o/w emulsion method and using two different lipid mixtures, stearic acid and Compritol® (SLN-C) or stearic acid and Gelucire® (SLN-D), both at a 4:1 proportion. Besides, the addition of stearylamine led to positively charged nanoparticles. The optimized lipid nanoparticles presented a 250–305 nm size and a positively charged zeta potential, i.e., ≈29–36 mV. SLN-C displayed higher EE values than SLN-D, 79 versus 47 %. The authors postulate that Compritol® together with stearic acid might form less perfect crystals, compared to Gelucire®, allowing the drug to incorporate within the spaces of the crystals leading to a higher encapsulation efficiency. Moreover, the drug released from both SLN-C and SLN-D showed a slower drug release profile compared to the commercially available one,

i.e., SLNs released 60–80 % of the drug in 12 h, whereas the commercial eye drop liberated the 80 % within the first two hours (Abul Kalam et al. 2013a, b). In a following article the authors compared eye drops based on the gatifloxacin-loaded SLN-C and SLN-D and the commercial eye drop Gate® formulation. The aim of the study was to test whether the SLNs were able to improve the residence time in the eye, enhance bioavailability and decrease ocular irritation. To cope with this, the authors performed a Draize test to measure potential irritation in eyes and pre-corneal retention of ^{99m}Tc-radiolabeled SLNs. In addition, an ex vivo goat permeability model was also carried out using gatifloxacin-loaded SLNs. This first ex vivo experiment showed that SLNs, as well as eye drops, were capable of maintaining corneal hydration at almost 80 %, meaning that the formulations did not exert eye damage. Furthermore, the corneal permeation profiles showed that the SLNs were able to provide a more sustained release of the drug. Moreover, the SLN-C displayed a slower release profile than SLN-D. In the following in vivo rabbit experiments, no sign of discomfort was detected, both at long-term and acute studies. Likewise, for cornea, conjunctiva and eyelids no irritation was detected, grade 0. After a repeated administration, the SLN group showed a slight mucoid discharge (grade 1), very likely related to the aggregation of the nanoparticles. Overall, this study suggests that SLNs represent a relatively safe drug delivery system for ocular administration. In the subsequent gamma scintigraphy studies, it was observed that 20 min after administration the SLNs presented an increased activity in the preocular area compared to the marketed eye drops. Nanoparticles might display an extended preocular residence time due to their positive charges that could interact with the negatively charged mucin layer of the corneal surface. Ocular pharmacokinetic studies were performed by analyzing the drug content by high-performance liquid chromatography (HPLC) at the aqueous humor, confirming that the SLNs were able to increase the relative bioavailability of drug 3.37-fold. In addition, the increase in drug half-life when encapsulated was 2.34-fold (Fig. 6). Altogether, these assays suggest that gatifloxacin incorporated in solid lipid nanoparticles leads to a significant improvement of the ocular bioavailability and residence time into the eye (Abul Kalam et al. 2013a, b).

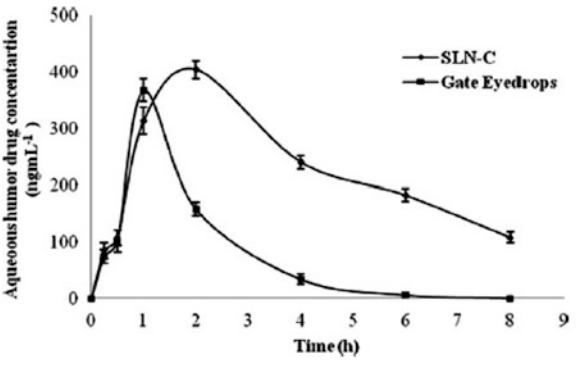

Fig. 6 Aqueous humour concentration–time profile of gatifloxacin after topical instillation of SLN-C and Gate® eyedrops to rabbit eyes (n = 3, ±SD) up to 8-h study period. The ocular bioavailability and relative bioavailability of GAT were significantly increased with the SLN-C as compared to Gate® eyedrops formulation. Reuse with permission Abul Kalam et al. (2013a, b)

A ciprofloxacin encapsulation approach was described by Garhwal and colleagues. In this occasion the authors chose pullulan and poli-ε-caprolactone as core polymer for the nanoparticles, embedding them after their preparation in hydrogel-based contact lens. Nanoparticles showed 142 ± 12 nm particle sizes and displayed a sustained release profile. Next, two different contact lens were elaborated, both using (hydroxyethylmethacrylate) HEMA polymer, one presenting a thin thickness (30 ± 4 mg) and the other displaying a thicker (≈60 mg) composition. These lenses incorporated the nanoencapsulated ciprofloxacin in the matrix as nanoparticles were added during the preparation process. The resulting lenses were clear and presented a prolonged drug release over 15 day. The microbiological assays revealed that both types of lenses were able to prevent *P. aeruginosa* and *S. aureus* growth, whereas the control group presented a log-phase growth within the first 2 h of incubation. In addition, when the culture media was replaced with fresh bacterial broth, the lenses inhibited their growth for other 24 h. When testing the thin lens against higher *S. aureus* inoculums, 10^9 CFU/mL, these lenses were able to prevent bacterial growth during 48 h, but failed to avoid proliferation for the third day. Yet, thicker lenses, which contained higher amount of nanoencapsulated ciprofloxacin, were able to inhibit microbe growth for three days. Therefore, the authors postulated that the thin lenses could be appropriate for the treatment of milder infection, counting 10^6 bacterial, whereas the thicker lenses could be useful for more severe infections such as corneal bacterial ulcers (Garhwal et al. 2012).

Levofloxacin nanoencapsulation has also gained much attention. For example, Gupta et al. described levofloxacin-loaded poly-L-lactic-co-glycolic acid (PLGA) nanoparticles as a successful approach for ocular administration. They described 190–195 nm size nanoparticles showing −25 mV zeta potential and 85 % of encapsulation efficiency. Nanoparticles were able to release the drug content during 24 h after an initial burst phase. Antimicrobial test showed that levofloxacin–PLGA nanoparticles displayed a similar inhibition area compared to the commercially available eye drops. The subsequent animal experiments revealed that nanoparticles displayed an appropriate spread and retention in the precorneal area. In addition, authors performed the HET-CAM test (Hen's Egg Test Chorioallantoic Membrane) in order to assess the potential irritancy of the formulation for the mucous membrane. Test score was 0.33 meaning that no irritancy was detected (Gupta et al. 2011). In a following study by the same group, the previously described levofloxacin–PLGA nanoparticles were embedded in an in situ forming chitosan gel in order to enhance the disposition of the formulation in the eye. This new formulation was also studied by γ-scintigraphy in rabbits and revealed that chitosan gels containing levofloxacio–PLGA nanoparticles presented good spread and retention properties in the precorneal area. Besides, the formulation clearance was slowed down by gel embedding and showed longer permanency compared to the commercial eye drops, labeled nanoparticles alone and labeled chitosan gel (Gupta et al. 2013).

4.2 Topical Administration

Nanoencapsulation has also been reported as a successful approach for the antimicrobial activity in the skin. In this regard, in the following part of this chapter, the latest nanoparticles as antibacterial and antifungal carriers for topical administration will be highlighted.

Sanchez et al. (2014) reported a very interesting work on amphotericin B encapsulation to fight against *Candida* spp in burn wounds. Pursuing this goal, polyethylene glycol and chitosan were selected for the preparation of the hydrogel/glassy composite ranging 91.3–101.9 nm in size. The drug was released in a sustained manner and the released amount of the drug was tested by means of MIC determination and confirmed 99 % of antifungal activity. Besides, the antiproliferative effect of nanoparticulated amphotericin B was tested by XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) cell viability assay against *C. albicans* SC5314. It was observed that after 4 h incubation 95.9 % of growth was inhibited and 82 % post-24 h. There were no differences between the amphotericin B solution and the nanoencapsulated drug, suggesting that the released drug was active. In a further experiment, clinical strains of non-albicans *Candida* were assessed and it was shown that the growth inhibition of the nanoparticles was 72.4–98.3 % after 4 h incubation. On the contrary, amphotericin B solution was not able to reach those inhibition levels, $P > 0.01$. Nanoparticles were also tested against biofilm bearing fungi and it was observed that amphotericin B-loaded nanoparticles were capable of reducing the metabolic activity of the fungi at 80–95 % level. Moreover, when tested in mature biofilms, nanoencapsulated amphoterin B showed enhanced inhibitory results compared to amphoterin B in solution ($P < 0.05$). It is worth it to mentioning that when *C. parapsilosis* strain #75 was tested these differences were only remarkable at lower concentrations (Fig. 7).

In a following step nanoparticles were tested in a murine infected burn wound model, where nanoparticulated amphotericin B showed improved antifungal effectiveness compared to amphotericin B solution at day 3, meaning that the nanoparticles were able to speed up the drug effect resulting in a lower CFU account. On the following days, amphotericin B solution and nanoparticles presented no statistically significant differences. However, as the histological studies revealed, the group treated with nanoparticles displayed a more advanced re-epithelialisation, organized dermal proliferation and appropriate dermal remodeling. Hence, it might be postulated that a faster *Candida* reduction plays a role in a proper healing process, although it did not accelerate the wound closure. This work underlined three important points of the usefulness of nanoparticles, (i) that encapsulation did not affect the drug activity, (ii) that nanoparticles were active against plantonik and biofilm *Candida*, and (iii) that nanoparticles exerted in vivo activity. Altogether, amphotericin B nanoparticles seem a feasible alternative to the oral administration of amphotericin B in burn wounds avoiding systemic exposure and side effects of the orally administered drug (Sanchez et al. 2014).

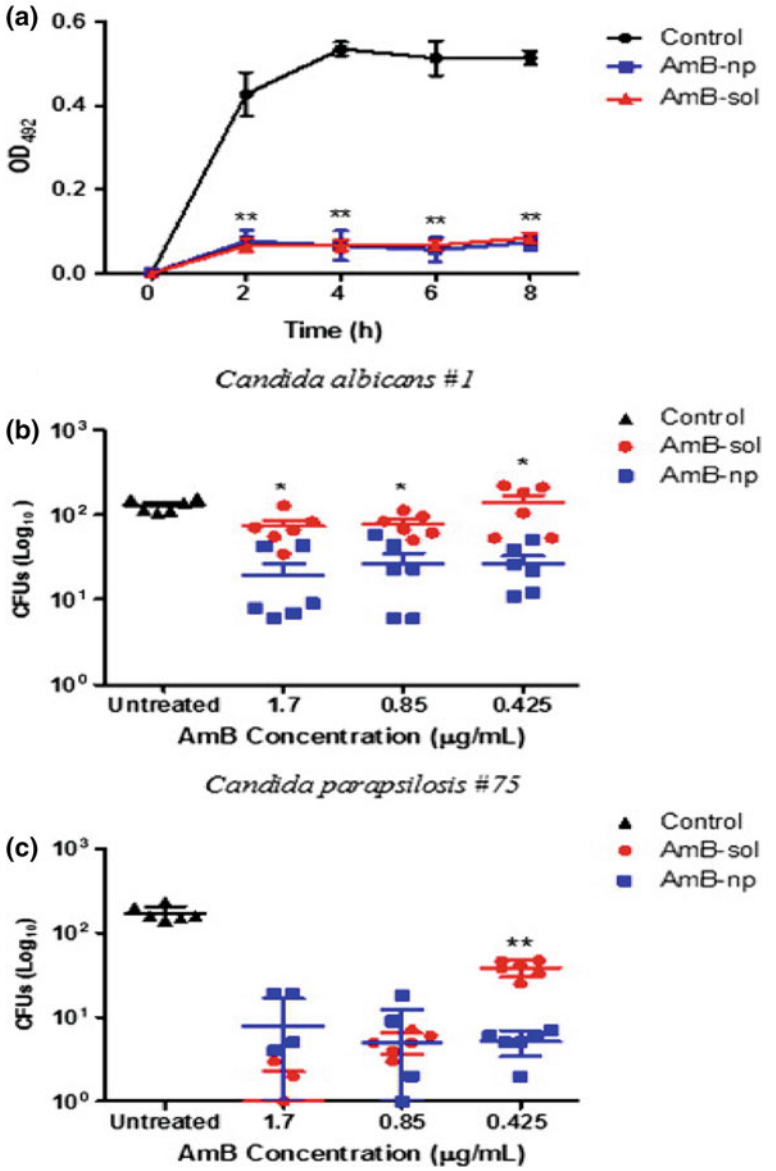

Fig. 7 Amphotericin B nanoparticle inhibits biofilm formation and reduces mature biofilm viability. **a** *C. albicans* biofilm development after being subjected to Amphotericin B nanoparticle, Amphotericin B solution, and no treatment. Outlined statistical differences comparing amphotericin B solution and nanoparticles to control. **b**, **c** Clinical strains of *C. albicans* (Ca#1) and *C. parapsilosis* (Cp#75) produced mature biofilms after 48 h. Subsequent to Amphotericin B nanoparticles, drug solution, or PBS application, samples were plated for CFU tabulations. *Asterisk* denotes *P*-value significance (*$P < 0.05$, **$P < 0.001$) calculated by two-way ANOVA statistical analysis. Both experiments were performed twice and similar results were obtained. OD, optical density reused with permission Sanchez et al. (2014)

Another approach against burn wound infection was reported by Krausz et al. (2015), but in this work MRSA, and *P. aeruginosa* were the pathogenic targets. For that purpose, curcumin was selected as nanoencapsulation candidate due to its antimicrobial, anti-inflammatory and antioxidant effect. Besides, nanoencapsulation could improve some of the drawbacks that curcumin presents, such as low oral bioavailability, poor aqueous solubility and rapid degradation. Chitosan, polyethylene glycol 4000 and tetramethyl orthosilicate were chosen as core polymers resulting in 222 ± 14 nm nanoparticles showing a sustained release profile of 81.5 % of the drug in 24 h. The cytotoxicity of nanoparticles was tested in cell cultures of PAM212 keratinocytes and by the embryonic zebrafish assay. Both studies confirmed the safety of the formulation as no toxic effect was detected compared to the controls. The in vitro bioactivity assay against MRSA and *P. aeruginosa* showed that nanoparticles were able to inhibit their growth from the 8th hour onwards. When tested against 10^7 CFU/mL inoculums, the nanoparticles were able to inhibit bacterial growth in 97 and 59.2 % for MRSA and *P. aeruginosa*, respectively. In order to get insight into the cell–nanoparticle relationship, 5 mg/mL of nanoparticles with and without curcumin were added to the bacterial strain and observed over time under transmission electron microscopy, TEM. Unloaded nanoparticles (with no curcumin) interacted with bacteria, but there were no observable changes in the cell (Fig. 8b). Whereas, when adding curcumin-loaded nanoparticles (Fig. 8c, d), after 6 h incubation the cell displayed a modified shape and oedema and lysis was undertaken by the 24th hour.

Next, the efficacy of the nanoparticles was assessed in an *in vivo* murine MRSA infected burn wound model. It was observed that on days 3 and 7, curcumin-loaded nanoparticle treated groups presented a lower CFU account. In addition, it was observed that curcumin-loaded nanoparticles were able to accelerate the healing process and to enhance re-epithelisation and granulation processes leading to a more mature epidermis/dermis compared to the control groups (Krausz et al. 2015).

4.3 Pulmonary Administration of Antimicrobial-Loaded Nanocarriers

Approaching the lung from an inhalatory point of view is very interesting as an anti-infective strategy due to the possibility of targeting lung infections in a direct manner, avoiding plasmatic high drug concentrations. In this regard, one successful example, approved by the FDA, has been already reported, which is tobramycin encapsulation by Pulmosphere™ technology that gives rise to a dry powder of porous microparticles (Geller et al. 2011). The success of these microparticles has encouraged many other groups to keep on working on other drug delivery systems, such as liposomes, for the administration of different antibiotics, i.e., amikacin and ciprofloxacin.

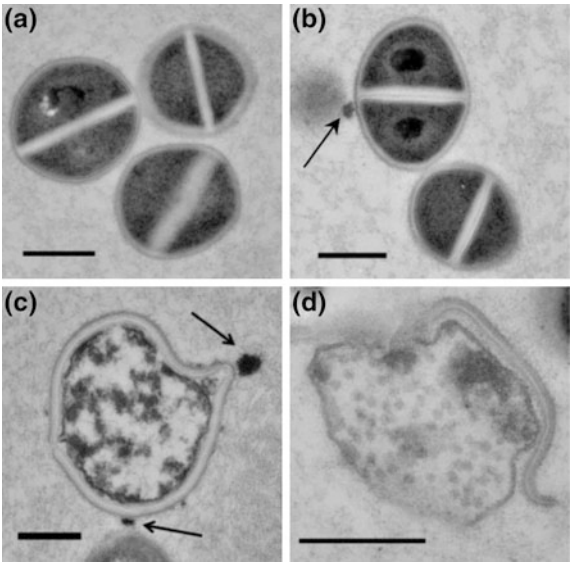

Fig. 8 Curcumin nanoparticles induce cellular damage of MRSA. High-power TEM demonstrated interaction of nanoparticles (*arrows*) with MRSA cells. **a** Untreated MRSA showed uniform cytoplasmic density and central cross wall surrounding a highly contrasting splitting system. **b** After 24 h, cells incubated with control nanoparticles at 5 mg/mL did not exhibit changes in cellular morphology compared to untreated control. **c** After 6 h, cells incubated with Curcumin-nanoparticles at 5 mg/mL exhibited distortion of cellular architecture and oedema, followed by lysis and extrusion of cytoplasmic contents after 24 h. **d** All scale bars = 500 nm. With permission from Krausz et al. (2015)

Liposomal amikacin consisting of DPPC and cholesterol is also known as Arikace™. These liposomes display ≈300 nm in size and after nebulization they are able to penetrate sputum in cystic fibrosis, as fluorescently labeled liposomes has revealed. Besides, these liposomes have shown to release amikacin in a sustained manner even in a *P. aeruginosa* environment. Furthermore, in vivo studies in rats revealed that the liposomes increased drug concentrations in the lungs compared to the free drug. In addition, when rats were infected using a mucoid strain of *Pseudomonas* (PA3064) embedded in agarose beads, and treated with 6 mg/kg three times a week with free amikacin, no CFU decrease was observed, whereas liposomal amikacin reduced bacterial account in two orders of magnitude. In a next study, a single administration of liposomal amikacin was tested against tobramycin twice daily. It was confirmed that liposomal amikacin was as effective as free tobramycin administered twice a day. Remarkably, it was also observed that amikacin liposomes administered every other day displayed similar results to tobramycin twice daily (Meers et al. 2008).

In a further study, Weers et al. administered radiolabelled Arikace™ to healthy volunteers, determining that the lung deposition was around 32 % of the emitted dose and that 24 h after administration the retention percentage was 60.4 %,

decreasing to 38.3 % after 48 h. Furthermore, no adverse effects such as cough or bronchospasm were observed (Weers et al. 2009).

A phase II clinical trial was carried out in order to evaluate short term, 28-day, once daily Arikace™ safety, tolerability, and effectiveness against chronically *P. aeruginosa* colonized cystic fibrosis patients. The design of this clinical trial was set as a randomized, double-blind, placebo-controlled, multiple-dose, multicentre trial including 105 patients. Four doses were tested, i.e., 70, 140, 280, and 560 mg against placebo. The adverse effects were similar for the placebo and treated groups, e.g., 20 % for 560 mg Arikace™ and 22 % for the placebo group. Lung function, studied by spirometry, presented an improvement only with the two high doses. In fact, the 560 mg Arikace™ group showed an enhanced lung function that was maintained until day 56, i.e., 28 days after the last dose. The microbiological results showed that especially the highest dose rapidly reduced the bacterial density in the sputum of patients (Clancy et al. 2013). In order to gain insight into a longer treatment period with cycles of 28 days on treatment followed by 28 days off treatment, a Phase III trial is currently being conducted with CF patients colonized by *P. aeruginosa* in Europe, Australia, and Canada (2011-000441-20). The goal of this trial is to study whether Arikace™ treated groups show an improvement in lung function and a reduction in *P. aeruginosa* counts compared to TOBI®. After the analysis of some preliminary results, the authors deduced that liposomal amikacin was effective and safe and its effectiveness was not reduced after three cycles of 28 days on and 28 days off treatment. A single daily dose of liposomal amikacin showed better results that TOBI® twice daily, especially in terms of the respiratory symptoms (Bilton et al. 2013; Ehsan et al. 2014).

Altogether, Arikace™ seems to be close to be released on the market in the EU and US. Hence, the efforts that Insmed Inc. (Monmouth Junction, NJ) has made are about to be a successful approach of a lab scale to clinical framework of a drug delivery system that could be defined as a milestone for the application of nanotechnology for infectious treatments.

Another successful example, which also reached clinical trial, is illustrated by Lipoquin® and Pulmoquin® from Aradigm Corporation (Hayward, CA). Lipoquin® is based on ciprofloxacin hydrochloride liposomes, that presents a 90 nm size and has already shown to be safe, capable of reducing *P. aeruginosa* CFU account and improving lung function in a Phase II clinical trial.

On the other hand, Pulmoquin® is composed of free ciprofloxacin and liposomal ciprofloxacin at a 1:1 volume ratio. Pulmoquin® is prepared using hydrogenated soybean phosphatidylcholine and cholesterol. Pulmoquin® underwent phase II B clinical trial, termed ORBIT-1, aiming at non-cystic fibrosis bronchiectasis patients. The study was designed as randomized, double-blind, placebo-controlled (empty liposomes) and focused on safety, tolerability and efficacy of either 150 or 300 mg of Lipoquin®. Liposomes were administered once daily either 300 or 150 mg dose with a 28-day treatment phase and a 28-day off stage with a follow-up period. It was observed that ciprofloxacin was safe and well-tolerated for both doses. Lung function presented no changes and adverse effects were mild to moderate and detected in two patients in the placebo group and two patients in the treatment

group (Bilton et al. 2011). Similarly, Pulmaquin® was studied in another Phase II-B clinical trial, ORBIT 2, designed as a randomized, double-blind, placebo-controlled trial aiming at the non-cystic fibrosis bronchiectasis population suffering from *P. aeruginosa* infection. For this trial, the 210 mg dose was selected and three cycles were fixed, consisting of 28 days of treatment and 28 days off. Besides, Lipoquin® patients presented a low incidence of adverse effects and the formulation was overall well-tolerated (Serisier et al. 2013). Pulmaquin® is currently ongoing another evaluation in a Phase III clinical trial (ARD-3150-1201, ORBIT-3).

4.4 Oral Route

Nanotechnology has been also applied for drug administration by the oral route, as it protects the drug from the acidic environment in the stomach and from enzymatic degradation throughout the gut. In addition, the nanocarriers themselves may also improve drug absorption (He et al. 2012).

A pioneering work was first reported by Cavalli and colleagues when tobramycin was encapsulated into SLNs in order to study its pharmacokinetic profile. SLNs showed a 85 ± 5 nm size, a negative zeta potential of around −20 mV and 2.5 % of drug loading. These nanoparticles were administered intraduodenally or intravenously to rats and compared to a tobramycin solution i.v. administered. Firstly, it was observed that i.v.- administered SLNs were able to increase the AUC (area under the curve) of the drug 4.85-fold compared to the free drug. More interestingly, when tobramycin-loaded SLNs were administered intraduodenally the AUC was increased 25-fold in comparison to the i.v.-administered tobramycin–SLN and this increase was 120-fold in contrast to the free tobramycin solution. Turning to the maximum drug concentration in plasma, i.v. SLNs showed the highest value, followed by the intraduodenally administered SLNs. The authors postulated that these differences among intra-duodenal and iv routes were related to the transmucosal transport of the SLNs mainly to the lymphatic system instead of to the bloodstream (Cavalli et al. 2000). In a following study, Bargoni et al. analyzed the tissue distribution of tobramycin after i.v. administration and tobramycin-loaded SLN after i.v. and intra-duodenal administration. They observed that the clearance of nanoparticulated tobramycin was tenfold slower than the clearance of the tobramycin solution. In addition, it was also determined that the intra-duodenal administration led to a lower kidney concentration of the drug compared to the i.v. administration of both, tobramycin solution and tobramycin–SLNs. It is also worth mentioning that 24 h after the administration, intraduodenally administered SLNs displayed the highest lung concentration. This finding could be very useful for the therapy of cystic fibrosis. Finally, it was also remarkable that SLNs were able to cross the blood–brain barrier, especially when administered intravenously. The authors hypothesized that the SLNs may aid masking the drug and improving its passive transport by protecting the drug from the e-flux pump or other type of mechanisms that may avoid drug absorption or passage (Bargoni et al. 2001).

Altogether, they demonstrated that intraduodenally administered SLNs were efficiently absorbed and the pharmacokinetic profile was improved, presenting extended AUC and half-life ($t½$).

The oral route is also the most appropriate approach for *H. pylori* eradication. Lin and coworkers explored the use of a chitosan/heparin nanoemulsion as an amoxicillin carrier candidate. The nanoemulsion prepared showed ≈300 nm particle size, positive zeta potential around 30 mV, and a 54.3 % encapsulation efficiency that gave rise to 19.2 % drug loading and a sustained drug release. Moreover, the particles were found to be stable at pH 1.2-6. Besides, confocal microscopy confirmed that the particles were able to interact with the bacteria. In order to assess nanoemulsion efficiency, mice were infected with 10^9 CFU/mL of *H. pylori* once daily for 7 consecutive days and treated for another 7 days with deionized water, amoxicillin solution or amoxicillin bearing nanoemulsion. It was determined that non-treated animals presented the highest *H. pylori* CFU account per stomach, 137.3, followed by animals treated with amoxicillin solution, 45.2, whereas nanoemulsionated amoxicillin showed lower CFU account, 11.3. Altogether this work represents a new strategy to fight against *H. pylori* infection (Lin et al. 2012).

4.5 Systemic Route

Sande and coworkers reported liposomal vancomycin encapsulation to fight against MRSA. Two different liposomal formulations were designed, DCP liposomes composed of DSPC, DCP and cholesterol and DMPG liposomes composed of DPC, DMPG and cholesterol. Both displayed ≈530 nm in size and showing 9 and 20 % of encapsulation efficiency for DCP and DMPG liposomes, respectively. Both formulations were able to decrease MIC values two- to four fold compared to free vancomycin against different hospital-associated MRSA. DCP liposome showed the most promising results, so these were further characterized by time–kill assays. Three different concentrations were assessed, 0.3, 0.6 and 1.25 mg/L against NRS-35, a hospital-associated MRSA (HA-MRSA) strain from NARSA; and LAC a CA-MRSA strain of the pulsotype USA300. The results revealed that after 3 h incubation all the three concentrations were active against LAC. Likewise, liposomes at 1.25 mg/L concentration were more effective than the free drug in killing NRS-35 at the 3rd and 6th hours. The animal studies using LAC infected mice showed that liposomal vancomycin was able to reduce 2–3 log the bacterial CFU in spleen and kidneys compared to the PBS control. Yet, when comparing with the free drug, the liposomes presented a 1-log reduction in the bacterial account for the kidney but spleen CFU fail to present statistically significant differences (Sande et al. 2012).

Van de Ven and colleagues designed PLGA nanoparticles and nanosuspensions to encapsulate amphotericin B by nanoprecipitation. The particles presented a 86–153 nm size and where freeze dried using 5 % mannitol cryopreservative. All the formulations displayed a negative zeta potential, with EE ranging from 54 to 63 %.

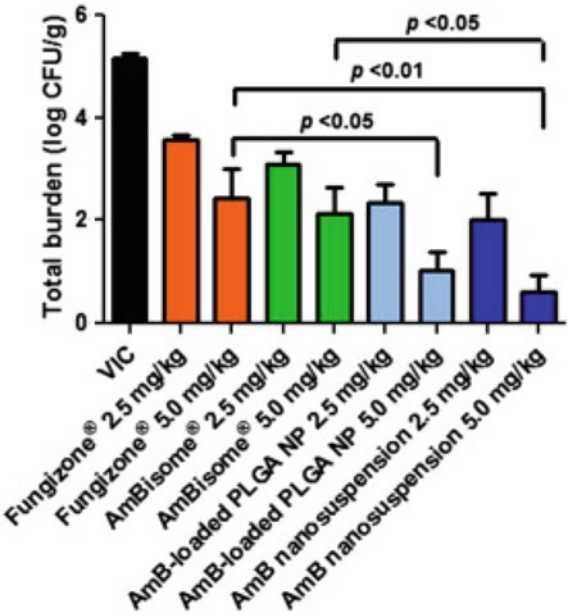

Fig. 9 Total burden (liver, kidneys and spleen) of mice infected with *A. fumigatus* and treated (IP) with either 5.0 % (w/v) mannitol (VIC: vehicle-treated infected-control group, sacrificed at day 4 postinfection) or the various amphotericin B nanoformulations at 2.5 or 5.0 mg/kg (sacrificed at day 10 postinfection) (data represented as mean ± sem; $n = 4$ for VIC and $n = 5$ minimum for the different treatment groups). Significant differences between groups are indicated; in addition, all treatment groups were significantly ($p \leq 0.001$) different from VIC (except $p < 0.01$ for Fungizone® 2.5 mg/kg vs. VIC), Fungizone® 2.5 mg/kg significantly ($p < 0.05$) different from amphotericin B-loaded PLGA nanoparticle 2.5 mg/kg, Fungizone® 2.5 mg/kg significantly ($p < 0.01$) different from amphotericin B nanosuspension 2.5 mg/kg. The percentage of reduction of fungal burden (compared to mannitol) amounted to 99.4 % for treatment with Fungizone® 5.0 mg/kg and 99.8 % for treatment with AmBisome® 5.0 mg/kg. With permission Van de Ven et al. (2011)

Nanoparticles showed an increased activity against intracellular *L. infantum* amastigotes compared to the free drug, IC_{50} 0.03 vs. 0.16 μg/mL, respectively. When testing the formulations against *C. albicans*, it was also observed that the nanoparticles (IC50, 0.04 ± 0.01 μg/mL) were 10 times more active against the fungal strain than the commercially available formulations, Fungizone® or AmBisome®, (IC50, 0.34 ± 0.12 and 0.40 ± 0.09 μg/mL). The nanoformulations also revealed to be less cytotoxic. The nanoparticles were next assessed against an *Aspergillus fumigatus* infection model and compared to Fungizone® or AmBisome® at the same dosing schedule. Amphotericin B-loaded nanoparticles and nanosuspension were more active than Fungizone® (Fig. 9). Indeed, the nanosuspension was more effective against fungi than PLGA nanoparticles. These experiments underlined that the nanosuspension was the most efficacious of all formulations and that it could allow a dose reduction from 5 to 2.5 mg/kg maintaining the same therapeutic efficacy than the free drug (Van de Ven et al. 2011).

5 Conclusions and Future Perspectives

Over the last years, the pharmaceutical industry has decreased its efforts to develop new antibiotics, mainly due to the low potential returns of this therapeutic group and to the different interests in the development of other drugs. As a consequence, it seems that breakthrough innovations are needed to effectively manage microbial infections. In this regard, the application of nanotechnologies to medicine seems as an interesting tool, with over 100 nanomedicine products being approved for clinical use, and 10 nanoparticulate formulations being already marketed.

The application of nanomedicines in antimicrobial therapy aims at combating resistance to antibiotics and reducing the appearance of adverse effects. In addition, they show promise especially in the treatment of chronic infections as they may inhibit biofilm formation and may also target intracellular microorganisms. Moreover, the delivery of antimicrobial agents could improve considerably the current therapy of infectious diseases, as they could decrease the systemic toxic effects of antibiotics, increase uptake and decrease the efflux of drugs, act on biofilm formation and be more effective in intracellular bacterial infections.

Besides that, DDS are quite versatile as allow the targeting to the infection site by the use of ligands, may improve the solubility of hydrophobic drugs, prolong drug systemic circulation and allow a sustained release of the drug, which leads to a reduction in the frequency of administration.

This review covers a range of diverse drug delivery systems in the submicron range such as liposomes, polymeric nanoparticles, solid lipid nanoparticles, or dendrimers that have been investigated in vitro for antibiotic delivery with successful results together with some in vivo studies confirming the enhanced activity against sensitive and resistant bacteria.

Taking into account the studies revised in this chapter, antimicrobial nanomedicine has a potential impact, although their clinical development still faces important challenges. The first one is the availability of biomaterials and the regulatory requirements that they have to meet before entering the market, especially in terms of biocompatibility and long-term safety. Another major concern is the scalability of the processes used in the preparation of DDS. They should allow a feasible translation into industry-scale manufacturing providing appropriate reproducibility.

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Bacterial Predation: Natural Guns to Control Infection

Mercedes Berlanga, Miguel Viñas and Ricardo Guerrero

Abstract From the Paleolithic until the twentieth century, infectious diseases were the main scourge of mankind. Vaccination, treatment of infectious diseases with antibiotics, and water purification have contributed to enormous improvements in the quality and length of the human life span. However, since it is difficult to design new vaccines to protect us against a wide variety of microbial pathogens and the spread of antibiotic-resistant pathogens poses additional difficulties, new alternative treatments for infectious diseases are needed. Environmental bacteria can be killed by predation from bacteriophages, heterotrophic protists, and predatory bacteria. We present in this review these bacterial natural predators to envisage their potential usefulness in fighting pathogens from the environment and/or from infected animal hosts. Protists are generalist grazers of prokaryotes, bacteriophages mostly highly host-specific and predatory bacteria collectively known as the *Bdellovibrio* and bdello-like organisms (BALOs), are neither true generalists nor hard-core specialists. Recently, "natural" non-predatory microorganisms are also being studied to treat a wide variety of human diseases, including gastrointestinal disorders, urinary infections, osteoporosis, and cancer. We "simply" should observe nature. As Joshua Lederberg remembered, taking August Krogh principle, "for any given scientific challenge there is a critter fittest toward its solution".

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1 Introduction

Although the real nature and biological meaning of microorganisms did not become well known until the end of the nineteenth century, the history of mankind has been determined on many occasions by their actions. Humans live in a dynamic state of coexistence with a myriad of microbial life forms that are (usually) mutually beneficial (they keep us healthy and we keep them alive), and show physiological symbiotic relationships. Indeed, humans still inhabit the Earth because the pathogens that have attacked us have an interest in our survival. The death of the host is not the primary marker of a microbe's fitness; rather, it can be considered as "collateral damage." In this context, humans survive because it is disadvantageous for a pathogen to eliminate its hosts. Instead, the aim of pathogens, the driver of their natural selection, is to "domesticate" their host. Prolonged interaction between the human host and infectious organisms carried out across many generations and among suitably numerous populations on each side, creates a pattern of mutual adaptation that allows both to survive. A pathogenic organism that kills its host quickly creates a crisis for itself, since it must find a new host often enough to keep its own chain of generations going; thus, this apparent initial advantage will typically lead to a Pyrrhic victory. Conversely, a human body that resists infection so completely that the would-be parasite cannot find any lodgment obviously creates another kind of crisis of survival for the infectious organism. The most successful parasites are the ones that persist in healthy or near-healthy hosts and promote and exploit their behavior for two principal goals: (i) to ensure continued carriage and viability, and (ii) to promote efficient dissemination to other hosts. Parasite maintenance and dissemination are more important for fitness, and morbidity is the more prevalent outcome of disease than mortality (Merrell and Falkow 2004).

There is a great diversity in the microbial world, manifested by a diversity of structures, modes of reproduction, ecological relationships both with their biocenosis and their biota, and evolutionary history that makes the subject of human exposure to them so challenging. The incredible diversity of the agents of most infectious diseases, i.e., bacteria, viruses, and protists, results in varied modes by which they encounter humans. The enormous gains made in public health during the twentieth century, through the prevention and treatment of infectious diseases, have contributed to dramatic improvements in the quality and length of the human life span. But, continued advances in medicine are dependent on addressing several challenges such as the increase in existing and new resistance to antibiotics. So, alternatives to antibiotics are urgently needed. The most useful replacements for antibiotics would be, like several antibiotics, natural compounds or agents that kill pathogenic microorganisms, such as virus (bacteriophages and virus of protists), predatory bacteria, or bacteriovorous protists.

In the present work, we present relevant ecological concepts and findings on bacterial predators such as bacteriophages and protists, to exploit their potential to remove pathogens and reduce microbial loads from their hosts. Protists are ubiquitous and overall generalist grazers of prokaryotes in marine, aquatic, and

terrestrial ecosystems. As opposed to protists, bacteriophages, mostly highly host-specific, and predatory bacteria collectively known as the *Bdellovibrio* and bdello-like organisms (BALOs), are neither true generalists nor hard-core specialists. Instead, prey range differs between isolates, being more or less broad (Johnke et al. 2014).

2 Microorganisms as Components of Natural Ecosystems

How many different forms of life exist and how they are evolutionarily related is one of the most challenging problems in biology. In 1962, Roger Y. Stanier and Cornelis B. van Niel defined "the concept of a bacterium" and thus allowed (micro) biologists to divide living organisms into two primary groups: prokaryotes and eukaryotes (Guerrero et al. 2002). Although invisible to the naked eye (except when they make up large masses), prokaryotes are essential components of the Earth's biota. The growth and survival of prokaryotes drives the geochemical cycling of the elements, detoxifies many organic and inorganic contaminants, makes essential nutrients present in the biomass of one generation available to the next, and maintains the conditions required by other inhabitants of the biosphere. Most of the Earth's prokaryotes reside in the oceans (1.2×10^{29} cells), in soil (2.6×10^{29} cells), in oceanic subsurfaces (3.5×10^{30} cells), and in terrestrial subsurfaces ($0.25–2.5 \times 10^{30}$ cells). Due to their large population sizes and rapid growth, prokaryotes have an enormous capacity for genetic diversity and rapid adaptation to subtle changes in environmental conditions (Whitman et al. 1998; Guerrero and Berlanga 2007; Prosser et al. 2007).

The Earth's habitats provide complex gradients of environmental conditions that include extreme variations in temperature, light, pH, pressure, salinity, and both inorganic and organic compounds (materials ranging from elemental sulfur to ammonia, hydrogen gas, and methane; and from cellulose and lignin to fats, proteins, lipids, nucleic acid, and humic substances). Each geochemical setting (for example, anaerobic peatlands, hydrothermal vents, deep subsurface, etc.) features its own set of resources that can be physiologically exploited by microorganisms (Madsen 2005). The free-energy governed interactions between these resources, their settings, the microorganisms themselves, and $\sim$3.5 billion years of evolution are probably the source of the metabolic diversity of the microbial world (Guerrero and Berlanga 2006).

Microbial populations rarely occur alone in nature but rather interact with each other forming complex communities. Communities can be regarded as assemblages of microbial heterogeneous populations living together at a given place or habitat. The community is the highest biological unit in an ecological hierarchy (Prosser et al. 2007). Community structures can be more or less stable depending on their degree of development. This usually involves the orderly and sequential succession of populations. The integrating mechanisms of the members of the community and the permanence of the community modify habitat conditions that allow the

evolution of new communities. Population identification is the first step to establish relationships between the whole (community) and its parts (populations). The study of microbial communities has raised questions about their composition, structure, and stability and about the activity and function of the individual inhabitants. In the past, knowledge of microorganisms in the environment depended mainly on studies of axenic cultures in the laboratory (Guerrero et al. 2002). In fact, microbial physiology and genetics can be investigated in great detail in cultivated isolates. All current information about prokaryotes is based on measurements performed on <5000 isolated species, which represent ~0.1 % of the total estimated diversity of prokaryotes in the biosphere (Madsen 2005).

Ecological diversity is considered to be a function of both the number of different types (richness or variety) and the relative importance of individual elements among these types (evenness or equitability). Species richness can be expressed by simple ratios between total species and total numbers. It measures the number of species in the community, but not how many individuals of a species are present. Equitability, which measures the proportion of individuals among the species, indicates whether there are dominant populations. Various indices have been proposed as measures of diversity that incorporate both aspects richness and evenness.

Competition for nutrients and other limiting resources is the major selective force that promotes bacterial adaptations, such as motility to search for nutrient patches, antibiotic production to inhibit competitors, and adhesion to stay in favorable environments. Success in the environment, however, is not only defined by growth and reproduction but also by the ability of organisms to avoid, tolerate, or defend themselves against natural predators. The basic principles of a biocenosis are: (i) the growth of one cell leads to N cells, (ii) N cells are a population, (iii) a population depletes nutrients and accumulates wastes, (iv) several populations associate in a guild or in a community, and (v) the community is the minimal unit of sustainable life. As a consequence, we (Guerrero et al. 2002) stated that the growth of each individual population can be expressed by the adaptation of the Monod's equation:

$$dP/dt = \mu P$$

where P is the population density in a given time (t) and μ is the specific growth rate of the population. The value of μ depends on both favorable conditions (K) such as nutrients, water, light, pH, and temperature, and deleterious conditions (ω) such as outflow, predation, lysis, and sedimentation, which reduce the numbers of cells in the population. If $K > \omega$, then $\mu > 0$, and the population increases, whereas if $K < \omega$, then $\mu < 0$, and the population decreases. In the second case, if ω is much higher than K, then $\mu \ll 0$, leading to the death of the population.

If we consider that an ecological community is the integration of the individual populations composing the community, we can propose that the growth, and hence

the permanence of a given community depends on the integration of the individual growth of each population, which can be defined as (Guerrero et al. 2002):

$$\mu \text{ community } = \mu P1 + \mu P2 + \mu P3 + \cdots + \mu Pz$$

Therefore, the size of a population of organisms in the environment is determined by the balance between their specific cell growth and mortality rates (Guerrero et al. 2002; Madsen 2005). Let us consider now the main factor contributing to ω: the mortality due to predation or to lysis. The three key groups of micro-predators include many protists (and nematodes), a few (but ecologically significant) predatory bacteria, and the omnipresent and abundant bacteriophages. It is well known that they greatly differ in size, in prey specificity, in hunting strategies, and in the resulting population dynamics.

3 Predatory Protists and Nematodes

Eukaryotic predators of bacteria include protists and nematodes. Protists represent one of the major factors accounting for the mortality of prokaryotes in the environment (Jonhnke et al. 2014) and therefore greatly affecting microbial nutrient recycling and regeneration. A key aspect of this predatory interaction is the removal of a high number of bacteria in a short amount of time. Bacterivorous protozoa hold several functional feeding groups, such as ciliates, heterotrophic flagellates, and amoebae. Each of these functional types has its own hunting characteristics and ecological niche (Pernthaler 2005; Jousset 2012) in aquatic systems heterotrophic nanoflagellates are the main consumers of bacteria, and amoebae dominate soil systems. Nematodes, such as *Caenorhabditis elegans* are common in compost, soil, as well as aquatic systems. Protists further differ in feeding strategies by showing prey preferences (depending on bacterial size, motility, shape, cell surface characteristics, and food quality (Pernthaler 2005; Dopheide et al. 2011; Saleem et al. 2013). For example, early biofilm colonizers including flagellates and ciliates, allowing fast surface feeding, while intermediate late colonizers (some ciliates and amoebas) are abundant in mature biofilms (Jousset 2012; Saleem et al. 2012) (Fig. 1).

Numerous bacteria from all phyla developed a collection of defense mechanisms reducing predation pressure. The similarities between predator resistance and pathogenesis have fostered research on this subject by both environmental and medical microbiologists (Adiba et al. 2010; Jousset 2012).

The steps involved during the predation process of a bacterial prey by a protozoan predator are: search; encounter; attack/ingestion, and digestion. Predators may spend a large fraction of their time searching for prey. Predators may move randomly through their environment until encountering potential prey. The search time can however be greatly reduced by chemotaxis. Protists and nematodes use chemical signals from their prey (bacteria) to locate and reach it more effectively.

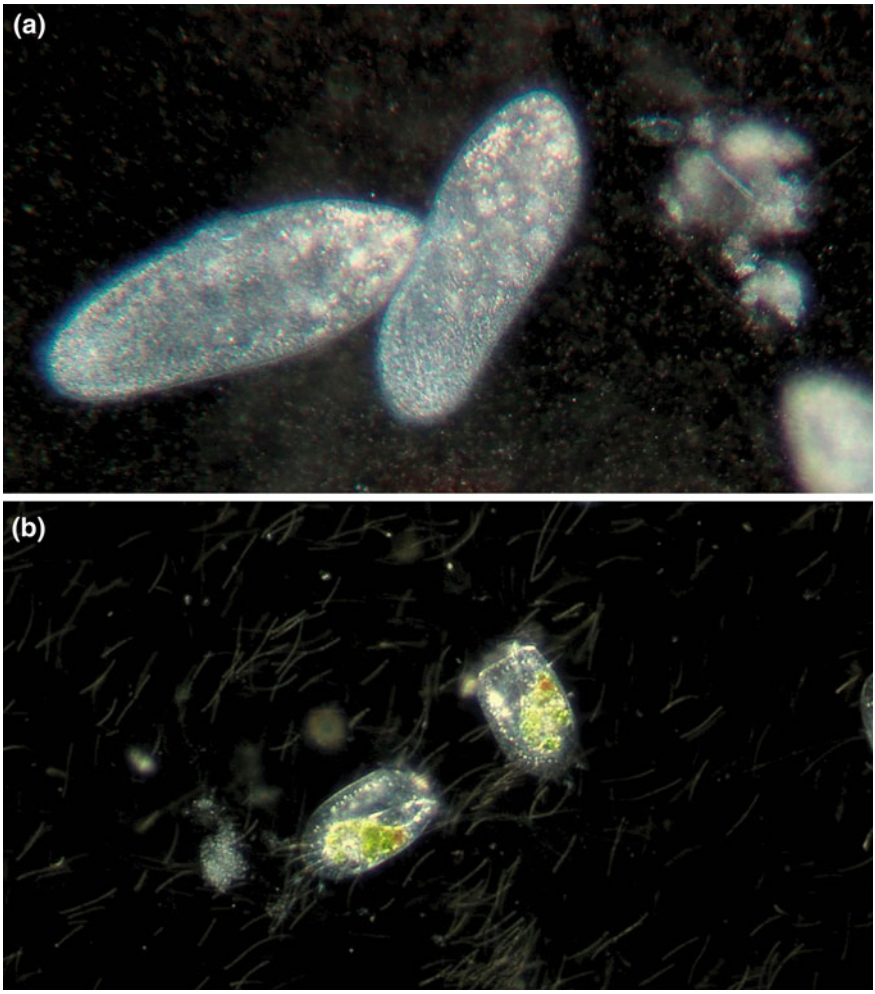

Fig. 1 Photographs obtained by dark field microscope. **a** Several *Paramecium* feeding on an unspecified bacterial mass of a pond. **b** *Euplotes* predating bacteria. Samples were obtained from the freshwater Sils reservoir (Girona). Photographs by R. Duro

For example, quorum sensing signals *N*-acylhomoserine lactones (AHLs) produced by Gram-negative bacteria, attract the nematode *C. elegans* as well as human neutrophils (Beale et al. 2006). Many bacterial strains further produce toxic secondary metabolites that inhibit predators before encounter, such as proteases in *Pseudomonas fluorescents* and *Vibrio cholerae* (Siddiqui et al. 2005; Vaitkevicius et al. 2006). Other bacteria like *Enterobacter intermedium* repel predators by acidifying their environment by excreting gluconic acid (Gómez et al. 2010).

Protists when encountering a potential prey must recognize it and avoid it from evading. Eukaryotes, including humans, use conserved receptors such as Toll-like

receptors (TLRs) to recognize molecular patterns associated with Gram-positive and -negative bacteria (Martin 2014). Protists "select" their prey by surface properties, such as flagella, cell wall components, or hydrophobicity. Bacteria to impair prey encounter can develop several strategies, such as high motility, masking surface, or secreting repelling metabolites that protists reject after their "taste." In general flagellate protists cannot ingest large prey, so several bacteria can form filaments that offer partial protection against predators. Tiny cells may elude also capture. Hydrodynamic calculations indicate that bacteria smaller than 0.5 μm in diameter encounter grazing protists four to six times less often than larger cells (∼1 μm), and filamentous cells or cells with diameters greater than 3 μm are often too large for protists to ingest. Therefore, cells in the intermediate size range are consumed more rapidly. The formation of microcolonies or aggregates reduces ingestion by flagellates and ciliates, but provides little protection against amoebae (Young 2006).

Bacteria ingestion by protozoa usually happens by phagocytosis, a conserved mechanism present in most eukaryotes, such as human defense cells, macrophages (Stuart and Ezekowitz 2008). Phagocytosis is a complex process involving the recognition and binding to specific receptors of bacteria and the engulfment in a digestive vacuole. Pathogenic bacteria can prevent phagocytosis by inhibiting the restructuration of the actin cytoskeleton, or by blocking the receptors responsible for prey binding and initiation of phagocytosis (Ernst 2000).

When bacteria have been taken up, the digestion process begins with the formation of a digestive vacuole, the phagosome. The phagosome is later acidified, and fused to lysosomes that provide lytic enzymes and reactive oxygen species that will degrade the bacteria. Several bacteria secrete toxins that inhibit digestion, delay development, and eventually kill the protist or nematode. Phage-encoded exotoxins apparently have evolved for the purpose of bacterial antipredator defense. These exotoxins kill mammalian cells by inactivating universally conserved factors and/or pathways (Stolfa and Koudelka 2012). Bacteria, in the disguise of a food source, function as a "Trojan Horse," carrying genes encoding an exotoxin into target organisms. This "Trojan Horse" mechanism of exotoxin delivery into predator cells allows intoxication of predators that lack a cell surface receptor for the particular toxin, allowing bacteria-bearing exotoxins to kill a broader spectrum of predators, increasing the fitness of the otherwise "defenceless" prey bacteria (Arnold and Koudelka 2014).

Strains of *Escherichia coli* shiga toxin-producing can sense phagosome-like conditions and kill ciliate predators by expressing the toxin after ingestion. *Pseudomonas aeruginosa* use the type III secretion system to deliver effectors and toxins into the predator's cytosol. Some bacteria can even poison their predators in a concerted manner: *Chromobacterium violaceum* specifically induced the eukaryotic apoptotic response after being ingested by a flagellate by the production of the toxic agent, violacein. The violet pigment violacein is an indole derivative isolated mainly from bacteria of the genus *Chromobacterium*, and exhibits relevant antitumoral, antimicrobial, and antiparasitic properties (Vaishnav and Demain 2011). Protist cells also offer an attractive nutrient source for bacteria to be exploited once the predatory

threat is overcome. For example, the survival and successful replication of bacteria inside the protist cell gave rise to several facultative and obligate intracellular pathogens, such as *Listeria*, *Rickettsia, Mycobacterium, Legionella,* and *Chlamydia*. It is well established that these intracellular pathogens normally persist in the environment in close association with protists, in particular with amoebae. These pathogens exhibit intracellular survival within both amoebae and human macrophages by using related mechanisms and amoebae and macrophages share similar phagocytic mechanisms (e.g., prey-recognition by cell surface receptors and prey-killing by oxygen radicals) supports the notion that resistance to amoebae is an important prerequisite and a driving force in the evolution of some bacteria as pathogens. Also, it is an important strategy to persist in the environment or to evade eukaryotic immune systems (Amaro et al. 2015).

4 Predatory Bacteria

The impact of prokaryotic predation play on bacterial processes, microbial community structure, cycling of various elements, and even health or medical issues remains to be fully investigated. Our understanding of prokaryotes is greatly impaired by our inability to identify predatory bacteria, and quantify their activity in nature. There are some reasons that difficult the study of predatory bacteria, such as predatory behavior can be observed by designing predation tests with isolated bacteria; most bacteria are not readily culturable, including predatory bacteria, which may be unable to grow in the absence of the right prey, e.g., *Ensifer* shows a strong specificity for attacking and consuming members of the genus *Micrococcus* (Casida 1982); another example is *Vampirococcus* and *Daptobacter* predators of *Chromatiaceae* (Esteve et al. 1983; Guerrero et al. 1986); and finally, the large and expanding metagenomic data obtained from the environment cannot be used to identify putative novel predatory prokaryotes because no molecular signatures specific to bacterial predation are known (Pasternak et al. 2013).

Predatory prokaryotes have evolved a unique strategy of obtaining energy and biosynthetic materials by acquiring them from other living bacterial cells. There are described several basic strategies by predatory prokaryotes: “wolfpack” group predation, epibiotic attachment, direct invasion, and periplasmic invasion (Martin 2002). In the wolfpack strategy, bacteria predators produce in common a variety of hydrolytic enzymes that degrade nearby bacteria. *Myxococcus* sp. (δ-Proteobacteria) and *Lysobacter* sp. (γ-Proteobacteria) are examples of these kinds of prokaryotes, which appear to be common in the soil. *M. xanthus* grow by scavenging nutrients from decomposing soil and detritus, or by predation of other microorganisms. *Myxoccus xanthus* cells lack flagella and are nonmotile in liquid growth media, but can move on solid growth substrates at speeds of 2–4 μm per minute. The clustering of cells into organized groups known as swarms facilitates predation and food gathering, because numerous bacteria cooperate to produce antibiotics and digestive enzymes. These antibiotics and lytic enzymes kill and digest prokaryotic and eukaryotic

microorganisms (Kaiser et al. 2010). In the epibiotic, the predator cell attaches to the outer surface of the host cell, and starts to degrade and assimilate host molecules. *Vampirococcus* attacks various species of *Chromatium* (Esteve et al. 1983; Guerrero et al. 1986). In direct invasion, the predator cell enters into the host cytoplasm. The bacterium *Daptobacter* preys specifically *Chromatium minus* (Guerrero et al. 1986). Finally, in the periplasmic strategy, the predator cell invades and grows within a specific compartment found in Gram-negative cells, the periplasm. The predatory organism that is best known to invade the periplasm of host cells, as part of its life cycle is *Bdellovibrio*. Next, we will center in on the predatory bacterium *Bdellovibrio* and *Bdellovibrio*-like organism (BALOs) that have been more extensively described and studied (Fig. 2).

***Bdellovibrio* and bdello-like organisms (BALOs)**. *Bdellovibrio* and like organisms (BALOs) prey many Gram-negative bacteria, and are ubiquitously distributed in terrestrial and aquatic environments (Sockett 2009; Jonhke et al. 2014). BALO belong to the α- and δ-Proteobacteria. BALO prey using either a periplasmic (they penetrate, grow, and divide within the prey; e.g., δ-Proteobacteria *Bdellovibrio bacteriovorus*, *Bacteriovorax marinus*, *Bacteriolyticum stolpii* and *Peredibacter starrii*) or an epibiotic predatory strategy (they attach to the cell wall, and grow and divide outside the prey, e.g., δ-Proteobacteria *Bdellovibrio exovorus*, and α-Proteobacteria *Micavibrio aeruginosavorus*) (Pasternak et al. 2014). By contrast to the generalist nature of protist predation, and the specific interactions of phages with their hosts, BALOs are neither true generalists nor hard-core specialists. Instead, prey range differs between isolates, being more or less broad, except for some particular cases described above (Chen et al. 2011).

Predatory bacteria are Gram-negative bacteria with complex life cycles with two phases separated metabolically and spatially. The first phase, the "attack phase" (AP), consists of free-living cells (Fig. 3). They are in a quiescent state, unable to replicate outside the prey, and actively swim (flagellum-propelled) by chemotaxis

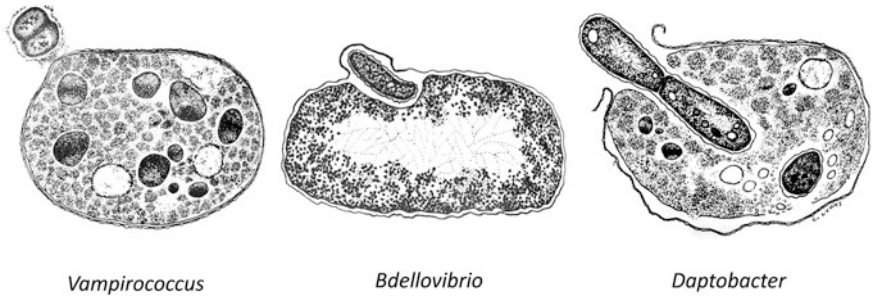

Fig. 2 Topological relations among predatory bacteria. (*Left*) *Vampirococcus* attaches to the cell wall of *Chromatium* sp bacteria. (*Middle*) *Bdellovibrio* attacks Gram-negative bacteria and stays in the periplasmic space of its prey. (*Right*) *Daptobacter* penetrates in the cytoplasm of *Chromatium* sp. bacteria. Drawings by Christie Lyons. Originally published in Guerrero et al. (1986) PNAS 83:2138-2142, and later in several editions of the General Microbiology book, by Prescott et al.

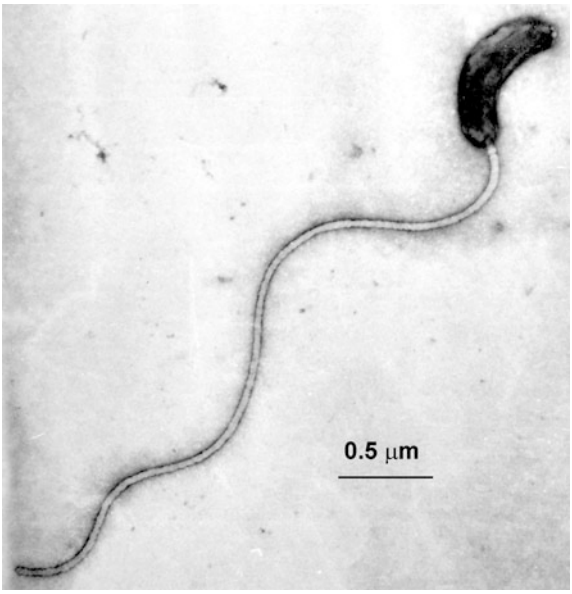

Fig. 3 Free-living cell of *Bdellovibrio*. Photograph by F. Torrella

to search for a Gram-negative prey. Flagellar motility is important in encountering prey, but it is not required to enter prey cells. Second, the "growth phase" (GP), is initiated by an AP cell attaching to or penetrating into the periplasm of a prey cell. During GP, the predatory cell used nutrients from the prey to multiply (Sockett 2009).

In the periplasmic strategy, *Bdellovibrio* cross the prey outer membrane quickly (1–2 min) through an area ~200 nm in diameter, where two moving living cells make contact. It seems that *Bdellovibrio* penetrates through a tight space in the outer membrane, no wider than the *Bdellovibrio* itself, into the prey periplasm. The means by which this is achieved is a mystery. It remains unanswered how *Bdellovibrio* makes contacts with the prey's cytoplasmic membrane to secrete degradative enzymes and how *Bdellovibrio* tolerates the changes in osmolarity, pH, or redox upon entry into the prey periplasm. The infected prey cell is now called bdelloplast. The invading predator initiates growth using the prey as a food source. The predatory cell forms a filament that then septates into individual attack-phase cells and bursts into the external medium (Rendulic et al. 2004; Sockett 2009). In the epibiotic strategy, predation is extracellular as the predators remain attached to the cell wall but do not penetrate the prey cell, consuming it from the outside before dividing into two daughter cells via binary fission (Koval et al. 2012; Pasternak et al. 2014).

The genomes of predatory bacteria may reflect the predatory phenotype in the distribution and abundance of known genes. Pasternak et al. (2013) hypothesized that the genomes of prokaryotic predators may be discernible from those of non-predators by a distinctive distribution of known and unknown protein families. These set of protein families were described as the "predatome." The predatome

exhibits deficiencies in riboflavin and amino acids biosynthesis, suggesting that predators obtain them from their prey. In contrast, these genomes are highly enriched in adhesins, proteases, and particular metabolic proteins used for binding to, processing, and consuming prey, respectively.

Bdellovibrio bacteriovorus strain HD100 have small dimensions of (0.2–0.5 μm wide and 0.5–2.5 μm long), but the complete genome sequence consists of 3,782,950 base pairs (bp) on a single circular chromosome and is predicted to code for 3584 proteins (Rendulic et al. 2004). This genome was surprisingly large and suggests that *Bdellovibrio* predatory life is far from obligate parasitic bacteria such as *Rickettsia*. *Bdellovibrio* genome contains ca. 1500 genes sequences that are not functionally characterized compared with other bacterial genomes (Rendulic et al. 2004; Gophna et al. 2006). Genomic analysis by Gophna et al. (2006) revealed that there had been a significant lateral gene transfer from other bacteria earlier in the evolutionary history of *Bdellovibrio*, including some 80 recognizable genes from spirochetes. It is plausible that spirochete lateral gene transfer allowed *Bdellovibrio* to penetrate membranes (Charon and Goldstein 2002).

Predatory bacteria with potential as biocontrolers and therapeutic agents. For successful therapeutic use, predatory bacteria must survive in the human body but not invade mammalian cells. These predatory bacteria have been readily isolated from feces of several mammals, including humans without evidence of harmful effects on the intestinal microbiota (Schwudke et al. 2001; Lebba et al. 2013). Studies in vitro have shown that *Bdellovibrio* can prey on many human pathogens, including *E. coli, Salmonella, Pseudomonas, Legionella* (Sockett and Lambert 2004; Kadouri et al. 2013; Lebba et al. 2014). A significant observation was that *Bdellovibrio* attacked both actively growing and VBNC cells (Markelova 2010). It is also observed that *B. bacteriovorus* HD100 can predate Gram-positive bacteria such as *Staphyloccus aureus*; in this case, they have an epibiotic predation behavior different from what is usually done against Gram-negative prey (periplasmic predation) (Lebba et al. 2014). The growth *Bdellovibrio* in eukaryotic cells was unsuccessful in laboratory assays (Lenz and Hespell 1978). BALOs have low immunogenicity in part because of a significantly lower binding affinity to the LPS receptors in human cells (Schwudke et al. 2003; Shanks et al. 2013). So, *Bdellovibrio* could be potentially used as a probiotic. Although predatory bacteria could be used to control human pathogens, there is still concern regarding the toxic effects of administering large numbers of Gram-negative bacteria as live antibiotics. Also, more research on this group BALO must be required to establish the effect on the beneficial commensal microbiota.

It was suggested that bdellovibrios could be used in the food industry as an additive when cleaning equipment, to reduce the incidence of food poisoning. Although there was a reduction in the number of pathogenic microorganisms, the incubation times required were prohibitively long and the use of detergents is likely to be more effective for the species (Fratamico and Whiting 1996). *Bdellovibrio* and like organisms (BALOs) could be used as possible biological agents against periodontitis diseases. It has already been evaluated with positive results in vitro with

suspensions of planktonic periodonto-pathogens. However, not all periodonto-pathogens were susceptible to BALO predation (Loozen et al. 2014). Treatment of local infections where the pathogens are easily accessible to topical or locally injected treatment would be ideal candidates to demonstrate that infections can be successfully treated with predatory bacteria. One such local bacterial infection that is treated by direct administration of BALO to the site of infection is burn wounds. *Bdellovibrio* can prey on *Pseudomonas* species that infect burn wounds, and it is possible that bdellovibrios could survive and control the numbers of *Pseudomonas* if applied topically to such wounds (Sockett and Lambert 2004). Another example is keratitis, infection of the cornea. Bacterial keratitis can be caused by both Gram-positive and Gram-negative pathogens. Common Gram-negative pathogens associated with keratitis are *P. aeruginosa* and *Serratia marcescens* (Shanks et al. 2013). The ability of *Bdellovibrio* to survive and prey in human fluids was also demonstrated in biofilms of *Aggregatibacter actinomycetemcomitans* in the presence of human saliva that contains many of the same antimicrobial compounds as do tears (van Essche et al. 2011; Loozen et al. 2014).

5 "Predatory" Bacteriophages

Bacteriophages (or phages) are bacterial viruses that infect bacterial cells. Typically, the life cycles of bacteriophages can be classified broadly into two categories, lytic (virulent) and lysogenic (temperate phage) cycles. Bacteriophages in aquatic ecosystems can reach densities of 10–100 times higher than bacteria (Breitbart 2012), with viral lysis being responsible for up to 71 % of bacterial mortality (Thomas et al. 2011).

Bacteriophages usually have a very narrow range of host specificity; the infectivity of each phage is usually limited by a specific range of bacterial strains, which belong to a single or several closely related species of bacteria. However, in certain cases bacteriophages can infect bacteria from different species and even different genera (Comeau et al. 2005). Bacteriophages predation may lead to oscillations in both predator and prey as suggested by the "killing the winner" hypothesis: in which bacterial populations are affected in a density-dependent manner (Thingstad 2000; Allen and Abedon 2014). Microbial communities are under constant predation pressure by bacteriophages that regulate microbial activity, including nutrient fluxes and food web dynamics, microbial diversity, and diversification (Weinbauer 2004; Weinbauer and Rassoulzadegan 2004). Bacteriophages are obligate parasites, and their diversity is limited by the presence of their prey in a given environment. If each bacterial species is infected by at least one phage, it is reasonable to consider bacteriophage diversity to be at least as high as bacterial diversity and probably higher (Weinbauer 2004) (Fig. 4).

In the lytic cycle, bacteriophages adsorb on the surface of a bacterium, which is followed by subsequent irreversible attachment. This ability to recognize and attach to receptor molecules on the cell surface generally dictates the host range of a

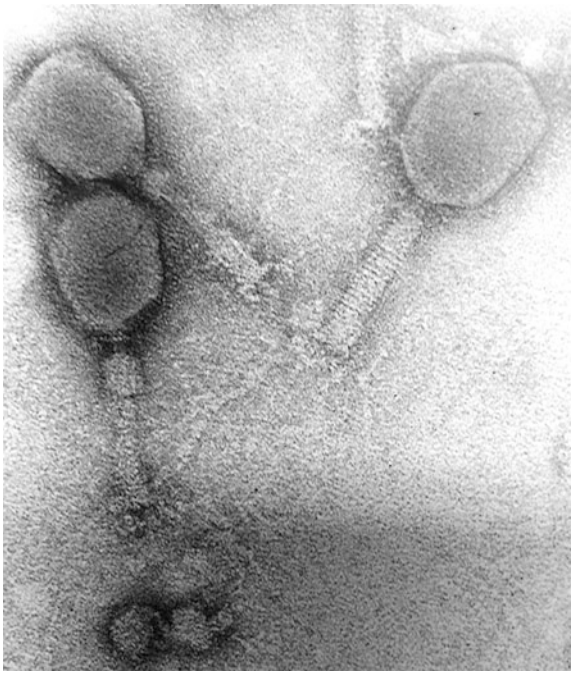

Fig. 4 Bacteriphages of natural environment, Lake Vilar, karstic system of Banyoles. Photograph by T. Figueras and R. Guerrero

phage. These receptors are usually liposaccharides, surface proteins, teichoic acids, the components of the bacterial capsule, and other surface structural elements of bacteria. This recognition is a passive physicochemical process, which is dependent on the collision frequency of phage particles and cells during Brownian motion. This process is described by a kinetic equation of the second order, which means that its rate depends on the concentrations of the bacteria and the phage (Letarov et al. 2010). The next step is the insertion of the phage genome into the cytoplasm of the host. Once inside the cell, the phage genome and proteins are synthesized to the host to generate new phage particles. At the end of the phage cycle, phage-encoded holins form pores in the cell membrane allowing phage-encoded peptidoglycan hydrolases (lysins) access to the peptidoglycan. This results in rapid cell destruction with the release of progeny phage, which then proceeds to infect neighboring susceptible cells (Endersen et al. 2014) (Fig. 5).

A phenomenon known as "lysis from without" can also result in lysis of cells without phage replication and progeny release. Lysis from without occurs as a result of high multiplicities of infections (>100) and a change in the membrane potential of the cell brought about by excess phage adhesion (Abedon 2011). Temperate bacteriophages, in addition to being capable of undergoing the lytic life cycle, have the ability to persist as a prophage within the genome of their host bacteria. In the replication cycle, temperate bacteriophages do not kill the bacteria that they infect and bacteria become immune to infection by the same or closely related bacteriophages (Guerrero and Carretero 1975; Gill and Hyman 2010).

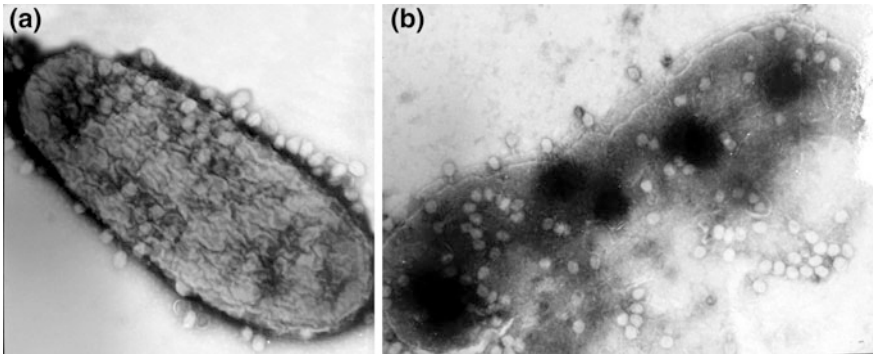

Fig. 5 Lytic cycle of *Alcaligenes* (*old name*) *eutrophus* bacteriophages. **a** Bacteriophages were adsorbed on the surface of a bacterium. **b** Lysis of the cell and leakage of new viral particles. Photographs by D. Xairó and R. Guerrero

Bacteriophages, in general, show lower propensity to induce resistance in their host bacteria, also, they do not display cross-resistance to antibiotics (Nobrega et al. 2015). However, the development of phage-resistant bacteria may occur (Labrie et al. 2010; Kutter et al. 2010). The mechanisms of resistance include blocking of phage adsorption due to loss or mutation of the bacterial receptor as well as horizontal acquisition of a restriction-modification system or development of adaptive immunity by interfering clustered regularly interspaced short palindromic repeats (CRISPR) sequences, both resulting in degradation of the injected phage DNA (Barrangou et al. 2007).

Numerous physical, chemical, and biological factors, such as temperature, light, pH, salts, and the presence of organic matter, influence the persistence of viruses in the environment. Generally physical conditions detrimental to protein structure and function could inactivate bacteriophages. Bacteriophages were usually stable at pH values between 5 and 8, and at low temperatures this range may be extended to pH values between 4 and 10. The effect of thermal stress on bacteriophages can be variable, but in general, bacteriophages may be more heat resistant than most vegetative bacteria (Greer 2005). The inactivating effect of UV light appears to be due to the accumulation of nucleic acid damage. In aquatic systems, sunlight (in particular UV-B) appears to be the major factor in the decline of phage numbers. Osmotic shock usually produces ghost particles, where the genetic material is prematurely lost from the otherwise intact particle (Greer 2005).

6 Bacteriophage Use as Biocontrol and Therapeutic Agents

Bacteriophage therapy for bacterial infections is a concept with an extensive but controversial history. At present, there is increased interest in potential application of bacteriophages to control bacterial pathogens and spoilage organisms, principally due to the increasing incidence of antibiotic resistance and virulent bacterial pathogens. The application of bacteriophage therapy must overcome some hurdles, including limited phage host ranges, possible evolution of bacterial resistance to bacteriophages, potential systemic side effects of phage therapy, and regulatory constraints placed on new clinical therapeutics in the western countries. However, recent advances in biotechnology, phage biology, etc., may help to overcome these drawbacks (Lu and Koeris 2011). The bulk of experience with human phage therapy was focused for many years in Eastern Europe and former Soviet Union.

Bacteriophages can have several applications, such as "therapy" that involves treatment of disease in infected individuals or individual disease-carrying host; as "biosanitizers" that involves treatment of equipment, contact surface, etc., with bacteriophages, this is the equivalent to disinfectant or antiseptic use; as "Biocontrol" that refers to treatment of some food products during processing (e.g., raw products and carcasses), and to extend the shelf life of perishable manufactured foods as natural preservatives ("biopreservatives"); and in addition to being used to kill bacteria, bacteriophages can be exploited for detecting and typing bacterial infections (García et al. 2008; Abedon 2014).

Bacterial virus use as antibacterial agents is comparable to drug, it can view bacteriophages during phage therapy in pharmacological terms. Pharmacology consists of two elements: pharmacodynamics and pharmacokinetics. Pharmacodynamics is the study of drug impact on the body. Impacts can be either positive (i.e., maintaining or restoring health) or negative (i.e., effecting toxic side effects). Phage preparations may exhibit minimal toxicity. Pharmacokinetics depends on the relationship established between the drug and patient. Generally pharmacokinetics deals with four processes: absorption, distribution, metabolism, and excretion (Abedon and Thomas-Abedon 2010). Phage therapy success depends on several factors such as proper dosing of bacteriophages; how many bacteriophages must be found target bacteria to achieve substantial bacteria killing; host microbiota; host physiology and immunity; production of antibodies specific to bacteriophages in patients can block the positive effect of phage therapy, etc. (Letarov et al. 2010; Abedon 2014). It would be necessary a deeper research on the eco-physiological pharmacology to gain a better mechanistic appreciation of phage therapy to improve their results.

7 The Biology and Future Prospects of Microbial Therapies

Most bacteria live in harmony with other inhabitants of the Earth. When a bacterial infection occurs, in most instances it is the result of the entry of bacteria into the body by chance. Antibiotics were initially developed for the treatment of infectious diseases in people. Over the last 75 years, relationships between bacteria and people have vastly changed. Today we are witnessing another change, that is, among bacteria themselves. The spread of antibiotic-resistant pathogens requires new treatments for bacterial infectious disease. Alternative treatments include bacteriophage therapy, bacteriophage lysins, bacteriocins, and predatory bacteria. Recently, "natural" microorganisms are also being studied to treat a wide variety of human diseases, including gastrointestinal disorders, osteoporosis, and cancer (Weiman 2014, see several chapters in this book).

Resistance to conventional anticancer therapies in patients with advanced solid tumors has prompted the need for alternative cancer therapies. Moreover, the success of novel cancer therapies depends on their selectivity for cancer cells with limited toxicity to normal tissues. Natural and genetically modified bacteria (e.g., *Salmonella*, *Listeria*, and *Clostridium*) are being evaluated as disease treatments to make them safer and to enhance specific disease-fighting attributes (Minton 2003; Forbes 2010; Swofford et al. 2015). Due to their selectivity for tumor tissues, these bacteria also serve as ideal vectors for delivering therapeutic proteins to tumors. The most potential and promising strategy is the use of bacteria, genetically engineered to express a specific therapeutic gene. Although it has shown successful results in model animal in vivo, yet further investigation about the targeting mechanisms of the bacteria are required to make it a complete therapeutic approach in cancer treatment (Wei et al. 2008; Patyar et al. 2010).

The progress in knowledge poses microbiology in a key position since it provides one of the bases in the fight of humans and infectious diseases in terms of new weapons. Research in medicine and pharmaceuticals is moving to explore the progressive use of microbes to treat infectious diseases. Thus, despite microbes being the origin and cause of infectious diseases, they can become allied to humans when used as weapons. The twenty-first century will see enormous progresses in the fight against illnesses and death, although, as Pasteur said: "Messieurs, c'est les microbes qui auront le dernier mot."

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The Biological Fight Against Pathogenic Bacteria and Protozoa

Tomás G. Villa, Angeles Sánchez-Pérez and Miguel Viñas

1 Intestinal Bacteria: Saprophytic Versus Pathogenic Organisms. A Historical Perspective

The animal gastrointestinal tract is a tube with two open ends; hence, from the microbial point of view it constitutes an open system, as opposed to the circulatory system that must be a tightly closed microbial-free environment. In particular, the human intestine spans *ca.* 200 m^2 and represents a massive absorptive surface composed of a layer of epithelial cells as well as a paracellular barrier. The permeability of this paracellular barrier is regulated by transmembrane proteins known as claudins that play a critical role in tight junctions (Pruteanu and Shanahan 2013; Barmeyer et al. 2015). Breaches in the integrity of either the epithelial or claudin barriers have profound effect on human health, causing a variety of diseases. The intestine is not only normally full of nutrients, but also at a constant temperature making it an ideal environment for microorganisms to grow. As a result, intestinal invasion by pathogenic bacteria or viruses causes a disease known as "gastroenteritis", a term believed to have been coined in *ca.* 1820. Until then this condition was often referred to as "typhoid fever," although the most common etiological agents were not *Salmonella typhi* or even *S. paratyphi.* The first typhoid fever reported (often disseminated by asymptomatic carriers such as in the case of the

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infamous Mary Mallon, aka "Thyphoid Mary") caused several disease outbreaks in the New York area from 1900 to 1910, while the second was an outbreak of paratyphoid fever (Bainbridge and Dudfield 1911). Gastroenteritis, nevertheless, can be caused by a variety of viral (norovirus, rotavirus and adenovirus), bacterial (*Escherichia coli* O157, *Salmonella* spp., *Shigella* spp., *Campylobacter,* or toxins produced by species such as *Vibrio cholerae, Staphylococcus aureus,* or *Clostridium difficile*), and parasitic pathogens (such as *Giardia lamblia*, *Cryptosporidium* spp., and *Entamoeba histolytica*). As pointed out by Schottmüller (1904), it is very difficult to assign the etiological agent for what we call "gastroenteritis": "*Bacillus paratyphosus B is capable of giving rise not only to paratyphoid fever, but also to acute gastroenteritis simulating "food-poisoning," a fact not hitherto observed in this country* (United Kingdom)*. Second, the distribution and dates of onset of the illness of the various cases were unlike those of ordinary "food-poisoning," and pointed to a human source of infection.*" It is currently estimated that there are three to five billion cases of gastroenteritis per year worldwide, and that they cause almost one and a half million deaths, with malnourished children as the population most under threat (Eckardt and Baumgart 2011).

The idea of using bacteria in antibiosis, i.e., to fight other microorganisms, originated with Vuillemin's work (1890) in the nineteenth century. He coined the term "antibiosis" to epitomize the tremendous fight constantly taking place in the microbial world in the interaction between predator and prey. Pasteur and Joubert (1877) previously described the existence of antibiosis affecting the development of *Bacillus anthracis*, a disease-producing microorganism which pathogenic effect could be counteracted by "normal bacteria." Some years later Cantani (1885) published an interesting article demonstrating the elimination of *Mycobacterium tuberculosis* from the lungs of a seriously infected patient by "insufflating" air containing a "normal nonpathogenic bacterium" By the beginning of the twentieth century, the actinomycetes bacterial group was seen as an important source of active principles that could be used against pathogenic bacteria. It was finally Greig–Smith who, in 1917, demonstrated that these bacteria play a relevant role in the control of other bacterial groups. Whipple et al. (1913) studied the origin of toxins in closed canine duodenal loops and suggested intestinal bacteria as the source of the toxins: "*The preceding experiments show that the material which accumulates in a closed duodenal loop is highly toxic when introduced intravenously. This material in the loop may be thin and soup-like or thick and pasty, but in all cases the toxic material can be demonstrated. Only a few cubic centimeters of this material (dogs S-37and 32) may be diluted, incubated at 38 °C under toluol for days, then heated at 60 °C for thirty minutes, and filtered without removing or destroying the toxic substance. Intravenous injection of this broth-like substance will cause an initial drop in blood pressure followed by a rise to normal and a prolonged secondary fall to one half or one third of normal. The heart beat is slowed and may become irregular at this time*" (Whipple et al. 1913).

Undoubtedly, intestinal microbiology is a complex matter in both animals and people. The intestinal flora includes hundreds, perhaps thousands, of

microorganisms such as bacteria, protozoa, fungi, and viruses; in normal circumstances, these organisms digest complex food materials, synthesize vitamins, and allow the animal to grow and be healthy. Probably, the first person to actually see intestinal bacteria was Anton van Leeuwenhoek; in l675 he examined a variety of samples, including his own diarrheic discharges, and wrote: "*With great astonishment I observed everywhere through the material which I was examining animalcules of the most minute size which moved themselves about very energetically*" (In Robert Hooke: Collected Memoirs of Anton v. Leeuwenhoek. See also Anton v. Leeuwenhoek: Memoirs to Royal Society of London, 1675 and 1683). If a pathogenic microorganism enters the intestinal tract, it must be eliminated before it produces illness or even death.

The intestinal microbiota found in feces has been a source of problems since the beginning of human settlements, as a continuous source of epidemic outbreaks. Once science became aware of the problems posed by these microorganisms, the different human societies tried to prevent them from contaminating drinking water, or at least to reduce the microbial contamination of drinking water through the development of sewage systems. Although Romans were the sewage pioneers, it was not until modern times that the sewage treatment systems fully developed. Weston and Kendall (1901) were some of the first researchers to systematically study the sewage microbiotas part of their work in the Laboratory of the Sewerage and Water Board of New Orleans (Louisiana, USA), an area of research often ignored by microbiologists. They wrote in their article: "*This paper, therefore, is an attempt to bring together scattered data, and to describe and tabulate into convenient form the more common bacteria of American streams.*" The following year Kendall (1902) proposed a general bacterial classification that contained most of the currently known bacteria present in sewage and fecal waters. He later reported (1910) the isolation of *Bacillus dysenteriae* from human stools and its characterization as the agent causing bacterial diarrhea. Kendall (1916) was interested in food as a transmission vehicle for intestinal bacterial pathogens and, while at the Department of Bacteriology in Northwestern University Medical School (USA), he stated: "*The public restaurant is a potential factor in the spread of certain types of disease because foods from many sources, manipulated by many hands, are dispensed to many patrons. The occasional dissemination of disease through food is well established; infected shellfish, milk, meat and vegetables have been shown to transmit typhoid bacilli and the viruses of other excrementitious diseases, botulism, and that large and somewhat poorly defined group of gastro-intestinal disturbances commonly classed as food poisoning to susceptible individuals who partake of them.*"

Kendall published an interesting article in 1911, while at the Department of Preventive Medicine and Hygiene at Harvard Medical School (USA), on the possible use of bacteria against the establishment of potentially pathogenic intestinal bacteria: "*The first logical attempt to modify the intestinal flora along definite lines was that of Metchnikoff. His work is too well known to need reviewing here, but inasmuch as he apparently failed to appreciate the full significance of his treatment, it will be well to go over the salient features in some detail. Metchnikoff was by no*

means the first to recognize the fact that the intestinal flora responds to dietary changes on the part of the host. Escherich as far back as 1886 noticed that there was a sudden, absolute and relative increase in the numbers of liquefying bacteria in the feces of dogs which were fed upon a purely protein diet" … "*In other words, utilizable carbohydrate in artificial media shields protein from bacterial attack, or, expressed in another way, fermentation takes precedence over putrefaction. This hypothesis expresses a* ***fundamental and important feature of bacterial activity. It plays a prominent part in nature, and it can be utilized to advantage in medicine***" (our highlighting) … "*His idea (Metchnikoff's) is to combat these proteolytic bacteria* (for example clostridia) *in their own field of action by the introduction of an* ***antagonistic flora, the lactic acid bacilli****. These lactic acid bacilli are said to be inimical to the proteolytic organisms in consequence of the considerable amounts of lactic acid which they generate under suitable conditions. Metchnikoff believes, furthermore, that the lactic acid bacilli themselves are necessary, since his experiments indicate that the mere feeding of lactic acid will not accomplish the same result.*" From these old publications it is clear that, even then, microbiologists were already setting the biological base for fighting pathogenic bacteria with "good" (saprophytic) microorganisms. The treatment of diarrheic syndromes in those days was particularly important, due to the lack of antibiotics or antimicrobials. This was expressed very well by Kendall (1911): "*The routine treatment for this disease in the past was: starvation for several days until the stools became more normal in appearance, until the temperature dropped, or until it became apparent that the patient could not be permitted to go longer without food.*" In that prolific year (1911) Kendall published yet another article on the principles concerning bacterial activity in the intestinal tract, in particular their use in therapeutics, his words constitute a prediction that microbiologists should be aware of: "… *the fact remains that medicine is still uninformed concerning many of even the more general principles which underlie the modes of attack and action of these microbes. The most potent factor which underlies the incompleteness of our knowledge is not difficult to determine: bacteriology, "the handmaiden of medicine," as it has been drolly expressed, besides contributing many of the most brilliant chapters of medicine, enters into so many fields of human activity and interest that it has been neglected as a pure science.*"

Regarding the action exerted by saprophytic microorganisms on pathogenic organisms, Sugg and Neill published an article in 1929 on the immunological relationship between one *Saccharomyces cerevisiae* strain and Type II *Streptococcus pneumoniae*. In that early publication, these authors showed that horse anti-yeast antibodies caused a potent agglutination of the bacterium. In addition, these antibodies protected mice challenged with the capsulated pathogenic type II bacterial strain to the same extent as rabbit anti-type II *S. pneumoniae* antibodies. The anti-yeast serum was found to be specific against Type II *S. pneumoniae* s and, hence, inactive against either type I or type III. This report, as many others in those years, focused on "vaccine" development mainly against secondary bacteria (some of them pathogenic) that invaded the upper respiratory

tract in humans after orthomyxovirus infection (as an example see Kneeland 1934); this topic however falls beyond the scope of this chapter.

Many pathogenic bacteria can alter the molecules on their surface during intestinal (or other organ) invasion, thus evading some host defenses. This process can either cause a long lasting disease or establish the host as a disease carrier, and these carriers became continuous microbial sources, capable of infecting other people (Saunders 1990). White described in 1929 three forms of antigenic variation in *Salmonella*, they included the "H" form ("O" variation described by Weil Felix; Wilson 1920), the smooth form (rough form variation of Arkwright 1920 and 1921) and the specific phase (non-specific variation of Andrewes 1922; see the introduction of Henning 1937) that appeared to be bacterial attempts to evade the host's defense mechanisms. Concerning the "H" variation, the alternative expression of *Salmonella g*enes H1 and H2, which specify different flagellar antigens (Silverman et al. 1979), results in the oscillation of phenotype known as "phase variation" or "Andrewes's" variation, by which *Salmonella* successfully evades the host's immune system. According to Silverman and co-workers this alternative antigen expression is controlled by the inversion of an 800-base-pair sequence of DNA adjacent to, or including part of, the H2 gene. But this interesting "evasion strategy" is also present in other bacteria. For instance, members of the *Neisseriaceae* family (i.e., *Neisseria gonorrhoeae*) express an opacity protein (Op, protein II), a major outer membrane antigen subjected to frequent phase transitions; this again represents a bacterial strategy to evade the host's immune system (Black et al. 1984; Stern and Meyer 1987). The evasion mechanism in *N. gonorhoeae* may be reinforced if the bacterium undergoes pilus phase and antigenic variation, in which the pilin gene is turned on and off at quite high frequencies; in fact the pilin gene is expressed by two loci on the gonococcal chromosome (pilE1 and pilE2; see Segal et al. 1985s). Antigenic variation in the genus *Shigella* (identified by Kiyoshi Shiga in 1897; for a review see Yabuuchi 2002) was finally recognized by Weil and co-workers in 1946, while working with *S. paradysenteriae.* In fact, the original work was carried out with *S. sonnei* (or Sonne's bacillus), a bacterial species isolated from human stools, possibly first isolated in the United States by Duval in 1904. Although the early history of this microorganism has indeed been confusing, as noted by Bojlén (1934): "*Certainly no other pathogenic microbe has been 'discovered' so many times as Sonne's bacillus*" (see Baker et al. 1949). Wheeler and Mickle concluded in 1945 that the different variants of *Sh. sonnei* probably represented three culturally and antigenically distinct types of *Sh. sonnei*, namely Phase I (smooth), Phase II and Rough (Baker et al. 1949). Some *Shigella* species, such as *S. flexneri* (with 6 serotypes; Brenner 1976) could go even further to evade the host's immune system; they modify the O antigen to resemble *E. coli* strains (Matsumoto 1964). *S. boydii* can also display antigen variations, but the origin and pathogenicity of this species in the human intestine is really complicated. In 1991 Albert and colleagues suggested an etiological role for *Hafnia alvei* in human diarrhea and that enteropathogenic *E coli* strains caused epithelial damage, these microorganisms were later reclassified as *Escherichia albertii* (Huys et al. 2003). Hyma et al. (2005) found that this species is closely related to strains of *S. boydii* serotype 13, a distant relative of *E. coli*

representing a divergent lineage in the genus *Escherichia*. They concluded that the *E. albertii-Shigella* B13 lineage had split from an *E. coli*-like ancestor some 28 million years ago, and eventually constituted a novel evolutionary branch of enteric pathogens; as these organisms share antigens with the saprophytic *E. coli* strains, it can explain why some enteric pathogens are able to override the host's immune system.

Vibrio cholerae is a non-sporulating Gram-negative enteropathogenic bacterium that moves using a single polar flagellum with a sheath. Filippo Pacini originally discovered this microorganism in 1854, which was again isolated by Robert Koch some 30 years later (see Howard-Jones 1984). *V. cholerae* O1 and O139 serogroups cause epidemic cholera and, while O1 causes the majority of outbreaks worldwide, O139 appears to be confined to Southeast Asia. *V. cholerae* O1 has two biotypes, classical and El Tor, and each biotype has two distinct serotypes, Inaba and Ogawa. The genes encoding cholera toxin are part of the genome of CTXϕ, a filamentous bacteriophage with a 6.9 kb ssDNA genome (McLeod et al. 2005). When this phage lysogenizes choleragenic strains of *V. cholerae,* the phage genome stably integrates into one of the host chromosomes, either chromosome 1 (with 2,961,149 base pairs and 2770 predicted open reading frames) or chromosome 2 (with 1,072,315 base pairs spanning 1115 open reading frames) (Fraser et al. 2000) and continually produces infectious viral progeny without lysing the bacterial cell wall. Viral progeny (including transducing particles) is discharged into the stool of infected people and released into the environment, thus amplifying the dispersion of the toxin genes. During interepidemic periods *V. cholerae* lives in various aquatic habitats, and recent findings (Joelsson et al. 2006) suggest that *quorum sensing* mechanisms control *V. cholerae* pathogenesis, biofilm formation, and protease production. These authors suggest that variations in *quorum sensing* systems are due to environmental selective pressures and could increase *V. cholerae*'s fitness in certain environments such as seawater. In contrast to other *Vibrio* species, *V. cholerae* does not require sodium chloride for isolation or growth but, according to V.P. Skulachev (Dibrov 2005), it must possess a Na^+ cycle that plays a key role in the colonization of the small intestine (Bakeeva et al. 1986) and the cholera toxin-induced [Na^+] increase in the intestinal lumen is in fact needed to maintain operative the sodium cycle in the relatively alkaline intestinal environment.

The huge genetic variation in the circulating *V. cholerae* strains (as an example see Tamplin et al. 1989 on the variation in epitopes of the B subunit of El Tor and classical biotype *V. cholerae* O1 cholera toxin) requires nearly 200 sera to biotype the bacterial strains. This problem was recently overcome using the simple sequence repeats (SSR), also termed VNTR (for variable number of tandem repeats), as a good and rapid means of bacterial typing (Danin-Poleg et al. 2007). In addition to the O1 and O139 serogroups, the non-O1 and non-139 strains can also cause acute diarrhea and, although they are normally non-toxigenic, some of them are becoming toxigenic (Chatterjee et al. 2009).

Stool transplant, from healthy people, could constitute an alternative, or even a complementary treatment to pseudomembranous colitis caused by *C. difficile* (Burke and Lamont 2013), but candidate vaccines are important, at least until stool

transplant becomes a generalized medical practice. Sanofi Pasteur has developed a novel bivalent candidate vaccine, a formalin-inactivated highly purified preparation of toxoids A and B, that when injected intramuscularly elicits protective antibodies against this bacterium (Foglia et al. 2012).

From the moment that a pathogenic microorganism enters a person's intestine, a frenzy battle begins, with the body synthesizing and secreting certain immunoglobulins (mostly A type). These abundant peptides with antimicrobial activity, produced by vertebrates (also known as AMPs), include the interferons (Isaacs and Lindenmann 1957) and the active calcium-binding protein (originally described in neutrophils as the L1 myelomonocytic antigen or the cystic fibrosis antigen) present in the cytoplasm of human neutrophils that is released when the neutrophils lyse (Sohnle et al. 1991), as well as the normal bacteria of the gut (including the vast and poorly known group of anaerobic bacteria).

Undoubtedly, an important mechanism regulating the complex relationships between the hundreds of different bacteria present in the animal intestine involves the *quorum sensing* signals. This *quorum sensing* depends on the production of one or more diffusible molecules, called "autoinducers" (*N*-acylhomoserine lactones in Gram-negative bacteria or "polypeptidic pheromones" in the Gram-positive world), which enable a microbial species to be aware of its population density (Hardman et al. 1998). As these authors propose "… *Irrespective of the chemical 'language' employed, interference with either the synthesis or transmission of a quorum-sensing signal molecule in pathogenic bacteria offers an exciting new strategy for controlling infection*…" The present book contains a specific chapter in which this *quorum sensing* mechanism is described in detail.

Antibiosis is a relationship between microorganisms common in nature, whether it involves prokaryotes suppressing the proliferation of eukaryotic microorganisms or vice versa. It occurs in many quarters, from animal intestines (Andremont et al. 1983) to the soil environment (Ayers and Papavizas 1963). We are still beginning to understand the complex microbial relationships taking part in the intestines of animals. The gut microbiota can even vary with the nature of the food intake, as the plant or animal origin of the ingesta could modify the microbial balance, which, in turn, could either block or facilitate the invasion by bacterial pathogens (Duncan et al. 1998). Even endoparasitic insects, such as the wasp *Pimpla turionellae*, have been reported to produce an anal hyaline secretion that strongly inhibits pathogenic bacteria and fungi (Willers et al. 1982).

2 Antibiosis in the Animal Intestinal Tract

2.1 Bacteriophage Activity Against Pathogenic Bacteria

This section is devoted to bacteriophage therapy in the intestines of warm-blooded animals, other phage therapies are described elsewhere in this book. The idea of

using bacteriophages to fight intestinal pathogenic bacteria originated from the work of the French–Canadian Felix d'Herelle (April 25, 1873—February 22, 1949). This researcher even experimented with the possibility of phage therapy and, during World War I, produced over 12 million doses of medication for the Allied forces. d'Herelle also used phages to successfully treat dysentery, probably representing the first use of bacteriophages as therapeutic agents. This biological approach soon died out in Europe under pressure from chemotherapists; the antibiotic industry mainly followed Paul Ehrlich's "magic bullet" concept, and the biological approach was forgotten under the powerful "chemical world." Nevertheless, the idea survived in Russia and other East European countries. In fact, George Eliava founded in 1923 the Eliava Institute in Tbilisi (Georgia) devoted, even currently, to the development of phage therapy. As a result of the cold war, the advances in bacteriophage therapy taking place in Stalin's Empire remained largely unknown in Western countries. Nevertheless, and despite this lack of communication, the West was also making slow advances in the field of intestinal bacterial infections, as epitomized by Klosterman and Small. In 1928, while studying a variety of stool samples in an attempt to control diphtheria, these authors isolated several bacteriophages from *Corynebacterium diphtheriae*. Much later Monsur et al. (1970) concluded that treatment of cholera with massive doses of the appropriate bacteriophage, although not as effective as tetracycline treatment, might selectively eliminate the majority of infecting vibrios without affecting the rest of the intestinal flora and without any apparent toxic effect on the patient. Years later, Smith and Huggins (1983) showed the effectiveness of phages B44/1 and B44/2 to control fatal diarrhea (caused by the enteropathogenic strain of *E. coli* O9:K30) in calves, piglets, and lambs. As it is well known, the most common pathogens associated with diarrhea in developing countries are *Vibrio cholera* (Chakraborty et al. 2001), the *Salmonella/Shigella* group, certain strains of enteropathogenic *E. coli*, and foodborne bacteria such as *Campylobacter* and *Listeria* (Mangen et al. 2007). Despite the fact that cholera is one of the oldest and most severe human diarrheal diseases, it is still widespread and little has been done recently on the use of vibriophages to control the disease, particularly in developing countries. In 2009, Bhowmick and co-workers reported the use of a mixture of five *V. cholerae* O1 biotype El Tor typing phages (ATCC 51352-B1, B2, B3, B4, and B5) as potential tools to control the disease; they achieved some success in a rabbit model of cholera. In 2010 Begum and colleagues described the isolation of one phage (IMM-001, with an isodiametric icosahedral head and long filamentous tail) that displayed a significant specificity toward CS7 fimbriae, with a high potential to control *E. coli* ETEC strains. More recently, three new *V. cholera* (O1 El Tor Inaba) DNA bacteriophages have been described, they are present in different water sources and represent good candidates for further bio-phage-control studies (Al-Fendi et al. 2014). In this species, RS1 satellite phage promotes the diversity of toxigenic strains by driving CTX prophage loss, and hence elimination of lysogenic immunity, thus contributing to the emergence of highly pathogenic strains (such as those associated with recent epidemic cholera outbursts in Asia and Haiti; Kamruzzaman et al. 2014). This means that it is now possible to develop effective bacteriophage therapy for cholera prophylaxis.

In the area of bacteriophage therapy against *Salmonella*, this approach was suggested, in the mid-1960s or early 1970s, to treat antibiotic-resistant recurrent gastroenteritis, as typhoid fever prophylaxis, to treat preschool children, and as a way to prevent secondary cases in typhoid affected areas (Courtieu et al. 1965; Nevskiĭ et al. 1965; Kiknadze et al. 1971, respectively). Years later Slopek et al. (1983) reported that phage therapy could be successfully applied in the treatment of septic infections by several bacteria, including *Salmonella and Shigella*. More recently Berchieri et al. (1991) reported that bacteriophages isolated from sewage, when concurrently inoculated into newly hatched chickens with any of three strains of *S. typhimurium*, resulted in a high reduction in bird mortality. Additionally, in 2005 Toro and colleagues demonstrated that the use of bacteriophages, in combination with competitive exclusion, indeed reduced the *Salmonella* bacterial load in infected chickens.

A serious drawback in bacteriophage therapy is the rapid clearance of the injected organisms from the fluids of warm blooded animals; but in 1996 Merril and co-workers managed to isolate long-circulating mutants of *E. coli* lambda phage and of *S. typhimurium* phage P22 that exhibited greater capability as antibacterial agents than the corresponding parental strain. In 2006 O'Flynn carried out a wide fecal screening program and isolated two lytic phages (st104a and st104b), these bacteriophages have the potential to be used in the control of *S. enterica* in pigs; additionally, st104a can be administered orally, as it is particularly resistant to porcine gastric juice. As is the case in pigs, poultry (mainly chickens) are the main reservoir for human transmission of *Salmonella* spp. and, although some progress has been made in lowering the *Salmonella* load by chemical treatment, it still remains a considerable health problem. Atterbury and co-workers made a further contribution to this field, in 2007 they developed a bacteriophage therapy for broiler chickens; out of the 232 bacteriophages tested, they found 3 capable of successfully controlling the cecal load of *S. enterica* serotypes (*enteritidis*, *typhimurium,* and *hadar*). Sillankorva and colleagues in 2010 investigated the task of controlling *S. enteritidis* in poultry, reaching the general conclusion that optimal host and growth conditions must be carefully studied and selected for the production of specific bacteriophages for animal therapy. The question of the amount of bacteriophage required to control the pathogenic *Salmonella* species is elusive, but there appears to be agreement in the utility of bacteriophages to biocontrol pathogens present in low numbers, given that a sufficiently high concentration of phages is used, and it appears that it is not even necessary to ascertain the concentration of pathogens (Bigwood et al. 2009). In addition, frequent treatment of the animals with bacteriophages, especially prior to colonization of the intestinal tract by *Salmonella* sp., is required to achieve effective bacterial reduction over time (Bardina et al. 2012). Problems such as (i) phages inducing neutralizing antibodies, (ii) phages being active only when administered shortly after bacterial infection, and (iii) the rapid emergence of phage-resistant bacteria during the course of therapy (Capparelli et al. 2010) have to be properly addressed before bacteriophage therapy can become of general use. Therefore, as the authors demonstrated, phage-resistant bacteria indeed constitute excellent vaccines, protecting against lethal doses of heterologous *S. enterica* serovars. The problem of

the appearance of phage-resistant bacterial strains can be aggravated by the phase-variable glycosylation phenomenon present in the *O*-antigen of *Salmonella,* as reported by Kim and Ryu in 2012. Kang et al. (2013) described the isolation of a novel DNA-containing bacteriophage (wksl3, relative of SEPT3 phage) belonging to the Siphoviridae family that does not encode any phage lysogeny factors, toxins, pathogen-related genes, or foodborne allergens, but capable of controlling the growth of *Salmonella enterica* (serovars *enteritidis*) and *typhimurium* in food.

Waseh et al. (2010) described an alternative to the use of whole bacteriophages, they found that P22 phage tail spikes are sufficient to elicit a specific *Salmonella* agglutination response in the animal, thus mimicking antibody agglutination and facilitating bacterial elimination through intestinal movements, and perhaps more importantly inhibiting bacterial intestinal translocation. Another approach, reported by Oliveira et al. in 2014, involves the direct use of bacteriophage endolysins with a modicum of thermal stability, as well as resistance to gastrointestinal pHs. These endolysins are very active in Gram-positive bacteria, but not in Gram-negative due to the outer membrane present in these bacteria that is only permeable to bacteriophage endolysins at low pH.

Although currently bacteriophages cannot be used in humans or farm animals, until appropriate protocols are developed, they could be used to control the horizontal transmission of pathogenic *Salmonella* species from infected to noninfected animals (Lim et al. 2011). There are some bacteriophages that have lost their specificity for infecting particular bacterial species and display polyvalent activity on a variety of bacterial genera. This is the case for phage phiKP26 (reported by Amarillas and co-workers in 2013), proposed as a putative biocontrol agent for both *Salmonella* and *E. coli*. However, one should bear in mind that polyvalent bacteriophage activity could result in undesirable side effects, by destroying saprophytic bacteria. While bacteriophage therapy is being profusely used in the food industry, to reduce the effect of food born pathogenic *Salmonella* species, as well as in the poultry (to treat laying hens) and pig industries, its application in human health is still rare.

Bacteriophage therapy against *Shigella dysenteriae* is also mainly lacking, although the description of new viruses for this species (such as bacteriophage WZ1 isolated from waste water in 2015; Jamal et al. 2015) could pave the way for the use of this kind of complementary therapy in the treatment of bacillary dysentery. As it is the case for enteropathogenic *Salmonella* species, the use of bacteriophage therapy against enteropathogenic *E. coli* strains is currently restricted to farm animals, such as calves, piglets, and lambs (Smith and Huggins 1983; Smith et al. 1987), or to the treatment of milk and meat products (Tomat et al. 2013a, b). Reports on bacteriophage therapy against *Campylobacter* are even sparser. To the best of our knowledge, no human has ever been treated with *Campylobacter jejuni* phage*s*, although this treatment is successful in broiler chickens, resulting in a drastic reduction in bacterial load (Loc Carrillo et al. 2005). This is also the case for *Listeria monocytogenes*; the fight against this bacterial pathogen is currently being experimentally applied in food science (Anany et al. 2011), but it has never been

employed in human therapy. This situation may change once Romulus and Remus (two phages belonging to the Twortlikevirus genus described in 2013) are fully understood (Vandersteegen et al. 2013).

2.2 Bacterial Activity Against Intestinal Pathogenic Bacteria

This issue has always been elusive, even to the most seasoned microbiologists, due not only to the number of bacterial and fungal species involved, but also to bacteriophages and other viruses (Velasco et al. 1984). Even long-term confinement generates changes in the human microbiota, as reported by Shilov et al. (1971). The authors showed that the intestinal microbiota of astronauts that spent over 1 year of isolation in space underwent drastic changes; these involved a severe reduction in the number of aerobic bacterial species (less than 6 %), whereas anaerobic bacteria increased to almost 90 %. Gut microbiota is, therefore, a very complex microbial community in unstable equilibrium. When this equilibrium is broken, the body suffers from disorders that range from diseases caused by microbial pathogens to vitamin or essential amino acid deficiencies. Human intestines contain *ca.* 100 trillion microorganisms, about 10-fold the number of human cells present in the body (Guarner and Malagelada 2003), representing between 300 and 1000 different species (Sears 2005), although it is probable that 99 % of the bacteria belong to only 30 or 40 species (Beaugerie and Petit 2004).

Microbial antagonisms have long been observed in the intestinal tracts of animals, including humans, and can be due to a defined mixture of strains fighting colonization by a species either belonging to the same (Duval-Iflah et al. 1981) or a different genus (Ducluzeau et al. 1977), as well as to complex endogenous microflora against pathogenic microorganisms (Wilson et al. 1981). It is often proposed that intestinal anaerobic bacteria control the growth of members the *Enterobacteriaceae* family, through the production of volatile fatty acids and colicins, as well as by modification of bile acids and competition gut nutrients (Andremont et al. 1985). Borriello and Barclay (1986) studied the role of volatile fatty acids in preventing the establishment of *C. difficile* and found that this inhibition could not be linked to specific volatile fatty acids or enzymes. In addition, as the number of bacterial strains harboring antibiotic resistance plasmids is increasing steadily, the use of antibiotics can affect these microbial relationships and cause unexpected end results (Andremont et al. 1983, 1985).

The genus *Lactobacillus* has been long recognized as an efficient tool for controlling other intestinal bacteria, including enteropathogens. Watanabe et al. (1977) investigated the effects of three indigenous *Lactobacillus* groups (group I, including *L. acidophilus* and related strains; group II, represented by *L. fermentum;* and group III, consisting of *L. murine* and associated strains) on other bacterial populations in gnotobiotic rats. The authors found that the indigenous bacteria present in the wall of the nonglandular part of the stomach (including the stomach and upper part of the small intestine) were controlled by groups I and II, but not by III; this implies

that the bacterial-controlling activity was linked to particular species (and not to a whole genus) and even, most likely, linked to particular bacterial strains. In addition, the species belonging to the *Lactobacillus* genus were reported to exhibit anticarcinogenic and hypocholesterolemic effects (Mital and Garg 1995). Other lactic acid bacteria, belonging to the *Pedicoccus* and *Lactococcus* genera, have also been reported to play antagonistic roles that control intestinal colonization by human enteropathogens in live poultry (Juven et al. 1991). In fact, *Lactobacillus casei* was shown to display in vivo and in vitro antagonistic activity against *Salmonella typhimurium* infections (Hudault et al. 1997). Coconnier et al. (1993) reported that even heat-killed human *L. acidophilus* inhibits the pathogenicity caused by diarrheagenic bacteria in cultured human intestinal cells. Blomberg and colleagues in 1993 studied the inhibition of *E. coli* K88 adhesion to piglet ileal mucus caused by *Lactobacillus* spp. strains, and concluded that *L. fermentum* 104R produced a soluble proteinaceous component (molecular mass above 250kD, as determined by gel filtration) that inhibited the adhesion of K88ab and K88ac fimbriae to ileal mucus by interacting with the mucus components. Jin and colleagues further confirmed this in 1996; they studied the antagonistic effects of intestinal *Lactobacillus* isolates on bacterial chicken pathogens. Their results showed that all of the 12 *Lactobacillus* tested could inhibit the growth of *S. enteritidis* 935/79, *S. pullorum, S. typhimurium, S. blockley*, and *S. enteritidis* 94/448, as well as that of three *E.coli* serotypes (O1:K1, O2:K1 and O78:K80).

More recently, Cleusix and co-workers reported that reuterin effectively controls the enteric microbiota. This compound is produced by *Lactobacillus reuteri* and present as 3-hydroxypropionaldehyde, its hydrate, or its dimer; it displays a broad-spectrum activity against enteropathogens, yeasts, fungi, protozoa, and viruses (Cleusix et al. 2007). However, Fetissov et al. suggested (2008) that the intestinal presence of a high number of *Lactobacillus*, or other probiotics, could produce oligopeptides resembling appetite-regulating peptide hormones, and alter the normal appetite/satiety equilibrium by generating autoantibodies.

In a classic paper, Barnes et al. (1979) reported several factors that influence the incidence and anti-*Salmonella* activity of the anaerobic caecal flora in young chicks (*Bacteroides hypermegas* and a *Bifidobacterium* sp.); these factor are mainly acidic conditions and the production of volatile fatty acids. Modulation of the bacterial microbiota can be achieved by selective elimination of the aerobic bacteria in the oropharyngeal cavity and intestinal tract, leaving the anaerobic microbiota intact to a large extent (van Furth and Guiot 1989); this treatment prevented colonization by resistant, but potentially pathogenic, bacteria or fungi, even in patients exhibiting severe granulocytopenia episodes. Hillman and co-workers in 1994 carried out in vitro experiments that demonstrated that the resident microflora of the porcine ileum (containing a balanced load of anaerobic and aerobic bacteria) actually inhibited the penetration of enterotoxigenic *E. coli* strains.

Indeed, dysbacteriosis, a series of illnesses that occur in the early postnatal period, can be avoided by simply instilling *Lactobacillus acidophilus* (with anti-*klebsiella* and anti *S. aureus* activity) into the mouth and nasal passages of

neonates. Following this treatment, the babies were discharged from the maternity ward with a normal intestinal microflora (Moshchich et al. 1989).

Kieckens et al. (2015) showed that enterohemorrhagic *Escherichia coli* (EHEC) strains (of which *E. coli* O157:H7 is by far the best-studied serotype, as it constitutes an important foodborne pathogen worldwide) could be easily controlled by rectal administration of bovine lactoferrin in cattle. This pathogenic *E. coli* strain can live for long periods of time in the intestine of affected animals without showing any clinical symptoms. The same authors concluded that this abiotic way of fighting pathogenic bacteria by rectal treatment could represent a useful strategy to preclude transmission of EHEC infections from cattle to humans, which currently represents the most common way of transmission. These pathological serotypes of an otherwise commensal species, together with the labile toxin producing enterotoxic *E. coli* (ETEC), are the most common pathogens isolated from diarrheal stools of hospitalized children and adults, closely followed by *Salmonella* spp (Mendis et al. 1995).

Weinack and co-workers in 1982 reported a reciprocal competitive exclusion between either *Salmonella thyphimurium* or pathogenic *E. coli* strains and the native intestinal microflora of chickens and turkeys, with the result that the native intestinal microflora of both birds were protected against the pathogenic species. Hence, the chicken and turkey microflora appeared to be equally effective in protecting the two species from *S. typhimurium,* but protection against *E. coli* was somewhat greater in the chicken than in the turkey. This appears to define a pattern of microbial protection against pathogenic microbiota somewhat based on the evolutionary relationships between saprophytic and pathogenic microorganisms. In this sense, Ducluzeau and Bensaada reported in 1982 that *Saccharomyces boulardii*, when given to monoxenic mice, was active against *Candida albicans*, *C. krusei* and *C. pseudotropicalis* strains, but unfortunately ineffective against *C. tropicalis*. Interestingly, the antagonistic effect totally disappeared when *S. boulardii* cells were heat-killed. Rodrigues et al. (1996) investigated the effect of the yeast *S. boulardii* on oral infection of gnotobiotic mice with *S. typhimurium* and *Shigella flexneri*. They found that the yeast rapidly colonized the intestines of the germ-free animals, and this protected them from diarrheal disease when challenged with a high numbers of either pathogen.

A series of in vitro studies confirm the role played by the "normal" gut microbiota to control the growth of pathogenic bacteria that use the gastrointestinal tract as the main point of entry. Accordingly, Ushijima and Ozaki reported in 1986 the antagonism of *E. coli, Bacteroides ovatus, Fusobacterium varium*, and *Enterococcus faecalis*, either alone or together, against enteropathogens (i.e., *Yersinia enterocolitica, Shigella flexneri, Salmonella typhimurium, Vibrio parahaemolyticus, V. cholerae* serogroup non O1, *S. aureus*, and *Clostridium perfringens*). They found that, in anaerobic continuous flow cultures, it only took mixed cultures of the four resident bacteria a few days to eliminate *Y. enterocolitica*. On the other hand, *E. coli* alone was sufficient to eradicate *Sh. flexneri,* and *E. coli* together with *B. ovatus* could eliminate *S. aureus, C. perfringens, V. parahaemolyticus,* and *V. cholerae* serogroup non-O1. In addition, *S. typhimurium* was

the species most resistant to elimination, and, depending on the nitrogen source available, *C. perfringens* (itself an anaerobic microorganisms) could even resist the action of the four resident bacteria together. It was Gorbach and co-workers who, in 1988, suggested that intestinal anaerobic bacteria represented an actual barrier against enteropathogens. These authors tested the intestinal microflora resistance to colonization, in human volunteers, and found that this resistance not only occurs but it is diminished by antibiotic administration, but is not dependent on the anaerobic microbiota. *C. perfringens* intestinal colonization has been extensively investigated in cesarean-delivered newborns, from birth to the two first weeks of life. Bezirtzoglou et al. (1989) found that breastfeeding directly modulates the numbers of *C. perfringens* present in the neonate's gut, and that *Bifidobacterium bifidum* indeed plays a role in the control of *C. perfringens*. Interestingly, it was also found that saprophytic species could eradicate pathogenic microorganisms. In this regard, Kuroiwa and co-workers reported in 1990 that *C. butyricum* M588 exerted a preventive effect against the proliferation of *C. difficile* during antimicrobial therapy. Bernet and co-workers in 1993 investigated the role of bifidobacterial adhesion to cultured human intestinal epithelial cells on the inhibition of enteropathogen–cell interactions; they found that both *B. breve* and *B. infantis* were able to inhibit epithelial cell association of either enterotoxigenic or enteropathogenic *E. coli*, *Yersinia pseudotuberculosis* and *S. typhimurium*, in a concentration-dependent manner. Indeed, Tazume et al. reported in 1993 that, in the "abnormal" intestinal flora of patients with severe diarrhea, there is a close correlation between a decrease in the number of anaerobes and a reduction in the level of short-chain fatty acids and free bile acids; this, in turn, causes an increase in pH and water accumulation in the intestine that may facilitate enteric infections.

In newborn babies, the intestinal microbiota is the result of a specific selection process influenced by many factors (Ducluzeau 1993). In breast-fed infants, *E. coli* and streptococci are the first bacteria to colonize the gut, usually followed by *Bifidobacterium* species, which soon constitute the main microbiota. On the other hand, the gut of bottle-fed infants has a bigger bacterial variety, including other enterobacteria as well as different anaerobes. Breast milk is known to contain some "bifidus factors" that promote the growth of *Bifidobacterium,* as well as providing immunoglobulins, which prevent intestinal colonization by pathogenic enterobacteria.

After weaning, as the variety of the infant's diet considerably increases, the gastrointestinal tract develops to harbor an enormous number of both aerobic and anaerobic bacteria, which exhibit a quasi-symbiotic relationship with the host. Regulation of the intestinal microbiota depends on a very complex network of interactions and factors that include immunoglobulins, gastric acid secretion and bacterial adherence to intestinal cells (Batt et al. 1996), which can exert either beneficial or detrimental effects on the host. The beneficial effects of the so-called "normal enteric microbiota" include the competitive exclusion of potentially pathogenic organisms, as well as the production of short-chain fatty acids and vitamins. The detrimental effects encompass competition for calories and essential nutrients, contribution to inflammatory bowel disease and colonization by transient

pathogens that could, in turn, interfere with the mucosal barrier; the latter could give rise to bacterial translocation and cause bacteremia or even septicemia. Jackson et al. (1990) described that this bacterial translocation across intestinal walls can involve both Gram-negative and Gram-positive microorganisms.

It is well documented that intestinal bacteria can translocate from the intestinal tract to several parts of the body and cause serious illness or even death (Wells 1990). Only a few aerobic/facultative species appear to have the ability to translocate, and it was originally proposed that anaerobic bacteria prevented such translocation (van der Waaij et al. 1971). Although the mechanisms controlling bacterial translocation remain unclear, they are known to involve both microbial and host factors. Strictly anaerobic bacteria do not appear to translocate in healthy hosts; but other organisms, such as *L. monocytogenes* or *Salmonella* species, can either enter macrophages and reach the Peyer's patches or distal organs or use host phagocytes to reach the draining mesenteric lymph node (Wells 1990). In some cases, the presence of fatal intestinal injuries, intense burns, or acute mesenteric ischemia can facilitate the translocation of both anaerobic and facultative anaerobic microorganisms.

Crohn's disease constitutes another example of a detrimental health effect involving intestinal microbes. Although other factors cannot be excluded for this disease, such as chronic infection with a specific persistent pathogen (Balfour Sartor 2007), or an overly aggressive immune response to normal commensal enteric bacteria, as well as host genetic susceptibility resulting in defective mucosal barrier function or lack of bacterial killing ability. All these aspects, and probably others as yet not described, lead to an overly aggressive T-cell response to normal bacteria that finally causes the tissue damage characteristic of Crohn's disease.

Colonic anaerobes, such as *Bacteroides fragilis* or *Peptostreptococcus* species, rarely cause infections as solitary pathogens; but, when accompanied by aerobic bacteria or in environments with an abundance of nitrogen source (dead tissue), they can cause quite severe infections in areas including the abdominal cavity. These mixed infections of aerobes and anaerobes must be treated by surgical drainage, in combination with antibiotic therapy (Fry and Schermer 2000). However, in general, anaerobic bacteria appear to play a key role in confining indigenous bacteria to the gut (Wells et al. 1987). Despite the fact that intestinal anaerobes include pathogenic species, they usually represent microorganisms beneficial to humans, since they are instrumental in restraining the growth of *C. difficile* in human carriers, as well as providing catabolic enzymes that allow digestion of organic compounds, which cannot be otherwise digested by eukaryotic enzymes. In addition, these microorganisms are essential for the catabolism of cholesterol, bile acids and steroid hormones, as well as for detoxification of certain carcinogens (Bokkenheuser 1993). These beneficial effects have also been reported by Shimizu and coworkers, who in 2006 noted altered gut microbiota in patients affected by severe systemic inflammatory response syndrome (i.e., significantly lower total anaerobic bacterial counts and two log higher "pathogenic" *Staphylococcus* and *Pseudomonas* bacterial counts than in healthy people).

Clostridium perfringens is another ubiquitous Gram-positive bacterium reported to include two types of strains, the necrotic enteritis-producing strains and the non-necrotic enteritis strains. Barbara et al. (2008) demonstrated that, at least in chicken intestines, the necrotic strains naturally displace the non-necrotic varieties. In addition to producing toxins, the bacterium proceeds to the digestion of epithelial tight junction proteins, thus contributing to bacterial translocation across the intestinal barrier and producing the necrotizing syndrome (Pruteanu and Shanahan 2013). This microorganism also produces many enzymes, including sialidases, which contribute to bacterial dispersion (Li and McClane 2014). One of the first, if not the first, defense mechanism in humans is linked to the mother's colostrum, that normally exhibits high titers of specific secretory IgAs against *C. perfringens* (Liem et al. 1979); but after weaning, the child's defense must rely on its own ability to produce those antibodies. As indicated above, the use of gnotobiotic animals has provided important insights into different research areas. These animals' response to different bacterial pathogen challenges has been important to elucidate, on one hand, the pathogenic action of a given pathogen and, on the other hand, the protection provided by a particular saprophyte or "normal" bacterial flora (Adremont et al. 1983). Consequently, Yurdusev and colleages reported in 1987 that gnotobiotic mice harboring a *Bacteroides thetaiotaomicron* strain, a *Fusobacterium necrogenes* strain and a *Clostridium* sp. strain were protected against challenge by pathogenic *C. perfringens* B, C and D serotypes, although the authors were unable to isolate any diffusible substance. The same authors 2 years later (Yurdusev et al. 1989) confirmed again the antagonism exerted by *B. thetaiotaomicron*, in association with *F. necrogenes*, against *C. perfringens*, both in vivo (in gnotobiotic mice) and in vitro (in fecal suspensions). Ramare and colleagues published an interesting paper in 1993, demonstrating the trypsin-dependent production of an antibacterial substance by a human *Peptostreptococcus* strain in gnotobiotic rats; this represented the first report of a potent antibacterial substances produced through a mechanism involving both intestinal bacteria and exocrine pancreatic secretions.

The in vivo role of intestinal bacteriocins as protective substances against pathogenic bacteria has not been clear, due to their narrow activity and their sensitivity to proteolytic degradation. Jennes et al. (2000) studied the intestinal bacteria in ostriches and found that *Enterococcus gallinarum* 012 synthesized a polypeptide (enterocin 012) active against *E. faecalis, Lactobacillus acidophilus*, *L. sake, Listeria innocua, Propionibacterium acidipropionic*i, *C. perfringens, Pseudomonas aeruginosa*, and *Salmonella typhimurium*; but enterocin 012 was ineffective against *Bacillus cereus, C. sporogenes, C. tyrobutyricum, Leuconostoc cremoris, Pediococcus pentosaceus, Staphylococcus carnosus*, and *Streptococcus thermophilus*. Finally, Crost and colleagues produced an interesting publication in 2011 that constituted the first report confirming the in vivo protective action of the *Ruminococcus gnavus* E1 bacteriocin; the compound, originally isolated from human stools, exhibited a clear protective role against intestinal colonization (and hence intestinal necrosis) by *C. perfringens.* Activation of the bacteriocin is trypsin dependent and its activity is comparable to the protective effect exerted by metronidazole. Namkung and colleagues demonstrated in 2011 that the protective

role of bacteriocins against this anaerobic pathogen (in particularly the strains producing intestinal necrosis) must be studied in combination with the intestinal production of n-butyric acid by other intestinal anaerobic bacteria. In addition, the inclusion of certain plants in the human diet could positively regulate the intestinal necrosis syndrome produced by this bacterium. This is the case for *Artemisia annua*, as its leaves can control necrotic enteritis in broiler chickens and compensate, to a certain extent, for the weight loss caused by the disease (Engberg et al. 2012).

The epsilon- (ε-) proteobacteria include one of the five classes in the Proteobacteria phylum (Vandamme et al. 1991) that can inhabit the gastrointestinal tracts of animals, as well as inhabiting water reservoirs or sewages, and cause serious illness (Bereswill and Kist 2003). Much of the interest in this bacterial group arises from the fact that some of its genera, such as *Campylobacter, Helicobacter,* or *Wollinella*, constitute human pathogens. While *Helicobacter pylori* is the causative agent of gastric and peptic ulcers (Ghose et al. 2005), and *H. hepaticus* could contribute to liver or gastric cancers (van Amsterdam et al. 2006), *Campylobacter* is usually a human pathogen; this Gram-negative microaerophilic bacteria is oxidase-positive and most of its strains are motile, with one or two polar flagella (Vandamme et al. 2006). Despite the undefined taxonomical situation of the bacterial genus, Holländer managed (1984) to characterize the main *Campylobacter* groups isolated from human stools. *C. coli* and *C. jejuni* are two very closely related species (sharing 28 proteins; Gupta 2006) and together represent the main source of bacterial foodborne disease in many developed countries (Ackerley and Jones 1985); in some cases, infection by these bacteria can cause sepsis or meningitis (Pequignot et al. 1973; Gubina et al. 1976). Escherich, in 1886, originally described the symptoms associated with "campilobacteriosis" (Kramer and Kanof 1960), but the genus itself was not described until 1963 (Debruyne et al. 2008). Hence, the microbiological history of this genus is short, in addition until 1971 *Campylobacter* was considered a vibrional species (Morris and Park 1971). The *Campylobacter* flagella (unusually composed of two types of flagellin) are involved not only in intestinal adherence, but also in translocation and cell internalization, thus playing an important role in bacterial virulence (Grant et al. 1993). Expression of flagella in *Campylobacter* spp. is subject to both phase and antigenic variations; in such a way that some strains can switch between flagellated and non-flagellated forms and other strains can even reversibly express flagella with different antigenic specificities (Caldwell et al. 1985; Alm et al. 1992). It appears that the central, surface-exposed region of the flagellar hook protein FlgE in *C. jejuni*, in fact displays hypervariability among strains (Lüneberg et al. 1998); this flagellar antigen variation is part of the defense mechanism of these pathogenic bacteria in the human intestine. Flagellar variations, however, could just represent one of the multiple variations displayed by epsilon bacteria, which include DNA uptake, DNA recombination, adhesion, and iron uptake; this is in contrast with more classical variations, such as those displayed by *E. coli* (Gilbreath et al. 2011). As it is the case for other mucosal pathogens (i.e., *Neisseria* or *Haemophilus*), *Campylobacter* lacks the *O*-polysaccharide repeating units in their outer core glycans, although they display structural diversity (Moran et al. 1996). These glycans are low-molecular

weight lipopolysaccharide variants, known as lipooligosaccharides, which also play a role in evading the host's defense mechanisms. Sugar *N*-formyltransferase is an important enzyme in *C. jejuni* that generates the LPS variations characterized by Thoden and co-workers in 2013.

Clostridium difficile is another bacterium that merits to be cited here, because of its ability to colonize the human large intestine and cause pseudomembranous colitis, a condition very hard to treat (Smith and King 1962; Larson et al. 1978). The bacterium was initially named *Bacillus difficilis* by Hall and O'Toole in 1935, because they found it difficult to isolate, but was later reclassified within the *Clostridium* genus as the soil constitutes its main habitat (for a comprehensive review of the phylogenetic status of this genus see Elsayed and Zhang 2004). While in the large intestine, pathogenic strains of *C. difficile* produce multiple toxins, such as enterotoxin (toxin A) and cytotoxin (toxin B). Both compounds are glucosyltransferases that target and inactivate the Rho family of GTPases, and this can produce diarrhea and inflammation of the large intestine, although the relative contributions of each of the toxins is not yet clear (Lyerly et al. 1986). In addition, toxin B induces actin depolymerization, by a mechanism that correlates with a decrease in the ADP-ribosylation of the low molecular mass GTP-binding Rho proteins (Just et al. 1995). Elimination of this bacterium from the large intestine is difficult, and is probably related to the unusual tetragonal structure exhibited by the outermost layer of the bacterial cell wall, formed by two proteins (Masuda et al. 1989). To the best of our knowledge, Wada et al. (1980) were the first to demonstrate, in the supernatants of cultured human colostral cells, a neutralizing activity against *C. difficile* toxin; this activity was mainly due to IgAs secreted by the macrophages found in the human colostrum. Neutralizing IgAs can also be found in stools of individuals infected with pathogenic *C. difficile* strains. In fact, Kyne and colleagues reported in 2000 that antibody titers against toxin A were higher in patients with mild *C. difficile*–associated disease than in individuals with prolonged or severe diarrhea.

Another clostridial species worth mentioning here is *C. perfringens*. This bacterium, formerly known as *C. welchii*, or even *Bacillus welchii*, is a Gram-positive anaerobe, ubiquitously distributed (including in human intestines), which can cause serious illness due to the production of several toxins, including an enterotoxin (synthesized in vivo during sporulation; Narayan 1982). Enterotoxin damages epithelial cells by binding to claudin family members, including claudin 3, 4, 6, 7, 8, and 14, but not 1, 2, 5, and 10 (van Itallie et al. 2008). Some circulating *C. perfringens* strains (such as that reported by Tilton and co-workers in 1981, isolated from the intestine of a dog which died from a parvoviral infection) secrete toxins similar to those produced by *C. difficile*. Since *C. perfringens* spores can survive the temperatures used for cooking food, this bacterium is a common cause of foodborne worldwide infections, producing a rarely fatal infection (necrotizing enteritis, known as pigbel). The pathogen can also contaminate surgical facilities, leading to postoperative infections (Parker 1969). Although rare, serious infections can cause gangrene of the entire large intestine during the seventh month of pregnancy (Jirán 1971).

C. welchii was originally described by Welch, who isolated it from the body of a 38-year-old male, in 1891 and 1892. He described the microorganism as *Bacillus aerogenes capsulatus*, and later on it was renamed *C. welchii* (Euzéby 1997).

The role of indigenous resident "normal" bacteria in the prevention of the "'sudden infant death syndrome" is progressively gaining acceptance; as indicated above, bacterial colonization of human infant colon is influenced by many factors, including age and antibiotic exposure. As the intestinal microbiome is known to influence the development of the immune system, it must play an important role in protecting infants from the bacteria and/or their toxins involved in the pathogenesis of the "sudden infant death syndrome" (Highet et al. 2014). These authors analyzed the intestinal bacteria in infants affected by this syndrome, and compared it to the flora found in infants not suffering from the syndrome; they concluded that *C. difficile* and *C. innocuum* were significantly associated with the syndrome's development.

It is generally accepted that probiotics exert beneficial effects in the gastrointestinal tract of warm blood animals, but Mangell and co-workers reported in 2012 that, at least in the case of *Lactobacillus plantarum* 299v, once the bacteria was established in the intestine of animals it had no effect on either enteric bacteria or bacterial translocation. The results were not consistent with others in the literature, such as the study by Cao et al. (2012) who reported the control of *C. perfringes*-induced necrosis enteritis by *Lactobacillus fermentum* 1.2029). In conclusion, the mechanisms behind the protective effects of probiotics in animals and humans, as well as the effects of bacteriocins, remain largely unknown and further research is required to ascertaining their utility in the fight against intestinal bacterial pathogens. Hence, it remains imperative to continue the research into antimicrobials as well as extend the available arsenal with new additions such as the semisynthetic antimicrobial thiopeptide LFF571 (Fig. 1). LFF571 was described as a translation inhibitor that binds bacterial elongation factor Tu (EF-Tu) and blocks the delivery of aminoacyl-tRNA (aa-tRNA) to the ribosome (Parmeggiani et al. 2006). In addition, essential oils, such as nerolidol, thymol, eugenol, and geraniol, could be exogenously added to the human diet, since they appear to effectively control the population of intestinal pathogenic bacteria, such as *E. coli* O157:H7, *C. difficile* DSM1296, *C. perfringens* DSM11780, *S typhimurium* 3530, and *Salmonella enteritidis* S1400 (Thapa et al. 2012).

Gastroesophageal reflux disease is a common condition that, according to Del Piano et al. (2012), affects 175 million people just in Europe. The disease is currently treated with proton pump inhibitors, such as omeprazole, that increase the pH of the gastric fluids but can create unwanted side effects, such as proliferation of potentially pathogenic bacteria. These authors demonstrated that *L. rhamnosus* LR06, *L. pentosus* LPS01, *L. plantarum* LP01, and *L. delbrueckii* subsp. *delbrueckii* LDD01 successfully restored the "gastric barrier effect" in patients chronically treated with proton pump inhibitors. Microbiologists from all over the world are constantly striving to find new strains of *Lactobacillus* species, with the potential constitute better probiotics to be used in the fight against pathogenic

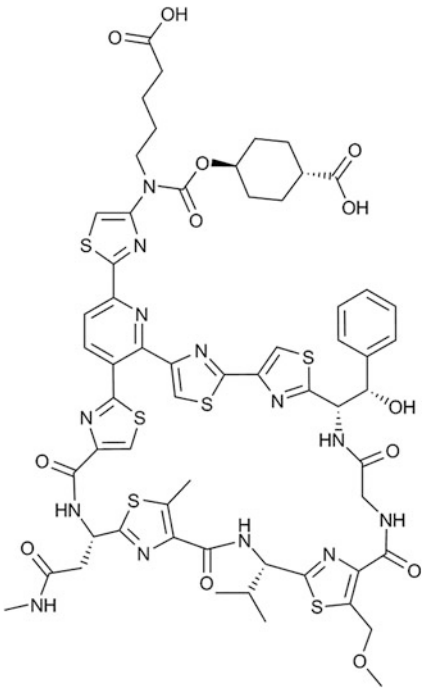

Fig. 1 Chemical structure of LFF571. LFF571, a semisynthetic thiopeptide (see Selva et al. 1991)

bacteria. One such example is the novel JSA22 strain of *Lactobacillus plantarum*, isolated from traditional fermented soybean, active against *S. enterica* serovar *typhimurium* (Eom et al. 2015)

2.3 *Bacterial Activity Against Protozoa and Other Intestinal Parasites*

Only a few specific examples of the protection exerted by the "normal" microbiota against protozoan infections have been documented. As described in the previous section, it appears that species belonging to *Lactobacillus* genus can protect the human gastrointestinal area not only from pathogenic bacteria but also protozoa. Alak et al. (1997) reported that *L. reuteri* could induce intestinal resistance to *Cryptosporidium parvum* infection in mice suffering from immunodeficiency syndrome. It is not clear if this report is, in any way, related to Langer and Riggs' findings (1999), concerning the apical complex glycoprotein CSL that contains a sporozoite ligand for intestinal epithelial cells. *L. casei* has also been reported to have anti *Trichinella spiralis* activity (possibly involving gamma interferon activity); Bautista-Garfias et al. (1999) demonstrated that treatment of mice with *L. casei* resulted in the elimination of adult worms from their intestines. Similarly, Singer and Nash (2000) reported that the "normal" flora in mice intestines protected them

against *G. lamblia* infections. *G. lamblia* is a flagellated protozoan that causes watery diarrhea worldwide and can be treated in a variety of ways, including with plant extracts, as reported by Rahimi-Esboei and co-workers in 2013. The host defense against *Giardia* infection involves several different immunological and non-immunological mucosal processes (Roxström-Lindquist et al. 2006), including the production of cryptdins (small intestinal defensins produced by the Paneth cells; Aley et al. 1994). Immunological responses include the production of secretory IgAs both in milk and saliva (59 and 52 %, respectively), although the antibody titer in milk was 50 times higher than in saliva. The antibodies generated targeted the trophozoite membrane, flagella and cytoplasmic antigens (Téllez et al. 2005); this aspect, however, falls outside the scope of this chapter.

When *G. lamblia*, a microaerophilic organism lacking catalase activity, infects the proximal small intestine mucosa, it must overcome the adverse oxygen and nitric oxide concentrations in the mucosa; it does this with the help of two yet uncharacterized 2-cys peroxiredoxins, GiPrx1a and GiPrx1b, suggested to play a role in the antioxidant defense of *Giardia* and thus a factor contributing to its pathogenesis (Mastronicola et al. 2014). *G. lamblia* can be infected with a dsRNA virus, although it is currently unknown if this virus could be eventually used to combat *G. lamblia* infections. This phage is a reovirus-like viral particle discovered by Wang and Wang in 1986, these authors described the phage as spherical virus-like particles (VLP) with a diameter of 33–35 nm and a dsRNA genome encompassing a region of 7 kilobase pairs. The capsid contains a major, highly antigenic, 100 kDa protein (Wang et al. 1988; Miller et al. 1988). Furfine and Wang (1990) used the dsRNA virus to infect other virus-free strains of the intestinal parasitic bacteria, and found that Giardia could even be infected by electroporation with purified viral ssRNA (for a comprehensive review see Wang and Wang 1991). More recently, Humen et al. (2005) used *Lactobacillus johnsonii* La1 as a probiotic, in a *Meriones unguiculatus* model infected with *Giardia intestinalis,* and found that the lactic acid bacterium antagonized the intestinal parasite to such extent that it protected the membrane integrity of the microvillus. The authors additionally concluded that the cellular response to *Giardia* antigens was stimulated in spleen cells. It appears that *Lactobacillus* species capable of producing bacteriocins, such as *L.acidophilus* (P106) and *L.plantarum* (P164), are able to affect *G. lamblia*. As a matter of fact, an ultrastructural examination proved that the bacteriocines produced marked changes in the cellular architecture of the trophozoites, with evident disorganization of the cell membrane, adhesive disk and cytoplasmic components (Amer et al. 2014).

Oral administration of recombinant acid lactic bacteria, such as Lactococcus lactis and *Streptococcus gordonii,* can stimulate the intestinal immune responses against *G. lamblia* cyst wall protein-2, and significantly increase the number of CD4(+) T-helper and B-cells in the mesenteric lymph nodes and Peyer's patches of treated animals (Lee et al. 2009). This indicates that probiotics could indeed constitute a good approach to control parasitic diseases.

Another successful strategy focuses on immunizing the animals with recombinant *E.coli* strains harboring the *Eimeria acervulina* trophozoite antigen (Kim et al.

1989), this approach resulted in the immunization conferring the animals with partial resistance to coccidiosis.

Mucin degrading bacterial glycosidases from "normal" colon microbiota in warm-blooded animals, in combination with colonic luminal proteases can degrade the key adherence lectin present on *E. histolytica* trophozoites, and this effectively decreases the pathogen's epithelial cell adherence and prevents *E. histolytica* from invading the intestinal mucosa (Variyam 1996). The use of bacterial proteins, known to be harmless to humans while maintaining high activity against worms (i.e., crytal proteins isolated from the parasporal crystal of *Bacillus thuringiensis*), is a new strategy worth mentioning here. Accordingly, Urban and colleagues demonstrated in 2013 that Cry5B protein could intoxicate *Ascaris suum,* thus triggering the activation of the p38 mitogen-activated protein kinase pathway and resulting in a near complete elimination of the intestinal parasite infection in pigs.

Not only intestinal bacteria can play a role in controlling parasites, Buske et al. (2013) recently suggested that certain fungi could share this ability. These authors reported that the fungus *Duddingtonia flagrans* systematically reduces *Haemonchus contortus* larval population.

Blastocystis hominis is a common human intestinal parasite that causes 4.3 % of diarrheal cases in humans (Mendis et al. 1995). It belongs to the *Stramenopiles* (Silberman et al. 1996; Tan 2008) and, although it was described over a century ago, little is still known about its pathogenicity, genetic diversity, or treatment (Roberts et al. 2014). In addition, some researchers claim that despite its high prevalence, it does not cause a diarrheic syndrome, at least not in Nepalese people (Shlim et al. 1995). The organism was originally classified as the cyst of a flagellate, as a vegetable, or even as a yeast, but it was subsequently reclassified as a protist (Tan 2008). Chandramathi et al. (2014) demonstrated that stress exacerbates the infectivity and pathogenicity of *B. hominis*, both in vivo and in vitro. *B. hominis* and *G. lamblia* have been found to preferentially infect people carrying the A > T transversional mutation (36 and 28 %, respectively; Mahdi and Ali 2002) that replaces the sixth amino acid of the β-globin chain and causes sickle-cell anemia. Although metronidazole is the most frequently prescribed antimicrobial for these infections (Gupta and Parsi 2006), one study showed the potential benefits of using *S. boulardii* to treat *Blastocystis* infection (Dinleyici et al. 2010). *Blastocystis* infections stimulate immunoglobulin G (IgG) and IgA production (Hussain et al. 1997; Mahmoud and Saleh 2003), apparently involved in the elimination of the parasite. Antibodies, or at least the cytotoxic monoclonal antibody 1D5, can trigger programmed cell death in the parasite *B. hominis*, and this cell death is independent from the caspase and mitochondrial pathways (Nasirudeen and Tan 2005). If this could be demonstrated as a general mechanism for *Blastocystis* eradication from the human gut, it would preclude intestinal bacteria participation, unless the presence of these microorganisms could induce heterophilic antibodies capable of initiating apoptosis.

Production of IgA against certain intestinal parasites (including bacteria) could in turn cause nephropathy, due to acute glomerulonephritis caused by deposition of IgA-containing immune complexes in the glomerular mesangium. The discovery

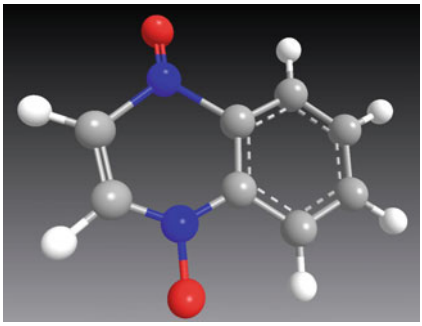

Fig. 2 Basic structure of heterocyclic *N*-oxides with antibacterial and antiparasitic activities

that the distribution of the six new risk loci in humans associated with acute glomerulonephritis can vary in different ethnic groups (it is most prevalent in East Asians, less frequent in Europeans, and relatively rare in individuals of African ancestry; Kiryluk et al. 2014) advocates that great care must be taken in the administration of probiotic bacteria that could result in hyperproduction of secretory IgA.

Microsporidia are opportunistic agents that infect immunocompromised patients, such as those suffering from AIDS (Atías 1995). Five strains of this pathogen are currently known: *Encephalitozoon, Enterocytozoon, Nosema, Pleistophora*, and *Septata* (van Gool and Dankert 1995) and the clinical symptoms they cause can range from hepatic necrosis, ocular infections affecting not only the cornea but also the eye surroundings (even including the paranasal sinuses), to multisystemic infection affecting the central nervous system. The diagnosis of microsporidiosis currently depends on morphological demonstration of the organisms themselves, either in scrapings or tissues and, unfortunately, there is yet no evidence on the efficacy of probiotic treatment with either bacteria or yeasts.

As mentioned above, until more is known about the effect of "normal" intestinal bacteria on the protozoa that colonize the human intestine, it would be wise to use probiotics in combination with other approaches, such as the novel antiparasitic compounds known as heterocyclic *N*-oxides (Fig. 2) (Mfuh and Larionov 2015), or the triazolyl-quinolone-based chalcone derivatives that are active against *G. intestinalis* (Bahadur et al. 2015).

2.4 Bacterial Activity Against Intestinal Viruses

One of the first reports on the use of bacteria to combat intestinal viruses comes from Loria et al. (1976); they studied the intestinal tract of mice and discovered that the group B coxsackievirus offers natural protection against peroral infection. They concluded that this protective effect consists of at least two separate components: (i) a barrier function that prevents virus from passing through the gut mucosa into the circulation, and (ii) a clearance mechanism that eliminates the virus from the

enteric tract after the infection. The authors however did not reveal the involvement of any microbial entity responsible for the clearance. In 1989 Ogra and colleagues studied the effect of oral immunization of the gastrointestinal tract, with bacterial and viral antigens, on mucosal immunity, but could reach a definite conclusion on the cross-protection provided. Koopman et al. (1989) reported that fusiform anaerobic bacteria caused the elimination of murine viral pathogens from the caecum of mice, and this resulted in the normalization of the intestinal microbiota content and function. Indeed, after the treatment the murine duodenal extracts exhibited high activity against the lactate dehydrogenase-elevating virus (LDV), but once again the authors could not associate the virucidal effect of a 10–100 kDa intestinal protein to any particular bacterial species (Broen et al. 1992). Finally, Duffy and co-workers were able to demonstrate in 1993 the protective effect of a human strain of *B. bifidum* against murine Group A rotavirus. Saavedra et al. (1994) further these studies by successfully using the combination of *B. bifidum* and *Streptococcus thermophiles* against human rotavirus, while Majamaa et al. (1995) demonstrated that lactic acid bacteria are effective in the treatment of acute rotavirus gastroenteritis. Worldwide, approximately 90 % of people have antibodies against Herpes Simplex Virus type 1 (HSV-1), and around 30 % of them will develop symptoms. An et al. (2012) showed that *Bifidobacterium adolescentis* SPM 0214 is active against HSV-1. This finding is fortunate, since not only the percentage of HSV-1 infected people is increasing, but also the virus resistance to antiviral drugs, such as acyclovir, is constantly on the rise.

Another function that the intestinal bacteria could carry out is the transformation of bioactive molecules into even more active metabolites with the potential to control the proliferation of rotavirus and even *H. pylori*. This is the case of *Eubacterium* L-8 and *Streptococcus* LJ-22 that can convert glycyrrhizin into two metabolites (18β-glycyrrhetinic acid-3-*O*-β-D-glucuronopyranosyl-(1→2)-β-D-glucuronide and 18β-glycyrrhetinic acid-3-*O*-β-D-glucuronide) with increased antiviral and antibacterial activities (Kim et al. 2000). Human breast milk contains all the elements necessary for normal growth and development of infants. It also has large amounts of sialic acid-containing, or sialo-conjugated, molecules that favor the growth of bifidobacteria and lactobacilli, while inhibiting bacterial toxins and enterovirus adhesion to intestinal epithelial cells (Nakano et al. 2001). Human breast milk additionally contains large quantities of fucosylated oligosaccharides, produced by enzymes encoded by the genes associated with the expression of the Lewis blood group system, that play a clearly protective role against both intestinal bacterial or viral enteropathogens (Newburg et al. 2005). Soon after the discovery that intestinal lactic acid bacteria possess antiviral activity, novel recombinant species harboring particular enteroviral genes were developed and used to elicit quick antiviral immunological responses (Ho et al. 2005). Commensal bacteria have also been used to cheaply express HIV antigens and efficiently control early stage HIV infection. Accordingly, Rao and colleagues described in 2005 the development of a highly colonizing probiotic recombinant strain of *E. coli* Nissle 1917 that secreted the HIV-gp41-hemolysin A hybrid antigen, and blocked HIV internalization. The authors reported that the bacterial strain could colonize mice for periods ranging

from weeks to months; the recombinant *E. coli* strain was predominantly found in the colon and cecum, with lower bacterial concentrations present in the rectum, vagina, and small intestine. Zhao et al. (2006) found that recombinant *Listeria monocitogenes* expressing the *gag* protein from HIV could offer mice vaginal protection against human immunodeficiency virus type 1. In a similar manner, Yang et al. (2005) orally used an attenuated strain of *S. flexneri* carrying the human papillomavirus (HPV) L1 capsid protein to protect the animals against papillomavirus infection. Shiga toxin is a toxin belonging to a large family of ribosome-inactivating proteins with *N*-glycosidase activity that causes rRNA depurination and is found in many plants and in some bacteria (Endo et al. 1987). Ferens and colleagues reported in 2006 that this toxin displays a protective role against leukemia-producing virus; they experimentally infected sheep with bovine leukemia virus and found that an intestinal Shiga toxin-producing *E. coli* strain mitigated the infection.

Pan et al. (2009) successfully used recombinant *S. thyphimurium* attenuated strains as a vehicle for DNA vaccines against avian influenza viruses.

As described above for other biological control purposes, *Bifidobacterium* or *Lactobacillus* probiotic bacteria have the ability to lessen the effect of vesicular stomatitis virus infection, by establishing an antiviral state in macrophages that results in the production of NO and inflammatory cytokines, such as interleukin 6 and interferon-gamma (Ivec et al. 2007). Similarly, Pant et al. reported that treatment with *Lactobacillus rhamnosus* strain GG, in combination with anti-rotavirus antibodies significantly reduced the diarrheal disease caused by rotavirus (Pant et al. 2007). Indeed, Pregliasco et al. (2008) carried out a 3-stage randomized study that showed the positive effect of a combination of 3 probiotics (*L. plantarum*, *L. rhamnosus* and *Bifidobacterium lactis*), lactoferrin and prebiotics (such as short-chain fructooligosaccharides or galactooligosaccharides), on lowering the impact (number of disease cases) of winter-associated diseases. Maragkoudakis et al. (2010) obtained similar results, although using cell monolayers and only probiotic bacteria (*L. rhamnosus* GG, *L. casei, Enterococcus faecium* PCK38, *L. fermentum* ACA-DC179, *L. pentosus* PCA227 and *L. plantarum* PCA236 and PCS22). They concluded that the bacteria protected the cells from invasion by rotaviruses and gastroenteritis viruses. Accordingly, Kawase et al. (2010) protected mice against influenza viruses by supplemented their diet with lactobacilli from the human intestinal tract. In fact, *E faecium* (strain NCIMB 10415) has been approved as a probiotic feed supplement for use in animals in the European Union; this strain is effective against the enteropathogenic coronavirus transmissible gastroenteritis virus (TGEV; Chai et al. 2013).

Vlasova et al. (2013) found that, in gnotobiotic pigs infected with human rotavirus, both lactobacilli and bifidobacteria could promote immune homeostasis by modulating the innate immune response against the virus. Muñoz et al. (2011) described that different *Bifidobacterium* species (such as *B. longum* subsp. *infantis* CECT 7210) could also display a probiotical effect against intestinal rotavirus, while maintaining the main properties required from a probiotic; these properties include resistance to gastrointestinal juices, biliary salts, NaCl, and low pH, as well

as adhesion to intestinal mucus and sensitivity to antibiotics. More recently, Liu et al. (2013) described that *Lactobacillus rhamnosus* GG on its own is moderately effective against rotavirus diarrhea, lessening the disease by partially preventing injuries to the intestinal epithelium. In fact, in a human gut microbiota transplanted neonatal gnotobiotic pig model, this microorganism enhances Th1 cellular immunity, without affecting the antibody responses (Wen et al. 2014). Some *Lactobacillus* strains (such as *L. plantarum* AYA) can induce pleiotropic responses when orally administered; they induce the production of high amounts of IgAs, which protects both the lungs and intestines from influenza viruses (Kikuchi et al. 2014). Marranzino et al. (2012) described that certain *L. rhamnosus* strains (such as Lr1505 and Lr431), when used as probiotics, can induce additionally effects; in fact, it was reported that regular consumption of this probiotic improves resistance to infections, even in body areas away from the gut, by increasing the macrophages activity at those sites.

Probiotic bacteria also play a role in the control of HIV infections in humans. According to Cunningham-Rundles et al. (2011), the alterations in the gut microbiota that occur early in HIV-1 infection lead to dominance of potential pathogens and reduce the levels of *Bifidobacterium* and *Lactobacillus* species, which consequently increase mucosal inflammation. The authors carried out pilot studies and concluded that probiotic bacteria, given as a supplement, protect against the loss of CD4+ T cells. According to Lehtoranta et al. (2014), probiotics also increase the general resistance against the *picornaviradae* that cause the common cold in winter.

Other lactic acid bacteria, such as recombinant *L. lactis*, have been successfully used as vehicles for coronavirus antigen delivery (as it is the case for the 70 kDa fragment of the N-terminal globular domain of the spike of coronavirus TGEV; Tang and Li 2009), and constitute an effective protection against this diarrhea-causing virus.

In conclusion, the information currently available indicates that either wild type or recombinant strains of lactic acid bacteria constitute a good means of controlling intestinal colonization by certain pathogenic viruses and that they represent a future means of designing more effective vaccines. Additionally, the unspecific response described above for lactic acid bacteria, such as the increased IgA production, merits further investigation and opens the door to further novel uses for these bacteria.

2.5 *Bacterial Activity Against Pathogenic Yeasts*

Gillot (1958) originally suggested the use of active substances produced by *L. acidophilus* against *Candida albicans*. Several years later, Balish and Phillips (1966) demonstrated, in germ-free chicks, the protective role of normal bacteria (such as *E. coli*) against the pathogenic yeast. Controlling the intestinal population of *C. albicans* is important since this yeast can promote food antigen sensitization in mice, by affecting the mucosal barrier (Yamaguchi et al. 2006). The anti-*Candida* activity

of the enteric bacterial microbiota is important to control yeast growth, additionally preventing their adherence to epithelial surfaces; but unfortunately little is known on the probiotic and biotherapeutic effects of the intestinal "normobacteria" against candidiasis (Balish and Wagner 1998). A number of bacterial species have the ability to inhibit *C. albicans* in vitro, these include *E. coli*, *Salmonella* spp and mainly *Lactobacillus* species (Isenberg et al. 1960; Caves et al. 1973; Hummel et al. 1975; Balish and Wagner 1998). The control of the intestinal growth of microorganisms such as *C. albicans, M. avium* subsp *paratuberculosis* and adherent-invasive *E. coli* strains is important, since they are suspected to play a role in the infectious etiology of Crohn's disease. This suspicion is based on the genetic susceptibility, which relates to impaired function in the defense against intracellular microorganisms (Pineton de Chambrun et al. 2008). In fact, Sendid et al. (2009) found that a high number of Crohn's disease biomarkers (mainly anti-cell wall glycan antibodies) were induced during *C. albicans* development in the intestine.

But the relationship between intestinal bacteria and yeasts can also work in reverse, this means that a yeast strain can also act as a probiotic that protects the animal against pathogenic bacteria. In this way, the ascomycetous yeast *S. boulardii* can grow at 37 °C (McFarland and Bernasconi 1993) and ameliorate different types of diarrheal diseases (Bartlett 1992; McFarland et al. 1995). This yeast was reported to modulate the immune system (Buts et al. 1990), degrade *C. difficile* toxins A and B as well as their respective receptors on the colonic mucosa (Castagliuolo et al. 1996), inhibit cholera toxin action (Brandão et al. 1998), and stimulate digestive enzymatic activities (Buts et al. 1986; Jahn et al. 1996). It can also inhibit the production of pro-inflammatory cytokines by inhibiting main regulators of inflammation (including nuclear factor κB (NF-κB), mitogen-activated protein kinases (MAP kinases), and ERK1/2 and p38), but it stimulates the production of anti-inflammatory molecules such as peroxisome proliferator-activated receptor-gamma (Im and Pothoulakis 2010), among others (for a more comprehensive review see Martins et al. 2005; Pothoulakis 2009).

S. boulardii can also interfere with *C. albicans*; in fact, both *S. boulardii* and its extracts significantly inhibit *C. albicans* adhesion to epithelial cell lines, as well as reducing cytokine-mediated inflammatory host response (Murzyn et al. 2010). In addition, Martins et al. (2005) isolated twelve different *S. cerevisiae* strains from a variety of sources and found that strain 905 was the best equipped to become a successful probiotic. This strain displayed a good ability to grow in the gastrointestinal tract of germ-free mice and it displayed a protective effect against experimental infection with *S. tithymurium* and *C. difficile.* Strain 905 was also shown to reduce the intestinal translocation of *S. enterica* serotype *typhimurium* and to stimulate the immune system in both gnotobiotic and wild type mice (Martins et al. 2007). *S. cerevisiae* has additional probiotic capabilities, such as the ability to modulate transcription of proteins involved in inflammation, and the capacity to inhibit the ETEC-induced expression of pro-inflammatory proteins IL-6, IL-8, CCL20, CXCL2, and CXCL10, as well as IL-6, and IL-8 (Zanello et al. 2011). In a more recent study, Martins et al. (2009) described a comparative study on the suitability of using four different microbial species (*Bifidobacterium animalis,*

E. coli, L. casei, and *S. boulardii*) as probiotics, with the conclusion that the yeast displayed better immunomodulation characteristics, whereas *B. animalis* and *L. casei* constituted better antagonistic microorganisms.

2.6 Bacterial Activity Against Protozoan Insect Vectors

The relationship between the bacteria found in the midgut of insects, intestinal protozoa and host arthropods has always been, to say the least, peculiar. This association has become so close that often the organisms are dependent on each other for survival, making it difficult to study them individually (Dillon and Dillon 2004). Learning how these gut bacteria interact with their insect host could give an insight into how to control the insect's life cycle and, perhaps, contribute to the biological control of vector-transmitted protozoan diseases (Chanbusarakum and Ullman 2008). Additional aspects could include: (i) the effect of the Rickettsia-like maternally inherited *Wolbachia*, known to alter the arthropod's sexual differentiation, resulting in female-biased sex ratios or parthenogenesis (Rousset et al. 1992), or (ii) the novel insecticidal toxins produced by the nematode-symbiotic bacteria *Photorhabdus luminescens* and *Xenorhabdus nematophilus* (Ffrench-Constant and Bowen 2000). Species of *Photorhabus* such as *P. temperate* are entomopathogenic bacteria that have a negative, although indirect, effect on the insect's microbiota. This is the case for the sugarcane stalk borer *Diatraea saccharalis* (Lepidoptera); soon after infection of the insect with *P. temperate* 90 % of its intestinal microbiota is killed, eventually resulting in the animal's death (Carneiro et al. 2008) The study of the intestinal bacterial population of hematophagous insects is gaining importance, as it could represent a biological way to control classic protozoal diseases of difficult eradication worldwide, particularly in third world countries. One of the insects targeted is the sand fly *Phlebotomus papatasi*, the vector for leishmaniasis, Carrión's disease, bartonellosis, and a variety of arboviral diseases (Depaquit et al. 2010). This emphasizes the fact that knowledge of the vector's intestinal microbiota is important in order to advance the fight against these diseases. In 2012 Mukhopadhyay and colleagues reported a wide range of variation in the aerobic flora inhabiting the sand fly gut, which possibly reflected the different breeding habitats; but there are common bacteria (such as *Bacillus pumilus* and *Bacillus flexus*), possibly involved in oviposition and therefore with the potential to controll the insect's life cycle. Maleki-Ravasan et al. (2013) continued the study of the sand fly microbiota, with a focus on the bacterial population found in *P. papatasi* midgut, and their findings disagreed with previous reports for *Phlebotomus duboseqi*. The authors found that, in addition to *Enterobacteriaceae* (with *Proteus mirabilis* and *P. vulgaris* as the most prevalent isolates) and non-enterobacteria (*Bacillus, Staphylococcus,* and *Pseudomonas*) species, the main bacterial strain for females was *Ochrobactrum* sp., a species acquired by *transstadial* passage (Volf et al. 2002). The Malpighian tubes of *P. papatasi* are often carriers of *Aspergillus sclerotiorum* and *S. cerevisiae* strains, and that makes the insect refractory to

artificial infections with the protozoan parasite *Leishmania* and even with bacteria such as *E. coli, S. aureus, Shigella sonnei, Streptococcus group A*, and *Pseudomonas aeruginosa* (Schlein et al. 1985). In addition, this particular sand fly, when challenged with bacteria such as *Erwinia carotovora* subsp. *carotovora*, synthesizes a novel defensin (with a molecular mass of 4095.5 MH^+) displaying a strong antiparasitic activity against the promastigote forms of *Leishmania major* (Boulanger et al. 2004). Chanbusarakum and Ullman (2008) found insect bacterial endosymbionts phylogenetically related to *Erwinia* in the western flower thrips (*Frankliniella occidentalis*).

Chaga's disease is caused by the protozoan *Trapanosoma cruzi* and uses triatomine insects, such as *Rhodnius prolixus*, as vectors. This protozoan interferes with the insect's immunity system, lowering its gut's microbiota and allowing the protist to permanently establish itself inside the insect (Castro et al. 2012a). The bacterial microbiota of *R. prolixus* can be increased by simply treating the insect with physalin B (a natural secosteroid, extracted from the plant *Physalis angulata*), causing a reduction in the number of *T. cruzi* parasite cells (Castro et al. 2012b). Current work involves the in vitro manipulation of one of the insect's gut bacterium (*Rhodococcus rhodnii*) to express antiparasitic agents in the gut, where the trypanosomes are also located as a means of controlling the parasite's population. According to Dotson et al. (2003) stable integrons are desirable to ensure that the bacterium constantly produces the antiparasitic drug. The surface of *T. cruzi* is covered by a thick mucin-like substance, involved in binding this protozoon to the insect's intestinal membrane; hence, disruption of these glycoconjugates by 1,3-β-D-glucanase-producing *R. rhodnii* strains could stop the parasite's development (Jose et al. 2013). In addition, *R. rhodnii* intestinal microbiota could be modified to deliver other molecules with antiparasitic activity. Accordingly, Taracena et al. (2015) successfully used a systemic RNAi strategy to control Chaga's disease; they fed *R. prolixus* nymphs and adults with transgenic *E. coli* HT115 (DE3) strains expressing dsRNA for the *Rhodnius* heme-binding protein and for catalase.

Despite all the research carried out, malaria still remains a health problem in African countries, where it still causes more than 1 million deaths annually (Boissière et al. 2012). The disease in these countries is caused by *Plasmodium falciparum*, transmitted by the mosquito *Anopheles gambiae*. The ability of the mosquitoes to transmit *Plasmodium* parasites is highly variable between individuals and this individual susceptibility appears to have a genetic basis (Blandin et al. 2009), but is also influenced by the bacteria colonizing the insect's midgut, which can provide a protective role against the malarial parasite (Dong et al. 2009); indeed, the use of antibiotics to disrupt the insect's gut microbiota can enhance *Plasmodium* infection. A low number of the bacterial species constituting the insect's gut microbiota have been isolated and identified by classical microbiological methods (Favia et al. 2007). On the other hand, Boissière et al. (2012) used pyrosequencing techniques to study the microbiota present in one mosquito. They found that the midgut microbiota in that adult mosquito included five dominant *Phyla* (*Proteobacteria, Bacteroidetes, Actinobacteria, Fusobacteria, and Firmicutes*), but the vast majority of sequence tags (>90 %) corresponded to just a

few taxa and only 21 bacterial families, while 28 genera only represented >1 %. Their conclusion was, therefore, that *A. gambiae* is colonized by a few dominant bacterial species (Boissière et al. 2012). Sharma and co-workers described similar results in 2013, working in Asian malaria transmitted by *Anopheles stephensi*. Akhouayri et al. (2013) suggested the use of the endosymbiont *Elizabethkingia meningoseptica*, either alone or in combination with other approaches, in the bio-control of *A. gambiae*.

Often the relationship between bacteria and protozoa within the insect's gut is entirely different, as is the case for protozoal endosymbiotic bacteria. Recent studies revealed that facultative bacterial symbionts could substantially affect various ecological traits in herbivorous insects (Hosokawa et al. 2007), such as resistance to pathogenic fungi (Scarborough et al. 2005), and even their ability to broaden the plant food range (Tsuchida et al. 2004).

In 2006, Kämpfer and colleagues reported the isolation of a new Gram-negative, rod-shaped bacterium (CCUG 49520T), from the midgut of the mosquito *Anopheles arabiensis*, which exhibited <93 % similarity to species of the families *Enterobacteriaceae* and *Vibrionaceae*, and contained phosphatidylethanolamine and phosphatidylglycerol as their major lipids. Kämpfer et al. (2006) proposed the creation of a new genus and species (*Thorsellia anophelis*) for this microorganism and its classification as a new member of the Gammaproteobacteria. Duguma et al. (2013) described *Thorsellia anophelis* as, by far, the most predominant bacterium in the midgut of not only *Anopheles* mosquitoes, but also in *Culex tarsalis*. Four major *Thorsellia* lineages had been thus far identified, all closely affiliated with an insect endosymbiont known as *Arsenophonus* (Briones et al. 2008). These authors suggest that: "*aquatically derived bacteria such as T. anophelis can persist through mosquito metamorphosis and become well-established in the adult mosquito midgut.*" The isolation and identification of culturable bacteria from different wild *Anopheles* species must be considered as a first step in the paratransgenic approach to fight malarial diseases worldwide (Chavshin et al. 2014).

2.7 Protozoan Activity Against Pathogenic Bacteria

To the best of our knowledge, very little research has been published on this topic; but perhaps one of the first well-documented studies, as mentioned above, refers to an insect anal secretion with strong antibacterial activity. Willers et al. (1982) reported a hyaline anal secretion from *P. turionellae* (a lepidopteran endoparasitic species) that exhibited antibacterial activity against microorganisms such as *E. coli, Micrococcus luteus,* and *Pseudomonas phaseolicola*, and was additionally able to control the entomopathogenic fungus *Beauveria bassiana*. However, the authors could not establish whether the active principle was synthesized by the parasitic insect or by a biological entity residing in its midgut.

Cystatins are cysteine protease inhibitors that play a great number of roles in insects and other organisms, such as pathogenic protozoa (Kang et al. 2012); most

of these roles relate to the differentiation of the insect's immune system of (Liu et al. 2010) and the completion of the protozoan's life cycle (Lee et al. 2013). The parasitic wasp *Cortesia congregate* injects its eggs into the caterpillar *Manduca sexta*, but a bracovirus is needed to ensure the success of the parasite; indeed, a virally encoded cystatin modulates the immune system of the wasp. This represented the first report demonstrating the bracoviral origin of a cystatin (Espagne et al. 2005). Once again, neither the wasp, and nor the proptozoan or bacteria were reported to be affected by the potent viral cystatin.

Kotsyfakis et al. (2010), while working on a murine model of Lyme disease, caused by *Borrelia burgdorferi* with the tick *Ixodes scapularis* as its vector, discovered two salivary cystains (sialostatins L and L2). Their results revealed that the structure of the two tick salivary cysteine protease inhibitors not only facilitated the vector blood feeding, but also that the compounds (in particular L2) were involved in the transmission of *B. burgdorferi* to the vertebrate host (Kotsyfakis et al. 2010).

Kang and colleagues in 2012 identified and characterized a new cystatin from *C. parvum*, with a low sequence homology to other cystatins belonging to the chagasin-family, capable of inhibiting papain, human cathepsin B, human cathepsin L, and cryptopain-1, with Ki values in the picomolar range; and, therefore, involved in the establishment of the disease (Kang et al. 2012). These authors, however, do not mention anything in their report concerning the action of this molecule on pathogenic bacteria; and this was also the case for the report published by Schwarz et al. (2012). In summary, although there are hints indicating that the protozoan parasite could be involved in the development of the insect's gut microbiota, little information is still available, and further research is required to reach a conclusion in this matter.

2.8 Bacterial Activity Against Uropathogenic Bacteria

Urinary tract infection (UTI) is the most common infection in patients with spinal cord injury (SCI) and it constitutes a major cause of morbidity and mortality in this population. SCI patients almost permanently need antimicrobial treatment and hence constitute a natural reservoir for the emergence of antibiotic-resistant bacteria. This is due, in part, to the fact that most of them need urinary catheters, which can be used by bacteria to penetrate the bladder. Some of the bacteria that colonize these neuropathic bladders do not produce symptoms, and they are classified as benign colonizers, and, theoretically, bladder colonization by these bacteria could prevent infection by pathogenic microorganisms. In 1999 Richard Hull and his group studied the urinary tract colonization mechanisms used by bacteria associated with asymptomatic bacteriuria (ABU). They examined the virulence properties of *E. coli* 83972, a strain isolated from a clinical ABU episode (Hull et al. 1999). They showed that this strain maintained the genetic potential to express both type P and type 1 pili, but it did not express either D-mannose-resistant or D-mannose-sensitive hemagglutinins (the main cause of uropathogenicity). They proposed that ABU

strains could represent a microbiological approach to prevent urinary tract infections in spinal cord injured patients, and reached the conclusion that *E. coli* 83972 can be safely used as a long-term asymptomatic bladder colonizer that could successfully prevent the establishment of uropathogenic bacteria. This means that bladder colonization by *E. coli* 83972 could reduce the incidence of urinary tract infection in patients with neurogenic bladder secondary to spinal cord injury (Hull et al. 2000; Darouiche and Hull 2000). Clinical trials demonstrated the feasibility of this technique (Darouiche et al. 2001), additionally demonstrating that the artificial colonization of urinary catheters by a benign *E. coli* completely prevented the adhesion and further colonization by not only *E. faecalis* (Trautner et al. 2002) but also many other uropathogenic species (Trautner et al. 2003, 2005a, b). The method is currently being improved; by combining it with additional strategies, such as bacteriocin impregnation (Trautner et al. 2007), increased expression of certain fimbriae (Trautner et al. 2008), and the addition of agents that prevent biofilm formation (Mansouri et al. 2013). This method is expected to be approved in the near future, and will probably represent a powerful weapon to treat UTI in people with spinal cord injuries.

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