Tomás G. Villa · Miguel Viñas *Editors*

Horizontal Gene Transfer

Breaking Borders Between Living Kingdoms

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Horizontal Gene Transfer in Bacteria, an Overview of the Mechanisms Involved

T. G. Villa, L. Feijoo-Siota, A. Sánchez-Pérez, JL. R. Rama, and C. Sieiro

Abstract Bacterial transformation, transduction, and "conjugation" can be considered the first horizontal transfer mechanisms in living organisms; these mechanisms have occurred since the origin of bacteria and are still current. Although mechanistically quite different, the three processes aim to achieve the main objective of all cells, the ability to survive and adapt to new environments. Transformation, transduction, and "conjugation" implement DNA recombination, creating genetic diversity and, hence, allowing bacteria to acquire new capabilities and evolve, resulting in additional, improved, environmental adaptations that enhance bacterial survival. This chapter summarizes old paradigms and novel findings in these three genetic processes and includes the latest research on the recently described vesicle-mediated bacterial communication mechanism.

Keywords Horizontal gene transfer · Bacterial transformation · Transduction · Conjugation · Bacterial vesicles

1 Introduction

Horizontal gene transfer (HGT) was not even suspected to occur in the bacterial world a century ago, although there were already a variety of data indicating its existence. It was then already established, for example, that bacteria can change their pathogenicity profile, alter their ability to utilize different nutrients, or modify their physiology to form biofilms in response to different factors (these biofilms were noted to occasionally breakdown, by some unknown mechanism). In the majority of

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cases, biofilm breakage is accompanied by the release of cytoplasmic contents, including genomic DNA (Ma et al. 2009), which can contribute not only to bacterial antibiotic resistance (Tetz et al. 2009; Sadykov and Bayles 2012) but also promote horizontal gene transfer (Molin and Tolker-Nielsen 2003).

When Avery, MacLeod, and McCarty unraveled, in 1944, the chemical nature of the Griffith's "transformation principle," they not only discovered the genetic basis of inheritance but also shed light into the many then unknown aspects of bacterial genetics. In fact, the main conclusion of their publication was: "The evidence presented supports the belief that a nucleic acid of the desoxyribose type is the fundamental unit of the transforming principle of Pneumococcus Type III."

The story of the bacterial transformation process started in England with Frederick Griffith (1879–1941) who, after 8 years of long and patient work, published (1928) one of the most pivotal papers in microbiology. His article, that is known worldwide as the Griffith's experiment, reported that he successfully transformed an attenuated, nonencapsulated (denominated R, for *r*ough colonies) bacteria into a fully encapsulated, virulent (known as S, for *s*mooth colonies) strain. He, nonetheless, could not identify the chemical nature of the transforming principle. Griffith's results were confirmed the same year by Dawson (1928), who 2 years later (Dawson 1930) further expanded his research on the transformation of *S. pneumococci*. These early publications on the streptococcal transformation process were followed by numerous others, many originating from Dawson's group at the Rockefeller Institute for Medical Research in the USA, which are currently overlooked by microbiologists (for instance, the publications in 1923 by Heidelberger and Avery and in 1923 by Avery and Heidelberger, as well as the 1925 article by Avery et al.). Genetic transformation is not identical in all types of bacteria, in fact there are profound differences between this process in Gram-positive and Gram-negative organisms, see below.

Research carried out in the second half of the twentieth century demonstrated a high complexity in bacterial genomes. In this manner, a typical bacterium can contain, in addition to its own chromosome, several replicons that are capable of interacting with one another and even form stable co-integrates. They include not only plasmids but also bacteriophages that can integrate into the bacterial chromosome either at specific points, such as bacteriophage λ, or at different positions (i.e., P2). In addition, some replicons can integrate virtually anywhere in the bacterial chromosome and can, hence, originate a variety of different mutations, some of which are lethal to the bacterial host (i.e., Mu bacteriophage).

For many years, bacterial transformation constituted the only horizontal gene transfer process fully demonstrated and documented in nature. But in the 1940s, a second process was described by Lederberg and Tatum (1946), who denominated it "bacterial conjugation", as it was reminiscent of the "true" eukaryotic conjugation; although it requires physical contact between both partners, it involves the transfer of a single chain of double-stranded DNA. It was initially thought that all bacterial conjugation followed the model of *E. coli* (Wollman et al. 1956), but, in fact, there are many types of bacterial conjugation, and some of them are exceedingly different from the process undergone by *Enterobacteriaceae*.

Transduction, the third classical way of HGT in bacteria, is mediated by bacterial viruses, known as bacteriophages or phages. Although bacteriophages were

discovered at the beginning of the twentieth century by Twort (1915) and D'Herelle (1917), their ability to transfer genetic material was not recognized until 1952. Norton Zinder and Joshua Lederberg (1952) demonstrated that these subcellular structures can transfer genetic properties between different bacterial strains, and even between unrelated bacterial species. Zinder and Lederberg concluded: "The mechanism of genetic exchange found in these experiments differs from sexual recombination in *E. coli* in many respects so as to warrant a new descriptive term, transduction." In addition, these new ultramicroscopic viruses, as Twort referred to them, were soon used in the therapy of certain bacterial diseases, such as in the treatment of bacterial dysentery. It must be noted that all three mechanisms, transformation, transduction, and conjugation, are limited by restriction-modification (R-M) systems. This means that, for naked DNA or a phage DNA to be protected from degradation by the recipient strain restrictases, it must be derived from a host possessing the same R-M system as the recipient bacteria, hence protecting the DNA by methylation. This, in turn, determines how quickly genetic markers are transmitted by HGT. Transmission is fast for DNA protected from degradation, while unprotected DNA is disseminated at a much slower rate. In accordance, the restriction-modification (R-M) system can potentially hinder bacterial HGT, as indicated by Johnston et al. in 2013. In fact, while the three HGT mechanisms increase genetic diversity, the R-M system is set to minimize bacterial genetic diversity, by degrading foreign DNA to protect bacteria from either bacteriophage infection or from naked DNA that could have a detrimental effect on the recipient strain.

2 Transformation

This chapter will only address "natural" bacterial transformation, defined as an exceptional ability of certain bacteria to accept naked DNA (either dsDNA or ssDNA; Miao and Guild 1970). This precludes the inclusion of bacteria, such as *Escherichia coli*, lacking that natural process, although they are of great importance to microbiologists and biotechnologists.

A review of the literature on genetics and microbiology in the early twentieth century reveals that the scientists of the time were constantly focusing their attention on the fact that bacteria in pure culture could undergo dramatic transformations, not only in their metabolic abilities but even in their morphological appearance, both at the cellular and colony level. As an example, here is an extract from the Duval and Couret (1912, Tulane University, New Orleans, Louisiana, USA) publication on the experimental production of leprosy in the monkey *Macacus rhesus:* "The experimental work serves not only to emphasize this fact, but is proof that a transformation from the slender beaded rods of the tissues to solidly staining diplococcoid forms of culture does occur for *Bacillus leprae*; and, conversely, that the coccoid forms of culture may again assume the slender beaded appearance by passage through warm-blooded animals." Advances in bacterial transformation started to gather momentum after the Frederick Griffith publication in 1928, although the chemical nature of the

"pneumococcal transformation principle" was not unraveled until 1944 (see Sect. 1). To date, transformation has been confirmed in more than 40 different Gram-positive, Gram-negative, and archaeal bacterial species (Lorenz and Wackernagel 1994).

It is not our intention to reiterate the famous "Griffith's experiment" but to give an overview on the basic principles that govern the natural process of transformation in both Gram-positive and Gram-negative bacteria.

2.1 Transformation in Gram-Positive Bacteria

Natural bacterial transformation in Gram-positive bacteria involves the recipient cells uptaking and incorporating into their genome not only homologous DNA but also heterologous DNA, although the second requires a certain degree of DNA homology, to allow heteroduplex formation with the recipient DNA. Gram-positive bacteria transformation follows two pathways, represented by *Streptococcus pneumoniae* and *Bacillus subtilis* that can be used as models for this process.

Natural transformation was first discovered in *S. pneumoniae*, and this organism represents the pivotal cornerstone upon which other bacterial transformation studies have been built for the last 90 years. After the publications by Griffith in 1928 and Avery et al. in 1944, mentioned above, there were a number of studies on the chemical nature of the "pneumococcal transforming principle" that corroborated the original findings of 1944 (i.e., McCarty and Avery 1946; McCarty 1946; Macleod and Krauss 1947). In the early bacterial transformations that converted R (rough) strains to S (smooth), it was established that the morphology of the two classical types of pneumococcal colonies, S and R, was due to the presence or absence, respectively, of capsular polysaccharides. This was soon followed by other studies that demonstrated that different capsular characteristics could be also transferred by transformation. For example, Pneumococci manifesting "binary capsulation" contain two closely related capsular polysaccharides originating from two different bacterial strains (Austrian et al. 1959); and the M protein, a streptococcal virulence factor, can also transferred by transformation (Austrian and MacLeod 1949). The M protein exhibits a conserved pentapeptide motif (LPXTG) that precedes a membrane-spanning hydrophobic C-terminal domain (Fischetti et al. 1990). This protein is present in many pathogenic bacteria and displays antiphagocytic activity, it binds to serum factor H, destroying C3 convertase and preventing opsonization by C3b in the complement activation pathway. Austrian and Colowick (1953) and Deutsch (1962) also described metabolic capabilities affected by transformation, such as modification of the fermentative ability of *S. pneumoniae*, the formation of amylomaltase after genetic transformation of pneumococcus (Lacks and Hotchkiss 1960), or the transfer of streptomycin resistance genes (Hashimoto 1957). Later on, it became clear that these new abilities could be transferred, not only between *S. pneumoniae* strains but also to different *Streptococcus* species, namely, *S. viridans*, *S. salivarius*, and *S. pneumoniae*. In accordance, resistance to optochin (ethylhydrocupreine), a characteristic of most

streptococci, can be transferred by transformation to *S. pneumoniae* (naturally susceptible) (Bracco et al. 1957). All those results confirmed the prediction that McCarty made in 1946, when he suggested that pneumococcal transformation, as well as its implication in bacterial biology, went far beyond *S. pneumoniae*. In just a few years, a variety of cellular characters and metabolic capabilities were shown to be mobilized by this form of bacterial recombination; and this was not just a characteristic restricted to *Streptococcus*, as it was demonstrated that other bacterial entities, such as *Bacillus* spp., also had a natural transformation process.

Figure 1a summarizes *S. pneumoniae* transformation process. An important characteristic of *S. pneumoniae* is that, although naturally transformable, it possesses either of two Restriction-Modification (R-M) systems, DpnI and DpnII, which restrict methylated or unmethylated double-stranded dsDNA, respectively. In addition, the DpnII system possesses an unusual single-stranded DNA methylase, DpnA, which is specifically induced during competence for genetic transformation (Fig. 2) (Johnston et al. 2013).

From an academic point of view, the genetic transformation in Gram-positive bacteria can be organized into four different stages: (1) development of competence, (2) foreign DNA fixation to the surface of the cell to be transformed and foreign DNA uptake, (3) eclipse, and (4) foreign DNA integration into the recipient DNA. This process is temperature sensitive, with an optimal temperature of 37 °C, and it takes up to 100 min until the transformed phenotype is apparent (Abe and Mizuno 1959).

2.1.1 Development of Competence

The term competence, as defined by Pakula and Walczak in 1963, refers to the ability of a bacterium to undergo transformation, although it has also been defined as the cellular capacity to uptake naked DNA. Although both concepts are basically concordant, the first term implies the development of a special metabolic cellular state that paralyzes most of the bacterial metabolism, except for the synthesis of specific proteins involved in transformation (Ephrussi-Taylor and Freed 1964). Competence is a frequent status in bacteria, and perhaps represents the first example of HGT in nature. Indeed, the comprehensive study of the molecular mechanisms underlying this physiological state in bacteria has the potential to unravel novel biochemical and genetic pathways that rule the HGT in bacteria (Solomon and Grossman 1996).

Pakula and Walczak were some of the first authors, if not the first, to suggest the existence of a special competence factor (CF) for streptococci. The *Streptococcus* CF is an exocellular, autocatalytic protein with average mass of 5–10 kDa that diffuses into the culture medium. This factor, when used to treat noncompetent cells, induces their transformability in a short period of time (~5–10 min), following a quasi-enzymatic kinetics comparable to a hormonal mode of action. The process is time- and temperature-dependent and entails all streptococcal cells acquiring a competent status; in other words, the CF acts via a "quorum sensing" mechanism. A main difference between the *Streptococcus* and *B. subtilis* mechanisms resides on

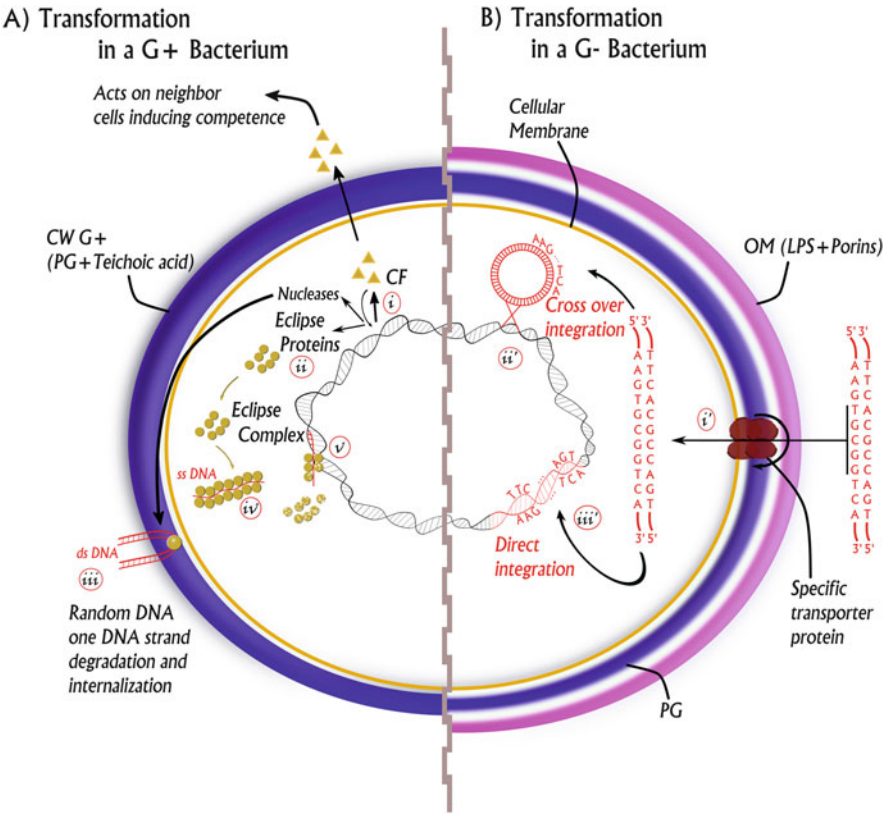

Fig. 1 Transformation in Gram-positive (**a**) and Gram-negative bacteria (**b**). (**a**) *Gram-positive bacteria*: (i) Induction of competence, competence factor (yellow triangle) is produced and either released into the culture medium (*Streptococcus*) or remains cell-bound (*Bacillus*); (ii) Nucleases and eclipse proteins are produced; (iii) Random dsDNA degradation to generate ssDNA that binds onto choline-rich regions; (iv) Formation of eclipse complexes in which ssDNA fragments are covered by special proteins that protect the ssDNA from cytoplasmic DNases; (v) Integration of the ssDNA in homologous regions of the bacterial chromosome. (**b**) *Gram-negative bacteria*: (i′) Binding and internalization of dsDNA by means of proteins that recognize specific DNA sequences (the figure shows the sequence from *Haemophilus influenzae*); (ii′) Integration of circularized dsDNA into the bacterial chromosome following a Calef-Campell mechanism; (iii′) Linear DNA integration in homologous regions of the bacterial chromosome

the fact that, in *B. subtilis*, the CF remains cell-attached and does not diffuse into the culture media. This means that, when the bacterium is grown nonsynchronically, only a maximum of 20% of bacterial cells acquire a competent state. This fact is not very relevant in laboratory conditions, since a counterselection in penicillin (also known as the "suicidal technique"; Lederberg and Zinder 1948) can result in 100% of the bacilli achieving a competent status (noncompetent cells are susceptible to β-lactam, whereas competent cells are not, since they are not actively growing); alternatively, competent cells can be isolated by centrifugation in Renografin. Hui

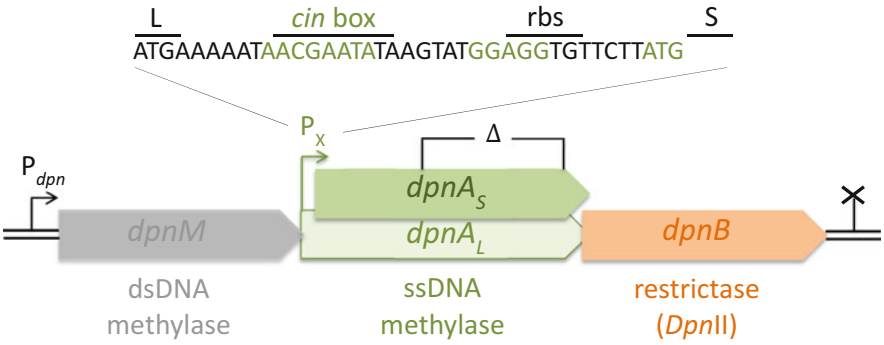

Fig. 2 *dpn*II locus organization in *S. pneumoniae* (adapted from Johnston et al. 2013). The start codons for DpnA and the competence-induced promoter are indicated by an arrow, while the terminator is represented by a cross. P_{dpn} and P_X are σ^{70} and σ^X dependent promoters, respectively; while L and S indicate the start codons for $dpnA_L$ and $dpnA_S$, respectively. Also depicted are the *cin* box, σ^X binding site, and ribosome-binding site (rbs); while Δ represents the limits of the deletion internal to *dpnA* used in the study by Johnston et al. 2013

et al. reported, in 1995, a new locus, *comAB* (encoding two proteins of 77 kDa and 50 kDa, respectively), involved in the synthesis of competence factor in *S. pneumoniae*. ComA protein is homologous to pediocin PA-1 and lactococcin A, whereas ComB shares homology with LcnD, a polypeptide required for secretion of the lantibiotic lactococcin A.

There are dramatic changes in the streptococcal biochemistry during competence; no less than ten new polypeptides are synthesized, eight of which are detectable after 5 min of entering competence. The remaining two polypeptides include a novel DNA binding factor, reported by Seto and Tomasz, in 1975, that is a protein of 16 kDa involved in the eclipse state, and the SsbB protein, shown to protect internalized ssDNA (Attaiech et al. 2011). The data suggest a reservoir function for these proteins, so multiple transformation events can take place in the same cell at the same time. During the competent state, the cells reduce their protein and RNA synthesis by approximately 90%, with the remaining 10%, producing essential molecules for transformation to occur (Tomasz 1970).

Development of competence is governed by the *com* genes. These genes have been grouped into two classes in *B. subtilis*, denominated "early" and "late" genes (Albano et al. 1987); the early genes are generally constitutively transcribed, whereas the late genes are only expressed in competence-inducing medium (Albano et al. 1989; Mohan et al. 1989). A *B. subtilis* late gene, *com*F, encodes a protein similar to ATP-dependent RNA/DNA helicases (Londoño-Vallejo and Dubnau 1993).

2.1.2 DNA Fixation to the Recipient Cell Surface and DNA Uptake

Although several texts consider DNA fixation and DNA uptake as two different stages, it is summarized here as a single stage, since the two steps quickly follow

each other in the transformation process. DNA fixation occurs rapidly, in the first 5–10 min of treatment at 30 °C, followed by a slower uptake for up to 30 min. Much of what is known of this process originates from the work of Seto and Tomasz in 1974 and Seto et al. in 1975a, b. In the case of streptococci, both homologous and heterologous naked dsDNA are fixed into the choline-rich equatorial region of the streptococcal cell (30–80 receptors per cell), highlighting the importance of the cell wall structure in the transformation process (Tomasz 1971). Hence, the presence of choline and continuous cellular incorporation into the teichoic acids is essential for proper dsDNA fixation, as well as for DNA uptake (Tomasz et al. 1971) and to obtain total cellular responsiveness to the competence factor. In accordance, the presence of ethanolamine instead of choline considerably diminishes the biochemical response to the competence factor (Tomasz and Westphal 1971).

After the seminal research by Abe and Mizuno in 1959 studying the fate of DNA in bacterial transformation, Seto et al. (1975a) produced a classic publication on the "Nucleolytic degradation of homologous and heterologous deoxyribonucleic acid molecules at the surface of competent pneumococci." In this article, they demonstrate that competent cells catalyze quantitative degradation of extracellular and cell surface-fixed dsDNA (nicked in both strands every ~6 kbp, although up to 9 kbp are not usually detected) due to the activity of cell surface-located nucleases (both endo and exonucleases, which are inhibited by ethylenediaminetetraacetate). The end result is that the dsDNA is rapidly converted into ssDNA, before entering the pneumococcal cell, which is the way for both homologous and heterologous DNA to enter the pneumococcal cell in natural transformation processes. Single-stranded DNA enters the pneumococci in a non-elutable, DNase-resistant form that requires Ca^{++}; in the case of *B. subtilis*, the endonuclease requires Mg^{++} instead of Ca^{++} (Garcia et al. 1978). In addition, Lopez et al. concluded in 1980 that there are two types of receptor sites on the membrane of competent *B. subtilis* cells.

According to Lacks et al. (1975), the membrane-bound endonuclease I (End A nuclease) is the main enzyme responsible for genetic transformation, to such an extent that *end*$^{-}$ mutants (strains with only 10% of the DNase activity) have their transformability compromised, while transformation ability was totally abolished in *noz*$^{-}$ mutants (displaying no detectable endonuclease activity). During uptake, either of the two DNA strands is randomly degraded, resulting in a 50% reduction on the efficiency of the natural transformation process. Uptake proceeds with 3′–5′ polarity (Méjean and Claverys 1988), and while some of the oligonucleotides released from the degraded strand make their way into nucleotide salvage pathways in the cytoplasm, others engage in an "attack" on the chromosome, displacing homologous resident DNA segments (Lacks et al. 1967; Morrison and Guild 1972).

2.1.3 Eclipse

Immediately after DNA fixation, conversion of dsDNA to ssDNA and uptake, the transforming DNA can be re-isolated from cells undergoing transformation, but the re-isolated DNA appears to have lost its biological activity, hence, it cannot

transform the recipient bacteria. This is known as the DNA undergoing an "eclipse" period. The DNA now remains as single-stranded (Lacks 1962) and recovers its biological activity in approximately 3 min (see integration step, Sect. 2.1.4). In *S. pneumoniae*, the ssDNA is associated ("protected," since this form of DNA is highly sensitive to the action of nucleases) with a specific protein of 19.5 kDa (previously synthesized during the competence state) that renders the ssDNA resistant to degradation by pancreatic, micrococcal, *Neurospora*, and P1 nucleases. The safeguarding protein is smaller (15.5 kDa) for *S. sanguis*. Protection from intracellular DNases results in the ssDNA having a lower affinity for diethylaminoethylcellulose, as well as displaying a faster sedimentation coefficient (Morrison 1977).

In a publication by Bergé et al. in 2003, the authors concluded: "Seventy-five years after the discovery of transformation with *Streptococcus pneumoniae*, it is remarkable how little we know of the proteins that interact with incoming single strands in the early processing of transforming DNA." These same authors clearly demonstrated that the transformation process in this bacterium relies on DprA- and RecA-dependent protection of incoming single DNA strands; this means that the incoming ssDNA needs to be actively sheltered prior to the RecA-driven search for homology.

2.1.4 ssDNA Integration into the Recipient dsDNA

Integration of the ssDNA requires little, if any, net DNA synthesis (Fox and Hotchkiss 1960), but entails a nucleic acid containing at least 900 nucleotides (Fox 1962). The process is complex yet efficient, as up to 50% of the ssDNA that enters the cell is rapidly integrated; donor/recipient DNA complexes start forming as early as 1 min after entering the cell, and the procedure is completed in 10 min. The incoming ssDNA (usually contains 3–9 kb, but it can span up to 30 kb; Fox and Allen 1964; Gurney and Fox 1968) must find a homologous place (or at least partially homologous) in the recipient dsDNA (Rec A-driven), dissociate the DNA strands, and hybridize with one of them (known as single-strand displacement mechanism; Guild and Robinson 1963). This process only requires a few minutes (~10 min at 37 °C; Fox and Hotchkiss 1960; Ghei and Lacks 1967), and, soon after ssDNA integration, the transforming ssDNA is covalently linked to the DNA in the recipient bacteria (Fox and Allen 1964), resulting in recovery from eclipse (i.e., restoration of donor DNA codifying activity). The DNA integration process can be dissected into four steps: (1) translocation of the eclipse complex to the vicinity of the chromosome; this process is inhibited by ethidium bromide; (2) formation of a complex between donor and recipient DNAs; (3) formation of a stable noncovalently bound complex; and (4) formation of a stable covalently linked DNA complex (this process is inhibited by coumermycin, which suggests that chromosomal supercoiling is essential to promote recombination). Variations in as little as a single base between donor and recipient DNA originate mismatches, that in pneumococci can be corrected either at low (i.e., transitions AT>GC), at medium, or at high efficiency (i.e., transversions AT>TA).

At this point of the transformation process, the novel phenotype is not displayed, and the heteroduplex (DpnA-protected) DNA must replicate at least once in order to obtain a homoduplex capable of expressing the new gene(s). Transformation finally gives rise to two bacterial populations, each descending from one of the strands in the heteroduplex. The surviving recipient's DNA strand will give rise to a wild-type population, whereas the lineage carrying the transforming DNA marker will have the characteristics transferred from the donor strain.

2.2 *Transformation in Gram-Negative Bacteria*

Transformation in Gram-negative bacteria (Fig. 1b) has been mostly studied in *Haemophilus influenzae* and, to a lesser extent, in *H. parainfluenzae*, *Neisseria*, *Acinetobacter*, and *Moraxell*a. Hence, making *H. influenzae* the paradigm for this recombination process in "naturally transforming Gram-negative bacteria".

Highly virulent strains of *H. influenzae* (originally known as Pfeiffer bacillus and *Bacillus influenza*) display a developed capsule, which is usually not present in hypovirulent strains, although Nizet et al. uncovered in 1996 a noncapsulated virulent strain of this bacterium. Pfeiffer's bacillus was discovered in 1892 amidst an influenza pandemic, since most of its strains are opportunistic pathogens that cause problems only when other factors, such as orthomyxovirus infections, create an opportunity. In fact, the association of this bacterium with a ssRNA virus resulted in illustrious authors like Twort and Twort (1921), who discovered the bacteriophage world, wrongly concluding: "*the influenza disease is caused by* Bacillus influenza"). It was only in 1933 that it was proven that a virus caused influenza and the Pfeiffer's bacillus was renamed as *H. influenza*. At that time, it was already known that this bacterium suffered a series of cellular and colony variations (Pittman 1931) reminiscent of the R>S transformations reported by Griffith in 1928 for *S. pneumoniae*. In 1951, Alexander and Leidy published that the R to S variations in *H. influenza* were completed in 15 min, after homologous DNA was added to a recipient R strain (Alexander and Leidy 1951); and Alexander and Redman reached the same conclusion for *Neisseria meningitidis* in 1953. Leidy et al. used, in 1959, the interspecific transformation indexes in different species of *Haemophilus* to stablish the relationship between them; they studied *H. influenzae* and *H. aegyptius*, whereas Nickel and Goodgal (1964) worked with *H. influenzae* and *H. parainfluenzae*.

Some *Haemophilus* species can enter a "natural" transformation state, although generally, transformation is only successful with homologous dsDNA (originating from strains belonging to the same species), since nonhomologous DNA is usually extracellularly hydrolyzed by endonucleases (Gromkova and Goodgal 1972); although Roszczyk and Goodgal discovered in 1975 that a methylase from *H. influenzae* could protect *H. parainfluenzae* dsDNA from restriction by endonuclease R.

Transformation requires a specific sequence, present in the dsDNA, which interacts with proteins on the bacterial surface, and results in DNA internalization.

Beattie and Setlow (1970) found that the transformation efficiency is reduced by sixfold if the dsDNA originates from different *Haemophilus* species. Steinhart and Herriott reported in 1968 that up to 2% of host DNA could be recovered after *H. influenzae* transformation; their conclusion was that the recovered DNA represented endogenous genetic material displaced by the transforming homologous dsDNA (corresponding to an estimated 16×10^6 Da of DNA). An interesting theory was proposed by McCarthy and Kupfer in 1987, when they suggested the existence of transformation hot spots (areas particularly rich in A+T) in the *Haemophilus* genome.

One important point before proceeding to analyze the different phases in the transformation of Gram-negative bacteria, exemplified by *H. influenzae*, is that, as per current knowledge, type IV pilus proteins and homologues of the ComEC/Rec2 membrane channel are universal requirements for all species (Chen and Dubnau 2004).

2.2.1 Development of Competence

Competence is internally regulated in *H. influenzae*, but no competence factors have yet been described (Herriott et al. 1970). Surprisingly, point mutations in genes unrelated to the transformation process can trigger this mechanism, although the activation pathways are yet unknown (Ma and Redfield 2000). In *Neisseria*, only pilated strains develop competence and this characteristic is fully inherited. Chloramphenicol, novobiocin, 8-azaguanine, erythromycin, and streptomycin strongly inhibit the development of competence, while not affecting cell viability; this suggests that competence requires continuous protein synthesis (Ranhand and Lichstein 1969). The number of competent cells in *H. influenzae* is normally low (~0.01%), but addition of lactate and inosine can increase the number to 2.5% (Ranhand 1969). In fact, in the last 30 years, a number of additional ways have been described to increase the number of competent cells in a nonsynchronic culture of *H. influenzae*. These include (1) blocking cellular division or following the aerobic growth period with anaerobic incubation (Goodgal 1961; Goodgal and Herriott 1961a); (2) increasing competence by up to 10,000 times by addition of cAMP to exponentially growing cells (Wise et al. 1973), although this was contradicted by Zoon et al. in 1975; and (3) requiring a competence development through determining a gene encoding cAMP receptor protein (CRP) which was established by Chandler (1992). Exogenous CRP, like that produced by *E. coli*, can replace the function of endogenous CRP in *H. influenzae*. In addition, a novel CRP-dependent regulon, Competence Regulatory Element (CRE), spanning up to 25 genes in 13 transcription units, was shown to be involved in the development of competence (Redfield et al. 2005); competence is totally inhibited by valine and NAD, as well as by the presence of certain temperate bacteriophages.

As is the case for Gram-positive bacteria, achieving competence requires substantial changes in the surface components of the recipient cell, including the dsDNA receptor proteins. It also requires an increase in the recombinant activity of the cell, in order to facilitate the integration of the foreign DNA. Additionally, transformation

in this bacterium necessitates a fully operational *HiTopA*, which encodes DNA topoisomerase I (Chandler and Smith 1996).

Zoon and Scocca (1975) were pioneers in the study of *H. influenzae* cell envelopes from both competent and noncompetent cells. They concluded that the envelopes from competent cells contain a higher amount of lipopolysaccharide; the authors also identified three previously unknown polypeptides, including one protein associated with the inner cytoplasmic membrane of competent cells. In addition, competent cells displayed a higher ability to transport deoxyribonucleic acid. Zoon et al. (1976) increased the number of novel polypeptides to six, isolating six proteins with estimated molecular weights of 95, 90, 80, 67, 64, and 43 kDa; describing them as characteristic of cell envelopes in competent *H. influenzae* cells, synthesized during the period of development of competence. The 43 kDa protein has a molecular mass similar to the product of a novel gene (*dprA*+), shown to encode a 41.6 kDa inner membrane protein; it is required for efficient chromosomal dsDNA fixation in *H. influenzae* (Karudapuram et al. 1995).

Indeed, mild treatments such as osmotic shock can release a protein from the periplasmic space that binds to either ssDNA or dsDNA, but not RNA, and is involved in transformation of *H. influenzae* (Sutrina and Scocca 1979). A genetic locus related to the development of competence in *H. influenzae* Rd was mapped to a 12.8-kb PstI region of the bacterial chromosome (Tomb et al. 1989), and in 1991, Tomb et al. obtained the nucleotide sequence of a cluster of genes and determined that at least three of those genes are involved in transformation. Competence in *H. influenzae* also appears to be regulated by genetically encoded functions (Caster et al. 1970) that could belong to a class similar to those found in *B. subtilis* (Larson and Goodgal 1991). Competence genes in *Haemophilus* are also denoted as *com.* Larson and Goodgal reported in 1991 the sequence and transcriptional regulation of *com*101A, a locus required for genetic transformation in *H. influenzae*, and concluded that this gene codes for a competence-specific protein, since mutations in the *com*101A gene cause the donor DNA to be degraded, consequently resulting in transformation failure (Larson and Goodgal 1992). Zulty and Barcak (1995) studied the early gene *tfoX*+ (encoding a 24.9 kDa polypeptide) and concluded that the *com*101A gene, mentioned above, is strictly *tfoX*+-dependent. Additional genes, such as the three *dprABC* genes (required for efficient processing of linear DNA during cellular transformation), constitute a competence-inducible operon that depends on the protein encoded by *tfoX*+ (Karudapuram and Barcak 1997).

Tomb described a novel gene, in 1992, *por* (*p*eriplasmic *o*xido*r*eductase), encoding a 20.6 kDa protein containing the sequence CPHC (Cys-Pro-His-Cys) characteristic of periplasmic proteins in different types of bacteria. In fact, the *Haemophilus* 20.6 kDa protein (Por) can complement the function of the endogenous polypeptides in *E. coli* cells containing the DsbA mutation, hence unable to produce the protein. Tomb concluded that Por is required for the correct assembly of envelope proteins involved in the development of either competence or subsequent transformation steps. In summary, ten operons (including the three described by VanWagoner et al. in 2004) appear to be involved in the development of competence, the first step in the transformation process of *H. influenzae*. These operons are

characterized by a conserved 22 bp regulatory element upstream of the first gene, and are activated when the cells encounter poor nutrient conditions and an increase in intracellular cAMP.

2.2.2 DNA Fixation to the Recipient Cell Surface and DNA Uptake

DNA fixation requires Ca^{++}, Na^{+}, and/or K^{+}, and binding requires naked dsDNA, although, as described by Postel and Goodgal in 1966, *H. influenzae* can also bind (and be transformed with) ssDNA at low pH values (4.5–4.8) in the presence of 1 mM EDTA. In 1976, Sedgwick and Setlow reported that about 15% of donor DNA was single-stranded immediately after uptake into competent wild-type *H. influenzae* cells, as determined by its sensitivity to S1 endonuclease. The molecular weight of transforming DNA was estimated as 16 million daltons by Sol H. Goodgal and Roger M. Herriott in a classic publication in 1961b, using sedimentation and diffusion techniques. The minimal amount of DNA necessary for transformation in *H. influenzae* is equivalent to the quantity of DNA present in a single bacterial cell (Zamenhof et al. 1953).

The binding of dsDNA to specific cell surface proteins is a rapid process, and about 20% of the DNA is degraded within 15 min (Stuy and Van der Have 1971). Double-stranded DNA binding in *H. influenzae* requires a specific sequence of 11 bp (5′-AAGTGCGGTCA-3′), later reduced to 9 bp (5′-AAGTGCGGT-3′; Kroll et al. 1998), while *Neisseria gonorrhoeae* requires 10 bp (5′-GCCGTCTGAA-3′; Graves et al. 1982). These sequence motifs are repeated every 1 kbp (Redfield et al. 2006), and, although initially it was thought that it was repeated 600 times over the entire chromosome (Sisco and Smith 1979; Smith 1980; Danner et al. 1980), the number was revised to 1465 by Smith et al. in 1995. The importance of the repeated sequence was established by Danner and colleagues, who demonstrated in 1982 that addition of the 11 bp DNA motif to a foreign DNA fragment is both necessary and sufficient for preferential DNA uptake. The rate of DNA uptake depends on the A+T content of the sequence flanking the 11 bp motif. Kroll et al. noted in 1992 the presence of pairs of palindromic sequences near matches to the specific sequence, shortly after the stop codon in three *Haemophilus* genes. This suggests that the uptake motif must be preferentially incorporated into gene termination signals, which has also been advocated for *N. gonorrhoeae.*

Other Gram-negative bacteria are different from *H. influenzae* and only transform with homologous dsDNA, as is the case for *Neisseria* species (Mathis and Scocca 1982). In fact, Graves et al. demonstrated the presence of sequence motifs in *Neisseria*, but they contain 10 bp instead of 9 bp. *Haemophilus* can uptake heterologous dsDNA, but this is only achieved by hypercompetent cells and at very low efficiency, less than 1% of the efficiency for homologous dsDNA (Goodgal and Mitchell 1984). A surprising discovery by Kroll et al. in 1998 revealed that *N. meningitidis* can uptake heterologous dsDNA. The authors uncovered a Neisseria gene (*sodC*) containing the typical 9 bp *Haemophilus* specific motif sequence, thus concluding that HGT had occurred between both bacterial genera.

The number of dsDNA molecules that can be fixed onto the cellular surface depends on the number of cellular receptors present (4–8 per cell; Deich and Smith 1980), as each receptor can only transport one DNA molecule. Concino and Goodgal, in 1981, used a surface-specific iodinating reagent 1,3,4,6-tetrachloro-3-α, 6-α-diphenylglycoluril, to obtain an iodinated 29 kDa polypeptide. The protein was identified as the primary receptor for the dsDNA because the protein iodination level was reduced in the presence of homologous DNAs. On the other hand, there are a variety of polypeptides involved in the transport of the dsDNA into the cytoplasm. As mentioned above, only dsDNA harboring the repeat sequence motif can achieve *H. influenzae* transformation, hence only homologous DNA can enter the cell. Once the membrane receptor proteins are attached to the dsDNA, they rotate and internalize the genetic material in a double-stranded form.

2.2.3 Eclipse

Gram-negative bacteria, such as in *H. influenzae*, do not have an eclipse stage. As opposed to Gram-positive, the re-isolated internal dsDNA transforming Gram-negative bacteria retains its biological activity. As indicated above, the nucleic acid penetrates the cell in a double-stranded form and, being homologous DNA, it has a similar methylation pattern to the host's genetic material and is, therefore, resistant to the recipient R/M system nucleases. The transforming DNA is stabilized by a protein, and the DNA/protein complex travels to the host chromosome by means of small vesicles (similar in composition to the *o*uter *m*embrane, OM) (Kahn et al. 1982). These vesicles were named "transformasomes" by Kahn et al. (1983); they contain open dsDNA (that soon starts degrading, originating 3′ tails of ssDNA) and perhaps covalently closed circular dsDNA. The open DNA is the first to exit the vesicles, followed by preferential homologous integration/recombination. As it is the case for Gram-positive bacteria, the transforming nucleic acid must integrate as ssDNA. R/M systems do not appear to play a role in Gram-negative transformation; this is supported by Stuy, who concluded in 1976 that restriction enzymes did not play a significant role in either *Haemophilus* homologous or heterologous transformations. In addition, the presence of single stranded regions at the 3′-end of the dsDNA (Barany and Kahn 1985) could facilitate, at least in *H. parainfluenzae*, the formation of circular DNA; this would facilitate the integration process, through a traditional double recombination event following a Calef-Campbell-like mechanism (Calef 1967; Campbell 1963, 1992).

2.2.4 ssDNA Integration into the Recipient dsDNA

Classic papers on *H. influenzae*, such as that of Voll and Goodgal (1961), reported that recombination in this species can occur in the absence of cellular growth and requires less than 15% synthesis of genetically functional DNA (the authors even suggested the possibility of not requiring any DNA synthesis). Notani and Goodgal

described in 1966 that, although the DNA enters *H. influenzae* as double-stranded, the actual integration into the recipient chromosome occurs as single-stranded DNA (the evidence was obtained by cesium chloride density gradient centrifugation of donor-recipient DNA complexes). An alternative would be that, when the dsDNA approaches the recipient chromosome, it forms closed circles via short monocatenary 5′ ends generated by an exonuclease specific for dsDNA (Gunther and Goodgal 1970; LeClerc and Setlow 1975). The DNA would then, after localizing relevant homologous regions, integrate by Campell- and Calef-like mechanisms into the recipient chromosome. Genetic studies in *H. influenza* revealed 3 *loci* for dsDNA integration, defined by studying their corresponding mutations. For example, the gene *comM*, induced during competence development in wild-type *H. influenzae*, when mutated, generates a bacterial strain with normal DNA uptake and translocation but apparently is unable to integrate into the recipient chromosome (Gwinn et al. 1998).

Helicobacter pylori, a totally different type of Gram-negative, is a microaerophilic bacterium that exhibits a "natural" transformation process (Wang and Taylor 1990). Baltrus and Guillemin (2006) studied transformation during both logarithmic and stationary growth phases in this microorganism, and demonstrated that the state of competence in *H. pylori* occurs, in an unprecedented pattern, during the growth curve, with no clear relationship to DNA release. On their part, Dorer et al. (2013) described that natural competence promotes *H. pylori* chronic infections. This topic is of considerable medical interest, because of the peculiar ecological niche occupied by *H. pylori* in the human body, entering the stomach lining where immune cells are unable to reach it, and its involvement in "bacterial-caused human tumors". The bacterium was isolated by Marshall and Warren in 1984 from patients with chronic gastritis, peptic and duodenal ulcers, as well as from mucosa-associated lymphoid tissue lymphoma, or even from people suffering from stomach cancer (Caruso and Fucci 1990; Veereman Wauters et al. 1990; Blaser 1992; Wotherspoon et al. 1993; Neelapu et al. 2016; Nguewa et al. 2016). In fact, Barry J. Marshall and J. Robin Warren were awarded the Nobel Prize in Physiology or Medicine in 2005 for their discovery of "the bacterium *Helicobacter pylori* and its role in gastritis and peptic ulcer disease" (Pincock 2005). The genome sequence of *H. pylori* was completed in 1997 by Tomb et al.

H. pylori is a curved rod (originally named *Campylobacter pylori*) that produces a hydrogenase capable of generating energy by oxidizing molecular hydrogen (Olson and Maier 2002). The bacterium is positive for oxidase, catalase, and urease and mobile by a group of 4–6 sheathed flagella, organized as a tuft, at one end of the cell. The O antigen in most strains in this bacterium lipopolysaccharide (LPS) is fucosylated and mimics Lewis blood group antigens, found on the gastric epithelium (Kusters et al. 2006). A fast-acting urease, in conjunction with a variety of adhesins and the vacuolating cytotoxin, are thought to be important virulence factors (Hofreuter et al. 1998).

Soon after its classification as a pathogenic bacterium, *H. pylori* was shown to possess a "natural" competent state and, hence, genetic transformation (Nedenskov-Sorensen et al. 1990; Tsuda et al. 1993; Dubois et al. 1994). This natural

transformation capability could explain the genetic diversity found in its circulating strains, although transformation of this bacterium has yet to be described in the stomach acidic mucosa.

H. pylori natural transformation does not depend on either type IV pili or type IV pilin-like proteins, but on the *comB* locus, as described by Hofreuter et al., in 1998. ComB proteins display significant sequence and structural homology to the basic components of a type IV secretion apparatus (Hofreuter et al. 2001). ComB1 is involved in the development of competence in the early stages of DNA uptake. The *comB* locus is integrated by four partially overlapping, tandemly arrayed genes (orf2, comB1, comB2, and comB3). These genes encode proteins spanning 37 residues (orf2), 29 kDa (comB1), 38 kDa (comB2), and 42 kDa (comB3). The *comB2* and *comB3* genes are homologous to HP0528 and HP0527, respectively, located on the *cagII* pathogenicity island of *H. pylori* strain 26695, suggesting that transformation via competence genes is important for pathogenic process. According to Hofreuter et al. (2001), ComB1, ComB2, and ComB3, now renamed as ComB8, ComB9, and ComB10, are absolutely essential for the development of natural transformation competence (a topological model for interaction of ComB proteins with the membrane can be found in Hofreuter et al. 2003). Corbinais et al. concluded, in 2017, that ComB proteins expression levels determine *Helicobacter pylori* competence capacity. In addition, all currently available data point towards the fact that pathogenic type I *Helicobacter pylori* strains contain two functional, independent, type IV transport systems; one is used for protein translocation (encoded by the *cag* pathogenicity island) and the other for DNA uptake in transformation (see also Smeets and Kusters 2002).

Ando et al. (1999) unraveled the role of the additional genes involved in the development of competence in *H. pylori*. They found that one of the genes involved, *DprA*, is present in a variety of bacteria without a known competence status, and that natural transformation can occur in *H. pylori* independently of *DprA*. These data led the authors to propose that the *DprA* gene product plays a much broader role in DNA processing. The following year, Smeets et al. reported two more genes, *comB3* and *dprA*, involved in chromosomal and plasmid transformation (Smeets et al. 2000), while Chang et al. (2001) identified the *virB4* homologue gene (HP0017) as essential in the natural competence process. On their part, Dwivedi et al. (2013) published that "DprA alleviates restriction barrier for the incoming DNA."

After discovering the existence of a natural transformation process in *H. pylori*, it was assumed the bacterium also required specific DNA uptake sequences, as is the case for *Haemophilus* and *Neisseria*; but Saunders et al. concluded in 1999 that *H. pylori* constitutes the first example of naturally transformable Gram-negative species lacking this transformation-targeting system.

Due to the harsh conditions, encompassing high acidity and protease activity, in the human stomach, where *H. pylori* lives, this bacterium undergoes DNA damage; but instead of inducing DNA repair mechanisms, this rod activates transcription and translation of natural competence genes, thus increasing its transformation frequency. One of the genes expressed produces a lysozyme-like protein that promotes DNA donation from intact cells (Dorer et al. 2010). *H. pylori*, however, has

restriction-modification (R-M) systems (Humbert et al. 2011) that act as a barrier to transformation, even with homologous DNA, by restricting inter-strain transformation events.

Transformation in *Pseudomonas* species was described as early as 1967 by Khan and Sen; the authors reported that it was possible to achieve transformation in this bacterium by changing the culture conditions at the end of the logarithmic phase of growth, as described for *H. influenza*. The same authors described in 1974 that *Pseudomonas* transformation is enhanced by divalent ions, such as Mg^{++}, Ca^{++}, and Ba^{++} (Khan and Sen 1974). The issue of *Pseudomonas* exhibiting or lacking a "natural" transformation process (i.e., no special treatment required to achieve competence) was finally resolved by Carlson et al. (1983), who found that not all species within this genus have this capability. For instance, *P. stutzeri*, *P. mendocina*, *P. alcaligenes*, and *P. pseudoalcaligenes* are easily transformed, mainly with dsDNA, but this ability was not detected in *P. aeruginosa*, *P. perfectomarinus*, *P. putida*, *P. fluorescens*, and *P. syringae.* In some cases, direct contact between donor and recipient cells enhanced transformation, without the presence of naked DNA in the environment (Stewart et al. 1983). Both types of transformation, normal (naked DNA) and natural, are present in *P. stutzeri*, capable of uptaking DNA bound on soil particles, even in the presence of competing microorganisms and DNases indigenous to the soil (Sikorski et al. 1998).

The detailed mechanism of transformation is less known in *Pseudomonas* than in other bacteria included in this chapter, although Graupner et al. described in 2000 that type IV pilus genes, *pilA* and *pilC*, are required in *P. stutzeri* for natural genetic transformation. This enables the bacterium to take up DNA from plants, in a clear example of HGT, provided that the DNA contains some short sequence homology (de Vries et al. 2001).

Graupner and Wackernagel finally identified and characterized, in 2001, two novel competence genes in *P. stutzeri*, *comA* and *exbB*. The *comA* gene is similar *to N. gonorrhoeae comA*, *H. influenzae Rec-2* gene, the *ComEC gene from B. subtilis*, and *S. pneumoniae CeIB* gene. Additional genes required for competence and natural transformation in *P. stutzeri* include *pilT* and *pilU*, described by Graupner et al. in 2001. The *pilT* gene contains a nucleotide-binding motif and is homologous to the equivalent gene found in other Gram-negative bacteria, including *N. gonorrhoeae* (67% homology). Under certain circumstances, *P. stutzeri* can be transformed by ssDNA, and the process requires type IV pili and the *comA* gene (Meier et al. 2002). It is generally assumed that natural transformation in this pseudomonas species starts with dsDNA, but integration into the recipient genome occurs via ssDNA, in a way similar to that previously described for *S. pneumoniae*. Berndt and colleagues reported in 2003 that DNA restriction constitutes an actual barrier to natural transformation in this taxon, creating a *P. stutzeri* strain cluster that is considerably different from the rest of pseudomonas species.

The complete genome sequence of *P. stutzeri* strain JM300 (DSM 10701) was reported by Busquets et al. in 2012, thus considerably facilitating a resolution, perhaps in the near future, of all the remaining questions on the mechanism of the natural transformation process in this bacterium.

3 Transduction

As indicated above, this method of bacterial recombination was described by Zinder and Lederberg in 1952, whilst studying the effects of bacteriophages on *Salmonella typhimurium*, concluding that this new method could transduce single traits at a time. Similar results were published in the same year by Berry et al., but they revealed that multiple transductions could occur from the filtrate of the donor strain to a susceptible recipient strain (Berry et al. 1952). The next year, Kauffmann expanded the knowledge by studying the involvement of transduction on the serological properties of *Salmonella* (Kauffmann 1953), while Stocker et al. (1953) discovered that even characteristics such as possession of flagella can be easily transduced via the appropriate bacteriophage. By 1955, Norton Zinder had already recognized the main properties of transduction, but those accomplishments were built on the deep-seated foundations established by Boyd in 1950 and 1951 (Boyd 1950, 1951; Boyd et al. 1951), Lederberg et al. in 1951 and 1952, as well as the insightful work on pseudoallelism by Lewis in 1951, and the idea of gene/enzyme relationship described by Bonner in 1951.

As a general rule, similar to the examples mentioned above, the restriction-modification (R-M) systems play a major role in transduction, as bacteriophage DNA is recognized as "foreign DNA" by the bacterium, unless the nucleic displays a similar methylation pattern. In fact, the R-M systems can jeopardize HGT; this was shown by Waldron and Lindsay, who in 2006 found that Sau1, a novel lineage specific type I R-M system, blocks horizontal gene transfer into *S. aureus* and between *S. aureus* isolates from different lineages. Soon after transduction was first described, it was discovered that some bacteriophages, upon lysogenization, transduce their genetic markers near the insertion point of the viral DNA on the bacterial chromosome; this is known as "specialized transduction". On the other hand, some bacteriophages can transduce any given genetic material from one donor to another with exactly the same frequency, in what is known as "generalized transduction". In any case, the frequency of genetic transfer is rather low; for one trait, it is between 1×10^{-5} and 1×10^{-8} per infecting phage particle (Adams and Luria 1958).

In addition, some bacteriophages, such as P1, display abnormal functions, when acting as transducing particles. Accordingly, introduction of transduced factors by a single P1 particle into a recipient cell is generally not accompanied by the genesis of phage-carrier cells, suggesting that either the transducing particles did not carry a phage genome, or that the phage genome was altogether too modified to retain the ability to form a stable lysogen. In fact, according to by Ikeda and Tomizawa (1968), prophage P1 behaves as an extrachromosomal replication unit, which could explain the anomalies observed in transductions mediated by this bacteriophage.

3.1 Specialized Transduction

This has been deeply studied in λ bacteriophage that infects *E. coli* through the porin LamB (Fig. 3). As a general rule, all bacteriophages have the ability to establish a lysogenic cycle with their bacterial hosts, and this normally requires the *attP* sites present in their genomes. These sites are short DNA sequences homologous to the *attB* DNA fragments located at specific points (nonrandomly distributed) in the bacterial chromosome. DNA insertion requires a minimum 21 bp in the *attB* segment, and a 234 bp supercoiled sequence in *attP* (Campbell 1992). Both *attB* and *attP* contain an identical region of 15 bp (GCTTTTTTATACTAA, only the plus strand is shown), and the integration reaction requires both a phage-coded protein (known as integrase) and a bacterial protein (named "integration host factor"). In

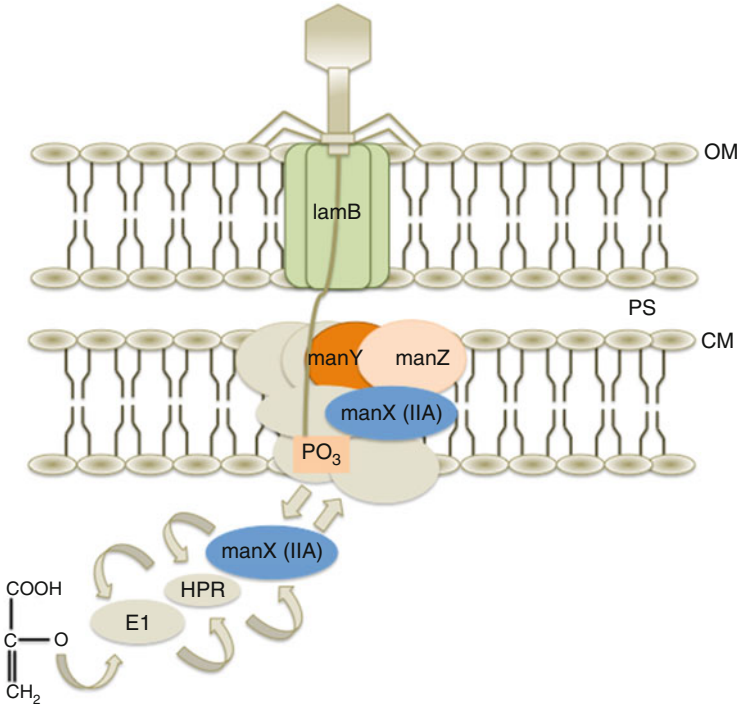

Fig. 3 The role of maltoporin LamB and PTS permease system (modified from Erni et al. 1987). Maltoporin LamB and PTS permease facilitate the injection of phage DNA into the bacterial cytoplasm. It was originally thought that lambda used the maltose regulon to infect *E. coli*, but Charbit et al. (1988) demonstrated that maltose transport and starch binding processes, although overlapping, have different genetic determinants. The figure depicts the bacterial outer membrane (OM), periplasmic space (PS) and cytoplasmic membrane (CM). LamB represents the porin involved in maltose uptake and binding the phage's tip tail J protein, while manY, manZ, and manX (IIA) correspond to genes of the mannose operon (Erni et al. 1987)

addition, the bacterial markers carried by transducing bacteriophages usually display a gene dosage effect, although this is not always the case.

Specialized transduction was first reported by Morse et al., in 1956, for *E. coli* K12, a bacterium lysogenized by the temperate bacteriophage λ. According to Morse et al., the transductants obtained were limited to a cluster of genes for galactose fermentation and were often heterogenotic; this means heterozygous for the *Gal* genes which they continued to segregate. It is currently known that transducing λ particles can contain the markers for biotin synthesis, and λ insertion in *E. coli* occurs between the *gal* and *bio loci*, at 17 min in the *E. coli* chromosome (Wollman and Jacob 1957; Rothman 1965), via the *attP* containing the 15 bp repeat, as indicated above (Leong et al. 1985). The λ bacteriophage was discovered in 1950, by Esther Lederberg, and belongs to the *Siphoviridae* group, order *Caudovirales.* It has an icosahedral (isodiametric) head and the wild type can only encapsidate 48,490 bp of double-stranded, linear DNA, containing additional single-stranded chains of 12 nucleotides at both 5′-ends on the dsDNA; these single-stranded segments are denominated "sticky ends" (Fig. 4). Because of this, specialized transducing particles can contain only one of the two neighboring clusters, *gal* or *bio*, but can never include both. When the transducing bacteriophage can complete its life cycle, it originates a new viral progeny, requiring excision of the phage genome from the bacterial chromosome. This excision is usually precise, but occasionally aberrant excision occurs and the resulting phage DNA can contain adjacent areas of bacterial DNA; this can give rise to specialized transducing phages, such as *λgal* and *λbio*, which only lack a small amount of viral DNA and carry the *gal* or *bio* bacterial gene, respectively. In some cases, however, a considerable portion of the viral DNA is left behind during DNA excision and the phage particle cannot complete its life cycle (unless helped by a wild type λ); these phage particles are called "defective" or simply "d", and the new transducing particles are denominated *λdgal* or*λdbio*, depending on the bacterial marker they contain. In some occasions, however, the bacteriophage can insert between loci *MetB* and *ArgH* (~87 min in the *E. coli* genetic linkage map; Johnson et al. 1977), and this can result in the generation of *λmetB*, *λargH*, *λdmetB*, and *λdargH* transducing particles. Abnormal phage excision is due to the existence of highly homologous phage-host DNA sequences, approximately 20 bp long; some of these sequences include *λplac 5* and *λplac10*, similar to the bacterial Z-Y spacer in the lac operon (Shpakovski et al. 1989). But all cases involve transduction of the same markers, hence the name "specialized transduction". Production of these abnormal viral particles, carrying either the *gal* or *bio loci*, are stimulated by oxolinic acid, an inhibitor of DNA gyrase; confirming that DNA gyrase participates in the abnormal excision of lambda prophage (Tomono et al. 1989).

In some instances, however, different specialized transducing particles are generated, such as *λdpolC*; this particle can transduce *E. coli* DNA polymerase III, but only when the recipient bacterial strain lacks the *attB* gene. But, even then, the bacteriophage is inserted by illegitimate recombination (Franklin 1971) near the polymerase gene (located at 4.4 min in the catalytic α subunit map). In this particular case, no gene dosage effect is observed, since induction of *λdpolC* does not increase

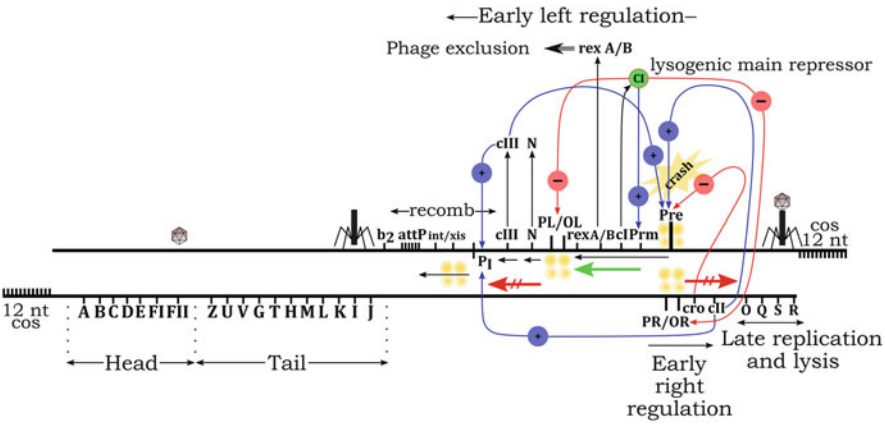

Fig. 4 Establishment and maintenance of a lysogenic state in bacteriophage lambda. Tetra-circles in yellow represent the *E.coli* DNA-dependent RNA polymerase. Promotors P_L and P_R are regular promotors with typical Pribnow-Schaller boxes, and hence instantly recognized by the *E. coli* RNA polymerase. Production of the anti-terminator protein N allows synthesis of protein CIII on the left strand of λ DNA, and simultaneously cro repressor and protein CII are produced, guided by the right strand. The positive effect (in blue) of CIII and CII allows production of integrase from P_I and also expression from Pre, provided the negative effect (in red) exerted by cro is overcome. In this situation main repressor C_I is produced and the surface exclusion genes are expressed, so no new co-immune bacteriophages may enter the lysogenized strain. The C_I main repressor then negatively controls from the left and right operators and whole lambda genome is silenced (interrupted thick red arrows) but the immune operon (genes on the left strands C_I, rexA and rexB; thick green arrow)

the activity of DNA polymerase III, as compared to un-induced controls. Another specialized λ transducing particle is *λdI857s7drif*^{d}*18*, a bacteriophage that carries the RNA polymerase mutation rif^{d}, a dominant rifampicin-resistant allele. In this case, prophage induction increases by twofold the amount of RNA polymerase β subunit, although it does not increase the amount of mature, active enzyme, as compared to the un-induced control (Kirschbaum 1973). Similar λ transducing phages, in particular λrif^{d}18, were found to carry a RNA transcription unit containing genes coding for 5S, 16S, and 23S RNA, as well as tRNAGlu/2 (Yamamoto et al. 1976).

Boulter and Lee described in 1975 another "abnormal" λ specialized transducing particle (*λdcI857S7*) carrying the genes for *E. coli* L-arabinose operon (at 1.42–154-min); as in previous cases, the recipient *E. coli* strain lacked the appropriate *attB* at minute 17. Two years later, Hennecke et al. were able to isolate an additional λ transducing particle, with the ability to transduce the genes *pheS* and *pheT*, encoding the *α* and *β* subunits of the phenylalanyl-tRNA synthetase (at 38, 71, 38,73, and 38,66 min, respectively) (Hennecke et al. 1977). The use of these "rare" λ transducing particles has been sometimes an invaluable aid to study *E. coli* molecular biology; for example, λCI857susN7N53 allowed Inoko and Imai (1976) to identify the transcription termination factor *rho* gen (*nit*A, at 85.45–85.47 min), and *λddapB-2* (Friesen et al. 1976) carries the structural genes for ribosomal protein S20 (*rps* T, at 0.45 min) and isoleucyl transfer RNA synthetase (*ile*S, minute

0.48–0.54). Other examples of these λ derivatives include the series λasn, containing the asparagine synthase marker (von Meyenburg et al. 1978) that permitted the allocation of oriC within 1.5 MDa of the asn gene, towards the *uncA* and *uncB* genes at 82 min on the *E. coli* genetic map. Indeed, using the same technology of illegitimate recombination between λ and *E. coli* DNA, Hansen and von Meyenburg were able to isolate, in 1979, new specialized transducing phages containing *tna* (tryptophanase, minutes 83.77–83.83) markers, as well as additional markers from the *dnaA* region (minutes 83.63–83.66) and the *gyrB* (DNA gyrase, minutes 83.53–83.59). Ream et al. (1980), also using this type of λ derivatives, were able to map *recF* (minute 83.59–83.61) and, the following year, the eight different subunits of the *unc* operon of the membrane-bound *E. coli* ATPase were mapped (minute 83.2) (Hansen et al. 1981; Nielsen et al. 1981; Lagoni et al. 1993).

Using the specialized transducing abilities of bacteriophage λ, MacNeil et al. isolated in 1980 bacteriophages carrying *Klebsiella pneumoniae nif* genes. This was achieved by means of bacteriophage Mu insertions in the *nif* region, that directed the of integration of lambda pMu phages into *nif*, via formation of lambda pMu-Mu dilysogens, hence rendering the DNA capable of being mobilized to other bacteria.

There are additional lamboid phages capable of exhibiting specialized transduction; one such phage is φ80, described in 1963 by Matsushiro, that integrates (the *att*P spans the 17 bp motif AGAACACTT TTCTAA AT; Leong et al. 1985) near the *E. coli* tryptophan operon (~27–28 min), thus originating transducing particles containing the neighboring *try* genes (Denney and Yanofsky 1974). The φ80 gene organization is very similar to λ, in fact, the essential genes are usually located in the same position on the genetic map on both bacteriophages. But the two phages display different immunity specificity, host range, conversion property, and temperature sensitivity (Rybchin 1984). The transduction frequency of φ80 resembles that of other specialized transduction systems, such as λ (see above), and the phage cultures are often heterogenotic, producing high frequency transducing (HFT) lysates that contain both active phage φ80 and defective transducing phage φ80 *dtry*. From these studies on specialized transduction of tryptophan markers, Sato and Matsushiro reached an interesting conclusion in 1965, when they asserted that the trp operon, at least in the *Enterobacteriaceae*, is regulated by the phage immunity system.

As is the case for bacteriophage λ, φ80 is an efficient lysogenic phage, and achieves this via a N protein that is transcribed from the general left promotor (PL) and works as an anti-terminator, hence allowing early transcription to overcome the ρ-dependent transcription termination point tL1 (Tanaka and Matsushiro 1985). This protein is slightly different from that of *E. coli*'s (~12 kDa instead of 11 kDa), although it is also basic in nature and contains the NH2-terminal sequence Met-Ile-Asp-Asp-Ile-Lys (Kanemoto et al. 1986).

Bacteriophage HK022, also a lamboid phage, has an 11 bp motif (CTTTAGGTGAA) within the *attB*. This phage is similar to λ in many respects, including the integrase system, but, although the proteins are fully interchangeable (Yagil et al. 1989), they recognize different, but overlapping, sequences by

alternating a succession of small, mutually compatible, changes in both protein and receptor site.

The lamboid phage 21 possess a 13 nucleotides long motif (TGCTGCGCCATAT; Campbell 1992) and inserts within the isocitrate dehydrogenase (*icd*) gene of *E. coli*, located 165 bp from the 3′ end (Campbell et al. 1992).

Campbell (1992) summarized and completed previous findings by Jacob and Wollman in 1961. He classified the 13 independently isolated coliphages with known *attB* sites into two categories; the first includes seven phages that are inducible by UV light and integrate in the same chromosome region; whilst the second groups the other six that are not inducible by UV light and map elsewhere. Regardless, all lamboid prophages are located between 6 and 44 min in the *E. coli* K12 chromosome.

In Gram-positive microorganisms, it is also common the presence of temperate bacteriophages that generate "λ-like" specialized transduction particles, such as IG1, IG3, and IG4 in *Bacillus subtilis* (Fernandes et al. 1986, 1989). Although low-frequency specialized transduction of *Bacillus subtilis* by bacteriophage ϕ105 was reported as early as 1974 by Shapiro et al., this phage probably still remains the most studied temperate bacteriophage in *B. subtilis.*

In conclusion, as summarized by Rutberg in 1982, the main transducing bacteriophages in *B. subtilis* belong to group I; this includes φ105 and derivatives that exhibit an attachment point within the bacterial DNA at, or very close to, the cohesive ends (Armentrout and Rutberg 1970). Group II, on the other hand, contains only bacteriophage SP02, isolated from soil samples by Okubo and Romig in 1965, and similar to φ105. The two phages only share 14% partial DNA homology in heteroduplex experiments (Boice 1969; Chow et al. 1972; Rutberg et al. 1972), in fact, SPO2 does not complement any of the 11 essential genes known in φ105. Finally, group III includes bacteriophages φ3T, ρ11, SPβ, and Z, and all of them contain much bigger DNA genomes than phages in groups I and II.

As it is generally known, *Bacillus* species contain the SPO operon that regulates the synthesis, maturation, and sometimes the release, of the bacterial endospore; this operon is absent, or at least nonactive, in the nonspore-forming bacteria. SPOA is of particular relevance among the SPO genes, since it plays a key role in the initiation of sporulation, constituting part of the apparatus known as a "phosphorelay switch," which can activate several key sporulation-specific genes. This gene was first genetically studied by Ikeuchi et al. (1985), using a φ105-derived specialized transducing phage, φ105-dspo0A+-1.

SP02 was described by Boice (1969) as a phage, heteroimmune to φ105, that uses different receptor molecules in *B. subtilis* 168 (Inselburg et al. 1969), and is capable of forming very stable lysogens through the synthesis of a cI repressor, similar to that described for λ. Many gene functions in this bacteriophage were patiently studied using a collection of *B. subtilis* suppressor-sensitive mutants, as indicated by Yasunaka et al. in 1970. Despite producing stable lysogens, SP02 is subrogated (i.e., not dominant) to other bacteriophages, such as SP82 and β22, in mixed infection experiments (Palefski et al. 1972). In addition, the bacteriophage is easily eliminated from lysogens by transformation with either plasmids pCM194, pUB110,

or pAM77 (Marrero et al. 1981); but despite this, sometimes unwanted property, SP02 has been commonly used to study interspecific plasmid transfers (Marrero et al. 1984).

Bacteriophage SPβ acts as a temperate phage in almost all transformable strains derived from Spizizen's *Bacillus subtilis* 168 (Spizizen 1958). SPβ is a large phage of combined symmetry, containing 62×10^6 Da of double-stranded DNA, with a chromosomal attachment site that lies between *ilv*A (requirement of Isoleucine-valine, 200°) and *kau*A (ketoacid uptake, 185°) in *B. subtilis* (Zahler et al. 1977; Fink and Zahler 1982). Many SPβ derivatives have played an invaluable role in mapping genetic markers in this bacterium (see Dubnau 1982). These include SPβc2dcitK1 (Rosenthal et al. 1979) used to locate, not only *kau*A, but also the *cit*K gene from the citric acid cycle (mapped at 185°). Another example is the SPβ degU that harbors gene*U*; this gene, in *B. subtilis*, exhibits co-dominance with gene *S* in a two-gene operon that positively controls, by epistatic interaction, the expression of a variety of genes encoding degradative enzymes (Podvin and Steinmetz 1992). Bacteriophage ϕ3T, that encodes a methyltransferase as is common for group III phages, was used by Terschüren et al. in 1987 to generate recombinant derivatives of bacteriophage Z, in order to study the expression and regulation of the enzyme in the lysogenic state. Another bacteriophage in this group, ρ11, can transduce *his*A or *lys* markers at considerably high frequency in *B. subtilis* (Dean et al. 1976; Kawamura et al. 1979). H2 (Zahler et al. 1987) is a bacteriophage isolated from *Bacillus amyloliquefaciens* strain H, that encapsidates a large dsDNA of 129 kbp (a characteristic that warrants inclusion in group III), also capable of lysogenizing *B. subtilis*, despite this bacterium's phylogenetic distance from *B. amyloliquefaciens* (Welker and Campbell 1967). Lysogenization occurs between the *tyrA* (mapped at 205°) and metB (mapped at 200°) genes, originating *metB* specialized transducing particles, as well as *ilvD* (200°) and *ilvA* (200°), at lower frequencies.

Streptococcus pyogenes (named by Friedrich Julius Rosenbach in 1884, who characterized it as the causative agent of Rosenbach's disease; Evans 1936) is another typical Gram-positive bacterium (group A, β-hemolytic) with a peculiar pathogenicity profile, responsible for diseases such as scarlet fever, pharyngitis, tonsillitis, myositis, impetigo, cellulitis, necrotizing fasciitis, toxic shock syndrome, rheumatic fever, acute glomerulonephritis, and recently identified as the cause of pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections (also known as PANDAS).

In streptococci such as *Streptococcus pyogenes* (see McShan and Nguyen 2016), as well as in the rest of species belonging to this genus, transformation is possibly the paramount HGT mechanism and occurs naturally while growing in biofilms (Marks et al. 2014), although already in 1968 Leonard et al. and in 1969 Malke had reported that the transduction by bacteriophage A25 (originally isolated by Maxted in 1952) was of the generalized type. This virus is the prevailing phage in streptococci; it belongs to the *Siphoviridae* family and has an isodiametric octahedral head of 58–60 nm that encloses a linear dsDNA of around 34.6 kbp (Pomrenke and Ferretti 1989); it also possesses a long flexible tail, of 180–190 nm in length and 10 nm in diameter, but it does not contain hyaluronate lyase in the tail fibers, although this is

the norm for *S. pyogenes* lysogenic phages. The bacteriophage uses peptidoglycan as the cell receptor and encodes a lysin active against group A, C, G, and H streptococci (Hill and Wannamaker 1981).

Transduction studies in streptococci have been mainly centered on the spreading of antibiotic resistance (Malke 1970, 1972; Stuart and Ferretti 1973, 1978; Ubukata et al. 1975; Hyder and Streitfeld 1978), but transduction has also been used to study the genetic determinant for streptolysin S production, particularly in group A streptococci (Skjold et al. 1982), and to study the role of hyaluronic acid in *S. pyogenes* virulence (Wessels et al. 1991).

S. pyogenes also has lysogenic bacteriophages, such as the ubiquitous toxin-carrying prophages, and as many as eight lamboid bacteriophages, with lysogenic control similar to λ, all sharing three or four genetic elements. This is the reason why HGT frequently occurs between *S. pyogenes* and *S. equi* bacteriophages, presumably through the sharing of these common elements (Holden et al. 2009). All these phages integrate via homologous recombination between *attP* and *attB* sites, at different points (3–6) located anywhere in the linkage map and, as the transducing particles always carry markers near their integration points, these markers can include a great variety of genes, such as the gene encoding the HU nucleoid scaffolding protein (McShan and Nguyen 2016). Interestingly, no prophages have been ever detected in the M protein gene; the M protein was identified as a major virulence factor, because it inhibits phagocytosis, and combined with its antibodies detonates the complement cascade that generates tissue damage. It is also noteworthy that the genes coding for some classical *S. pyogenes* enzymes, as is the case for the gene encoding "streptodornase" (a DNase), are activated upon interaction of *S. pyogenes* with human pharyngeal cells (Broudy et al. 2002). This possibility, however, does not clarify the results reported by Vlaminckx et al., who described in 2007 that, during a four-decade period, *S. pyogenes* strains (such as M28) acquired prophages on at least eight occasions and that all of the prophages carried the genes for streptodornase and for a superantigen.

As described in the chapter on bacteriophage-encoded toxins produced in the bacterial world, this type of phages is also present in *S. pyogenes*. A classic example of bacteriophage-encoded toxins is the scarlet fever toxin. As early as 1927, just 1 year before Griffith's paper on *S. pneumoniae* and his "transformation principle" was published, Frobisher and Brown reported that a filterable agent isolated from scarlatinal strains of hemolytic streptococci, when transferred to non-scarlatinal strains, could give rise to bacteria capable of producing scarlet fever. The new toxin was rapidly neutralized by standard antitoxins, a concept already used 3 years before in the Dick's test (Dick and Dick 1924), that produced an anti-scarlet fever vaccine scarcely used due to its low effectivity. Once Freeman stablished bacteriophage β as the carrier for the diphtheria toxin-encoding gene (Freeman 1951; Freeman and Morse 1952), and Krause (1957) demonstrated that lysogeny frequently occurs in Group A β-hemolytic streptococci, Zabriskie (1964) definitively confirmed that the scarlet fever toxin is encoded by a bacteriophage (speA), currently known as T12, that integrates into the bacterial chromosome at a gene coding for a serine tRNA (McShan et al. 1997). Related bacteriophages include

φ436 and φ49. Specialized transduction mediated by this type of bacteriophages is of particular importance in *S. pyogenes*, since this species does not appear to undergo either conjugation or transformation; this phenomenon would certainly explain the periodic shift in severity and the predominant clinical syndromes found in *S. pyogenes* (Quinn 1982). If the bacteriophage undergoes recombination prior to bacterial infection, it would transduce into the bacterium a variety of "toxic alleles" (Banks et al. 2002; Krause 2002). As a matter of fact, bacteriophages with horizontally acquired genes account for the emergence of novel pathogenic, and highly prevalent, *S. pyogenes* strains (Aziz et al. 2005). In addition, Broudy and Fischetti reported in 2003 the in vivo lysogenic conversion of nontoxic *S. pyogenes* into toxic strains, by means of a toxin-encoding bacteriophage, and concluded that "the eukaryotic host serves as an essential component in the phage-mediated evolution of virulence within the microbial." Further progress in understanding the subject of phage-mediated virulence could be achieved by analyzing the complete genomic sequence of a macrolide-resistant M serotype *S. pyogenes* strain (1900,156 bp in length, and 8 prophage-like elements; Banks et al. 2004), as well as studying a group A serotype M23 (emm23) *S. pyogenes* strain (M23ND), isolated from an invasive human infection (with a 1,846,477 bp DNA and containing 6 phage-encoded and 24 chromosomally inherited virulence factors; Bao et al. 2014). Banks et al. proposed that "GAS prophage-encoded extracellular proteins contribute to host-pathogen interactions in a strain-specific fashion."

S. pyogenes also produces streptolysin S (SLS) and streptolysin O (SLO), two potent cytolytic toxins involved in pathogenesis. SLS is an oxygen-stable cytotoxin produced by *S. pyogenes* GAS strains that affects immune cells, including lymphocytes and polymorphonuclear leukocytes; SLS is specifically inhibited by trypan blue and is primarily responsible for the β-hemolysis area surrounding GAS colonies (Feller and Stevens 1952). SLO, on the contrary, is an oxygen-sensitive, thiol-activated, cytotoxin that lacks homology to other thiol-activated toxins (Kehoe and Timmis 1984) and interacts with the cholesterol present in the membrane of eukaryotic cells, such as red and white blood cells, macrophages, and platelets. Skjold et al. (1982) were some of the first authors to suggest that SLS production could be experimentally transduced by bacteriophages (A25ts1-2), which would explain the isolation, on rare occasions, of nonhemolytic strains of *S. pyogenes.*

McMillan et al. (2007) finally proposed that, of the 62 known virulence factors specifically associated to *S. pyogenes* GASM-types, 32 genes are bacteriophage-related (originating from 6 to 8 different bacteriophages, probably related), while the 30 nonbacteriophage-related genes are located in chromosome 11, at a locus near the classical GAS virulence factors.

Lactococcus lactis (formerly *Streptococcus lactis*) is another industrially and economically important (with an estimated value of 55 billion euros; de Vos 2011) Gram-positive bacteria. This is a nonmotile coccus with a GRAS status (*g*enerally *r*ecognized *a*s *s*afe), that is commonly used as a starter culture in the manufacture of fermented dairy products. This bacterium contains an AT rich genome and exhibits homofermentative metabolism, producing L-(+)-lactic acid from sugars; although it can produce D-(-)-lactic acid under low pH conditions (Åkerberg et al. 1998). The

complete *L. lactis* genome was sequenced in 2001 by Bolotin et al. and spans 2,365,589 bp, encoding 2310 proteins; it also contains 293 protein-coding genes belonging to 6 prophages, and a 43 bp insertion sequence (IS) element. The analysis of the sequence revealed the existence of DNA transfer, by HGT, from *Lactococcus* to Gram-negative enteric bacteria belonging to the *Salmonella-Escherichia* group, opening the possibility of novel fermentation pathways; it also became clear that streptococcal bacteriophages can transduce DNA from *Streptococcus thermophilus* to *L. lactis* (Ammann et al. 2008).

Lysogeny is widespread among the lactic acid bacteria (LAB), and most of the lysogens can be induced by either UV irradiation or treatment with mitomycin C (Davidson et al. 1990). Reiter was the first to show the existence of lysogens in *L. lactis*, as early as 1949; but it took almost 30 years to demonstrate that transducing bacteriophages can change the metabolic abilities of *L. lactis* (McKay et al. 1973; McKay and Baldwin 1974), including the loss of proteinase- and lactose-utilizing enzyme activities. Klaenhammer and McKay (1976) finally isolated and studied two bacteriophages, c2t1 and c2t2. Additional dsDNA temperate bacteriophages found in this species include BKS-T, ϕT187, ϕ, T188, and φT189; these phages display an isometric head, with diameters varying from 56 to 78 nm, and tail lengths ranging from 120 to 182 nm (Davidson et al. 1990). Phage φLC3 is another bacteriophage with great potential to genetically manipulate *L. lactis* via specialized transduction; this virus has a genome encompassing 33 kb of dsDNA with cohesive ends that extend 13 nucleotides from the 3′-end. This property allows φLC3 to achieve similar basic genetic manipulations as those described above for λ phage (Lillehaug et al. 1991; Birkeland and Holo 1993), and this could result in the introduction of novel valuable traits in LAB. Unfortunately, this transduction system is limited to certain lactococcal strains, and often even those strains become bacteriophage-resistant (a property mediated by plasmids). This subject has been extensively investigated and analyzed by a number of researchers, including Labrie et al. (2010).

There are additional LAB organisms, also belonging to the *Lactobacillus* genus, that emulate the industrial and economic relevance of *L. lactis*. *Lactobacillus* represents one of the largest groups of lactic acid bacteria, containing approximately 80 species, that can be classified into three groups, according to the environment they usually live in and their contribution to the food transformation industry (i.e., milk, wine, and meat); an additional group is constituted by the lactobacilli present in the gut of both mammals and other homeotherms. Other species, such as *L. delbrueckii*, are mainly used in the dairy industry for the production of yogurt (subsp. *bulgaricus*) and Swiss-type hard cheeses (subsp. *lactis*) (Ravin et al. 2006). In general, the use of transformation as a means of genetic manipulation is a hard, often frustrating, task; but transduction of chromosomal markers, as well as plasmids and plasmid DNA, is a well-known phenomenon originally described by Luchansky et al. (1989) for *L. acidophilus* ADH (temperate bacteriophage φadh), and later reported for numerous LAB species tested (Chandry et al. 2002; Ravin et al. 2006); this process could even include transduction by the virulent pac-type phages, such as bacteriophage LL-H (Mikkonen et al. 1996). Research into transduction in *Lactobacillus* has been essential for the development of bacteriophage-resistant starter

cultures, of great importance for the dairy industry (Fitzgerald and Gasson 1988). An unexpected outcome of these studies was the development of anti-HIV microbicide engineered lactobacilli, that can topically control HIV-1 transmission (Pusch et al. 2006).

3.2 Generalized Transduction

Bacteriophages capable of generalized transduction (all genetic markers are transduced with approximately the same frequency) also exist in other members of the *Enterobacteriaceae* family. For example, in *Serratia*, Regué et al. isolated in 1991 a novel bacteriophage, belonging to the *Myoviriae* family, with the ability to transduce a variety of Tn5 insertions.

The P22 phage is a classic lamboid bacteriophage that causes generalized transduction in *S. typhimurium* (Chan and Botstein 1976; Ackermann 2015). Its head encloses a blunt-ended linear dsDNA molecule of 44–48 kbp (although the "wild-type" is of only 42 kbp, i.e., 27×10^6 Da); it is circularly permuted and contains terminal direct repetitions (Rhoades et al. 1968; Rhoades and Thomas 1968; Tye et al. 1974a, b). The head of P22 is icosahedral in shape and measures 60 nm, with a triangulation number $T = 7$; the phage also has a short tail and infects *Salmonella typhimurium.* P22 was the "work horse" used by Zinder and Lederberg (1952) to stablish the concept of transduction; the authors concluded that this phage could transduce a single trait (or more) at a time, with basically the same frequency. Therefore, P22 has been, and still constitutes, an important tool for studying *Salmonella* genetics. This bacteriophage belongs to the order *Caudovirales*, family *Podoviridae*, and, hence, it is similar in many aspects to the bacteriophage λ mentioned above; the similarities include the control of early and late operons and the immunity system, and it has emerged through extensive recombination events with other viruses.

As is the case for λ bacteriophage, P22 uses an identity region of 15 bp, present in both the *attB* and *attP* (ATTCGtAATGCGAAG), to integrate into the threonine tRNA gene of *S. typhimurium* (Campbell 1992). Some noncanonical transducing particles (with different genetic markers near the integration point) arise from DNA packaging into the phage heads; the bacteriophage genome does not fill all its head's space, allowing packaging of up to 48 kbp of additional bacterial DNA. This process is denominated "headful packaging" and allows the spontaneous formation of novel transducing particles, since the probability of containing any bacterial marker is approximately the same. In addition, the size of the DNA to be packaged is intrinsically the same, as described by Ozeki as early as 1959. This author reported that almost all of the DNA fragments involved in the transduction of a specific genetic locus were of uniform size; although Enomoto (1967) presented evidence that the transducing fragments of a given genetic marker had some degree of heterogeneity. This apparent contradiction arose from the existence of a mixture of DNA host fragments, resulting from unequal breakage of the *S. typhimurium* DNA. In other bacteria, such as *S. aureus*, the chromosomal fragments present in the

transducing particles are, however, highly uniform (Pattee et al. 1968). Anyhow, the head of bacteriophage P22 must be completely filled with DNA to be stable, hence, it requires up to 48 kbp of bacterial DNA.

Staphylococcus aureus, another well-known Gram-positive bacterium, is believed to lack a natural competent state, hence, it cannot take up naked DNA by genetic transformation. This means that HGT in this microorganism is based on transducing bacteriophages; these include *Siphoviridae*, *Myoviriade*, and *Podoviridae*, which use wall teichoic acid, but not lipoteichoic acid, as a receptor (Xia et al. 2011). Bacteriophages have been known for many years in this taxon, partially due to the fact that microbiologists used bacteriophages for staphylococcal typing (for pioneering typing efforts see Rountree in 1949 and Rippon in 1952). Morse described in 1959 the transduction of streptomycin and novobiocin resistance by bacteriophage 53; and later works including Ritz and Baldwin (1961) reported on the transduction by typing phages 29, 52A, 79, 53, and 80, and Mitsuhashi et al. (1965) stated that tetracycline resistance in staphylococci was mediated by lysogenic bacteriophages. The importance of these pioneering efforts was that they stablished that antibiotic resistance eventuates entirely in vivo during the course of infection (Jarolmen et al. 1965), although the authors were unable to demonstrate that it occurred by specialized transduction. Even though there were reports stating that the *S. aureus* transducing fragments were homogeneous, the results were inconclusive (Pattee et al. 1968). But these studies demonstrated that antibiotic resistance could be directly transmitted from wild-type staphylococcal strains, isolated from cattle, to laboratory strains (Pereira et al. 1997). In 1975, however, Schwesinger and Novick reported that certain *S. aureus* bacteriophages (i.e., φ11) could integrate into plasmids (or vice versa) and the two together integrated at or near the phage's attachment site, hence behaving as a specialized transducing particle. Unfortunately, this could not be confirmed, and, in 1985, Dyer et al. reported that phage ϕ11 contained the plasmid pC194, randomly inserted as a circularly permuted linear concatemer, and that both were transferred together by general transduction. In 1976, Parisi et al. localized the gene encoding coagulase on the bacterial chromosome; the reported transducing frequency suggested that a specialized transducing bacteriophage was involved, but, again, no definitive proof was provided.

Staphylococcus aureus produces several enzymes, such as coagulase, hyaluronidase, lipase, DNase, and staphylokinase. In addition, the bacterium can produce a variety of toxins, including three types of exotoxins (Dinges et al. 2000), enterotoxins, and exfoliative toxin (known to produce the so-called staphylococcal "scalded skin syndrome" and the leading contributor to the toxic phenotype of impetigo in *S. aureus* strains); additional toxins include α toxin, β toxin, δ toxin (that acts on eukaryotic cell membranes), and Panton-Valentine leucocidin, known to be encoded by tox genes of bacteriophage Φ-PVL. Several PVL phages have been identified and sequenced (Kaneko et al. 1998; Ma et al. 2008; Sanchini et al. 2014), and there are currently six PVL phages known (ΦPVL, Φ108PVL, ΦSLT, ΦSa2MW, ΦSa2USA, and ΦSa2958) that contain single-nucleotide polymorphisms (Boakes et al. 2011). Phage ΦSa2USA is the most ubiquitous, as it is present in the highest number of different lineages; but the chromosomal loci at which lysogeny of

the PVL phages occurs have yet to be identified in the different staphylococcal lineages tested.

In addition, *S. aureus* exhibits an immune evasion cluster, located on β-hemolysin-converting bacteriophages (βC-Φs), that Verkaik et al. (2011) reported to encode staphylokinase and enterotoxin A, regulate the interference with complement, and affect cellular chemotaxis. It must not be forgotten, however, that *S. aureus* can eliminate (either naturally or by genetic manipulation) a particular transducing bacteriophage and incorporate a different one, rendering this microorganism capable of switching from one animal species to another; Resch et al. reported in 2013 the loss of a beta-hemolysin-converting prophage and the acquisition of a new staphylococcal chromosomal cassette in this bacterium.

The majority of the staphylococcal temperate bacteriophages included here contain both integrase and the regulator proteins CI (inhibits development of lytic cycle) and cro (prevents Cl expression), as is the case for λ bacteriophage; this means that the phage will remain in a lysogenic state as far as expression of the CI repressor is higher than that of cro, but if cro is predominant, the bacteriophage will undergo a productive cycle (Xia and Wolz 2014). Resistance transfers in mixed *S. aureus* cultures indicate that generalized transduction is prevalent in this bacterial species, even in in vivo experiments, as indicated above; all that is required for this transduction is a lysogenic donor strain, carrying a transducing phage of a given serotype and a transducible plasmid, as well as an acceptor strain lacking both strong restriction and an incompatible plasmid (Meijers et al. 1981). Some of these generalized transducing bacteriophages, such as 80α, have been valuable in staphylococcal gene mapping and in the transduction analysis of transposon Tn551 (encodes resistance to erythromycin) in the *S. aureus* chromosome (Schroeder and Pattee 1984); they can also generate transducing particles harboring the pathogenicity island SaPI1 (Tallent et al. 2007; Tormo et al. 2008). In fact, moonlighting bacteriophage proteins can derepress staphylococcal pathogenicity islands such as SaPI (Tormo-Mas et al. 2010); SaPIs can also be transduced by bacteriophage 80α (Spilman et al. 2012).

In addition, in an experiment to use phage therapy for bovine mastitis, it was observed that the pathogenicity island was transferred between *S. aureus* and *Listeria monocytogenes* in raw milk; this was totally unexpected and suggests that bacteriophages can play a wider role in HGT exchange among different bacterial species than it was originally thought (Chen and Novick 2009). An important aspect of this would be the ability to override the R/M system, with the consequent implications in the overall HGT process.

Pathogenicity islands play an important role in bacterial pathogenesis in animals and plants; they appear to have been acquired during the transition of the bacteria from nonpathogenic to pathogenic. This process can be easily accelerated by horizontal gene transfer mechanisms mediated by temperate bacteriophages (Schmidt and Hensel 2004). The majority of *S. aureus* strains carry three genomic islands, νSaα, νSaβ, and νSaγ, conserved in the different bacterial lineages, which can be easily mobilized by bacteriophage ϕSaBov (including νSaα and νSaγ) and inserted into the *S. aureus* chromosome far away from the bacteriophage insertion

point (that lays near to νSaβ) (Moon et al. 2016). Although massive DNA sequencing of many *S. aureus* strains has identified many virulence factors, the key determinants for infection are still unknown. The differences between commensal and pathogenic bacterial strains are yet to be determined, although it appears that phage dynamics and transcriptional shifts could explain this transition (Feng et al. 2008).

Staphylococcal biofilms can also undergo HGT. It is known that cellular death is mainly controlled by operons *cidABC* and *lrgAB* (Rice et al. 2007; Sadykov and Bayles 2012), possibly by affecting the activity of staphylococcal murein hydrolase and proteins similar to the holins and antiholins encoded by the bacteriophage. Hence, acquisition of these genes by HGT during biofilm formation would result in the death of the staphylococcal biofilm, even in the absence of transducing bacteriophages.

Bacteriophage Mu is another example where generalized transduction occurs. This bacteriophage was discovered by Larry Taylor in 1963, who described the phage after several years of noting that Mu *E. coli* infection generated a bacterium with a strong tendency to develop new stable auxotrophies, thus reminiscent of mutation, which earned the phage the name of "*Mu*tator bacteriophage". This bacteriophage has been essential to localize bacterial genes (Faelen and Toussaint 1976) and to study the *nif* genes in *Klebsiella pneumoniae* (Bachhuber et al. 1976). Bacteriophage Mu inserts its DNA into the host chromosome via a transposition mechanism.

This phage contains ~37 kbp of dsDNA, with noncohesive ends and a G+C content of 48.1%; the adenine is substituted by N^6-(1-acetamido)-adenine, and its genome is divided into several segments, including the 3 kbp invertible G segment that is flanked by inverted repeats and is responsible for selecting the bacterial host. Mu belongs to the Myoviridae family and can develop a "normal" lytic cycle; it is also capable of high frequency transposition and can integrate at different points in the bacterial chromosome in either sense or antisense orientation, relative to the polarity of the bacterial gene where it inserts (Boram and Abelson 1973). This characteristic is not exclusive of Mu, and can also happen in related bacteriophages such as D108. Generally, the integration is guided by two main principles: (1) the larger the bacterial gene, the higher probability that Mu genome gets inserted into it; and (2) active genes, that are being transcribed, exhibit lower frequency of Mu insertion (Bukhari and Zipser 1972). The gene order remains the same whether the phage is in vegetative state or integrated; this confirms that integration is not mediated by a Calef-Campbell mechanism (Wijffelman et al. 1973). When the bacteriophage genome is injected into the host, it circularizes in a noncovalent manner; circularization is mediated by the phage's N protein that also enters into the bacterium and protects the phage DNA ends from host nucleases. At this stage, Mu can undergo replicative transposition; this produces multitude of DNA copies (can be in excess of 50) generating several types of mutations, including deletions and inversions. An interesting mutation originates when Mu integrates near the *acrA* and *mtc loci* (at ~10.5 min on Taylor and Trotter 0/90 map), the mutation increases both cell wall and membrane permeability, rendering the host cells sensitive to a variety of detergents and antibiotics (Aline and Reznikoff 1975).

Mu transduction cannot be classified as either "all specialized" or "all generalized"; when the phage is abnormally excised from its integration point, transducing particles with very distant markers appear, although there is an increase in the number of particles carrying markers linked to the thrA and the *nusB locus*. Mu can infect not only *E. coli* K12 but also *Citrobacter freundii* and *Shigella dysenteriae*; but it cannot infect *Salmonella typhimurium* (unless it carries the mutation hisG9424::Tn10 delta 4 delta 11; Faelen et al. 1981); *Salmonella typhi*; *E. coli* C, B, S, or W; and *Klebsiella pneumoniae.*

Bacteriophage T1 is another phage, where generalized transduction is well documented; this organism was used by molecular biologists for many years to study this process and merits to be included here. In addition, T1 is also a useful tool to monitor decontamination of laminar-flow biological safety cabinets (Jones et al. 1981). The virulent coliphage T1 is similar to T5 and φ80 viruses, as well as to colicin M, in the fact that it adsorbs to the tonA porin on the outer bacterial membrane (Braun et al. 1973). Furthermore, Lundrigan et al. isolated in 1983 a novel putative siphovirus, named UC-1, which is entirely unrelated to T1, T5, and φ80, although it also uses tonA porin as receptor; phage comparisons were carried out by restriction analyses, DNA-DNA hybridization experiments, and guanine-plus-cytosine determinations.

A few seconds after infection, bacteriophage T1 dramatically alters the bacterial plasma membrane permeability, by inducing complete and irreversible loss of K^+ and Mg^{++} (which in turn causes inhibition of respiration) and concomitant uptake of Na^+, Li^+, or choline (Keweloh and Bakker 1984).

T1 efficiently destroys the bacterial chromosome, using the DNA breakdown products in the formation of its progeny. This phage transduces many bacterial markers, but with varying efficiencies (Drexler 1970; Drexler and Kylberg 1975; Kylberg et al. 1975). This virulent coliphage T1 is not believed to be related to the typical temperate bacteriophages mentioned here; as noted by Drexler (1970), around 70% of the phage's DNA is derived from the host (or even from another bacteriophage; Bendig and Drexler 1977). In fact, Drexler described in 1970 that certain characteristics of T1 are incompatible with its potential existence as a temperate phage, as the average latent period for this virus is only 13 min (Delbrück 1945). In that same year, Drexler (1970) demonstrated that amber mutants can transduce many genetic markers from permissive to nonpermissive K strains of *E. coli.* These and other amber mutants (Wagner et al. 1977) were invaluable to assign the function of ten genes and to define early, early-late, and late proteins; they also helped define at least three mechanisms, such as the cessation of host gene expression and discontinuation of the early class gene expression during the late phase of the viral cycle. Although no lysogenic operons or immunity markers have been defined in this phage, the observation that T1 could transduce some bio^+ markers with far higher efficiencies than others convinced Drexler (1977) to postulate the existence, under specific circumstances, of specialized transduction in the biotin region in *E. coli* chromosome. This specialized transduction, however, had to be reconsidered due to the fact that T1 can package large sections of λ phage (Drexler and Christensen 1979).

This bacteriophage displays a series of oddities, many of which have been already addressed in long forgotten papers, such as the one by Theodore Puck in 1949, claiming that T1 existed in reversible forms and that it constituted a system in which, at least, two forms of the bacteriophage could coexist in equilibrium; one of these forms is more stable, but the other is rather unstable, depending on the availability Ca^{++}, and can quickly lose its ability to complete the lytic cycle, thus leading to abortive infection. In addition, as opposed to other dsDNA bacteriophages such as λ, the T1 dsDNA displays an erratic and anomalous behavior in the presence of phenol and ether (Brody et al. 1964). T1 can infect *E. coli* protoplasts, albeit at a low rate, and can generate complete, infective, bacteriophage particles that are released in a single protoplast burst (Brody et al. 1967). This bacteriophage also exhibits an anomalous rate of transcription, as noted by Male and Christensen (1970); the transcription rate for T1 is estimated to be between the values reported for infections by virulent phages, such as T-even phages (T4), and typical viruses like λ. In addition, superinfection with this phage induces premature lysis in λ-lysogenized *E. coli* cultures; this is hypostatically regulated by the *rex* A/B system and the λ N gene protein (Christensen and Geiman 1973; Christensen et al. 1978). An interesting oddity in T1 concerns the ability of this virus to grow interchangeably on different *E. coli* strains (C, B, and K); T1 DNA is methylated independently of the degree of host DNA methylation, suggesting that the phage encodes its own DNA methyltransferase, hence the viral DNA sequences are effectively protected from host restriction modification systems (Wagner et al. 1979; Auer and Schweiger 1984). The phage-encoded methyltransferase was finally purified and characterized by Scherzer et al. in 1987, who demonstrated that this enzyme methylates adenine in 5′-GATC-3′ sites in vitro.

T1 played an important role in stablishing the number of areas in the bacterial cell wall (between 200 and 400) susceptible to be irreversible bound by a bacteriophage; these areas are very precise locations where plasma membrane and cell wall distance is minimal (Bayer 1968).

The T1 genome was fully sequenced by Roberts et al. (2004), revealing that it contains 50.7 kbp of terminally redundant, circularly permuted dsDNA, containing 48,836 bp of nonredundant nucleotides and 77 open reading frames.

Listeria monocytogenes (with a GC content of 38%, according to Stuart and Welshimer 1973) is a Gram-positive, food-borne intracellular pathogen, that causes listeriosis in humans (*L. ivanovii* or *L. grayi* can also cause similar infections) characterized by affections of the central nervous system (meningitis, meningoencephalitis, brain abscess, cerebritis). It can also cause bacteremia in both newborns and elderly people, and gastroenteritis in healthy people. This bacterium undergoes actin-based propulsion inside the eukaryotic cells, as is the case for *Shigella dysenteriae* (Pantaloni et al. 2001), and represents a real health threat to consumers, as it survives high salt concentrations and can grow at even refrigeration temperatures (6–8 °C).

The first *Listeria* bacteriophage was described by Schultz in 1945 and, by 1986, more than 200 bacteriophages, belonging to either the *Myoviridae* or *Siphoviridae* families, had been isolated from different *Listeria* species, including

L. monocytogenes (Rocourt 1986). These bacteriophages were mainly used in *Listeria* phage typing and as a means of genus identification, but also, either as a substitute or in combination with serological typing, in epidemiological investigations (Sword and Pickett 1961). A recent review of *Listeria* bacteriophages by Klumpp and Loessner (2013) indicates that there are currently more than 500 bacteriophages described, all belonging to the two families mentioned above; interestingly, no Podoviridae phages have yet been isolated. Almost all of these bacteriophages contain short dsDNA (30–65 kbp), although in some cases the DNA can be as long as 140 kbp. Despite all this wealth of knowledge, general transduction was not demonstrated on these bacteria until 2000, by Hodgson; they contain wide and narrow range bacteriophages, but their molecular biology is considerably less-known than that of other Gram-positive bacteria. Phage A118 (*Siphoviridae* family) is a temperate bacteriophage that causes general transduction, specific for *L. monocytogenes* serovar 1/2 strains; it was isolated after UV induction of a bacterial strain isolated from Camembert cheese and fully sequenced by Loessner et al. in 2000. Its DNA is 40,834 bp long, circularly permuted, and terminally redundant without cohesive ends, and contains 72 open reading frames with genes clustered and organized into three modules. The phage contains an *attP* site that recombines with the bacterial *attB*, following a Calef-Campbell mechanism, and integrates at the *comM* (ATPase) *Listeria* marker.

Three years later, Zimmer et al. fully sequenced the bacteriophage PSA, also a temperate phage for *L. monocytogenes* strain Scott A. PSA head contains a linear 37,618 bp DNA, with cohesive ends of 10 nucleotides at the 3′ termini, and 57 open reading frames (Zimmer et al. 2003). An industrial development resulting from *Listeria* bacteriophage studies is the generation of several phage cocktails (also known as enzybiotics) approved by the FDA for application on comestibles and surfaces in food production facilities (Leverentz et al. 2004).

Epsilon 15 (ε^{15}) is a short, tailed bacteriophage that contains a genome spanning 39,671 bp of DNA, encompassing 49 open reading frames, capable of infecting *Salmonella* species such as *S. anatum*; it belongs to the *Podoviridae* family within the order *Caudovirales*. For many years, it has been known that this phage can transduce resistance to chloramphenicol, streptomycin, and sulfonamide to group E *Salmonella* (Harada et al. 1963; Kameda et al. 1965) and is involved, together with bacteriophage ε^{34}, in antigenic conversions in *S. anatum* (Le Minor 1965; Robbins et al. 1965; Losick and Robbins 1967; Bray and Robbins 1967). Hedges in 1971 studied the general system of transduction, concluding that the bacteriophage can achieve transduction by both replacement of homologous DNA with the recipient *S. anatum* sequences or by the formation of defective transducing particles. However, recent comparisons between the two bacteriophages indicate that little, if any, genetic exchange has occurred within the procapsid assembly gene cluster, and that both ε^{15} and ε^{34} are major contributors to the *O*-antigen serotype of their *Salmonella* hosts (Villafane et al. 2008). Robbins et al. reported in 1965 that, after infection and subsequent lysogenization of *S. anatum* by the temperate bacteriophage ε^{15}, chemically defined changes occur in the structure of the polysaccharide *O*-antigen. Accordingly, while uninfected cells contain an *O*-antigen with an *O*-acetyl-α-D-

galactosyl-mannosyl-rhamnosyl repeating sequence, cells carrying the prophage ε^{15} have a polysaccharide with a β-D-galactosyl-mannosyl-rhamnosyl sequence. In addition, bacteriophage tail-spikes, through the action of either exo-or endo-acting rhamnosidases, can contribute to the modification of such antigens, in addition to promoting the partial depolymerization of capsules (Eriksson et al. 1979). The bacteriophages of this group could then use HGT to transfect these metabolic abilities to other members of the *Enterobacteriacea*, thus advancing the overall modification of the external bacterial antigens.

4 Conjugation

Bacterial conjugation is the third type of HGT reviewed here; the main difference between this mechanism and both transformation and transduction is that conjugation requires cell to cell contact via the formation of a pilus bridge. Bacterial conjugation was discovered by Joshua Lederberg and Edward Tatum (1946), and Tatum and Lederberg (1947); although some pioneering work was carried out by Sherman and Wing in 1937, Gowen and Lincoln in 1942, Smith in 1944, and even Wollman and Wollman in 1925. While the word conjugation is often used to refer to the eukaryotic sexual process, bacterial conjugation is a rather different form of recombination, since it normally involves the transfer of plasmids or transposons. In addition, in particular in *Enterobacteriaceae* strains (i.e., *E. coli* K12 group strains, such as in *Hfr*, see below), it can eventually mobilize the hemi-chromosome, thus usually originating merodiploids and rarely complete diploids. This type of genetic recombination has been the base to stablish the first genetic maps in many bacteria, in particular in the *Enterobacteriaceae* family and related organisms.

After the original report by Lederberg and Tatum in 1946, there were a series of publications (Wollman et al. 1956; Jacob and Wollman 1956, 1958a; Wollman and Jacob 1957; Anderson et al. 1957) that culminated on the first 0/90 or 0/100 min *E. coli* linkage genetic maps, obtained by interrupted mating according to Wollman and Jacob in 1958. The momentum to unravel the bacterial conjugation process was so intense in those years that, by 1962, this process had been already described in *E. coli*, *Serratia*, *Salmonella*, *Pseudomonas*, and *Vibrio*. Moreover, by 1962, there was even evidence that intergeneric conjugation was possible between bacteria such as *Escherichia-Shigella*, *Salmonella-Vibrio*, *Escherichia-Serratia*, *Salmonella-Serratia*, and *Escherichia-Salmonella* (Clark and Adelberg 1962), and even *Shigella flexneri-Vibrio cholerae* (Kuwabara et al. 1963).

Mating in Gram-positive bacteria, however, appears not to be as frequent as in Gram-negative, although this possibility was described for *Streptomyces coelicolor* by Sermonti et al. (1966). Nevertheless, it was clear from the pioneering studies that this type of genetic recombination in bacteria requires permanent DNA synthesis (Bouck and Adelberg 1963). For recent reviews on this topic, see Grohmann et al. (2003), Guglielmini et al. (2011), and Arutyunov and Frost (2013).

The classic process of *E. coli* sexual recombination is addressed below, starting with the mating between two cells, one displaying a fertility factor (F^+) and the other

lacking it (F^-), represented as $F^+ \times F^-$; this will be followed by the conjugation ("cross") of high frequency recombination cells (Hfr) with F^-, indicated by Hfr $\times$ F^-.

4.1 $F^+ \times F^-$ Crosses in E. coli

Certain *E. coli* strains are classified as F^+ (male; Fig. 5a, b), because they contain a fertility factor (F stands for *f*ertility). This genetic factor, or "episome", can be easily eliminated from the F-harboring strains by treatment with acriflavine (Hirota and Lijima 1957). F^+ can contact strains lacking the F factor, known as F^- (female), through a bridge created by a sex pilus (F pilus). This F pilus is different from other sex-related pili, such as the P, N, W, and X pili, and it is constituted by spherical units of F pilin stoichiometrically complexed with phospholipid (Costa et al. 2016); it generates a hollow tube with a narrow diameter that allows the passage of single stranded nucleic acids, including filamentous bacteriophages containing positive sense single-stranded RNA, such as the leviviruses Qβ and MS2, hence resulting in the transfer of either one or several genetic markers. The name fertility factor was coined by Esther Miriam Zimmer Lederberg and Luigi L. Cavalli-Sforza (although the first observation was made by Cavalli-Sforza in 1950), in a three-author classic paper in 1952 (Lederberg et al. 1952), followed by a second publication in 1953 (Cavalli et al. 1953); the articles also included Esther's husband Joshua Lederberg as the first and second author, respectively. The observation was corroborated in 1953 by Hayes (Hayes 1953a). Two classic papers on the possibility of mating in *E. coli* include the publications by Jacob and Wollman (1958a) and by Wollman and Jacob (1958). These authors described that *E. coli* fertile strains contained a plasmid (Fig. 5a, b), "Plasmid F," formed by dsDNA. Although it should be referred to as "Episome F," a name crafted by Jacob and Wollman in 1958b to indicate a dsDNA that, being a replicon, can enter and exit the bacterial chromosome. On the other hand, the term "plasmid", coined by Lederberg in 1952, should only refer to autonomous replicons unable to interact with the chromosome. This F genetic element belongs to the conjugative class of large molecular weight plasmids, which remain in low copy number in the bacterial cells, as they are under stringent control (Kline and Miller 1975) and "complexed" with the chromosome. The copy number can, however, increase up to sevenfold in *pcn* mutants, which allow F to stay in a noncomplexed status (Cress and Kline 1976).

To further complicate matters, it appears that conjugative plasmids enable the maintenance of nonconjugative and nontransmissible plasmids; the prerequisite for this is the co-occurrence of an incompatible and energy-costly conjugative plasmid, which indirectly facilitates the preservation of the nontransmissible nucleic acid (Werisch et al. 2017).

The F plasmid currently remains as the best known of a large group of conjugative plasmids (IncFI), very common in the *Enterobacteriaceae*, that, apart from being involved in bacterial sexuality, are frequently associated with virulence, antibiotic resistance and similar genes. Recently, Fernandez-Lopez et al. (2016)

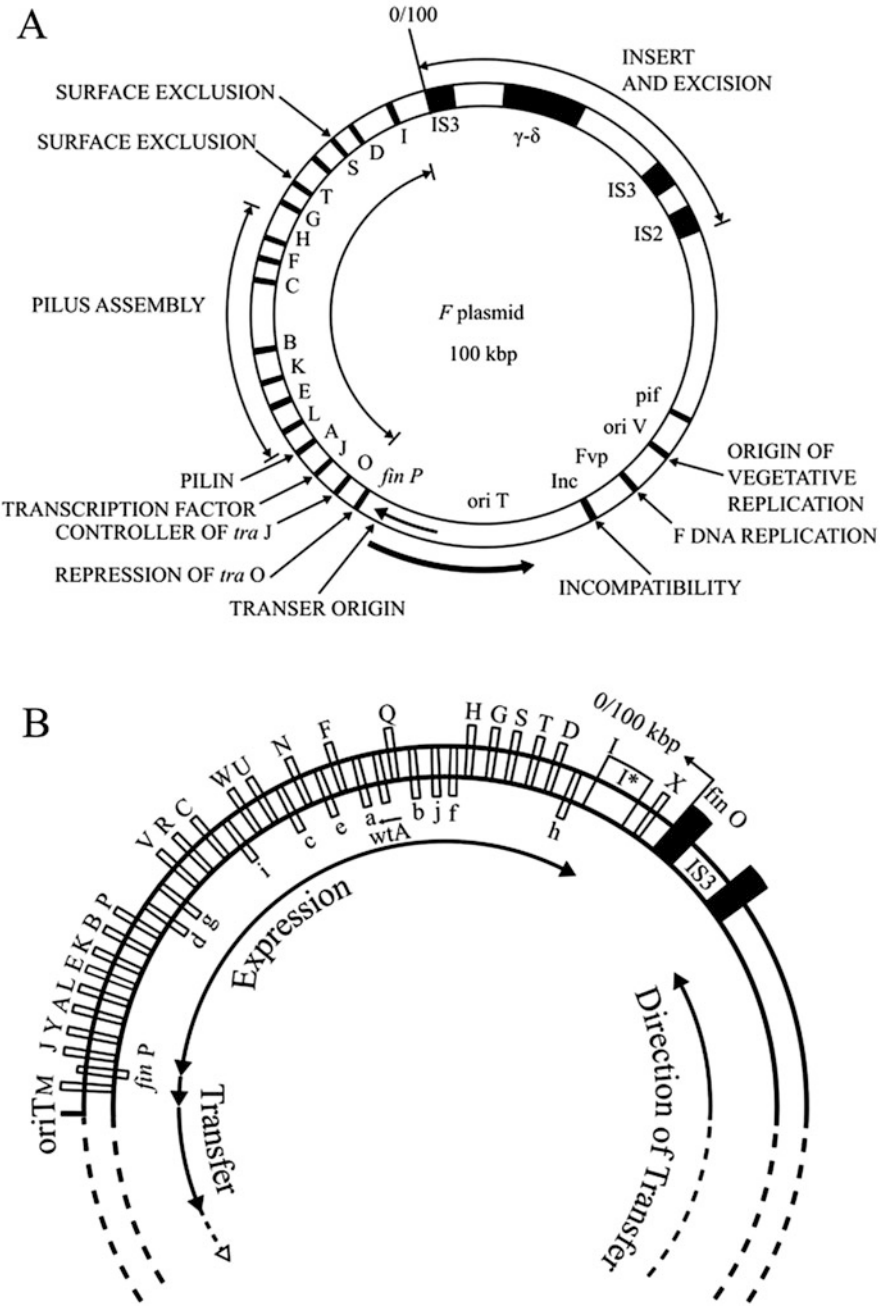

Fig. 5 Conjugation in *E. coli* (**a**) simple map of F plasmid showing the transfer operon and insertion elements; (**b**) detailed map of the 33.3 kbp *tra* operon, based on the review of Frost et al. in 1994. Capital letters refer to the *tra* genes. Lower case letters refer to the *trb* genes, *art A*, and *finP*. *OriT* corresponds to the origin of transfer. IS3 is the insertion element disrupting *finO*

identified five major groups of F-like plasmids, each of them with its particular operon structure, alternate regulatory systems, and a huge variety of different genes (Johnson et al. 2016); according to these authors, the five groups represent radiations from an ancestral MOBF conjugation system.

The F plasmids are capable of self-transmission, either alone or carrying other genetic markers, and therefore control the sexual functions of bacteria possessing a fertility inhibition (*Fin*) system.

The proteins encoded by the F-based conjugation plasmid successfully accomplish the ssDNA transfer. This transfer occurs in ~5 min, through a multiprotein type IV secretion pore, also called transferosome by Waksman and Fronzes (2010). TraD, a hexameric ring ATPase protein that generates the needed energy to transport the ssDNA, is located on the cytoplasmic face of the pore. To generate the ssDNA to be transported, the F plasmid must undergo nicking and unwinding at specific sequences, recognized by the relaxase/helicase TraI and a variety of additional DNA binding proteins that form a complex near the DNA cuts; this protein complex is known as "the relaxosome" (Wong et al. 2012; Peng et al. 2014).

The main components (see Fig. 5 and Table 1) of F plasmid include OriT (Origin of Transfer), the point where DNA transfer begins, and OriV (Origin of Replication), which is the point where DNA replication starts when the F plasmid ssDNA reaches the recipient cell; this process does not require de novo protein synthesis, as it uses the existing DNA polymerases. The third main component is the tra-region (transfer genes), also known as the *tra* operon, formed by 40 different genes spanning 33.3 kbp, with 20 of the genes being essential for conjugation (see the review by Frost et al. 1994). It also contains a number of genes encoding the fertility pilus and the transfer process itself, as well as a number of insertion elements (one copy of ϒ-δ (Tn1000), one copy of IS2, and two copies of IS3, that provides the chromosomal integration site in *Hfr* strains). There are, in addition, surface exclusion genes and a set of genes controlling the insertion and excision processes during the genesis of *Hfr* strains.

F factor replication is neither coupled to the host chromosome replication cycle, nor to the bacterial cell division (Pritchard et al. 1975). Furthermore, F plasmid promotes turnover of stable RNA in *E. coli* (Onishi 1975). An aspect often forgotten is that infection of *E. coli* F^+ strains with bacteriophage T7, results in an immediate cessation of bacterial growth, but T7 infection ends abortively and normal liberation of progeny phages does not occur. This phenomenon, known as "F-factor-mediated restriction of T7 development", is caused by blocking the synthesis of T7 late proteins (Morrison and Malamy 1971; Yamada and Nakada 1975).

Before the development of high-throughput DNA sequencing, gene mapping was typically tedious hard work in bacterial genetics. Willetts et al. started mapping the F plasmid in 1975 and managed to pinpoint the genetic locations for *traO*, *finP*, and *tra-4* on the *E. coli* K12 sex factor F.

The F plasmid can be artificially eliminated from bacteria by treatment with substances such as acriflavine and acridine orange (Hirota 1956; Hirota and Lijima 1957), hence converting an F^+ bacterial strain into an F^-. *E. coli* possesses another type of fertility plasmids, R plasmids, that confer resistance to tetracycline,

Table 1 Genes present in the 30.3 kbp *tra* operon, listed according to their position in the circular F plasmid, starting from *oriT*

Gene	Function	Amino acids encoded	Isoelectric point	Comments/References
traM	Mating signal	127	5.17	DNA binding ability. Involved in ssDNA transfer (Abo et al. 1991)
finP	Regulation *tra* operon	?		Antisense RNA cooperates with FinO to repress *tra* expression (van Biesen et al. 1993) Association with FinO prevents its degradation by RNase E (Jerome et al. 1999)
traJ	Regulation	229	6.78	Positive control of *tra* operon expression (Cuozzo and Silverman 1986) Stability or instability of *TraJ* (Gubbins et al. 2002) Dimerization of TraJ is essential for bacterial mating (Lu et al. 2012)
traY	*oriT* nicking	131	10.19	Required for λ-F *oriT* nicking (Everett and Willetts 1980) A ribbon–helix–helix DNA-binding protein that binds oriT and promotes nicking of plasmid DNA prior to conjugative transfer (Lum et al. 2002) Conservation of TraY integrity for bacterial mating (Williams and Schildbach 2007)
traA	F pilin subunit	121	10.13	Pilin maturation depends on *traQ* and acetylation at Ala-52 on *traX* (Laine et al. 1985) The TraA relaxase autoregulates type IV secretion system in *Streptococcus agalactiae* (Kurenbach et al. 2006) TraA interacts with bacterial DNA (Folli et al. 2008)
traL	F pilus assembly	91	10.64	First characterization of *traL* cistron (Willetts 1973) Hydrophobic nature and possibly peripheral location of TraL (Frost et al. 1984) Sequence determination of *traALE* region (Frost et al. 1984)
traE	F pilus assembly	188	10.06	Hydrophilic-membrane anchorage (Frost et al. 1984)
traK	F pilus assembly	242	5.38	Hydrophilic protein
traB	F pilus assembly	475	5.18	Hydrophilic-membrane anchorage (Moore et al. 1987; Anthony et al. 1996)

(continued)

Table 1 (continued)

Gene	Function	Amino acids encoded	Isoelectric point	Comments/References
traP	Minor role in conjugation	196	9.37	Interacts with TraB, stabilizing the transmembrane complex (Anthony et al. 1996)
trbD	Unknown	65	5.73	Gene product not yet described
trbG		83	6.48	Previously known as *traR*, *it* shares amino acid homology with proteins from the bacteriophages 186 and P2 and with the dosage-dependent dnaK suppressor DksA (Doran et al. 1994)
traV	F pilus assembly	171	9.38	Lipoprotein sensitive to globomycin. Putative anchor of F pilus (Doran et al. 1994; Arutyunov et al. 2010)
traR	Control of cellular stress during mating	73	7.20	Activates transcription of σ(E)-dependent promoters. It prepares the F^+ cell to endure periplasmic stress during mating (Grace et al. 2015)
traC	F pilus assembly	875	5.90	Membrane associated protein with an ATP-binding motif (Maneewannakul et al. 1987; Schandel et al. 1990, 1992) TraA, TraC, and TraD autorepress two divergent quorum-regulated promoters (Cho and Winans 2007) Purification of TraC protein (Hellstern and Mutzel 2016)
trbI	Influences pilus outgrowth and/or retraction	128	10.12	An intrinsic IM protein (Maneewannakul et al. 1992b) It constitutes an interaction group with TraH, TraF, TraW, TraU, and TrbB of type IV secretion system (Harris and Silverman 2004)
traW	F pilus assembly	210	8.39	Maneewannakul et al. (1987) It constitutes an interaction group with TraH, TraF, TraU, TrbI, and TrbB of type IV secretion system (Harris and Silverman 2004)
traU	F pilus assembly	330	7.68	Sequence Ser-24-Ala-25-Cys-26 resembles the signal peptidase II recognition sequence in lipoproteins. Hydrophobic in nature (Moore et al. 1990) It constitutes an interaction group with TraH, TraF, TraW, TrbI, and TrbB of type IV secretion system (Harris and Silverman 2004)

(continued)

Table 1 (continued)

Gene	Function	Amino acids encoded	Isoelectric point	Comments/References
trbC	F pilus assembly	212	81.2	*trbC* function is essential to the F plasmid conjugative transfer system (Maneewannakul et al. 1991)
traN	Aggregate stability	602	7.57	TraN interacts with OmpA and LPS moieties. Mating pair stabilization (Klimke and Frost 1998)
trbE	Unknown	86	4.39	Intrinsic inner membrane protein, although not essential for F transfer under standard mating conditions (Maneewannakul et al. 1992a) TrbE is essential for conjugation and phage adsorption (Rabel et al. 2003)
traF	Pilus assembly	247	9.49	TraF and TraH are required for F pilus assembly and F plasmid transfer. Components of an OM complex (Arutyunov et al. 2010) TraF constitutes an interaction group with TraH, TraW, TraU, TrbI, and TrbB of type IV secretion system (Harris and Silverman 2004)
trbA	Unknown	115	8.36	Inner membrane protein (Kathir and Ippen-Ihler 1991) TrbA operator depends on KorB (a transcriptional repressor protein in *E. coli*) (Bingle et al. 2003)
artA	Unknown	104	8.42	Encoded by an anti-*tra* strand. It lies on the DNA strand complementary to that of the F *tra* operon. The *artA* gene is expressed from its own promoter (Wu and Ippen-Ihler 1989) The *Staphylococcus aureus* pSK41 plasmid-encoded ArtA protein is a master regulator of plasmid transmission genes (Ni et al. 2009)
traQ	F pilin synthesis	94	9.38	Involved in pilin maturation (Maneewannakul et al. 1993) Single mutations in *TraQ* render *E. coli* partially resistant to Qβ infection (Kashiwagi et al. 2015)
trbB	Disulfide bond isomerase and reductase activities	179	8.10	Involved in correct folding and maintenance of disulfide bonds within F plasmid encoded proteins in the bacterial periplasm. Member of the thioredoxin-like superfamily (Hemmis et al. 2011, 2014) TrbB constitutes an interaction group with TraH, TraF, TraW, TraU and TrbI

(continued)

Table 1 (continued)

Gene	Function	Amino acids encoded	Isoelectric point	Comments/References
				of type IV secretion system (Harris and Silverman 2004)
trbJ	Unknown. Probably surface exclusion	83	6.80	It could be also involved in transfer efficiency (Maneewannakul and Ippen-Ihler 1993; Lyras et al. 1994)
trbF	Unknown	126	8.40	Intrinsic inner membrane protein
traH	F pilus assembly	458	7.22	Nucleotide sequence of *traH* gene (Ham et al. 1989) TraF and TraH are required for F pilus assembly and F plasmid transfer. Components of an OM complex (Arutyunov et al. 2010) Similar protein in *Enterococcus faecalis* (Fercher et al. 2016)
traG	F pilus assembly. Aggregate stability	938	6.30	Inner membrane protein that helps form stable mating aggregates resulting in stabilization (Manning et al. 1981) Characterization of the *traG* gene (Firth and Skurray 1992) Essential motifs of TraI and TraG proteins involved in conjugative transfer (Balzer et al. 1994)
traS	Surface exclusion	149	10.37	*traS* and *traT* are independently responsible for surface exclusion; TraS reduces DNA transfer within stable mating aggregates (Achtman et al. 1977) Nucleotide sequence of the surface exclusion genes traS and traT (Finlay and Paranchych 1986) Recognition between TraG and TraS in cell exclusions (Audette et al. 2007)
traT	Surface exclusion	244	9.04	*traS* and *traT*, are independently responsible for surface exclusion; TraT reduces the ability to form stable mating aggregates (Achtman et al. 1977) Properties of the TraT protein (Manning et al. 1980) Purification and characterization of the F traT protein (Minkley and Willetts 1984) Lipoproteinaceous nature of TraT (Perumal and Minkley 1984) TraT protein is involved in bacterial resistance to phagocytosis (Agüero et al. 1984) Nucleotide sequence of the surface

(continued)

Table 1 (continued)

Gene	Function	Amino acids encoded	Isoelectric point	Comments/References
				exclusion genes traS and traT (Finlay and Paranchych 1986) TraT as a powerful carrier molecule for the stimulation of immune responses (Croft et al. 1991)
traD	DNA transport	717	5.27	Nucleotide sequence of the traD gene (Jalajakumari and Manning 1989) TraD is an inner membrane protein that shows non-specific DNA binding ability (Panicker and Minkley 1992) Involvement of the TradD carboxyl terminus in F-plasmid conjugative transfer (Sastre et al. 1998; Lu and Frost 2005) Characterization of recognition betweenTraD and TraM (Lu et al. 2008)
trbH	Conjugative transfer protein	239	8.95	Estimated molecular weight of 26 kDa for *Salmonella enterica* subsp. *enterica* serovar Typhimurium protein (McClelland et al. 2001)
traI	*oriT* nicking unwinding	1756	5.94	The protein helicase I nicks *oriT* and attaches to the 5′ end of the DNA, unwinding it in the 5′ → 3′ direction (Abdel-Monem et al. 1983) Nucleotide sequence of the traI gene (Bradshaw et al. 1990) Essential motifs of relaxase (TraI) and TraG proteins involved in conjugative transfer (Balzer et al. 1994) Mechanisms of initiation and termination reactions in TraI reactions (Pansegrau and Lanka 1996) TraI can separate double-stranded DNA at a rate of ~1100 bp per s; it acts as a highly processive monomeric helicase (Sikora et al. 2006). Different ways of folding in TraI relaxase (Guogas et al. 2009) Regulation of TraI protein (Dostál and Schildbach 2010) Structure of TraI protein (Wright et al. 2012) Molecular Basis of traI-mediated DNA unwinding during bacterial conjugation (Ilangovan et al. 2017)

(continued)

Table 1 (continued)

Gene	Function	Amino acids encoded	Isoelectric point	Comments/References
traI∗		802	5.41	Same polypeptide sequence as TraI c-terminus (Traxler and Minkley 1987)
traX	F pilin acetylation	248	8.93	Synthesis of F pilin (Maneewannakul et al. 1993, 1995; Moore et al. 1993)
finO	Regulation	186	10.48	RNA chaperone that facilitates sense-antisense RNA interactions (Arthur et al. 2003)
Hfq				*E. coli* Hfq (not included in episome F) regulates *traM* and *traJ* transcript stability by a mechanism different from FinOP-mediated repression (Will and Frost 2006)

Streamlined and updated from Frost et al. (1994), and including data from Arutyunov and Frost (2013)

chloramphenicol, streptomycin, and sulfonamide, and are similar to plasmid F; but unlike F, R plasmids are not cured during growth by treatment with acridine orange, and usually have the ability to direct the synthesis of the repressed F pilus. Yoshikawa and Akiba (1961) described an interrelationship between the two types of plasmids, as well as reporting that the frequency at which resistance is transferred is independent of the F-polarity displayed by the donor and recipient strains. In R plasmids, the fertility determinant cannot be separated from the determinant responsible for infectivity, although they are both independent from the drug-resistance genes (Sugino and Hirota 1962). The F and R plasmids can undergo recombination to originate an FR hybrid that encodes the F^+ phenotype and can be transmitted to F^- strains (Harada et al. 1964). In fact, the two plasmids are related in their origin and some R plasmids, such as R386, have derepressed the pilus synthesis capacity, as it is the case for the F plasmid (Dennison 1972); although pilins that integrate the two types of plasmid are clearly differentiated by specific antisera (Lawn and Meynell 1970).

Fertility plasmid F has been the scaffolding used to construct the renowned bacterial artificial chromosome (BAC), a cloning vector with insert sizes in the range of 150–350 kbp. BAC's counterpart in yeasts is the Yeast Artificial Chromosome (YAC) that allows cloning of even larger dsDNA fragments (O'Connor et al. 1989).

The $F^+ \times F^-$ cross, originally suggested by Havekes et al. in 1976, starts with the tip of the F pilus contacting a proteinaceous receptor (OmpA porin) on the outer membrane (OM) of the F^- cell wall. Although, in 1998, Klimke and Frost posited that it is the TraN protein, and not the tip of the F pilus, the first to interact with the OmpA porin and LPS moieties present on the outer membrane, resulting in mating pair stabilization, the first step in efficient mobilization of DNA. After establishing

contact, the F pilus retracts, apparently by depolymerization of F pilin units; this brings together the male and female cells that are linked through the narrow pore that permits the passage of ssDNA. This depolymerization step may be critical for bacteria undergoing conjugation, since toxin CdiA-CT (Contact-dependent growth inhibition) mimics the pilin-binding maturation proteins of leviviruses, allowing the toxin to bind F pili and become internalized during pilus retraction (Beck et al. 2014). As indicated, the mating pair is stabilized by TraN (Klimke and Frost 1998), and the mature protein expressed in maxicell systems is located in the OM fraction, and partly exposed to protease K digestion (Maneewannakul et al. 1992a).

The conjugative transfer of the F plasmid relies on TraM protein, which forms a tetramer (Verdino et al. 1999) and contacts DNA by a pair of dimeric RHH (ribbon–helix–helix) DNA recognition modules, thus contributing to the unwinding of the DNA. This protein recognizes multiple DNA sites, such as *sbmA* and *sbmC*, in order to recruit the plasmid to the conjugative pore (Peng et al. 2014). The next step involves helicase TraI that nicks the DNA to open up the nucleic acid and facilitate replication. Then, a rolling-circle replication occurs and one strand of the F plasmid begins its passage through the pore, in a 5′–3′ orientation; as the ssDNA enters the female cell, the recipient's DNA polymerase III synthesizes the complementary strand; this process takes place in the absence of protein synthesis (Hiraga and Saitoh 1975). At the same time, the male strain also uses its polymerase to replace the missing complementary strand. The end result of this cross is two male cells, as both bacteria contain the F plasmid. Female strains emerge from male strains by F plasmid loss, as the replication rate of the F plasmid does not match that of the chromosome.

This seemingly simple process is finely regulated, mainly by F plasmid-encoded proteins, although there are some *E. coli* genes, such as *sfrA* and *sfrB*, which are required for *tra* gene expression (Beutin and Achtman 1979). Transcription of the *tra* operon requires previous *traJ* gene expression, since TraJ is a positive regulator of the transfer operon and its synthesis can be prevented by the trans-acting *finO* gene product. FinO is an RNA chaperone that facilitates sense-antisense RNA interactions (Arthur et al. 2003; Mark Glover et al. 2015), for example, interaction with FinP; FinP encodes an antisense noncoding RNA that binds *traJ* mRNA, blocking its translation, and that is effectively protected by FinO from being degraded by ribonuclease E (Willetts 1977; Jerome et al. 1999). However, in the best known typical *E. coli* F^+ strains, such as K12, there are constitutive expression of the *tra* operon because the *finO* gene is interrupted by an IS3 insertion (Fig. 6). Inhibition of the *traJ* function, by either mutation or recombination events, results in inhibition of the *tra* operon and, hence, the ability of F^+ to mate.

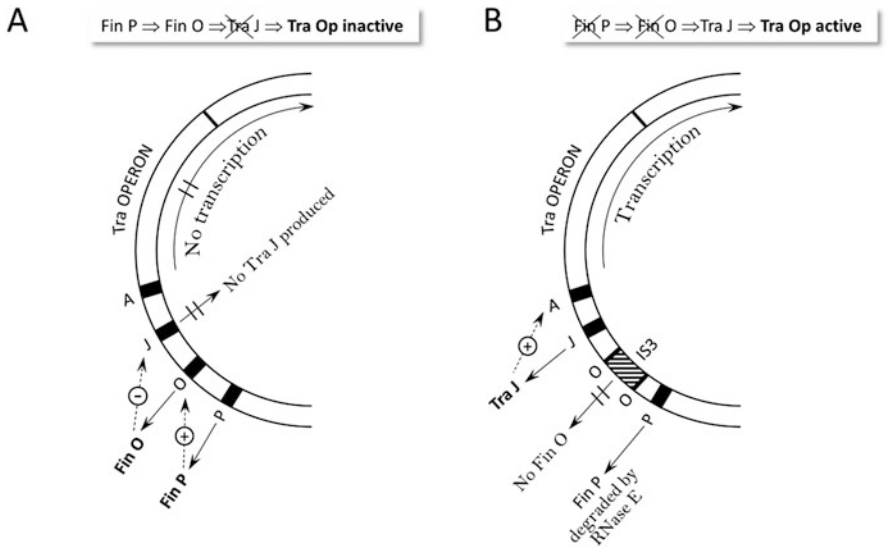

Fig. 6 Regulation of *Tra* Operon expression by the *FinP/FinO* system. (**a**) Negatively controlled by Fin O, the strains of *E. coli* are unable to mate, since no positive activator TraJ is produced; (**b**) Typical *E. coli* strain K12 with deregulated F plasmid by IS3 insertion into Fin O gene. In this case the positive activator TraJ is produced

4.2 *Genesis of* Hfr *Strains and* Hfr *(F^+) × F^- Crosses in* E. coli

Hfr (High frequency recombination) strains were first characterized by Luca Cavalli-Sforza in 1950, although William Hayes independently isolated another *Hfr* strain in 1953 (Hayes 1953b). These *Hfr* strains are phenotypically F^+ bacteria in which the conjugative F plasmid, or a similar one, is integrated into its chromosomal DNA at different points; this integration is usually, but not always, carried out by homologous recombination, due to the existence of homologous sequences in both DNAs (i.e., IS3); the same process used in some bacteriophage DNA integrations. Chromosomal integration occurs following a Calef-Campbell mechanism, thus resulting in inversion of the genetic markers. In the *Hfr* (F^+) × F^- cross described here, and due to the F factor's inherent tendency to transfer itself during conjugation following a rolling circle replication event, the first DNA to enter the recipient cell is a portion of the F plasmid, followed by one complete strand (hemi-chromosome) of chromosomal DNA, and finally by the rest of the F plasmid, in a manner similar to the F^+ × F^- conjugation. However, in *Hfr* cells, due to the length of the bacterial chromosome, it is rare for the entire hemi-chromosome to be transferred into the F^- cell; the time required for this process to be completed is too long and the bridge between donor and recipient cells breaks down. Therefore, if the conjugative transfer is not completed, the recipient cells do not receive the complete F factor plasmid (although the number of allelic merozygotes increases with time) and, hence, remain as female (F^-) due to its inability to form the F sex pilus. There are different points at

which the F plasmid integrates into the bacterial chromosome (including *Hfr H*, *Hfr 2*, *Hfr 3*, *Hfr 4*, *Hfr 5*, *Hfr 6*, and *Hfr 7*) and, as transfer always starts at the oriT and drags with it whatever DNA is behind, the order of genes transferred in *Hfr* × F^- crosses can vary according to the point of F insertion into the bacterial chromosome. This means that a given gene that enters first in a particular mating, can be the last to enter in another cross. This was indeed the rationale followed by Wollman and Jacob in their classic papers of 1956, 1957, and 1958 that led to the publication of the first genetic map of *E. coli* divided into minutes (0–100 min), representing the time required for all genetic markers to enter the recipient F^- strain. These authors concluded that the *E. coli* genome is a genetic circle with all its markers contained within a single linkage group. For many years, this method was used to obtain a rough map of a huge variety of genes in both *E. coli* and *S. typhimurium*; fine mapping, however, required stablishing recombination indexes in interrupted mating.

There are many *Hfr* strains that can be used in genetic crosses (Low 1972; Feinstein and Low 1986), some of these integrate through the IS, or via Tn 1000, while others use different, yet unknown, genetic recombination events. Mating two *Hfr* strains can occasionally result on the integration of two, or even three, copies of the F plasmid; this gives rise to the strains known as "double or triple males". These strains are fully active (contain a single linkage group) and can transfer F-adjacent chromosomal markers at high frequency (Clark 1963; Falkinham and Clark 1974).

In addition, in certain cases F^+ donor cells can mate with *Hfr* strains under the conditions of phenocopy mating, in which the male recipient cells (either F^+ or *Hfr* strains) can accept F^+ DNAs as if they were F^- (Saitoh and Hiraga 1975). As described above, the single-stranded F^+ DNA, synthesized via a rolling circle replication, is converted into a covalently closed circular dsDNA upon entering the *Hfr* cells, but this process renders the DNA unable to replicate, and the nucleic acid is diluted out during further cell multiplication (Saitoh and Hiraga 1975). An exception occurs when the mating involves an F' plasmid carrying the *dnaA-bgIA* region of the *E. coli* chromosome; this DNA contains a specific site, known as poh^+, that allows the plasmid to replicate (it contains the E. *coli* OriC) in *Hfr* environments, thus behaving as a mini chromosome (Hiraga 1976). This male to male form of mating, although rare, constitutes a useful tool in basic bacterial genetics.

Using a direct enrichment and screening procedure, Feinstein and Low (1986) performed *Hfr* × F^- crosses and managed to isolate several *E. coli* mutants with higher recombination activity (up to 20 times higher) than the parent strains; these mutants include mutS, mutL, mutH, and mutU.

Salmonella typhimurium is an enteric bacterium closely related to *E. coli*, with a similar mating process also mediated by F^+ or *Hfr* strains; this process was fortuitously found on a *S. typhimurium* strain that behaved as a genetic recipient (Baron et al. 1959; Zinder 1960a, b). Interrupted mating experiments and analysis of recombinant index, revealed that this bacterium possesses a single linkage group of 138 min (Johnson et al. 1964; Sanderson and Demerec 1965; Sanderson 1967). In 1995, Sanderson et al. published "edition VIII of the genetic map of *Salmonella typhimurium* LT2"; in this map, including a total of 1159 genes, they located 1080

genes on the circular chromosome and 29 on the 90 kbp SLT plasmid, while the remaining 50 genes were not mapped. The authors noted that the coordinate system was unusual, as it was not organized in minutes but in "centisomes" and kilobases.

As *S. typhimurium* is closely related to *E. coli*, it was originally thought that it would be easy to achieve intergeneric mating between the two bacteria. However, crosses between *E. coli* and *S. typhimurium* displayed low fertility; this was due to differences in the cell surface of female strains, the existence of restriction systems on male DNA and, finally, differences in the DNA sequences (Mojica-a and Middleton 1971). Although it was later discovered that the fertility can be substantially increased, by obtaining the appropriate mutants in the female restriction modification system.

Sexuality was also confirmed in *Pseudomonas aeruginosa* that, like *E. coli*, exhibits an F factor (Holloway 1955, 1956; Holloway and Jennings 1958); although further analyses on the fertility agents in both bacterial species revealed that there is some degree of behavior heterogeneity within each system. Furthermore, the evidence presented by Holloway and Fargie in 1960 indicated that male strains in *P. aeruginosa* can display different transfer frequency to female strains.

In summary, conjugation has been described in many different bacterial species, such as *Agrobacterium tumefaciens* (Li et al. 1999), *Proteus* (Hedges 1974), mycobacteria (Konícek and Konícková-Radochová 1975), *Haemophilus influenzae* (Stuy 1980), *Enterococcus faecalis* (Nakayama et al. 1998), *Helicobacter pylori* (Kuipers et al. 1998), and *Streptomyces coelicolor* (Vivian and Hopwood 1970). Nevertheless, and despite the data presented above, it appears that conjugation in bacteria only plays a major role in HGT between related species within a same genus, but its importance decreases as the strains are more phylogenetically diverse. Bacterial HGT would therefore mostly depend on transformation and transduction; in the cases where the bacteria do not have a "natural" transformation mechanism (such as *E. coli*), HGT would depend mainly on bacteriophages to transfer genes from species to species, or even from genus to genus, helping bacteria acquire novel metabolic abilities and, in turn, facilitate bacterial speciation.

5 Bacterial Vesicles as Possible Source of HGT

Vesicles derived from either the plasma membrane or the outer membrane (OM; in Gram-negative bacteria) are not uncommon in the prokaryotic world and may play a role in the active transport of a variety compounds, including amino acids and sugars (Short et al. 1972); they could even represent a mechanism by which, in environments such as the open ocean, a variety of relevant genes can be released, transported to great distances, and taken up by far away bacteria (Biller et al. 2014; Soler et al. 2015; Tashiro et al. 2017). The OM-derived vesicles can even play a DNA protective role in thermophilic environments, as shown by Blesa and Berenguer in 2015 for *Thermus* spp.; they can also contribute to the virulence potential in *Acinetobacter baumannii* (Jha et al. 2017), or induce protective immunity against salmonid rickettsial septicemia in *Piscirickettsia salmonis* (Tandberg et al. 2017). The

extracellular vesicles are often released (at least in Gram-negative bacteria) to alleviate cell surface stress (McBroom and Kuehn 2007). On the other hand, intracellular vesicles in *Azotobacter vinelandii* (Cagle et al. 1972) are involved in the reproduction of cell wall-deficient, L-form bacteria (Briers et al. 2012), or in intracellular compartmentation in the *Planctomycetes* (Fuerst 2005; Feijoo-Siota et al. 2017).

The vesicles can contain different bacterial components, including DNA, RNA, proteins, and parts of the bacterial cell wall, such as peptidoglycan or lipopolysaccharides, possibly resulting from cellular lysis (Habier et al. 2018).

Recently, bacterial vesicles have been involved in the modulation of gene expression in colon carcinoma cells (Vdovikova et al. 2018), and even in gene communication with human cells in a clear example of HGT between bacteria and higher organisms (Shen et al. 2012). Kahn et al. published in 1979 one of the first reports describing that *Haemophilus parainfluenzae* can produce outer membrane-derived vesicles with DNA binding; and in 1982 Concino and Goodgal described that DNA-binding vesicles, released from the surface of *H. influenzae*, could attach to homologous DNA. In 1999, Kolling and Matthews showed that *E coli* O157:H7 could export both virulence genes and Shiga toxin by membrane-derived vesicles, thus establishing that this mechanism can perform HGT in bacteria. Accordingly, Renelli et al. (2004) reported DNA inside natural membrane-derived vesicles in *Pseudomonas aeruginosa* PAO1, and involved them in the transfer of genetic information between cells. Vesicle-contained genetic information is also believed to be involved in biofilm formation in this bacterium (Nakamura et al. 2008). In 2011, Velimirov and Hagemann provided evidence that outer membrane-derived vesicles, in *E. coli* AB1157, can carry DNA fragments of at least 370 kbp. In addition, Nakayama-Imaohji et al. suggested, in 2016, that the type of outer membrane vesicle generated, at least in *Bacteroides fragilis*, can be controlled by specific DNA inversions in the bacterial genome.

Outer membrane-derived vesicles can also contain RNA; in fact, it was known since 1977 that artificial lipid vesicles could incorporate high molecular weight RNA (Ostro et al. 1977). After this finding, it was only a matter of time to discover that microbes could communicate by means of membrane-derived vesicles loaded with RNA (Blenkiron et al. 2016). At least, this is the possibility proposed by Tsatsaronis et al. in 2018. The authors suggested that this type of transmission could represent a universal communication mechanism, used not only in the prokaryotic world but also in eukaryotic organisms; they even proposed that pathogenic microorganisms can use this process as a means to modulate the host immune response. Both eukaryotic exosomes and bacterial outer membrane-derived vesicles can incorporate small RNAs, similar to microRNAs, that could play transcriptional or translational inhibitory roles. All facts taken together, this HGT mechanism could represent a novel cellular communication pathway, and even an inter-kingdom communication highway (Lefebvre and Lécuyer 2017; Choi et al. 2017).

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Alternative Ways to Exchange DNA: Unconventional Conjugation Among Bacteria

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Abstract In addition to asexual reproduction, many prokaryotes contain specific apparatus evolved to exchange DNA through their envelopes by direct cell-to-cell contacts, allowing the horizontal transfer of genes, usually between phylogenetically related species in a sexual-like process. These contact-dependent processes are called "conjugation" to differentiate them from other ways of DNA acquisition like transformation, mediated by DNA import systems, or transduction, mediated by defective phages.

The conjugation apparatus has been studied in great detail in *Proteobacteria*. As described in previous chapters, it is based on a DNA mobilization apparatus that selects and cuts one of the strands of the transferred DNA in the donor strain at a specific site (*oriT*), binds a protein covalently to the 5′ extreme, and delivers the ssDNA–protein complex to a type IV secretion system (T4SS), which is responsible for its injection into the recipient cell. However, the absence of homologues to essential components of these elements in the genomes of many bacteria in which conjugation has been described to occur soon revealed the existence of unconventional conjugation mechanisms. Conjugation in *Streptomyces* involves the transfer of dsDNA instead of ssDNA and depends on a single DNA membrane translocase. In mycobacteria and mycoplasma, conjugation leads to the simultaneous transfer of large DNA fragments from several points in the chromosome and the generation of diverse chimeric progeny. Also in *Thermus thermophilus*, a transformation-dependent conjugation process allows the simultaneous transfer of every gene in a bidirectional process. In this chapter, we will focus on these so-called "unconventional" pathways of conjugation which, at the end, could be much more frequent as originally thought.

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1 Introduction

As described in the previous chapter of this book, the availability of thousands of genomes and the development of increasingly powerful algorithms for its bioinformatics analysis have allowed the phylogenetic examination of individual genes and gene clusters among a widely diverse and large number of genomes. As a consequence, several genes have been identified in individual genomes that show different phylogeny from the core genes of the species, like those encoding ribosomal RNA and ribosomal proteins, leading to the conclusion that they have been acquired horizontally from other organisms. In some cases, these genes still present a DNA composition (G+C content, tetranucleotide composition, etc.) different from that of the core genome and are flanked by repeated sequences, in many cases derived from tRNA genes, or insertion sequences. Actually, many of these genetic islands are part of active or inactive mobile genetic elements (MGE), the main factors that allow the establishment of phylogenetically distant genes into the new host.

Among these mobile elements, conjugative plasmids (CPs) and integrative and conjugative elements (ICEs) play a major role in HGT in well-described model bacteria. In their more complete version, both encode all the components required for the recognition and conjugative self-transfer to a recipient cell, which has a passive role in the process. Upon entrance, CPs circularize and replicate in the new host, whereas ICEs integrate into specific, usually a tRNA gene, or unspecific sites through self-encoded specific virus-like integrases or IS-like transposases. As both elements are recruited during their spreading history through transposition-related events (insertion sequences and transposons), acquisition of these elements provide the new host with selectable properties to thrive in hostile environments, contributing, for instance, to the diffusion of antibiotic resistances within the population.

The use of bioinformatics tools for the search of homologues to the most conserved components of the conventional conjugation systems, that is, relaxases, coupling and secretion ATPases, together with other structural components of type IV secretion systems, has allowed the identification of conjugation apparatus in many bacteria. However, in some bacteria for which conjugation-like transfer has been reported, no homologues to these elements appear codified in their genomes. This is the case of, for example, species from the genera *Mycobacterium*, *Streptomyces*, *Mycoplasma* or *Thermus*, where different conjugation models exist which share little to no similarities at all to the conventional known ones. In this chapter, we will review the most relevant information known at present regarding these unconventional mechanisms of conjugal transfer.

2 Conjugation in *Mycobacterium*

Rather closer to Gram-negative di-derm architecture than to typical Gram-positive (*Firmicutes*) cell envelopes, the mycobacteria harbour unique external lipid-rich structures known as mycomembranes, formed by mycolic acids bound to the peptidoglycan layer, which determine many of their biological properties (Chiaradia et al. 2017). This hydrophobic layer shows minimal permeability to hydrophilic substrates and constitutes a strong barrier that protects the cells from the environment, not only preventing the entrance of toxic antimicrobial agents but also hindering the transport of nutrients and posing great difficulties to the transport of complex molecules such as DNA. Actually, the comparative analysis of the genomes of different isolates of the *M. tuberculosis* group, which includes slow-growing pathogenic isolates, shows a high degree of identity among them, supporting both their recent clonality and the absence of efficient mechanisms of HGT (Gray and Derbyshire 2018; Gray et al. 2013). In contrast, different isolates of *M. smegmatis* show a greater diversity with large quantities of SNPs along the genome and strain-specific genes which point to the existence of frequent recombination events at the chromosomal level. Regarding plasmids, few of them have been identified among the mycobacteria, especially scarce among different non-pathogenic fast-growing strains (Stinear et al. 2004; Kirby et al. 2002), but they are apparently not transferable. Actually, only one plasmid has been described that can be transferred by conjugation (Ummels et al. 2014) through a yet unknown system that could be related with that of the chromosomal transfer, as it encodes homologues to T4SS and also to mycobacteria ESX secretion systems.

The main mycobacterial model to study HGT is *M. smegmatis*. Its relatively fast-growing capability, the possibility of transformation of certain strains, its character as non-pathogenic, and the high gene synteny with several of the slow-growing pathogenic strains favoured its use in laboratories (Gray and Derbyshire 2018; Gray et al. 2013; Parsons et al. 1998). On top of laboratory-conducted episomal conjugation in this species, a new unconventional model for conjugation-like mechanism was identified by Parsons et al. (1998). In this study, the authors showed that large fragments of chromosomal DNA could be mobilized in an unidirectional fashion from a donor to a recipient cell grown on a solid medium, in a way that initially resembled the chromosomal transfer of high frequency of recombination strains (*Hfr*) of *E. coli*. However, contrary to the ordered transfer of genes from the *oriT* locus inserted in the chromosome of the *Hfr* strains, which means that every gene has an associated transfer frequency that decreases with the distance from the *oriT*, all the genes of the *M. smegmatis* donor were transferred basically at similar efficiencies, regardless of their location in the chromosome (Wang et al. 2005). This suggested the presence of multiple chromosomal *cis*-acting sites from where DNA transfer was simultaneously initiated. Further whole genome analysis of the transconjugants based on SNPs between donor and recipient chromosomes revealed an unexpected mosaic structure, where DNA segments clearly belonging to the donor strain were randomly scattered in fragments of different sizes (69 bp to >200 kbp) around the

chromosome of the recipient cell, generating an outstanding genetic variation (Gray et al. 2013). In some cases, these chimeric genomes contained up to 25% of the donor chromosome distributed over 30 separate *loci* of the recipient cell, leading to the name of Distributive Conjugal Transfer (DCT) to describe this unconventional conjugation process (Gray et al. 2013; Derbyshire and Gray 2014). Actually, the transconjugants that risen from this process also show micro-mosaicism, generated by minor changes at the level of nucleotides or small number of nucleotide replacements that are not easy to explain, but that could be produced by DNA repair processes, interspersing donor and recipient genomes as templates (Derbyshire and Gray 2014).

From the diverse progeny obtained from a single mating experiment, a minor fraction acquires the capability to become donors, thus allowing them to transfer their genome to the parental recipient strain and with other transconjugants, increasing the complexity of the population. This feature also contrasts with the *Hfr* conjugation, where the recipient cells do not acquire the capability to act as donor cells (Wang et al. 2005).

The molecular mechanism resulting in DCT is not yet described. In silico studies have evidenced the absence of homologues to the T4SS in the genome of *M. smegmatis* donors. The identification of the genes needed for DCT was carried out by transposon mutagenesis of both the donor and the recipient strains, which showed that in all the cases reported the DCT mutants were affected in the ESX1 and ESX4 secretion systems (Gray and Derbyshire 2018; Coros et al. 2008). ESX secretion systems, also known as type VII secretion systems, are typical of mycobacteria where different types of these sophisticated effector complexes exists, in some cases in multicopy (Gröschel et al. 2016). Each ESX encodes several proteins including integral membrane proteins, ATPases, chaperons, and specific protein substrates. One of the most interesting observations from the analysis of these mutants was the asymmetry of the results: whereas mutants of either the ESX1 or ESX4 systems in the recipient cells blocked the DCT, mutants in the donor had no effect (ESX4 mutant) or produced a hyper-conjugative phenotype (ESX1 mutant). Thus, instead of a conventional system in which the donor is actively transferring the DNA to a passive recipient cell, in mycobacterial DCT it is the recipient cell which plays a major active role in the process (Fig. 1a).

It has also been demonstrated that transcription of genes encoding the EXS4 system in the recipient cell is greatly activated by direct contact with the donor in a yet unknown pathway that depends on the ESX1 system which apparently encodes a repressor of the system (Flint et al. 2004). This suggests the existence of some class of signalling after contact with the donor which is detected by the recipient cell that sparks the regulatory circuit, as described, for example, for the regulation of competence in Gram-positive bacteria. It is also noteworthy that *sex* determination (donor vs. recipient) in mycobacteria actually depends on the variant genes encoded by the ESX1 secretion system, as the small percentage of the transconjugants that acquire the ability to behave as donor cells are those in which the donor ESX1 system has replaced that of the recipient cell (Coros et al. 2008; Flint et al. 2004).

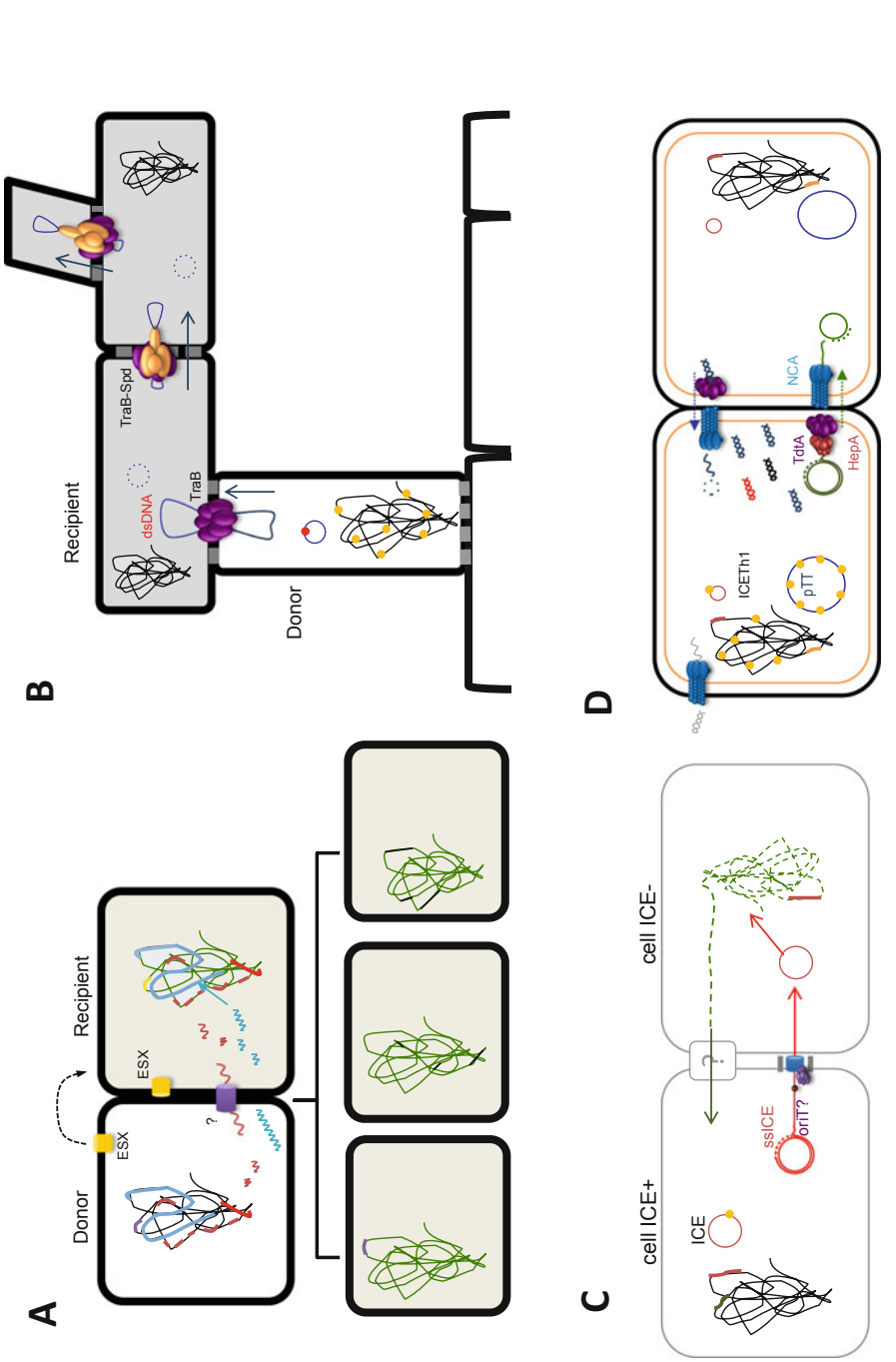

Fig. 1 Unconventional conjugation models. (**a**) In *Mycobacterium* an unknown signal is produced by the donor upon contact with a recipient cell that induces the overexpression of the ESX4 secretion system in the recipient cell. The ESX4 of the recipient cell is responsible for further actions, including those leading to the formation of a DNA transport channel. Chromosomal DNA of the donor is recognized at multiple points by an unknown system, allowing the simultaneous transfer of different chromosomal regions. The progeny has different chimeric genomes containing up to 25% of genes transferred from the donor. (**b**) In

Despite all this knowledge, the actual mechanism involving the *sex* determination, the selection of the sites along the chromosome to initiate the transfer, the mechanism by which these sites are cleaved (and further repaired), the actual substrate for the transfer (ss- or dsDNA), and the way through which such DNA is secreted, crossing the complex envelopes of both donor and recipient strains, are not known at all.

3 Conjugation in *Streptomyces*

Another relevant actynobacterial genus that shows a quite different mechanism of conjugation is *Streptomyces*. Conjugative *Streptomyces* and related genera contain CPs and ICEs of different sizes that are self-transferable on solid medium to plasmid-free recipient strains with efficiencies close to 100%. Also, the presence of these cryptic plasmids allows the transfer of chromosomal genes, although with and efficiency two to three orders of magnitude smaller than those of the plasmid (Kieser et al. 1982).

The transfer of these plasmids was initially detected by the formation of "pock structures", defined as circular inhibition spots of 1–3 mm of diameter within a lawn of recipient cells devoid of plasmid (Thoma and Muth 2015). These pocks are the consequence of the delayed growth of recipient cells that received the CPs due to the inhibitory effects produced by some of the proteins that they encode (Thoma and Muth 2012, 2015).

The sequence analysis of these CPs did not show genes encoding homologues to the proteins described as essential for canonical conjugation in any of the *Proteobacteria* models. Even, neither relaxases nor T4SS components were encoded in these plasmids (Thoma and Muth 2012). Actually, deletion studies demonstrated that a single DNA region of around 3 kbp was sufficient for conjugation, although other regions in the vicinity of this essential one had some influence in the size of the

Fig. 1 (continued) Streptomyces, conjugative plasmids provide the TraB translocase that recognizes the origin of transfer in the plasmid (red dots) and in the chromosome (orange dots). The DNA is cleaved by unknown proteins, and dsDNA is translocated to the recipient cells. There, the plasmid is spread through intramycelial walls with the help of TraB–Spd protein complexes. Chromosomal DNA is likely to recombine with the chromosome, and no spreading is expected. Figure adapted from Grohmann et al. (2017). (**c**) In *Mycoplasma*, the presence of an ICE provides the cell with a simple DNA transport machinery related to T4SS. In the recipient cell, the ICE likely replicates and integrates at multiple points allowing the retro-transfer of different chromosomal fragments that further recombine leading to chimeric progeny. Figure adapted from Citti et al. (2018). (**d**) In *T. thermophilus*, transjugation depends on the presence of ICEth1, which encodes a membrane translocase (TdtA). Multiple sites are recognized in the genome likely by a restriction enzyme, and DNA is extruded from the donor cell. In the recipient cell, the NCA is used to import the DNA, and homologous recombination leads to the new progeny. Figure adapted from Blesa et al. (2018). See the text for details

pocks, as observed in several conjugation experiments. This region encoded a protein—TraB—with domains of significant homology to FtsK, the DNA translocase involved in chromosome segregation during septation of *Proteobacteria* (Thoma and Muth 2012; Reuther et al. 2006).

In addition to TraB, the conjugative transfer of plasmids in *Streptomyces* spp. required a 50-bp sequence that did not encode any detectable protein (Thoma and Muth 2012). This *cis*-acting *l*ocus of *t*ransfer, or *clt*, seemed to play a role similar to that of the *oriT* in conjugative plasmids of other bacteria. Interestingly, the *clt* locus was not required for the transfer of chromosomal genes, supporting the existence of an alternative mechanism of chromosome transfer different to that of the conjugative plasmid (Vogelmann et al. 2011). Noteworthy, homologues to TraB and *clt* sites are found in most CP and ICE from the *Actinomycetes* phylum (Te Poele et al. 2008). Despite these TraB proteins have low sequence identities, structural predictions support a similar architecture and domain distribution. Moreover, in all these proteins, the N-terminal domain is likely involved in membrane binding, an intermediate ATPase domain similar to that found in FtsK with the corresponding Walker A and B motifs could provide the energy for the translocation and a C-terminal domain with a DNA helix–turn–helix (HTH)-binding motif is responsible for the identification of specific sequences at the *clt* sites (Vogelmann et al. 2011; Te Poele et al. 2008).

Electron microscopy of purified TraB protein from plasmid pSVH1 of *S. venezuelae* revealed a ring-shaped hexameric structure similar to that of FtsK, with a central pore of 31 Å (Reuther et al. 2006). A pore of such size could allow the use of dsDNA as substrate. Indeed, based on these data, it is assumed that TraB functions in a similar way to FtsK, promoting the translocation of dsDNA (see below). However, clear differences in the respective way of action are derived from the comparative analysis of their domains. Whereas FtsK does not require the passage of the DNA across the membrane as it functions before the septum has been completed, TraB has to promote the transfer of DNA across two complex cell envelopes: those of the donor and the recipient cells. In this sense, it has been shown that TraB spontaneously forms pores in lipid bilayers, thus supporting its capability to allow the transfer of DNA through a membrane. Also, its ability to bind peptidoglycan (Vogelmann et al. 2011) suggests the existence of a periplasmic domain not yet identified, involved in fixing this pore-forming DNA translocase to a rigid structure, likely limiting its lateral diffusion to other cell locations. A second difference between TraB and FtsK is related to the presence of the C-terminal HTH motif in TraB. It has been shown that this motif is responsible for the specific recognition of the *clt* locus required for the mobilization of its coding CP (Reuther et al. 2006). Actually, the exchange of this motif produces the exchange in plasmid specificity (Thoma and Muth 2015; Vogelmann et al. 2011). Analysis of the *clt* locus of different plasmids reveals the existence of aprox. 8-mer sequence repeats, with only two of them actually needed for the binding of TraB in in vitro assays (Vogelmann et al. 2011).

As stated above, the *clt* locus is needed for the mobilization of the plasmid but has no effect in the transfer of the chromosomal genes. Bioinformatics analysis revealed

the presence of 25-bp sequences homologous to the *clt* locus of plasmid pSVH1 distributed along the chromosome of *S. coelicolor* (Thoma and Muth 2015; Pettis 2018). Therefore, the hypothesis was that the transfer of chromosomal genes is the consequence of the recognition of these chromosomal sites, named *clc*, by the TraB protein and not due to the integration of the plasmid into the chromosome, as it happens in *Hfr* strains of *Proteobacteria*. Following this hypothesis, the transfer of the plasmid and the chromosomal genes could be the consequence of the independent binding of TraB to *clt* and *clc* sites, likely with different affinities, and the promotion from these sites of the gene transfer in a way similar to the formation of a relaxosome at the *oriT* site in conventional conjugation (Thoma and Muth 2015; Pettis 2018; Sepúlveda et al. 2011). However, it is noteworthy within this context that, contrary to the relaxase, binding of TraB does not produce any cut in the DNA, thus suggesting that unknown helper proteins could be involved at this step.

A major difference between conventional conjugation and the equivalent process in *Streptomyces* spp. is that the translocated DNA substrates are apparently double stranded. The evidence for this lies in the employment of a strain expressing a restriction enzyme (*SalI*) as the recipient cell and conjugative plasmid derivatives in the donor with or without a restriction site for this enzyme in conjugation experiments (Possoz et al. 2001). As only the plasmid without the restriction site could be transferred, the conclusion was that conjugation involved the transfer of dsDNA instead of ssDNA, used as substrate in canonical conjugation processes. Actually, this conclusion is in agreement with the abovementioned data regarding the size of the pore formed by TraB hexamers, wide enough as to accommodate dsDNA as substrate (Thoma and Muth 2015).

However, the main unsolved problem to understand the conjugation of *Streptomyces* is the mechanism used by this dsDNA to cross the cell walls of donor and recipient cells. Experiments involving GFP-based subcellular localization of TraB led to the conclusion that the protein was basically found at the tip of the hyphae, supporting that intermycelial conjugation takes place at these contact points (Thoma and Muth 2012, 2015). However, more recent evidences through combination of fluorescent markers suggest that conjugative intermycelial transfer takes place via the lateral walls (Thoma et al. 2016) with further spreading across the filaments. Nevertheless, and independently from its location, the multilayered cell walls of *Actinomycetes* hinder the understanding of the translocation of dsDNA by a single DNA translocase across the thick peptidoglycan layers of donor and recipient cells and the further transport through the cytoplasmic membrane of the later (Pettis 2018; Sepúlveda et al. 2011). Despite this, it has been clearly shown that intermycelial transfer of conjugative plasmids requires only TraB and the *clt* locus (Vogelmann et al. 2011). A more likely explanation includes the participation of other proteins in the process, encoded by the chromosome of the donor and/or recipient strains that could facilitate the generation of intercellular bridges through peptidoglycan-free spots in the cell walls (Thoma and Muth 2015). In this scenario, it is possible that peptidoglycan-remodelling enzymes could locally digest the cell walls of donor and recipient cells at the points of contact, being these the tip of the hyphae or the lateral walls (see above), thus providing an opportunity for the TraB protein to generate

membrane fusions between both partners. Actually, its protein homologue SpoIIIE of *Bacillus subtilis* has been shown to generate membrane fusions (Sharp and Pogliano 1999), although this property has not been shown yet for the TraB protein itself. Otherwise the requirement of only the TraB protein for intermycelial transfer could not be explained.

In contrast to this TraB-only dependence of intermycelial transfer, the intramycelial spreading of the CPs from *Streptomyce*s spp. requires a group of the so-called Spd proteins encoded by the conjugative plasmids in addition to TraB (Thoma and Muth 2015; Reuther et al. 2006). The use of bacterial two-hybrid assays and protein cross-linking has provided evidence of the interaction between many of these proteins from plasmid pSVH1 and also with its encoded TraB protein, suggesting the formation of a complex DNA translocation machinery at the intercellular walls of the plasmid-infected mycelium (Thoma and Muth 2015).

Finally, it is worth to mention the existence of an ever-growing number of ICEs and CPs in *Actinomycetes* which encode homologues of TraB and other plasmid genes found in well-known conjugative plasmids of *Streptomyce*s spp. (Pettis 2018), suggesting that the unconventional mode of conjugation that we have described above is the rule and not the exception in this large group of bacteria. A scheme of the conjugation process in *Streptomyce*s spp. is depicted in Fig. 1b.

4 Conjugation in *Mollicutes*

Mollicutes or mycoplasmas (trivial name) are a bacterial class that have originated from low G+C Gram positives by losing the capability to synthesize peptidoglycan during adaptation to parasitic or symbiotic lifestyle within eukaryotic hosts (Razin et al. 1998). In addition to the lack of peptidoglycan, the evolution of mycoplasmas has led to the loss of several biosynthetic capabilities including that of amino acids, vitamins and nucleotides or even pathways for DNA repair (Rocha et al. 2005). Actually, the main evolutionary selection for mycoplasmas was thought to be for many years this gene reductionism. However, once the genome of different mycoplasmas was available the existence of relevant amounts of genes transferred from other mycoplasmas was evident, pointing to the existence of HGT mechanisms that could facilitate their rapid adaptation to new hosts by the acquisition of specific traits (Sirand-Pugnet et al. 2007; Pereyre et al. 2009). Indeed, far from being marginal, these genomic comparative studies have revealed massive HGT events among mycoplasmas, even between phylogenetically distant ones that share similar habitats, showing, for example, that 18% of the genome of *Mycoplasma agalactiae* had been likely acquired from the Mycoides subgroup of mycoplasmas (Sirand-Pugnet et al. 2007).

In addition to these bioinformatics-based conclusions, the actual laboratory demonstration of HGT between mycoplasmas was first described for the transfer of resistance mutations in *Spiroplasma citri* (Barroso and Labarere 1988) followed by that of transposons in *M. pulmonis* (Teachman et al. 2002). More recently

M. agalactiae has been adopted as the laboratory model for the study of HGT within mycoplasmas due to its easier cultivation under laboratory conditions (Citti et al. 2018). Based on the use of this laboratory model combined with whole genome sequencing, a major conclusion arose that the HGT events detected depended essentially on cell-to-cell conjugative processes which somehow resembled that of those found in mycobacteria: transconjugants also showed a mosaic-like genome with fragments from both the donor and the recipient cell. As mentioned before, in mycobacteria this type of conjugation is called Distributive Conjugal Transfer or DCT and responds to an unconventional mode of conjugation capable of initiating the transfer from several sites in the chromosome. Actually, *M. agalactiae* can transfer large chromosomal fragments including any gene from multiple sites in the chromosome, likely in a simultaneous manner, with further integration by homologous recombination (Dordet-Frisoni et al. 2013). However, under laboratory conditions the frequency of these conjugative events is quite low (10^{-7} per donor cell), sowing doubts on the actual biological relevance of HGT between mycoplasmas in nature. In contrast, in species such as those found within the *Ureaplasma* genus bioinformatics have shown that 40% of clinical isolates of *U. parvum* and *U. urealyticum* were actually genetic mosaics from different serovars, supporting that conjugation has a major role in the adaptive process of these clinical strains (Xiao et al. 2011).

The mechanism underlying conjugation in mycoplasma is basically unknown. The first hypothesis raised was the existence of fusions between the cytoplasmic membranes of donor and recipient cells, taking advantage of the absence of cell walls (Barroso and Labarere 1988). However, more recent electron microscopy studies have revealed close contacts between both membranes, but not the presence of fusions or any other structure that could resemble a T4SS-related system (Citti et al. 2018), supporting that a less complex DNA transfer system may be acting. In this context, bioinformatic analysis of the genomes of conjugative versus non-conjugative strains of mycoplasmas revealed the presence in the former of homologues to ICE-encoded proteins. In fact, human parasitic species such as *M. pneumoniae* and *M. genitalium* that are genetically very homogeneous do not contain any ICE in their chromosome, in contrast to the highly diverse *Ureaplasma* genus that contains a panoply of ICEs (Dordet-Frisoni et al. 2014).

In other systems, ICEs encode the genes required for the integration, excision and conjugal transfer of the element to a passive recipient partner, where they integrate as transposons and phages (Burrus and Waldor 2004; Wozniak and Waldor 2010). Comparative studies revealed a similar overall organization in most ICEs from mycoplasmas (MICEs), which have a size between 20 and 30 kbp and encode around 20 genes, all in the same orientation. These mycoplasma ICEs are bordered by inverted repeats alike those recognized by transposases (Tardy et al. 2015). In fact, a DDE transposase is always present among the common genes or minimal backbone of these ICEs, likely participating in the excision and subsequent random integration of the ICE in the host chromosome (Dordet-Frisoni et al. 2014). Regarding other ICE-encoded genes, only a few of them show homology to conjugation-related proteins of conventional systems, such as the coupling TraG protein, a

ssDNA-binding protein, and a TraE homologue that likely participates in DNA transport (Citti et al. 2018). Other genes present in ICEs encode a set of membrane proteins that could be required for the generation of a channel between the donor and the recipient cells. Therefore, and despite our current knowledge on the functionality of these ICE-encoded proteins is very limited, it is likely that these elements play a role in mycoplasma's conjugation similar to those conventional ICEs that play in proteobacterial models (Dordet-Frisoni et al. 2014).

Based on the present data, a tentative conjugation model has been proposed for mycoplasmas (Fig. 1c) in which, upon unknown activation signals, the resident conjugative ICE excises from the chromosome of the donor, putatively replicates through a rolling circle mode, and, through a yet unidentified relaxase-like protein, mobilizes a ssDNA copy to an ICE-encoded secretion channel, reminiscent but much simpler than T4SS, which concomitantly transfers a putative ssDNA–protein complex to the recipient cell. In this process, cell-to-cell attachment involves an ICE-encoded surface lipoprotein instead of a type IV pili (Dordet-Frisoni et al. 2014). Therefore, such system could be a basic form of a conventional conjugation apparatus, despite no putative *oriT* sites have been yet identified in these mycoplasma ICEs.

A major difference between mycoplasma's and conventional ICEs is the fact that the conjugative transfer seems to be bidirectional in mycoplasmas and involves not just the ICE but also several chromosomal genes. Moreover, contrary to the expectations, the chromosomal segments are apparently transferred from the ICE-less recipient cell to the ICE-bearing initial donor (Dordet-Frisoni et al. 2013). Therefore, it has been hypothesized that the transfer of ICE from the ICE+ donor to the ICE− recipient was followed by its extensive replication in the new host and integration of the copies into the chromosome in a random basis. Further retro-transfer from the insertion site could involve the mobilization of chromosomal genes. However, other alternative mechanisms could exist to explain this apparently retro-transfer of chromosomal DNA from the recipient to the donor cells.

The lack of efficient repair systems together with the frequent mutations and deletions attacking these minimal genomes might shed light onto the relevance of conjugative HGT as a means to rescue genetic information from a pangenome reservoir in mycoplasma subpopulations living in common environments, sowing doubts on the actual concept of minimal cell to the broader context of flowing information.

5 Conjugation in *Thermus* spp.

We have just witnessed that small genomes can conceal an ample diversity of traits by erecting an extensive accessory pangenome preserving genetic information. This becomes critical for organisms living under high temperatures where the size of their genome is limited by the burden of accurate DNA replication and the possibility of

acquisition of genes from other organisms could be essential for rapid adaptation to environmental conditions (Brüggemann and Chen 2006).

The phylogenetically ancient genus *Thermus* is an example of adaptive success in thermophilic environments, as representatives have been isolated everywhere a thermal environment is set, regardless this being natural or artificial (Da Costa et al. 2006). In addition to thermal adaptation from moderate to high temperatures (50–75 °C), different strains show a panoply of metabolic capabilities, with aerobic and facultative strains able to use different electron acceptors (Da Costa et al. 2006). The genomes of most of the already sequenced strains consist of a small chromosome of around 2 Mbp and at least one megaplasmid (>200 kbp), although there are exceptions without (Gounder et al. 2011) or with another smaller megaplasmid (60–100 kbp). The comparison among *Thermus* spp. genomes has revealed a high degree of synteny of chromosomal genes and a major diversity concentrated in the megaplasmid(s), including genes involved in anaerobic respiration and the use of carbohydrates (Brüggemann and Chen 2006). In addition, phylogenetic analysis support that the genus has acquired many genes from thermophilic archaea that are likely involved in the adaptation to the extreme thermophile lifestyle (Brüggemann and Chen 2006; Averhoff 2009). Indeed, these and more recent studies have addressed a massive presence of mobile genetic elements such as *i*nsertion *s*equences (IS), both active and inactive, that could facilitate the acquisition of genes with lower or no sequence identity to resident ones and contribute to the selection of new phenotypic traits (Blesa et al. 2019).

The extreme thermophilic strains *T. thermophilus* HB8 and HB27 have been used as the main laboratory models to experimentally explore the HGT processes in the genus due to their fast growth under laboratory conditions and their genetic amenability, related to their highly efficient natural competence system (Cava et al. 2009). Both strains harbour a well-conserved chromosome of around 2 Mbp and a megaplasmid of around 250 kbp (pTT27) that concentrates the highest genetic differences between both strains (Brüggemann and Chen 2006; Blesa et al. 2019). An additional megaplasmid (pVV8) and a small plasmid (pTT8) are specific of the HB8 strain. The *n*atural *c*ompetence *a*pparatus (NCA) of *T. thermophilus* HB27 has been studied by a combination of both genetic and biochemical analysis (Averhoff 2009). In contrast to other naturally competent bacteria, the capability to incorporate DNA in this strain and in other *Thermus* spp. so far studied is expressed in a constitutive manner along the bacterial growth phases (Koyama et al. 1986), showing DNA incorporation rates of 40 kbp/s/cell in HB27 strain, the fastest ones so far measured for any bacteria (Averhoff 2009). Also, this NCA is not discriminative at all and can transport inside the cell any DNA, independently of its origin (Averhoff 2009).

The NCA of *T. thermophilus* HB27 is quite complex with at least 16 proteins encoded in 5 different chromosomal *loci* involved in the control and assemblage of a type IV pili (T4P) required for both twitching motility and natural competence, in addition to a series of proteins specifically required for natural competence. A few of these proteins show homology to components of the natural competence machinery described in Gram-positive bacteria [for a detailed review, see Chen and Dubnau

(2004) and Averhoff and Friedrich (2003)], suggesting that the incorporated molecules in the cell are actually ssDNA, despite this point has not been experimentally addressed yet. It has also been shown that the T4P apparatus are mainly located at the cell poles (Wall and Kaiser 1999).

In addition to its role in the transport of extracellular DNA, the NCA has been shown to be required by two other forms of HGT in *T. thermophilus*: vesicle-mediated HGT and cell-to-cell conjugative-like processes (Blesa et al. 2018). HGT based on vesicles is a low-frequency process under laboratory conditions but could have a major relevance in thermal environments due to the protective role that membranes have over the DNA under these conditions, not only against DNAses but also against the thermal unfolding, allowing the resilience of vesicle-protected eDNA in time and distance (Blesa and Berenguer 2015).

The first report on conjugal DNA transfer among *T. thermophilus* strains was described by Ramirez-Arcos et al. (1998) who demonstrated the transfer of the ability to respire nitrate from a partial denitrifying strain (NAR1) to the aerobic strain HB27 in a DNAse-resistant process that required direct cell contacts between the donor and the recipient. Interrupted mating experiments showed a delay between the transfer times of nitrate respiration cluster with respect to other genes, and a cryptic replicative origin was identified immediately downstream of the nitrate respiration genes, supporting the hypothesis that conjugation was similar to that of high frequency of recombination (*Hfr*) strains of *Escherichia coli* (Ramirez-Arcos et al. 1998), in which a nitrate respiration conjugative element (NCE) inserted into the chromosome of the donor strain was responsible for the transfer. Later sequencing of the DNA transferred to the new nitrate respiring HB27 derivative revealed that the NCE apparently encoded just the genes for the nitrate respiration and its control, but no homologues of conjugation-related genes (Cava et al. 2007). Further experiments involving matings between HB27 (recipient) and a complete denitrifying *T. thermophilus* strain (PRQ25) also lead to the isolation of HB27 derivatives encoding the capability to denitrify from nitrate to N_2O, and NGS-based sequencing revealed the acquisition of a large *d*enitrification *i*sland (DI). Such DI included a homologue of the NCE and a cluster of genes encoding nitrite and nitric oxide reductases (Alvarez et al. 2014). Sequence revealed also that the DI was not inserted in the chromosome as firts hypothesized (Ramirez-Arcos et al. 1998), but was located in a highly variable region of the pTT27 megaplasmid containing several IS (Alvarez et al. 2014). In addition, these sequence analyses revealed the absence in the recipient and in the donor strains of homologues to conventional conjugation genes associated with the DI or with the genome, thus suggesting the existence of a conjugative apparatus completely different from the conventional one.

Actually, matings involving only derivatives of the HB27 strain revealed the existence of contact-dependent DNA transfer of antibiotic resistance markers independently of being located in the chromosome or in the megaplasmid. The efficiency of the transfer for a given gene marker was close to 10^{-4} per recipient cell, which is higher to that provided by the natural competence of this bacterium. Further detailed analysis of the system revealed several differences compared to conventional conjugation. First, it was shown that DNA could be mobilized in both directions without

the existence of any mechanism of surface exclusion, in such a way that both partners act simultaneously as donor and as recipient in the mating experiments, making the parenthood analysis of the progeny extremely difficult. Second, the transfer was not gene-specific, mobilizing any gene within the chromosome or the megaplasmid, but with a tenfold preference for genes located in the megaplasmid compared to genes encoded in the chromosome (Blesa et al. 2015). However, there was no detectable order in the transfer of genes located in the same genomic element, as if all the genes were transferred simultaneously. Finally, it was shown that the transfer was dependent on the activity of the natural competence apparatus of the recipient cell, in such a way that matings between two mutants in relevant components of NCA produced no transconjugants (Blesa et al. 2015). This led to the proposal of a "push and pull" model in which an unknown component of the system in the donor cell somehow selected and cut the genome at several points and pushed the fragments out of the donor cell, whereas the recipient cell actively incorporated the transferred DNA in a DNAse-resistant form (Blesa et al. 2015, 2018). The new model was further renamed as "transjugation", for *trans*formation-dependent *conjugation* (Blesa et al. 2017a).

Under the likely hypothesis that such an efficient process of DNA secretion in the donor would require energy, multiple orphan proteins harbouring ATPase motifs were checked for their putative involvement in the push step of transjugation (Blesa et al. 2017a). Finally, a putative DNA translocase (TdtA) belonging to the FtsK protein family was shown to be required in the donor but not in the recipient cells, being its null mutants similar in any other phenotypes assayed to the wild-type parental (Blesa et al. 2017a). Further electron microscopy of pure TdtA revealed that upon incubation at high temperatures with ATP the protein oligomerizes to form an hexameric ring with a central pore of around 32 Å, which is wide enough as to accommodate dsDNA (Blesa et al. 2017a), similar to what had been reported for the TraB protein required for conjugation in *Streptomyces* (see above).

However, sequence comparisons revealed the absence in TdtA of the C-terminal DNA-binding motif required in TraB for the recognition of the *clt* and *clc* sites. Also, there could not be detected in TdtA a membrane-binding domain that could presumably be required for the DNA translocation through the membrane. Despite this, the TdtA protein was shown to attach to the membrane, meaning that this capability exists. Also, the use of fusions to thermostable fluorescent reporters supported a subpolar localization for TdtA (Blesa et al. 2017a), a position close to that of the T4P involved in motility and competence in this bacterium (Averhoff 2009; Averhoff and Friedrich 2003; Wall and Kaiser 1999).

The absence of a DNA-binding motif that could help the TdtA protein to identify and bind to putative origins of transfer in the chromosome or in the megaplasmid supports the requirement for (a) helper protein(s) performing such task. TdtA is encoded by an operon that includes four proteins: a type IIG restriction endonuclease identical to *Tth111*II (Zhu et al. 2014), a putative nuclease of the NurA family, the *tdtA* gene, and a putative DNA methylase. Mutants in the restriction enzyme or in the nuclease but expressing TdtA show a severe decrease in conjugal transfer frequencies (three to four orders of magnitude) supporting that they are required for the

DNA transfer. As the recognition sequence for the restriction enzyme is known, it has been possible to map them in the genome showing a random distribution within the chromosome and the megaplasmid. Thus, the present model for the pushing step suggests that the restriction enzyme recognizes these sites immediately after replication, when they are hemi-methylated and use them as *oriT* for the transfer (Blesa et al. 2017a). However, there are many specific points in the model that require further investigation. In particular, the preference of the system for genes encoded in the plasmid which does not seem related to a higher density of restriction sites compared to the chromosome, but likely to a higher frequency of appearance of hemi-methylation events.

The four-gene cluster encoding TdtA and the capability for DNA donation are located within a small (approx. 14 kbp) ICE-like element bordered by 47-bp direct repeats corresponding to the 3′ extreme of a tRNA gene (Blesa et al. 2017a). This element, called ICEth1, can excise at low frequency from the chromosome and be transferred to the ICEth1-free HB8 strain, conferring the derivative the capability for DNA donation in transjugation experiments. However, the details of how frequent this event is with respect to the transfer of other genes are not yet clear.

In addition to the proteins encoded by the ICEth1, the pushing system also requires the help of at least other DNA-related processing enzyme (HepA) which is not encoded within ICEth1. The HepA protein is a homologue to the archaeal helicase HerA, which is involved in the repair of double-strand breaks, a role conserved in its eukaryotic homologues. In *T. thermophilus*, this protein of archaeal origin is required for the survival to high temperatures and UV radiation, but also for an efficient transjugation in the donor cells (Blesa et al. 2017b). The HepA protein has been purified and studied, showing an hexameric structure with an internal pore smaller than that of TdtA, clearly suggesting ssDNA as a substrate, as it has been shown for its *Sulfolobus* homologue (Byrne et al. 2014). Thus, it can be speculated that upon recognition of sensitive restriction sites by the restrictase, the strand breaks generated have to be repaired, playing HepA a role for it. Alternatively, this putative helicase could participate in the DNA pushing procedure at an unknown step (Fig. 1d).

Despite the proteins involved in DNA donation are under study, a major problem arises in order to explain how this apparently simple mechanism allows the transport of DNA not only through a membrane but also across the complex envelope of the donor cell, which includes a peptidoglycan layer and an outer membrane (Cava et al. 2007). From this envelope complexity it is clear that additional proteins are required to form a tubular channel for the transfer of, likely, dsDNA. Also, the NCA of the recipient cell has to be nearby located to be coupled to this secretion system, as the transferred DNA is resistant to DNAses (Blesa et al. 2015). Electron micrographs of mating pairs reveal a perfect contact between the cells (laboratory data), but do not reveal the existence of structures that could correspond to a mating channel. Future structural work involving the employment of cryo-electron microscopy will be required to solve this question.

Finally, it is worth to mention that the efficient transfer of DNA by transjugation somehow depends on the inactivity of a DNA surveillance system. This quality

control of the eDNA takes places through the Argonaute protein (ThAgo) encoded in the megaplasmid of many strains. Under transformation conditions, the ThAgo is active and, by a yet unknown mechanism, acquires ssDNA guides complementary to the entering eDNA and based on base pairing identifies the complementary strand where it generates a cut with a high efficiency (90% of entering DNA is eliminated by this system) (Blesa et al. 2015). In contrast, when the same DNA enters the cell via transjugation, the ThAgo does not interfere, allowing the incorporation of the entering DNA to the cells (Blesa et al. 2015, 2018). Therefore, by this way the ThAgo surveillance system distinguishes between putatively dangerous eDNA acquired from the environment and trustable DNA provided by a mating partner, making transjugation as the likely major HGT pathway for the rapid spread of traits among *Thermus* isolates. This way the actual pangenome of the genus could be much wider than expectable from the small size of the individual genomes (Blesa et al. 2015). The role of ICETh1 in this scenario could be to use this population sharing and spreading of selectable traits as a way for its co-selection among the transjugants (Blesa et al. 2017a).

6 Concluding Remarks

Among the classical modes of horizontal gene transfer among bacteria, conjugation is, by far, the most relevant in terms of environmental spreading capability of new traits due to the large amount of DNA that can be transferred after a single mating and to the ability of the recipient strain to become donor vehicles in many cases. Actually, CPs and ICEs are the main spreading platforms for the dissemination of antibiotic resistances that jeopardize our present arsenal of antimicrobial weapons.

Bacterial conjugation has been widely studied in a few laboratory models, especially in *Proteobacteria*, and our current knowledge on the mechanism is easily found in most textbooks of Microbiology, taken as the "conventional" mode of conjugation described in chapter "Horizontal Gene Transfer in Bacteria: An Overview of the Mechanisms Involved".

However, the availability of whole genome sequences from thousands of microorganisms and their detailed analysis reveal that proteins regarded as essential for conjugation in the classical conventional view are absent in the entire phylogenetic groups of non-model bacteria. These include bacteria of high relevance in the industry or in medicine such as *Streptomyces* spp. and *Mycobacterium* spp., for which conjugation has been clearly demonstrated and in which it likely plays a pivotal role in their evolution. In this review, we have focused our attention on the existence of alternative conjugation models in these bacteria which notably diverge from classical conjugation.

In mycobacteria, conjugation depends on signal interplay between donor cells, characterized by a set of protein variants encoded by its EXS1 secretion system, and a recipient cell encoding a different set of proteins in the equivalent ESX1 system. This interplay produces the activation in the recipient cell of a signal cascade that

leads to the formation of a mating pair with the donor and to the recipient-driven synthesis of a DNA channel between them. The donor then transfers chromosomal DNA from different sites spread in the chromosome to the recipient cell where they recombine, leading to a diversity of progeny, each containing a different mosaic-like genome. In this way, this Distributive Conjugal Transfer or DCT has a tremendous potential for the generation of strains with new properties, as it has been proposed for the appearance of the *M. tuberculosis* group (Gray and Derbyshire 2018).

Similar to this DCT, *Mycoplasma* can also generate a diversity of mosaic-like genomes among the progeny. However, in these minimal bacteria, the process depends on the presence of what seems to be conjugative transposons integrated in the genomes of the donor strains which encode a minimal set of proteins that are required for the recognition of the recipient and the concomitant DNA transfer. However, in this case, the recipient cell becomes the actual donor of genome fragments, likely by retro-transfer, leading to a recombinant progeny with mosaicism in their chromosome, meaning that the process becomes bidirectional.

The mechanism of transfer in *Streptomyces* is completely different, as it relies essentially on a single DNA translocase encoded by a conjugative plasmid. Moreover, this model is the only one in which it has been clearly proven that the substrate that is transferred is dsDNA and not ssDNA. The system can also transfer chromosomal DNA from different *clc* sites at lower frequencies and without specificity, meaning that every gene in the chromosome could actually act as substrate for the transfer.

A mechanism also relying on a single DNA translocase operates with high efficiency in *T. thermophilus*, but in this case an active role of the recipient cell is required, being the NCA of the recipient responsible for the acquisition of this DNA, leading to the term transjugation. As in *Mycoplasma*, the system is bidirectional and seems to generate mosaicism in the progeny (laboratory data).

Thus, the above examples clearly suggest that textbook model of conjugation is only conventional because of the few microorganisms used currently as models in Microbiology. As far as we look into details in other groups of prokaryotes, completely different mechanisms of conjugation emerge. Nature is always providing surprises.

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Horizontal Gene Transfer Between Bacteriophages and Bacteria: Antibiotic Resistances and Toxin Production

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Abstract Antibiotic resistance genes (ARGs) are ubiquitous among microorganisms living in a wide variety of environments and can be detected by several molecular techniques. Similarly, toxins and genes encoding toxins are also widespread among organisms. Bacteriophages are bacterial viruses found wherever bacteria exist, and their concentration is particularly high in aquatic environments. The age of the "omics" truly revolutionized this field, establishing the phylogenetic affiliation and function of phages, as well as the role they play in microbial communities and horizontal transfer of bacterial genes. Genomics, transcriptomics, proteomics, and metabolomics have highlighted the role of phages and their interaction with bacterial populations. It is now generally accepted that horizontal gene transfer regularly occurs between bacteriophages and their hosts, either by generalized or specialized transductions or possibly by controlling certain bacterial populations of donors or recipients. This means that phages not only play a major role driving bacterial evolution but also influence their own evolution. Phage infection can result in the bacterial host quickly acquiring (or loosing) novel genes and thus biochemical properties, a process otherwise extremely slow that usually requires long periods of time. This chapter will focus on the role of bacteriophages in the transfer of both antibiotic resistance genes and genes encoding novel toxins to new bacterial species. This knowledge is essential not only to understand the current challenges experienced in medicine but also to prevent, or at least lessen, future clinically relevant threats resulting from gene transfer between microorganisms.

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1 Introduction

Bacteriophages represent the most abundant entities on earth, in particular the phages belonging to the *Caudovirales* order, tailed viruses harboring double-strand DNA, that account for an estimated 96% of all phages known (Ackermann 2009; Niu et al. 2014). These bacteriophages are believed to infect an estimated 10^{23}–10^{25} bacterial cells each second (Keen et al. 2017). Horizontal (or lateral) gene transfer (HGT) is the sharing of genetic material between organisms by means other than vertical (parent to offspring) transmission and plays a major role in the evolution of many organisms (Gyles and Boerlin 2014). McDaniel and coworkers documented in 2010 that there is a high frequency of horizontal gene transfer in costal and oceanic environments, with as high as 47% of the cultivable natural microbial community confirmed as gene recipients. They demonstrated that least part of this HGT was carried out by viral-like particles released by the bacteria and suggested that it facilitated the adaptation of marine bacteria to changing environmental conditions.

HGT is perhaps the main factor spreading antibiotic resistance in bacteria (Kay et al. 2002), contributing also to the maintenance of bacterial virulence (Keen 2012). Moreover, bacteria with a lysogenic bacteriophage infection can acquire toxigenic properties. Victor Freeman demonstrated in 1951 (Freeman 1951) that avirulent strains of *Corynebacterium diphtheriae* can become virulent when infected by the bacteriophage β carrying the *tox* gene, which encodes the diphtheria toxin. This discovery is essential to understand the virulence and toxigenicity displayed by the different Park-Williams strains of *C. diphtheriae*, currently circulating worldwide. Ochiai and coworkers already identified in 1959 (Ochiai et al. 1959) that interspecific antibiotic resistance transfer can take place between *Shigella* and *Escherichia coli*, and this transfer is frequently episome-mediated (Watanabe and Lyang 1962). The mammalian intestine is colonized by a multitude of different microbial species, as well as a variety of bacteriophages, making it a favorable environment for bacterial gene transfer. As early as 1964, Kasuya reported the transfer of antibiotic resistance genes for chloramphenicol, streptomycin, tetracycline, and sulfathiazole in mouse enteric bacteria.

Recent reports refer to “rampant” HGT in bacteria, even involving genes for the small subunit of ribosomal RNA; this is of particular concern as these RNA sequences are used to compile phylogenetic trees, questioning the validity of at least part of the current tree of life (Simonson et al. 2005).

HGT increases genome variety by introducing new genes, with novel functions, that help improve adaptation to specific environmental niches. This is particularly patent when HGT results in the acquisition of antibiotic resistance in bacteria, giving rise to pathogenic lineages.

There is increasing evidence of the existence of HGT in animals and higher plants. For example, plant grafting could involve the transfer of not only viroids, but also chloroplasts and/or mitochondria from graft to host. In the animal kingdom, Gasmi described how some wasp genes were transferred to Lepidoptera by a symbiotic virus (Gasmi et al. 2015). Accordingly, the impact of HGT is probably much higher than first envisioned, and although it is not yet fully established the extent of its contribution to the reticulation of life evolution in this planet, it is now known that HGT affects all life kingdoms (Woese 2004), not just prokaryotes and lower eukaryotes. Genetic transfer in prokaryotes is not only achieved by HGT, it can also be accomplished by transformation (approximately 70 species of bacteria show natural genetic transformation), transduction, or conjugation (see below). In addition, although in transformation the DNA is normally integrated into the recipient genome by homologous recombination, heterologous recombination can also occur.

But HGT can also occur between microorganisms and higher animals, for instance between *Plasmodium vivax* and *Homo sapiens* (Bar 2011), and between *Wolbachia* species of bacterial endosymbionts and certain beetles (Kondo et al. 2002) (see appropriate chapter in this book). In fact, the prevalence of HGT could explain why certain taxa exhibit traits totally different from closely related members of the same taxonomic group. Moreover, HGT can also take place between bacteria or viruses and plants. In fact, plant surfaces, such as roots, that are colonized by bacterial, viral, or fungal species constitute hotspots for horizontal gene transfer (Pontiroli et al. 2009; Maumus et al. 2014; Richards et al. 2009). De la Cruz and Davies (2000) even propose that HGT is responsible for speciation and subspeciation in bacteria. Hence, the study of HGT in bacteria has elucidated the caveats of traditional phylogeny based exclusively on the 16S rDNA gene sequence comparison (Lal et al. 2008). In fact, Beumer and Robinson demonstrated in 2005 that a new broad-host-range bacteriophage (SN-T), capable of generating generalized transduction, can transfer the 16S rRNA genes from either *Sphaerotilus natans* or *Pseudomonas aeruginosa* to unrelated bacteria. In the future, HGT may precipitate a review of the overall bacterial taxonomy. In addition, the haploid nature of the bacterial genome results in any new genetic material being manifested as a novel phenotype. If the new trait helps bacteria adapt to a new environment, it could result in the prevalence of a particular allele, considerably expediting the evolution of the bacterial species.

Antibiotic resistance in bacteria was already reported in 1945 by Waksman and coworkers who stated: "*This phenomenon has an important bearing upon-the utilization of the substance for chemotherapeutic purposes, where knowledge of the sensitivity of the particular strain of a given organism responsible for a certain disease becomes of paramount importance.*" The following year Fleming and Queen (1946) reported strain variations in penicillin sensitivity among bacterial species encountered in war wounds and infections. It is currently generally accepted that the antibiotic resistance phenomenon is, at least in part, caused by HGT and usually involves bacteriophages, transformation, transduction, or conjugation. This chapter only deals with antibiotic resistance involving bacteriophages, although bacterial HGT can also entail other genetic elements, such as transposons or plasmids (Boyd et al. 2001; Lindsay and Holden 2006). Even the early publications on the

acquisition of bacterial resistance to antibiotics by pathogenic bacteria suggest the possibility of horizontal gene transfer (Laurell and Wallmark 1953; Knight and Holzer 1954); these studies involved *Staphylococcus aureus* and their bacteriophages, and applied bacteriophage-typing. In fact, the research established a relationship between antibiotic resistance and the type of phage harbored by the pathogenic bacteria (Barber and Burston 1955; Schmidt and Lenk 1958; Griffith et al. 1961). In 1998 Jiang and Paul clearly demonstrated transduction as an important mechanism for horizontal gene transfer in marine bacteria.

More recently Vanessa and coworkers (2011) used metagenomic analyses of ancient DNA, from 30,000-year-old Beringian permafrost sediments, to identify several genes encoding resistance to β-lactam, tetracycline, and glycopeptide antibiotics, thus concluding that antibiotic resistance in bacteria is indeed ancient.

Bacteriophages are diverse viral entities, classified by their genetic material, morphology, and host range; some phages have a narrow host range, while others are described as having a broad host range or being polyvalent. This chapter will concentrate on the latter, since this group of phages can enter either a lytic or a lysogenic cycle and can produce generalized transducing viral particles, which can considerably increase HGT and provide a vast supply of genetic material. Balcazar in 2014 and Lekunberri and colleagues in 2017 analyzed more than 30 viromes from a variety of environments and revealed that human-associated bacteriophages, contrary to general belief, rarely carried antibiotic resistance genes. On the other hand, sewage and marine environments, or even freshwater, represent good reservoirs for antibiotic resistance genes, leading the authors to conclude: "*phages could play a part on the spread of antibiotic resistance.*" This statement agrees to a certain extent with the bioinformatic studies by Enault and coworkers in 2017 that concluded that antibiotic resistance genes in the human gut microbiota are rarely encoded in bacteriophages and that this matter should be taken with extreme caution.

Undoubtedly, massive DNA sequencing will be required (Monier et al. 2011) to decipher and interpret what Wright denominated "resistome" (collection of all antibiotic resistance genes and their precursors in both pathogenic and nonpathogenic bacteria; Wright 2007), present in a variety of environments. In 2015, Brown-Jaque and colleagues produced a comprehensive review that helps understand the role of bacteriophages in HGT resulting in antibiotic resistance in the bacterial world. Undoubtedly, the use of antibiotics at sublethal concentrations (a common form of antibiotic misuse) can substantially increase HGT mediated by bacteriophages, since it can cause prophage induction. On the other hand, combination therapy with antibiotics at lethal concentrations can lower antimicrobial resistance by curtailing phage packing (Stanczak-Mrozek et al. 2017).

Bacteriophage-mediated HGT is also documented to play a crucial role in the production of exotoxins by bacteria, and some classical examples will be reviewed in this chapter. But the fact remains that it was only recently understood that the environment can act as a gene reservoir and that the DNA stored in an organism can be transferred to a totally different host. Consequently, Casas and colleagues reported in 2010 the isolation of an enterotoxin A gene, typical from *S. aureus*, in the unrelated bacterium psychrophilic *Pseudomonas*, hence providing the first evidence for an environmental pool of exotoxin genes in bacteria.

2 The Spread of Antibiotic Resistance by HGT

2.1 Gentamicin Resistance

Drug resistance in *Staphylococcus aureus*, including resistance to gentamicin (Fig. 1), is frequently mediated by either nonchromosomal DNA or R plasmids, as reported by Soussy and colleagues in 1975. Gentamicin was isolated in 1963, by Luedemann and Brodsky, as a new broad spectrum antibiotic from the culture broth of *Micromonospora purpurea.* This antibiotic is useful in the treatment of bone infections, endocarditis, pelvic inflammatory disease, meningitis, pneumonia, and other sepsis. As an aminoglycoside, it can cause hearing problems (including tinnitus), especially if used during pregnancy. Already in 1971, Yourassowsky and colleagues reported high incidence of *Pseudomonas* strains highly resistant to gentamicin in different hospital wards (Belgium). Four years later Smith et al. (1975) isolated several plasmids carrying gentamicin resistance genes, and 2 years after that, Krcmery et al. (1977) demonstrated that transduction with either bacteriophage F116 or G101 provided resistance to gentamicin. In 1979 an additional bacteriophage (AP 34) was discovered as a new transducing agent for this resistance (Výmola et al. 1979), and Knothe et al. in 1981, added two new bacteriophages, F 116 and AP 19, as transducing agents for gentamicin resistance in *P. aeruginosa*. A breakthrough in the understanding of the role bacteriophages play in spreading gentamicin resistance in this bacterium came from the research by Bräu and Piepersberg, and they reported in 1983 that co-integrational transduction and mobilization of the gentamicin resistance plasmid pWP14a was mediated by IS140.

Witte and Dünnhaupt also described transduction in *S. aureus* mobilizing the gentamicin resistance phenotype in 1984, although the resistance gene they studied was chromosome-located and was mobilized after lysogenization followed by bacteriophage induction. Blahová and coworkers, in 1997, similarly reported that several wild-type bacteriophages in *P. aeruginosa* could effectively transduce not only gentamicin resistance but also resistance to carbenicillin. More recently, Mazaheri and coworkers (2011) confirmed that *Enterococcus faecalis* can also transduce resistance to gentamicin into *E. faecium*, *E. hirae*, and *E. casseliflavus*, and Eggers et al. (2016) demonstrated this ability in *Borrelia burgdorferi*. Hence, corroborating that resistance to gentamicin by HGT via transduction is more common than initially thought.

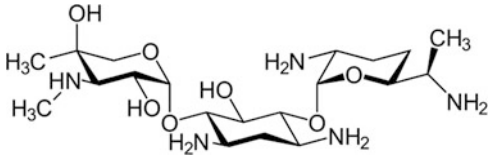

Fig. 1 Gentamicin structure

2.2 *Rifampicin Resistance*

Rifampicin (Fig. 2), also known as rifaldazina (Moncalvo and Moreo 1966), is an antibacterial compound (although it can also inhibit the maturation of poxviruses at a discreet step in envelope formation; Grimley et al. 1970) belonging to the ansamycins that contains a naphthoquinone chromophore that gives this antibiotic its peculiar red color. It was isolated by Sensi and coworkers in 1959, who named it "rifomycin"; in fact, rifampicin is a semisynthetic derivative of the antibiotic used to control infectious diseases in 1965 (Sensi 1983). Rifampicin inhibits the multicomponent ($2\alpha\beta\beta'$) bacterial DNA-dependent RNA polymerase by interacting with the β-subunit of the enzyme, thus inhibiting transcription and preventing RNA synthesis. The antibiotic does not inhibit T7 RNA polymerase. The *rpoB* gene encodes the β-subunit and is located at 89.5 min in the *E. coli* chromosome map. Resistance to rifampicin occurs by mutation in the *rpoB* gene (for a recent review, see Goldstein (2014)). The β-subunit spans 1342 amino acids (second-largest polypeptide in the bacterial cell), and mutations lowering the affinity of this polypeptide for the antibiotic originate resistant phenotypes. On the other hand, the bacteriophage Mu can generate a rifampicin-resistant phenotype by lysogenic insertion at a point located between 28 and 37 min in the *E. coli* chromosome (Kollenda et al. 1986). Matsiota-Bernard and coworkers, in 1998, studied *Mycobacterium tuberculosis* and characterized three distinct *rpoB* preferential mutations, in codons Ser531, His526, and Asp516, as the main source of rifampicin resistance in this bacterium, with Ser531 as the most frequent mutation (Ochang et al. 2016; Pérez del Molino et al. 2016; Ullah et al. 2016). Recent *M. tuberculosis* studies (Ismail et al. 2016) revealed that 80% of the isolates harbor mutations at codons 119 (His119Tyr), 135 (Arg135Trp and Ser135Leu), and 548 (Arg548His; Horng et al. 2015).

In another species, *Mycobacterium leprae*, a mutation at position 2275405 in its genome, creates a G→C transversion; this changes glutamic acid to histidine at position 442 (Glu442His) of the β-subunit of the RNA polymerase and generates a phenotype that is resistant to rifampicin (Hasanoor Reja et al. 2015; Vedithi et al. 2015).

In some bacteria, such as *E. coli*, the majority of mutations occur in three clusters in the *rpoB* gene, cluster I (from amino acids 509 to 533), cluster II (spanning amino acids 563 to 572), and cluster III (amino acid 687) (Goldstein 2014; Metcalf et al. 2016).

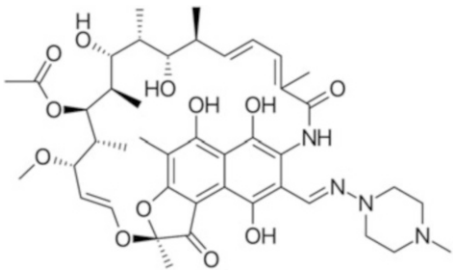

Fig. 2 Structure of rifampicin

Recent results suggest that incorporating a nonstandard amino acid at the recoded amber stop codon in the β-subunit of RNA polymerase (codon capture model of genetic code evolution; Hammerling et al. 2016) confers robust rifampicin resistance. They found that a variety of mutations, involving nonstandard amino acids in the essential part of RNA polymerase protein, generate alternative ways to rifampicin resistance.

Rickettsia prowazekii, an obligate intracellular parasitic bacterium that is the causative agent of epidemic typhus and is suspected to be linked to the origin of the eukaryotic mitochondrion (Andersson et al. 1998), can originate rifampin-resistant mutants by a single point mutation that results in an arginine-to-lysine change at position 546 of the RNA polymerase beta-subunit (Rachek et al. 1998).

Early reports on the involvement of bacteriophages in the transmission of resistance to rifampicin were contradictory, some authors even concluding that the antibiotic inhibited the development of many phages, even when the bacterium was resistant to the antibiotic. This is the case for phage D29 that infects *Mycobacterium smegmatis*, but the viral progeny is released without causing cell death (Jones and David 1971), and also for phage Qβ that replicates in *E. coli*. Rifampicin inhibits synthesis of the phage Qβ, although both viral ribonucleic acids and viral proteins are made in nearly the same amount as in the absence of rifampin; the antibiotic only appears to affect viral assembly (Passent and Kaesberg 1971). However, rifampicin does not inhibit the development of the phage PBS2 infecting *Bacillus subtilis* (Price and Frabotta 1972).

The first publications demonstrating that resistance to rifampicin could be positively transduced by a bacteriophage did not appear until circa 1973. Consequently, Stuart and Ferretti reported that rifampicin-sensitive strains of group A streptococci (*Streptococcus pyogenes*) could be transduced into resistant strains by bacteriophage A25. Similarly, Konrad et al. (1973) described the same role for bacteriophage φ80, and Kirschbaum and Konrad EB, also in 1973, reported the isolation of a specialized lambda transducing bacteriophage carrying the beta-subunit gene for *E. coli* RNA polymerase. In 1977, Lathe R and Lecocq described the *firA* gene (locus involved in the expression of rifampicin resistance in *E. coli*) and characterized a lambda-firA transducing phage able to transduce the rifampicin-resistant phenotype. However, Wall and coworkers described, in 1975, the total inability of the phages infecting *Rhodopseudomonas capsulate* to transfer genes.

2.3 Streptomycin Resistance

Streptomycin (Fig. 3) is a broad-spectrum antibiotic known to affect bacteria at different levels; these include stimulation of RNA metabolism, inhibition of protein synthesis (Erdos and Ullmann 1959), and changes in the membrane integrity (Anand et al. 1960).

Nearly half of the antibiotics currently in use target the 70S bacterial ribosome (in particular, the 16S rRNA of the 30S subunit), thus interfering with the binding of formyl-methionyl-tRNA to the 30S subunit and consequently inhibiting the

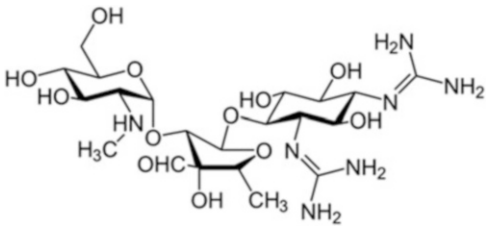

Fig. 3 Structure of streptomycin

translation process, resulting in an overaccumulation of mistranslated products. These antibiotics, that include streptomycin and kanamycin, belong to the aminoglycoside group. Early publications on the mode of action of streptomycin include Speyer et al. (1962) and Cox et al. (1964). Streptomycin mainly targets the S12 ribosomal protein, affecting the protein elongation process (Zengel et al. 1977). Streptomycin can cause negative side effects in humans that affect the VIII cranial nerve (vestibulocochlear nerve) producing ataxia (lack of coordination in muscle movements) and tinnitus (hearing of sound when no external sound is present) and, in serious cases, can cause permanent deafness (due to human mutations in the mitochondrial DNA; for a recent review, see Villa et al. (2016)). The antibiotic can also be nephrotoxic; but despite this side effect, it was the first randomized trialed antibiotic against pulmonary tuberculosis (between 1946 and 1948), and, since then, it is widely accepted as an effective treatment against this disease (Metcalfe 2011).

Streptomycin was isolated in 1943 by Albert Schatz, in the laboratory of Professor Waksman, at Rutgers University in the USA (Jones et al. 1944), and was soon established to exhibit anti-tuberculosis activity (Emmart 1945; Kingston 2004). But already in 1945, Waksman and coworkers reported the appearance of resistant strains to streptomycin within both Gram-positive and Gram-negative bacteria (Waksman et al. 1945), and 1 year later Youmans and colleagues suggested an increase in the antibiotic resistance of the tubercle bacilli when exposed to streptomycin (Youmans et al. 1946). Shortly after, streptomycin resistance was reported in other bacteria (Herrell and Nichols 1945), gonococci and meningococci (Miller and Bohnhoff 1946), members of the *Enterobacteriaceae* (Klein and Kimmelman 1946), staphylococci (Wolinsky and Steenken 1946), *Brucellaceae* family members (Sutliff and Mason 1947), *Bacteroides* species (Foley 1947), and *Haemophilus influenzae* (Alexander and Leidy 1947). In particular, Klein and Kimmelman, in 1946, examined up to a billion *Shigellae* not previously exposed to streptomycin, and were able to isolate six variant strains that were highly resistant to the antibiotic. These early reports, although abundant in number, did not shed any light on the mechanism(s) of streptomycin resistance. Despite that, the publication by Newcombe and Hawirko in 1949 merits comment here, as it was perhaps the first to estimate the rates of spontaneous mutation (dominant, according to these authors) of *E. coli* to streptomycin resistance as 1×10^{-10} per bacterium, per division cycle. *H. influenzae* also displayed similar rates of mutation, even in the absence of the antibiotic. Joshua Lederberg, in 1951, studied heterozygous strains of *E. coli* and found that the mutations conferring streptomycin resistance were recessive.

The interpretation of the mechanism of action of streptomycin can be equivocal, particularly since Gorini and Kataja (1964) demonstrated that streptomycin can act as a phenotypic suppressor altering the reading of the genetic code. In clinical isolates of *E. coli*, streptomycin resistance occurs by enzymatic inactivation, either adenylation or phosphorylation, of the drug (Davies et al. 1971) and can be encoded either in the bacterial chromosome or by plasmids (for a good article on this subject, please see Spotts and Stanier 1961). Streptomycin affects genetic systems in usual ways, one example of this is that the antibiotic can suppress both amber (UAG) and ochre (UAA) mutations in bacteriophage T4 (Orias and Gartner 1966).

Watanabe and Fukasawa (1961) and Harada et al. (1963) were probably the first to report antibiotic multiresistances in *Salmonella typhimurium* and *E. coli*; these species were resistant to streptomycin, chloramphenicol, tetracycline, and sulfonamide, and the resistance was mediated by two epsilon bacteriophages (15 and 34) and by bacteriophage P22, respectively. In 1964, Alexander von Graevenitz described that streptomycin resistance in *S. aureus* was also mediated by phages.

The involvement of bacteriophages in the propagation of streptomycin resistance is currently unclear, and it appears to differ according to the bacterial system studied. For instance, early publications on *Mycobacterium* revealed that the antibiotic induced bacterial lysis preferentially in bacteria infected with the bacteriophage (Tokunaga and Sellers 1965). In addition, Mankiewicz and coworkers studied several mycobacterial bacteriophages and reported, in 1969, that the phages were unable to transduce resistance to streptomycin, while the lysogenized bacterial strains displayed a decreased ability to reduce nitrate or transform ammonium ferric citrate. However, Jones and colleagues, in 1974, found positive transduction of a streptomycin R-factor from *Mycobacterium smegmatis* to *M. tuberculosis* H37Rv.

Nevertheless, in other bacterial systems, bacteriophages are able to transduce streptomycin resistance, as it is the case for PLT-22 and *Salmonella typhimurium* (Watanabe and Watanabe 1959; Săsărman and Antohi 1965). Additionally, bacteriophage 5006M can produce high-frequency transduction of streptomycin and sulfonamide resistance markers to *Proteus mirabilis* (Coetzee 1976). Chakrabarti and Gorini (1975) described an unusual consequence of the acquisition of streptomycin resistance in *E. coli*; they noticed that a female (F^-) strain of *E. coli*, originally sensitive to bacteriophage T7, turned into a viral exclusion status upon acquiring a mutation that rendered it streptomycin resistant, thus exhibiting a *Hfr* phenotype.

The fact that acquisition of resistance to streptomycin by *M. tuberculosis* usually results in resistance to bacteriophage infection has allowed the design of a low-cost assay, of widespread applicability, for the rapid screening of drug resistance in this pathogenic bacterium, particularly in developing countries (Eltringham et al. 1999). Rondón and colleagues, in 2011, further developed this assay by evaluating the use of fluoromycobacteriophages for detecting drug resistance in *M. tuberculosis.* On their part, Cairns and coworkers (2017) reported that sublethal streptomycin concentrations, combined with lytic bacteriophages, can promote streptomycin resistance.

2.4 Kanamycin A Resistance

Kanamycin A, or simply kanamycin, was isolated by Hamao Umezawa in 1957 from the culture media of *Streptomyces kanamyceticus* (Umezawa et al. 1957). This microorganism also produces additional forms of the antibiotic, such as kanamycin B, kanamycin C, kanamycin D, and kanamycin X. All the kanamycin varieties belong to the aminoglycoside group of antibiotics that also includes streptomycin, neomycin, and tobramycin (Fig. 4). Although kanamycin is not a first-line antibiotic, and it is recommended only for short-term treatments, it is active against a variety of bacteria, including *M. tuberculosis*. The antibiotic affects the 30S ribosomal subunit and interferes with protein translation, resulting in bacterial death.

The protocol for kanamycin production was established by a variety of authors (i.e., Okami et al. 1959; Basak and Majumdar 1973), and, soon after that, Okanishi et al. (1966) transfected bacteriophage PK-66 DNA into *S. kanamyceticus* protoplasts and reported the production of mature bacteriophages.

The mechanism of kanamycin resistance involves the enzyme aminoglycoside-3′-phosphotransferase (APH,3′) that catalyzes the addition of phosphate from ATP to the 3′-hydroxyl group of a 4,6-disubstituted aminoglycoside, such as kanamycin, thus reducing its binding affinity for nucleic acids. The enzyme is produced by a variety of Gram-positive bacteria (Gray and Fitch 1983).

Drabble and Stocker published, in 1968, an early report on kanamycin resistance (as well as tetracycline, streptomycin, sulphonamides, and chloramphenicol) in *Salmonella typhimurium* and found that it was mediated by the transducing bacteriophage P22. Some years later, Coetzee et al. (1973) reported that *Proteus mirabilis* can acquire kanamycin resistance with the assistance of bacteriophage 34. An in-depth study by Coetzee described, in 1974, that this phage, as well as other closely related, could generate a high-frequency transduction of kanamycin resistance into *P. mirabilis*. In addition to P22, bacteriophage P1 was also reported to spread resistance to kanamycin in different *Enterobacteriaceae* (Takano and Ikeda 1976). Occasionally, the acquisition of kanamycin resistance via bacteriophage infection is mediated by specialized transduction, as is the case for bacteriophages PL25 and *Providence* (Coetzee 1975). In 2000 Barbian and Minnick reported that even members of *Rickettsiaceae* (i.e., *Bartonella bacilliformis* and *B. henselae*, the causative agents of Oroya fever and cat scratch disease in humans, respectively) can produce bacteriophage-like particles capable of spreading kanamycin-resistant phenotypes.

To exacerbate matters, kanamycin resistance can also be transmitted between different bacterial species as transposable elements (Tn903; Oka, Sugisaki and Takanami in 1981) encapsidated within naked viral capsids, as is the case for the

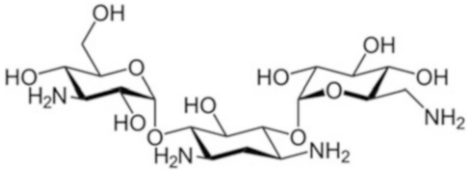

Fig. 4 Structure of kanamycin A

filamentous fd bacteriophage (Nomura et al. 1978). Tn5 is another transposon, found in *Shewanella* and *E. coli*, that provides resistance to kanamycin and other aminoglycoside antibiotics, and it is routinely used as a model to understand transposition mechanisms in bacteria (Reznikoff 2003). Tn5 can occasionally be encapsidated in P1 viral capsids produced in *E. coli*, thus transferring kanamycin resistance to far-distant bacteria such as *Myxococcus xanthus* (Downard 1988); it additionally produces a variety of polar mutations in the recipient strains (Merrick et al. 1978).

Clinical isolates of *P. aeruginosa* can also acquire resistance to kanamycin by transduction with bacteriophage F116, as reported by Blahová and colleagues in 1994.

Lutz et al. (2004) reported that some bacteriophages, such as phiC31, can even transfer kanamycin resistance to plant plastids, hence contributing to the spread of this resistance into the higher eukaryote world.

Bacteriophages have even been suggested to transfer antibiotic resistance by HGT into the human food chain, as reported by Shousha and coworkers. These authors concluded in 2015 that the appearance of resistance to several antibiotics (kanamycin resistant is the most common) in chicken meat could be mediated by bacteriophages.

In addition to transduction, certain lytic bacteriophages that cause intense bacterial DNA degradation could produce transformable DNA segments that can enter bacteria exhibiting "natural" transformation processes. Keen and coworkers confirmed this possibility in 2017, describing two "superspreader" bacteriophages (SUSP1 and SUSP2) that lysed *E. coli*, released segments of DNA from the bacterium encoding kanamycin resistance, and promoted the acquisition of kanamycin resistance by a soil-derived *Bacillus* strain not infected by the phages.

2.5 Resistance to β-Lactams

The β-lactam group are a class of broad-spectrum antibiotics with a nitrogen in β-position on the second carbon in the lactam ring; one of its members, penicillin, is the first antibiotic discovered and probably the most famous, but the term "penicillin" is often used generically to refer not only to benzylpenicillin (penicillin G, the original penicillin found in 1928) but also to procaine benzylpenicillin, benzathine benzylpenicillin, and phenoxymethylpenicillin (see Fig. 5 for the basic structure of penicillins). β-Lactam antibiotics, including penicillins, cephalosporins, monobactams, and carbapenems, currently account for over 50% of the antibiotics prescribed worldwide (Livermore 1996; Hall et al. 2004). Unfortunately, soon after the introduction of

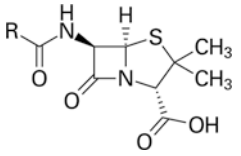

Fig. 5 Basic structure of penicillins, showing the β-lactam and thiazole rings. The various penicillins originate by substituting R for different radicals

these antibiotics in the market, resistant bacteria appeared. Demerec demonstrated in 1945 that the development of penicillin resistance is due to enrichment of single bacterial cells not completely inhibited by the penicillin concentration used, and Salvador Luria, in 1946, indicated that there are two types of penicillin resistance in staphylococci: one type is due to a mutation and the other results from the microorganism producing penicillinase. Finally, an early publication by Eagle (1954) already outlined the different pathways of penicillin resistance in bacteria. These phenomena should not be confused, however, with the antipenicillin effect exerted by a variety of amino acids (Shwartzman 1946). Fleming and Queen concluded in 1946: "*From these data, it is evident that species of a given organism vary greatly in their tolerance or response to penicillin, and that strains within species show considerable variation in their sensitivities. All strains of bacterial species usually considered sensitive to penicillin are not actually sensitive to this antibiotic (and vice versa).*"

Bacterial resistance to penicillin can result from the acquisition of the enzyme penicillinase, a type of β-lactamase (EC 3.5.2.6), that hydrolyzes the antibiotic by opening the β-lactam ring (Fig. 6). This enzyme was first isolated, from *E. coli*, by Abraham and Chain (1940), even before penicillin entered clinical use, although over 200 different penicillinases are currently known. Bush and coworkers (1995) and Bush and Jacoby (2010) provided a functional classification of these enzymes. Briefly, β-lactamases are molecularly classified into class A, C, and D enzymes, which utilize serine for beta-lactam hydrolysis, and class B metalloenzymes, which require divalent zinc ions for substrate hydrolysis. They can also be functionally classified by taking into account both substrate and inhibitor profiles, in an attempt to group the enzymes according to their phenotype in clinical isolates. The class B β-lactamases were originally identified by Sabath and Abraham in 1966, and they constitute a major threat because they are not inactivated by β-lactamase inhibitors and display activity toward carbapenems (not hydrolyzed by classes A, C, or D lactamases; Hall et al. 2004). These are ancient enzymes that, at least some of them, are believed to have originated 2 thousand million years ago (serine β-lactamases; Hall and Barlow 2003) and they include subclasses B1 and B2, believed to have evolved 1 thousand million years ago, whereas subclass B3 evolved an estimated 2 thousand million years ago (i.e., before the divergence of eubacteria into Gram-positive and Gram-negative) (Hall et al. 2004).

In vivo-acquired penicillin resistance also appeared early in a variety of pathogenic bacteria, like *Neisseria meningitidis* (Miller and Bohnhoff 1946), and there were hundreds of reports on penicillin resistance during the 1940s and 1950s,

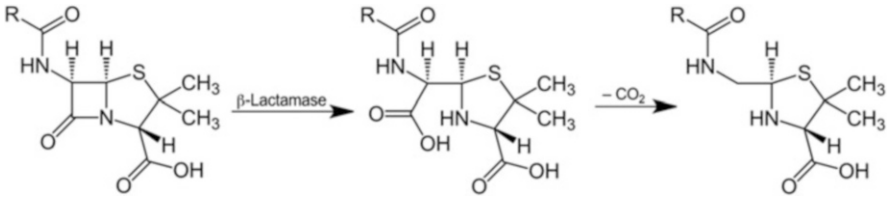

Fig. 6 Typical mode of action of β-lactamases

although none of the publications considered the possibility of bacteriophages causing the antibiotic-resistant phenotype. There have been a number of interesting reports linking the metabolism of amino acids in bacteria and their resistance/sensitivity to penicillin (as an example, see Gale and Rodwell (1949)) that supported the antipenicillin effect of certain amino acids, as mentioned above.

It is worth highlighting here the early reports by Voureka (1948) and by George and Pandalai (1949), who considered the possibility of resensitizing a penicillin-resistant bacterium by simply growing it together with penicillin-resistant strains, or even in the presence of their acellular products, but the authors did not elaborate on the nature of the mechanism(s) involved. However, Hotchkiss, in 1951 and 1952, shed some light on this matter, suggesting the involvement of a desoxyribonucleate derived from resistant cultures, possibly influenced by Avery and coworkers' (1944) findings on the nature of the "Griffith's transformation principle" in pneumococcus.

One of the first reports that used the term "transduction" was published in 1953 by Saz and Eagle, when they hypothesized that, in addition to the more classic "genetic transformation process,", there were other possible ways of genetic cross-talk between penicillin-resistant/sensitive *Streptococcus pneumoniae* strains. In addition, Blair and Carr (1961) described that lysogenization of particular strains of *S. aureus* by the appropriate phages affected bacterial antibiotic resistance, even to the point of reversing their penicillin susceptibility to yield mutants completely sensitive to the antibiotic (this bacterium displays a natural tendency to produce penicillin-resistant strains). The β-lactam-resistant genes in these Gram-positive cocci are, at least in some of the strains, probably of chromosomal origin, although under the control of phage recombination genes (and thus independent from the recA1 control system), making them acquiescent to transductional transmission (Sjöström et al. 1975); this transduction, however, only takes place after the bacterium is lysogenized with phage φ11 (Cohen and Sweeney 1973).

The origin of the penicillinase-encoding genes (*bla*) is a bit elusive, but extensive bibliographic research uncovered the report by Heffron and coworkers (1975) that describes the origin of the TEM beta-lactamase gene (TEM stands for *Tem*oneira, the family name of a patient in Greece from whom the lactamase producer was isolated; Datta and Kontomichalou 1965). It is possible that beta-lactamase genes could be mediated by a specific DNA sequence that makes possible their translocation from one replicon to another, as previously suggested (Datta et al. 1971). This transposable DNA sequence was named *transposon* (Hedges and Jacob 1974). Whether the lactamase genes are located on the bacterial chromosome or in a plasmid is probably unimportant (and does not shed much light into their origin), as long as they are contained within one of these genetic translocatable elements. Similarly, it is not important if these enzymes are inducible (i.e., BLA-B) or not (BLA-A), as their inducibility is likely the result of a random recombination event that originated the regulated mechanism. In addition, lactamase hyperproduction (hypertranscription) by a particular bacterial strain is also a conjunctural event that strictly depends on the appropriate mutations taking place in the Pribnow's boxes located at the promoter site on DNA, such as the A→G transitional mutation at nucleotide −28, or the GT insertion between positions −14 and −15 (Siu et al. 2003). In any event, once the *bla*

genes are included into those genetic elements, they can be horizontally transmitted, or even encapsidated during viral-generalized transduction, and contribute to the spreading of the resistance markers.

β-Lactamase genes can be found in bacteriophages isolated from sewage, as reported by Muniesa and coworkers in 2004, hence it remains possible that phages could play a role in the transmission of chromosomal genes bla_{OXA} and bla_{PSE} between *Pseudomonadaceae*, *Enterobacteriaceae*, and *Vibrionaceae* strains.

The original role of the β-lactamase enzymes is probably unrelated to the hydrolysis of β-lactam antibiotics, but rather a side effect. The conserved domains of bacterial lactamases display homology to a number of bacterial proteins, such as the division polypeptide FtsI, penicillin-binding proteins, D-alanyl-D-alanine carboxypeptidase, β-*N*-acetylglucosaminidase, serine hydrolases, D-aminopeptidases, the ABC1 family of polypeptides, Serine-type D-Ala-D-Ala, and peptidoglycan biosynthesis proteins, which is not unexpected from such an ancient group of proteins (see Geer et al. 2002). Under this premise, it is also possible that natural spontaneous mutations could account for the β-lactamase phenotype, as the mutations would drive the mature protein toward the hydrolysis of β-lactam antibiotics (for instance, by lowering the values of the Michaelis constant, KM) instead of its original role. Indeed, TEM-type penicillinases can be modified by point mutations to broaden their substrate spectrum to include almost all beta-lactams (Tham and Guesdon 1992), and substitution of aspartic acid for asparagine has been shown to reduce the susceptibility of β-lactamases to mechanism-based inhibitors (Giakkoupi et al. 1999).

From all the above, it is possible that every time a new β-lactam antibiotic is produced by the pharmaceutical industry, spontaneous mutations on the β-lactamase genes could, in a short time, provide antibiotic resistance and, by means of the bacteriophage world, transfer this resistance between pathogenic organisms, thus quickly rendering the antibiotic useless. Accordingly, Hendrix and coworkers reported in 1999 that lambdoid phages in *E. coli*, phage phiC31 in *Streptomyces*, *Mycobacterium* phages, phiflu in *H. influenza*, as well as two small prophage-like elements, phiRv1 and phiRv2, in *M. tuberculosis*, share common ancestry (and possibly all of the dsDNA-tailed phages have a common ancestor), making them putatively the main culprits for transferring (through transduction/lysogenization), by HGT, antibiotic resistance in bacteria. Lysogenization does not always require recombination of the phage and bacteria dsDNAs; in fact, the DNA of some bacteriophages (i.e., P1 and derivatives) remains in a plasmid-like manner. In 2014, Billard-Pomares and colleagues described that a RCS47 P1-like bacteriophage, containing two IS26 elements and thus the blaSHV-2 gene (an inhibitor-resistant lactamase), isolated from a clinical strain of *E. coli* displayed a high prevalence of spreading into other *E. coli* strains of both human and animal origin. The consequences of a P1-type lysogenic phage having either resistance or sensitivity to β-lactam antibiotics are difficult to interpret, since it is well known that this bacteriophage, via its Ref endonuclease, can affect bacterial survival under SOS conditions (Ronayne et al. 2016).

The existence of spontaneous zygogenesis (Z-mating) in bacteria (i.e., complete genetic mixing in the absence of a conjugative plasmid, even between far distant species; Gratia 2007) could also further the spreading of β-lactamase genes, and although to the best of our knowledge no bacteriophages have yet been described to effectuate these processes (apart from the preliminary study by Gratia in 2017 analyzing evolving lysogenized products of spontaneous zygogenesis in *E. coli*), it is worth considering as a putative spreading mechanism.

3 Role of HGT in Generating Toxigenic Bacterial Strains

3.1 Diphtheria Toxin

Antibiotic resistance is not the only bacterial trait (Fig. 7). Horizontally transferred between bacteria via bacteriophages, phages also play a very important role in the transmission of bacterial toxin production.

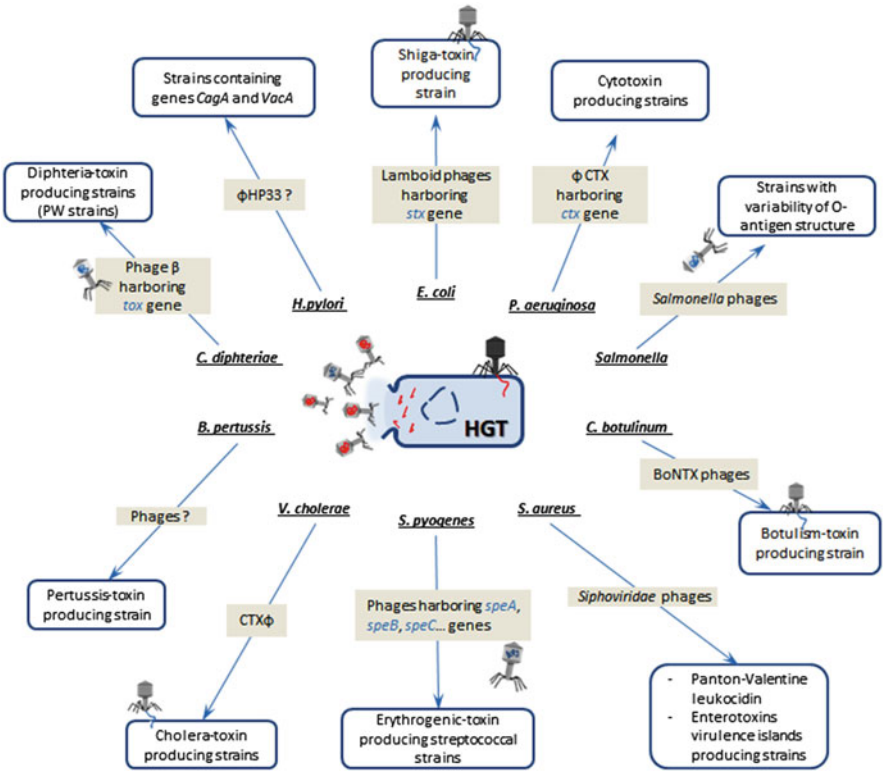

Fig. 7 Some examples of HGT mediated by bacteriophages in pathogenic bacteria

Diphtheria toxin is perhaps the most notable toxin of bacterial origin, and it spans a single polypeptide chain of 535 amino acids, consisting of two subunits (A and B) linked by disulfide bridges. Subunit B serves as a carrier (pore-forming activity) that allows subunit A to enter the target eukaryotic cell (Pappenheimer 1977; Bell and Eisenberg 1997). The toxin is not as poisonous as botulinum toxin, requiring a dose of around 0.1 μg of toxin per kg of body weight to be lethal to humans. The A subunit, while in the cytosol, ADP-ribosylates the unusual amino acid diphthamide, present in the eukaryotic elongation factor 2 (EF-2). This prevents EF-2 from playing its role in the translational apparatus, therefore arresting protein synthesis in the affected cells that subsequently die; the heart and liver are the main organs affected. The gene encoding diphtheria toxin is located in the bacteriophage β, a phage capable of lysogenizing *C. diphtheriae.* The three classical strains of *C. diphtheriae*, *mitis*, *intermedius*, and *gravis*, originated in this manner (indeed, *gravis* strains can contain up to four tandemly arranged β-genomes) and are normally referred to as the Park-Williams variants. The amount of toxin produced by these bacterial strains is, therefore, directly linked to the number of *tox* genes harbored in the phage genome.

The classical publication by Paula Maximescu (1968) revealed that, during the course of lysotyping, certain strains of *C. diphtheriae* spontaneously liberated bacteriophages that, in turn, infected two *Corynebacterium ulcerans* strains (9304 and 298 G) converting them into toxigenic strains. Hence concluding that HGT via bacteriophages can occur not only between different strains of the same species, but also between different species of *Corynebacterium* (Maximescu 1968), which considerably exacerbates the epidemiology of this disease caused by *Corynebacteriaceae.*

3.2 Shiga Toxin

Shiga toxin is another well-documented example of a toxin that can be horizontally transferred by bacteriophages to different members of *Enterobacteriaceae*, particularly *E. coli.* This transfer can be achieved by bacteriophage Φ24B, or even by other lambdoid phages; a lambdoid phage is a bacteriophage that can recombine with lambda phage and produce viable progeny. Shiga toxins are classified into two groups, Stx1 and Stx2 (or SLT1 and SLT2), and are encoded by lambdoid bacteriophages (Huang et al. 1987; Friedman and Court 2001; Herold et al. 2004). They are named after Kiyoshi Shiga, one of the microbiologists involved in the isolation of the etiological agent of bacterial dysentery, *Shigella dysenteriae* (although some sources claim that it was actually A. L. Kendall who first isolated this bacterium; Kendall and Walker of 1910). The toxin is also present in Shigatoxigenic *E. coli* (STEC) serotypes, including O157:H7, O104:H4, and some enterohemorrhagic *E. coli* (EHEC) (Spears et al. 2006). In fact, the Shiga toxin-producing *E. coli* O157:H7 (STEC O157:H7) strain remains one of the leading causes of foodborne disease in North America (Niu et al. 2014).

In 2001, Strauch and colleagues reported the characterization of a Shiga toxin (Stx)-encoding temperate bacteriophage 7888; this phage usually infects *Shigella sonnei*, but can also infect *E. coli*, and produce toxigenic strains of the two microorganisms (Fig. 8). All Stx bacteriophages share a regulatory region, involved in Q-dependent expression, that maps upstream of *stx1* and *stx2* genes (or A and B, respectively) in phage 7888 (similar in morphology to bacteriophage 933W, exhibiting a hexagonal head and a short tail). This led the authors to propose that the *stx* genes are naturally transferable between different *Enterobacteriaceae* species. EHEC O26 strains can revert into non-toxigenic enteropathogenic strains (EPEC O26) and vice versa; this is achieved by either losing or gaining the appropriate lysogenic bacteriophage harboring Shiga toxin, and it can even occur during the course of the disease in humans (Bielaszewska et al. 2007). This matter is even further complicated by the findings published by Döpfer et al. (2010); they reported that ovine strains of *E. coli*, which can also infect humans, are susceptible to HGT via transducing bacteriophages harboring the *stx* genes.

The Shiga toxin has two subunits (A and B_5) and is released by decaying bacteria in the gut, where it binds to the cellular receptor globotriaosylceramide and enters the enterocyte by macropinocytosis and transcytosis; it then migrates through the intestinal barrier and is transported in the bloodstream to target organs, like the kidney. Once inside the cell, the toxin depurinates the 28S rRNA in the 60S eukaryotic ribosomal subunit by cleaving the glycosidic bond of a single adenine, thus halting protein synthesis (Lukyanenko et al. 2011).

Bacteria belonging to the *Enterobacteriaceae* family are abundant in fertilizers that use animal manure, and this has always been a matter of concern as it could represent a source of infection for humans and animals. Indeed, Johannessen et al. (2005) studied the survival of Shiga toxin-encoding bacteriophages in a compost

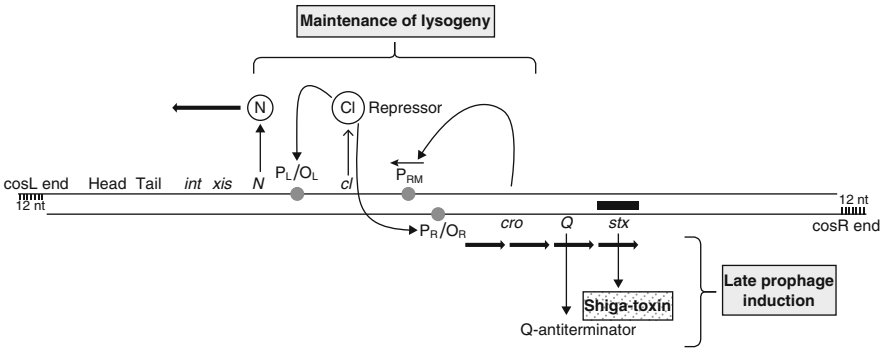

Fig. 8 A simplified model of late regulation of *stx* gene expression in a lambdoid phage. Stx gene is located in the late portion of the lambdoid genome and is controlled by the late Q-like antiterminator. This explains why lysogenized cells only produce Shiga-like toxin during prophage induction process. CI represents the main repressor of lysogeny and exerts a strong negative control on both right and left operators (OR/OL), so inhibiting expression of all lambdoid genes, but the immunity operon. N represents an antiterminator that allows early expression of integrase from the *int/xis* overlapping genes

model and found that it remained functional at low temperatures, but composting the animal manure for 40 days at a temperature of >60 °C is an effective way of eliminating both *E. coli* and the bacteriophages harboring the Shiga toxin gene. On the other hand, if the temperature is not high enough to rapidly kill *E. coli*, it could have the negative effect of contributing to a fast dispersion of stx bacteriophages to non-toxigenic commensal *E. coli* strains (Yue et al. 2012). In addition, high hydrostatic pressure treatment, often used in the food industry to preserve foods, can trigger lambda prophage induction in *E. coli* lysogens; hence, if the food contains lambdoid prophages harboring the Shiga toxin gene, it could cause food intoxication (Aertsen et al. 2005).

In 2006, Dumke and collaborators investigated the presence of active bacteriophages carrying the *stx* genes both in waste water and in rivers; they found that these bacteriophages were still present in the water, even after purification procedures, thus confirming the persistence of these viruses in aquatic environments. Similar results were also reported by other authors, such as the study by García-Aljaro and coworkers in 2006 on new bacteriophages with the stx_{2g} Shiga toxin gene isolated from *E. coli* strains present in polluted waters.

Despite the fact that bacteria can be destroyed at relatively high temperatures, Shiga toxin-producing *E coli* strains, such as O104:H4, represent new challenges for microbiology, as seen in Germany in 2011. Muniesa and coworkers (2012) reported that in 2011 the country "*experienced the largest outbreak with a Shiga toxin-producing Escherichia coli (STEC) strain ever recorded.*" The outbreak was spread through fecal-oral transmission, and the *E. coli* strain involved harbored a lambdoid prophage carrying the Shiga toxin gene (Figs. 7 and 8).

Tóth et al. (2016) recently isolated a new bacteriophage (Shiga phage 75/02 stx, *Podoviridae*) that harbors the Shiga toxin gene and can transduce *S. sonnei* into a toxigenic strain, as it was the case for *Sh. dysenteriae.* Similar results were previously reported by Beutin and coworkers (1999) for *Sh. flexneri* (Gray et al. 2014).

Shiga toxin can also be transferred to animals, as described above for antibiotic resistances and virulence factors, via bacteriophages. According to Petridis et al. (2006), this toxin can be horizontally transferred via bacteriophages (i.e., H-19B:: Ap1) into *E. coli* strains that colonize the intestine of the housefly (*Musca domestica*). The bacteria propagate in the fly's gut, and the fly can contaminate people and fomites with its stools. The contamination danger is aggravated by the fact that, whether transmitted by insects, fomites, contaminated waters, or beverages, the lysogenized and Shiga toxin-producing *E. coli* strains are far more resistant to acid environments than *E. coli* strains not infected by phages, as reported by Veses-Garcia and colleagues in 2015.

In conclusion, the three-classical species of *Shigella* can exhibit horizontal gene transfer via lambdoid phages, resulting in the production of the cytotoxic Shiga toxin. Effective toxin production occurs only after prophage induction; this results in lysis of part of the *Shigella* population, releasing the toxin, and the free toxin in turn kills eukaryotic cells and provides new nutrients for the surviving *Shigella* cells, in what has been termed as "altruistic behavior" (Loś et al. 2013).

In addition, it was recently highlighted the potential risks involved in the formation of new strains from species currently considered quasi-commensal, such as *Enterobacter*. The threat is that these species could acquire Shiga toxin-encoding *stx* genes and generate new Stx-producing strains (via bacteriophage transduction), either in the human gastrointestinal tract or in food production environments (Khalil et al. 2016).

In contrast, bacteriophages can also play a role in the eradication of *E. coli* strains harboring Shiga toxin genes; Niu and colleagues reported, in 2012, the characterization of four T1-like lytic bacteriophages that efficiently lysed Shiga toxin-producing *E. coli* O157:H7 from cattle, and suggested that this approach could be used in the environmental control of these dangerous strains.

3.3 Salmonella *Toxins*

Salmonella is Gram-negative, facultative anaerobe rod that belongs to the *Enterobacteriaceae* family and is similar, in many aspects, to *E. coli*; but, unlike *E. coli*, most species of this bacterium cannot ferment lactose but can generate hydrogen sulfide (H_2S) producing clear colonies with a black center (fish-eye colonies) when plated in Hektoen enteric agar. *S. enterica* is the type species for this genus, and exhibits more than 2500 different serotypes, based on the expression of two antigens, O (somatic) and H (flagellar). Some species, such as *S. typhi* that causes typhoid fever, are exclusively human pathogens, causing diseases that include paratyphoid fever and food poisoning, also known as "salmonellosis." Although the bacteria were originally discovered by Carl Eberth in 1880, they were named after Daniel Elmer Salmon, a veterinary pathologist. Pathogenic species of *Salmonella* produce a variety of toxins and can also be lysogenized by various bacteriophages (Fig. 7). In fact, the generalized transducing bacteriophage P_{22} of *S. typhimurium* was associated with the initial discovery of transduction by Zinder and Lederberg (1952), which makes *Salmonella* a highly relevant subject to study bacteriophage-related HGT. *Salmonella* viruses are currently classified into five groups (P_{27}-like, P_2-like, lambdoid, P_{22}-like, and T_7-like) and three outliers (ε^{15}, KS_7, and Felix O1; Kropinski et al. 2007).

As early as 1957, Silliker and Taylor described a sort of relationship between *Salmonella* bacteriophages and the bacterial O-antigens. Another relevant discovery, reported by Robbins and coworkers in 1965, demonstrated that lysogenization of *Salmonella analum* by the temperate bacteriophage ε^{15} altered the composition of the O-antigen; it contained the recurrent sequence β-*D*-galactosyl-mannosyl-rhamnosyl instead of the usual O-acetyl-α-galactosyl-mannosyl-rhamnosyl. Fuller and Staub published similar results 3 years later (Fuller and Staub 1968); they reported that conversion by phage 14 resulted on a simple displacement of glucose, from one mannose residue to the mannose residue that immediately precedes *N*-acetylglucosamine. In 1971 Andrew Wright studied structure variations on the O-antigen by lysogenization with bacteriophage ε^{34} and found that the bacteriophage induced glucosylation of the O-antigen, thus confirming the effect of HGT in

Salmonella [for a comprehensive review on the role of bacteriophage P22-mediated transduction analysis of the rough A (*rfa*) region of the chromosome of *S. typhimurium*, as well as the involvement of HGT via bacteriophage transduction, see Sanderson and Saeed (1972)].

In 1981 Peterson and colleagues reported that several isolates of *Salmonella*, when treated with mitomycin, produced a bacteriophage and released a cholera-like toxin; however, in the following year, Houston and coworkers claimed that toxin production was totally unrelated to the presence of temperate bacteriophages (Houston et al. 1982). At the time, therefore, it appeared that *Salmonella* lysogenic conversions only affected the structure of the O-antigen and the lipopolysaccharide composition, which in some cases resulted in a total loss of virulence (Chart et al. 1989) on oral inoculation, although the bacteria remained virulent by parenteral administration (Nnalue and Lindberg 1990). In this century, however, Bacciu et al. (2004) reported that different serovars of epidemic isolates of *S. enterica* could be lysogenized by Gifsy-like lambdoid phages carrying known virulence genes, including the heat-stable toxin astA, or even the *art* genes (encoding ADP-ribosylating enzymes), and that the toxins could be transduced into the bacteria (Saitoh et al. 2005).

3.4 Pseudomonas *Toxins*

Pseudomonas is a Gram-negative, non-fermenting, rod-shaped bacterial genus, with a versatile adaptive metabolism. *Pseudomonas* comprises a variety of species, some of which are of industrial interest, while others are plant or animal pathogens. *P. aeruginosa* is the most notorious among the latter, and it was described as a pathogen by Liu (1964) who identified a variety of extracellular products (i.e., hemolysin, lecithinase, and protease) that play a role in the pathogenesis. Pavlovskis discovered in 1972 that this species can produce exotoxins that affect eukaryotic mitochondrial respiration. The bacterium uses this type of toxins (exotoxin A) to inactivate eukaryotic elongation factor 2 by ADP-ribosylation, thus halting protein synthesis in the host cell. In addition, *P. aeruginosa* secretes an exoenzyme, ExoU, that degrades the plasma membrane of eukaryotic cells. Pollack ad coworkers reported in 1977 that the majority of clinical isolates of *P. aeruginosa* produced exotoxin, as shown by their capacity to cause dermonecrotic lesions in guinea pigs. Transduction in *P. aeruginosa* has been extensively studied (Loutit 1958; Holloway and Monk 1959), and Holloway (1969) provides an early and excellent review, while Krylov et al. (2016) supply a contemporary publication.

It is currently known that the frequency of lysogeny is variable in *Pseudomonas* species (Fig. 7). It was originally thought that lysogeny did not occur in *P. putida* (although it was suggested as possible by Chakrabarty and Gunsalus in 1970), although it had been described in *P. fluorescens* (Patterson 1965) and *P. aeruginosa*, with the latter lysogenized by at least one phage (Holloway 1960; Zierdt and Schmidt 1964). Lysogenization results in dramatic antigenic changes in *P. aeruginosa*, that can involve loss of sensitivity to several typing phages (Lányi and Lantos 1976). On the other hand, generalized transduction has considerably

simplified the development of early linkage genetic maps for *P. aeruginosa* (Watson and Holloway 1978). To the best of our knowledge, it was not until 1994 (Hayashi et al. 1994) that cytotoxin-converting phages, φCTX and PS21 (pyocin-related phages), were found in cultures of particular *P. aeruginosa* strains, but this topic did not attract as much attention as the toxins previously discovered. In conclusion, there is still need for considerable research into the effect of transducing bacteriophages on the production of exotoxin A and related polypeptide toxins.

3.5 Vibrio cholerae *Toxins*

Vibrio cholerae is a Gram-negative, facultative anaerobe, rod-shaped bacterium, with a single polar sheathed flagellum. It has two circular chromosomes spanning 4×10^6 million DNA base pairs that are estimated to encode over 3880 genes. The bacterium was first identified as the causative agent of cholera in humans by Pacini (1854) and subsequently studied by Robert Koch (Lippi and Gotuzzo 2014). The species contains four biotypes, *cholerae*, *El Tor*, *proteus*, and *albensis*, easily distinguishable by the Voges-Proskauer reaction; this assay detects acetoin and is positive for El Tor. Cholerae and El Tor are the most relevant to humans, in particular cholerae, that in turn displays three serotypes, Ogawa, Inawa, and Hikojima.

V. cholerae is responsible for several pandemics worldwide, with the first six taking place in the state of West Bengal, India, and Bangladesh (Cook et al. 1984). The first four pandemics occurred between 1817 and 1875, but there is scarce information about the etiological agent involved. Microbiologist, however, succeeded in isolating the classical biotype during the fifth (1883) and sixth pandemics (1898–1923). El Tor, rather than the classical biotype, was responsible for the seventh pandemic, starting in 1960 (Cook et al. 1984).

Many *V. cholerae* strains can produce a potent toxin, choleragen, encoded within a pathogenicity island described as a "toxin co-regulated pilus" (TCP). This is a large genetic element (around 40 kb), that is flanked by two repetitive regions, a typical structure for an integrated bacteriophage. The pathogenicity island spans two gene clusters, involved in toxin production, that activate the adenylate cyclase of intestinal cells and display the typical AB structure of a bacterial toxin. The B subunit of the toxin initially binds the ganglioside GM1 and, following internalization, activates adenylate cyclase; this process requires NAD, ATP, GTP, and a calcium-dependent regulatory protein (Vaughan and Moss 1978).

Most of *V. cholerae* infections are asymptomatic; however, an estimated 5% of patients will develop cholera gravis, with symptoms that include abrupt onset of watery diarrhea (a gray and cloudy liquid, in some countries referred as rice-like depositions), vomiting, and abdominal pains. Water loss can be massive, up to 15–2 L per day, and originates intense thirst, decreased skin turgor, hypotension, tachycardia, tachypnea oliguria, renal failure, and coma and results in death. The location of the *tox* gene in bacteriophages is controversial and hence the horizontal gene transfer to nonproducing *V. cholerae* through transduction. Gerdes and Romig (1975) studied the genetic basis of toxin production in *V. cholerae* El Tor and

reported evidence against toxin production in this serotype originating from a phage conversion phenomenon (as is the case for *C. diphtheriae* and bacteriophage β); they stated that production of choleragen appeared to be independent from lysogenization by the "Kappa-type" bacteriophage. In 1987, however, Siddiqui and Bhattacharyya reported that a bacteriophage induced toxigenic changes in *V. cholerae*; the phage could increase toxin production in low-producing strains.

It is currently accepted that bacteriophage CTXφ contains the genes for cholera toxin (*ctxA* and *ctxB*). CTXφ is a filamentous phage, resembling M13, that harbors a positive-stranded ssDNA of 6.9 kb, that integrates at specific points in both bacterial chromosomes (Waldor and Mekalanos 1996; Boyd 2010); integration at a *dif* site is normally required for resolution of chromosome dimers generated by homologous recombination (Blakely 2004). CTXφ is unusual among filamentous phages because it encodes a repressor that enables it to form stable lysogens in *V. cholerae* (Kimsey and Waldor 1998a). Horizontal gene transfer via CTXφ transduction can fail due to a growth phase-regulated factor, characterized as a secreted hemagglutinin/protease produced by stationary-phase *V. cholerae* cells (Kimsey and Waldor 1998b). CTXφ in natural habitats could infect additional *Vibrio* species, thus transducing them into choleragen-producing strains. This has been demonstrated for the non-toxigenic species. *Vibrio mimicus* could, therefore, contribute to the propagation of the bacteriophage and, in turn, to the spread of cholera disease (Faruque et al. 1999). This horizontal gene transfer was finally demonstrated by Boyd and coworkers in 2000 (Fig. 7).

In addition to CTXφ, two further filamentous phages, designated fs1 (6.4 kb) and fs2 (8.5 kb), have been found in strains of *V. cholerae* O139; fs1 originated from clinical isolates of *V. cholerae* O1 (Ehara et al. 1997). In 1998, Jouravleva and colleagues isolated a new filamentous phage, named 493, from the O139 strain AJ27-493; this phage did not affect the Cholera biotype, but can infect El Tor. This novel bacteriophage contained a closed circular ssDNA genome of 9.3 kb, spanning a 0.4 kb double-stranded stem supporting a 2 kb single-stranded loop. In 2010, Campos and coworkers discovered a new filamentous bacteriophage isolated from *V. cholerae*, designated VEJϕf, that could also transduce the cholera toxin genes. This suggests that the variety of phages involved in the horizontal transmission of cholera toxin genes could be more diverse than initially thought (Campos et al. 2010). The bacteriophage is released from *V. cholerae* without bacterial lysis and infects non-lysogenized cells inducing them to produce more choleragen, and the toxin is then secreted, together with the bacteriophage, by the type II secretion system (Davis et al. 2000).

3.6 Bordetella *Toxins*

Bordetella, a genus named after the microbiologist Jules Bordet, is a non-sporulating, Gram-negative bacterium, with normally aerobic metabolism, that includes three classical species (*B. pertussis*, *B. parapertussis*, and *B. bronchiseptica*). *B. pertussis* is the main cause of whooping cough in humans, while *B. parapertussis* rarely produces the disease, and *B. bronchiseptica* causes several diseases in other mammals,

including kennel cough (infectious tracheobronchitis) and atrophic rhinitis in dogs and pigs, respectively. *B. pertussis* exhibits unique properties and secretes a variety of bioactive compounds, including the pertussis toxin, that until the end of the 1980s was not recognized as the main pathogenicity determinant. The toxin (that follows the typical AB structure of bacterial exotoxins) is secreted as an inactive precursor that is activated after being taken up by the cells (the B subunits act as transporters), most likely through the action of glutathione and ATP. Once activated, it ADP-ribosylates the α-subunit of the heterotrimeric G proteins, which results in an increase in the cellular concentration of cAMP (Burns 1988). The toxin can then produce severe neurological complications.

Considering that the other AB bacterial toxins described in this chapter are horizontally transmitted to non-toxigenic strains via bacteriophages (Fig. 7), the expectation would be that this was also the case for pertussis toxin. In fact, in 1980 and 1982, Lapaeva and coworkers reported this to be the case. Unfortunately, this has yet to be confirmed, although some publications demonstrated the occurrence of lysogeny in this bacterium (Holzmayer et al. 1988; Siniashina and Karataev 2006).

3.7 *Botulinum Toxin*

Botulinum toxin is a neurotoxic protein produced mainly by the anaerobe *Clostridium botulinum*, that was first described in 1820 by Justinus Kerner (1786–1862), based on clinical observations of the case referred to as the "sausage poisoning" (Erbguth and Naumann 1999). The bacterium was further studied and fully described by van Ermengem in 1897. The genus includes species such as *C. baratii* and strains of *C. butyricum* that can produce similar proteinaceous neurotoxins; they prevent the release of the neurotransmitter acetylcholine from axon endings at the neuromuscular junction, thus causing flaccid paralysis (Burgen et al. 1949), known as "the three Ds disease" (dystrophy, dysarthria, and dysphagia; this is not to be confused with pellagra (lack of niacin or tryptophan) that is also described as "the three Ds disease" (diarrhea, dermatitis, and dementia in this case).

Botulinum toxin type A, first crystallized by Lamanna and coworkers in 1946, has proved to be an effective and safe, if applied appropriately, treatment for diseases causing unwanted skeletal muscle contractions, such as strabismus, hemifacial spasm, focal dystonia, spasticity, and some autonomic disorders (Erbguth and Naumann 1999; Magid et al. 2015). It even has some unexpected applications, such as preventing excessive sweating (Bushara and Park 1994) or migraine (Ashkenazi 2010).

C. botulinum strains can be divided into four physiological groups that are sufficiently divergent to be considered as separate species; the sequencing of the type III genome was completed in 2011 by Skarin and coworkers. *C. botulinum* produces seven antigenically distinct neurotoxins, referred to as A, B, C_1, D, E, F, and G, with an eighth recently described, H (Sugiyama 1980; Barash and Arnon 2014; Dover et al. 2014). All these neurotoxins are rather similar, exhibiting a molecular mass nearing 150 kDa; they comprise a heavy chain, spanning

approximately 100 kDa, linked by a disulfide bridge to a light chain of roughly 50 kDa. Despite its biological potency, botulinum toxin is thermosensitive and is denatured by boiling (Licciardello et al. 1967).

Early reports (Eklund et al. 1969; Sugiyama and King 1972; Hariharan and Mitchell 1976) described bacteriophages in *C. botulinum* type isolates recovered from different sources, suggesting an active role for phages in bacterial toxigenicity. Indeed, phage conversion, and thus toxigenicity, in *C. botulinum* types C and D was first reported by Inoue and Lida in 1970 and 1971, and verified by Eklund et al. (1971, 1972) and Oguma et al. (1973). By 1972, Sugiyama and King had already reported the isolation of seven phages, belonging to three morphological groups (tailless, hexagonal head and sheathless flexible tail, and sheathed tail). In all cases, the hosts were restricted to non-proteolytic *C. botulinum* strains producing type B, E, or F neurotoxins (Fig. 7). None of the phages described was active on proteolytic *C. botulinum* producing types A, B, or F toxins. These studies, together with the work by Fujii et al. (1988), confirm that the toxigenicity of types C and D strains requires the continued participation of Tox^+ phages. On the other hand, Kinouchi et al. (1981) established that A toxin production does not require the involvement of bacteriophages.

In fact, the genes for type A, B, and E toxins are believed to be chromosomally located, whereas the corresponding genes for types C and D appear to be located in pseudolysogenic bacteriophages (Eklund et al. 1969; Eklund and Poysky 1974). Botulinum type G toxin gene is encoded within an 81 MDa plasmid (Eklund et al. 1988). Transmission of the botulinum toxin is exacerbated by the fact that it can be transferred between *Clostridium* species; Zhou and colleagues reported in 1993 the transfer of neurotoxigenicity from *C. butyricum* to a previously non-toxigenic *C. botulinum* type E-like strain, suggesting that the transfer could result from a transduction event caused by a defective phage rendered infective by a helper strain.

Toxigenic *Clostridium* species other than *C. botulinum*, such as *C. novyi* types A and B, are also affected by bacteriophages ($NA1^{tox+}$ and $NB1^{tox+}$). Eklund and coworkers reported in 1976 that specific bacteriophages induced the production of the lethal alpha toxin in *C. novyi* type A; indeed, when the type A strain 5771 was reverted to the phage-sensitive state, it also ceased to produce alpha toxin, although it continued to produce the gamma and epsilon antigens. Recently, Skarin and Segerman (2014) reported that plasmidome interchange between *C. botulinum*, *C. novyi* (the causative agent of gas gangrene/black disease), and *C. haemolyticum* (causing bacillary hemoglobinuria) converts strains of independent lineages into distinctly different pathogens. This implies that different interspecies recombinational events involving *Clostridium* plasmids and tox-harboring bacteriophages could contribute to the appearance of totally new toxigenic strains.

The putative HGT origin of the toxigenic capacity has also been studied in other pathogenic clostridia, including *C. tetani* and *Clostridioides difficile* (formerly *Clostridium difficile*; Lawson et al. 2016). *C. tetani* exhibits a genome spanning 2,80 Mbp, containing 2,373 ORFs (Brüggemann et al. 2003). This species produces a potent neurotoxin that, if released in deep wound infections, can cause spastic paralysis and death in humans and other vertebrates; this is achieved by blocking the

release of neurotransmitters from presynaptic membranes in interneurons of the spinal cord, thus preventing muscle relaxation. The tetanus toxin is encoded (*tetX*) on a plasmid of around 74 kb, that contains 61 genes (Brüggemann and Gottschalk 2004). To the best of our knowledge, no bacteriophage has ever been associated with gene spreading in this bacterium. *Clostridioides difficile*, on the other hand, has a recently identified bacteriophage (φSemix9P1), that supplies it with a fully functional binary toxin, obtained by HGT (Riedel et al. 2017).

3.8 Staphylococcus *Toxins*

Staphylococcus is a classical genus, with over 40 species, that is well known in Microbiology; it was first identified in 1880 by Sir Alexander Ogston (1984) in an infected knee joint. It is a Gram-positive coccus, with a strong tendency to form grape-like groups (its name originates from Greek word *staphylē*, meaning grape). *Staphylococcus aureus* is normally oxidase positive, while other species are negative; it has 30–40% G+C content in the DNA and is catalase positive.

The increasing incidence of antibiotic resistance in *S. aureus* has led the scientific community to believe that horizontal gene transfer within this genus must be much higher than previously expected (Chan et al. 2011). This bacterial species produces several enzymes, such as coagulase, hyaluronidase, lipase, DNase, and staphylokinase. In addition, the bacterium can produce a variety of toxins that include three groups of exotoxins (Dinges et al. 2000), enterotoxins, exfoliative toxins (that produce the denominated "staphylococcal scalded skin syndrome" and are the leading contributors to the toxic phenotype of impetigo in *S. aureus* strains), α-toxin, β-toxin, δ-toxin (that act on eukaryotic cell membranes), and Panton-Valentine leukocidin (a toxin that represents a growing threat worldwide; Sheikh et al. 2015). These toxins are encoded by *tox* genes in bacteriophage Φ-PVL; in fact, leukocidin is encoded by two co-transcribed genes, lukS-PV and lukF-PV, carried by temperate phages from the *Siphoviridae* family (i.e., double-stranded DNA, with an icosahedral or elongated-head morphology, and a noncontractile tail; Canchaya et al. 2003). Several PVL phages are currently identified and sequenced (Kaneko et al. 1998; Ma et al. 2008; Sanchini et al. 2014), and six PVL phages (ΦPVL, Φ108PVL, ΦSLT, ΦSa2MW, ΦSa2USA, and ΦSa2958) have been described to contain single-nucleotide polymorphisms (Boakes et al. 2011).

Most *S. aureus* strains harbor at least one prophage (Botka et al. 2015), which replicates as part of the bacterial chromosome. To date, as described by Botka and colleagues, more than 1880 complete genomes of bacteriophages have been reported, 96 belonging to staphylococcal bacteriophages that are directly involved not only in toxin production, but also in many aspects of the bacterial metabolism, including enhancing bacterial virulence and mediating horizontal transfer of pathogenicity (Novick et al. 2010).

Staphylococcal intoxication by contaminated food is one of the most common cases of gastroenteritis originated by enterotoxins (enterotoxin A is one of the most important and is encoded by the *entA* gene of *Staphylococcus*) that, in the USA

alone, generates a burden of disease affecting nearly a quarter of a million people every year (Scallan et al. 2011; Zeaki et al. 2015). Already in 1985, Betley and Mekalanos reported that pathogenic *S. aureus* can carry a polymorphic family of phages (some of which could be defective), enabling the bacterium to produce enterotoxin A (Fig. 7). In 1989, Coleman and coworkers described an entirely novel group of serotype F bacteriophages (φ42) that interestingly simultaneously mediated a triple-lysogenic conversion of enterotoxin A, staphylokinase, and beta-lysin; according to the authors: "*The phages were recovered from methicillin-resistant strains of S. aureus isolated in Irish hospitals between 1971 and 1988 and from strain PS42-D, which has been used as the propagating strain for the S. aureus typing phage 42D since before 1965.*" Although Smeltzer and coworkers had already described in 1994 that the genome of *S. aureus* strain S6C contains a prophage inserted within the beta-toxin structural gene, the full characteristics of this phage were not understood until Dempsey and colleagues reported, in 2005, that φ42 is a quadruple-converting phage that encodes a truly novel restriction/modification system (named Sau42I).

Zeaki and colleagues reported in 2015 that, up to date, there are 22 staphylococcal enterotoxins or enterotoxin-like substances identified, differing on the encoding genetic element (plasmids, prophages, or pathogenicity islands). Enterotoxin A accounts for nearly 80% of all staphylococcal intoxications (Hennekinne et al. 2012) and is encoded by a CC;-like bacteriophage that can perform either a lysogenic or a lytic cycle. The regulation of the *tox* gene, and hence the amount of enterotoxin A produced, can vary according to the bacterial strain (Zeaki et al. 2015).

Two novel bacteriophages (ϕB166 and ϕB236) have been recently identified as responsible for the production of exfoliative toxins, and they belong to two yet uncharacterized *Siphoviridae* lineages that produced massive outbreaks of *pemphigus neonatorum* in Czech maternity hospitals. The bacteriophages were confirmed to harbor the impetigo toxin gene and could positively transduce this capability to prophageless *S. aureus* strains that started producing the toxin upon phage infection (Botka et al. 2015).

Concerning virulence islands, Novick and Subedi described in 2007 the SaPIs mobile pathogenicity island (a paradigmatic model of molecular parasitism; Ram et al. 2012) in S*taphylococcus*, and it spans 15–17 kb and encodes at least two superantigens. This island is highly conserved, integrated in a specific chromosomal site from which it can be induced to excise and replicate by specific bacteriophages; it was described by Guinane et al. (2011) to encode a novel von Willebrand factor-binding protein with ruminant-specific coagulase activity. SaPIs can also be mobilized by helper bacteriophages if they can inactivate the SaPI repressor Stl (Dearborn and Dokland 2012); this is the case for bacteriophage 80α that can mobilize several SaPIs, including SaPI1 and SaPIbov1.

The genomic island νSaβ encodes staphylococcal superantigens, as well as proteases, leukotoxins, and bacteriocins; it was shown to become transferrable between different bacterial strains via transduction by bacteriophages carrying overlapping νSaβ segments (Moon et al. 2015). According to the authors: "*Our findings solve a long-standing mystery regarding the diversification and spread of the genomic*

island νSaβ, highlighting the central role of bacteriophages in the pathogenic evolution of S. aureus."

Finally, there are some bacteriophages that, when undergoing a lysogenic cycle, integrate the phage DNA inside the bacterial toxin gene, preventing toxin production and converting a previously toxigenic staphylococcal strain into a non-toxigenic bacterium; one such an example is bacteriophage φSa3 that inserts into the reading frame of the chromosomal β-toxin gene (Salgado-Pabón et al. 2014).

3.9 Streptococcus *Toxins*

Streptococcus is another well-characterized Gram-positive genus in Microbiology; they grow in chains or pairs and are typically oxidase and catalase negative. Streptococci are divided into six groups according to their 16S rDNA sequences, *S. anginosus*, *S. bovis*, *S. mitis*, *S. mutans*, *S. pyogenes*, and *S. salivarius* (Kawamura et al. 1995). Streptococci are also classified into group A (*S. pyopeges*), group B (*S. agalactiae*), group C (*S. equi*), group D (*Enterococcus faecalis*), group F (*S. milleri*), and group G (*S. dysgalactiae*). *S. pneumoniae* (formerly known as *Diplococcus pneumoniae*, a member of the *S. mitis* group) is associated with apes, including humans (Fox and Soave 1971). It is the main causative agent of pneumonia in humans (>93% of pneumonia cases), causing such an impact that it is often referred to as "Captain of the Men of Death." Without doubt, *S. pneumoniae* is the most successful pathogen, endemic in all human societies, that, in addition to the classic pneumonia (although quite often it is group A *S. pyogenes* the responsible pathogen), produces a variety of diseases. These diseases include pink eye, tonsillitis, meningitis, sinusitis, arthritis, renal failure through acute glomerulonephritis, rheumatic fever, endocarditis, erysipelas, necrotizing fasciitis, and scarlet fever. When first isolated, the bacteria are largely capsulated, with the capsule enhancing the bacterial ability to cause disease. In fact, immunization with specific capsular polysaccharides prevents many of the streptococcal diseases (MacLeod and Hodges 1945). *S. pneumoniae* represents the first living organism in which the chemical nature of a given genetic trait was demonstrated (Avery et al. 1944), although Griffith publication in 1928 already provided an important hint into this matter.

Streptococci secrete a variety of products, many of which are enzymes (such as streptokinase and streptodornase), several are toxins (i.e., erythrogenic toxin, characteristic of scarlet fever), and a variety of antigens that, either alone or in combination, enhance bacterial virulence and/or help the bacterium evade the host defense mechanisms (Reglinski and Sriskandan 2014). Many of these traits are, at least in principle, amenable to be transferred between streptococcal species, not only by the "natural" genetic transformation process, but also through the relevant bacteriophages.

Bacteriophages (such as DP-1; McDonnell et al. 1975) are very common in the streptococcal world, with nearly 20 currently known. These phages belong to *Siphoviridae*, *Podoviridae*, *Myoviridae*, and *Inoviridae* viral families, and display genomic sizes between 38 and 41 kb. In 1981 Ronda et al. (1981) described a new

family, which they named Complutense (Cp). Already in 1927, Frobisher and Brown, using doubly marked bacterial strains, demonstrated the existence of a filtrable agent that could transfer the ability to synthesize erythrogenic toxin from a toxin-producing streptococcal strain to nonproducing strains. In fact, it was first Bingel (1949) and later Zabriskie (1964) who proved that toxin production was related to the formation of mature bacteriophages, while it did not involve an increased production of other extracellular products, such as streptodornase. However, Quinn and Lowry reported in 1974 that lysogeny and erythrogenic toxin production occurred simultaneously in only half of the bacterial strains tested, suggesting that erythrogenic toxin production did not necessarily require a bacteriophage. These results were inconsistent with a later publication by Johnson and coworkers (1980), who clearly demonstrated that pyrogenic exotoxin production was due to lysogenic conversion of the bacterium by the relevant temperate bacteriophage. Finally, McKane and Ferretti (1981) and Weeks and Ferretti (1984) proved that infection of a non-toxigenic *S. pyogenes* strain with bacteriophage T12 resulted in the production of either erythrogenic toxin or streptococcal pyrogenic exotoxin. In 1984, Johnson and Schlievert demonstrated unequivocally that phage T12 also encodes the structural gene for pyrogenic exotoxin type A. This was followed by the discovery that other pyrogenic streptococcal toxins were also encoded by bacteriophages, as is the case for pyrogenic exotoxin C (Goshorn and Schlievert 1989). As pointed out by Beres et al. (2002), and also in 2002 by Banks and colleagues, recombination can result in the production of a variety of chimeric phages, with uncharacterized virulence factor genes, including pyrogenic streptococcal exotoxins, making bacteriophage T12 a quintessential phage type.

In addition, Group A *S. pyogenes* can acquire, via transduction, genetic capabilities other than the pyrogenic and erythrogenic toxins described above. Skjold and colleagues showed in 1982 that the genetic determinant for streptolysin S production was transferred to this bacterium by a bacteriophage; streptolysin S was named so because its activity is stable to atmospheric oxygen (as opposed to streptolysin O), and hence is responsible for the hemolytic zone visible around streptococcal colonies. *S. equi* (the etiological agent of zoonotic meningitis in humans) uses the bacteriophage-encoded hyaluronate lyase to degrade hyaluronan, the main polysaccharide component of the host connective tissues, and related tissue components, to facilitate pathogenic spread (Singh et al. 2014).

Lyt A is the major pneumococcal autolysin (*N*-acetylmuramoyl-*l*-alanine amidase; EC 3.5.1.28) that triggers the denominated "bile solubility test." Interestingly, all pneumococcal prophages reported to date encode a 318 amino acid-long NAM amidase that resembles the bacterial LytA (Morales et al. 2010); this strongly suggests a role for streptococcal bacteriophages in the horizontal transfer of this enzyme (Fig. 7).

Similarly, Group A *S. pyogenes* secretes streptodornase (an extracellular DNA-hydrolyzing enzyme) to degrade the DNA from neutrophils and facilitate infection. These DNases are encoded by bacteriophages (phage SF370.1; Korczynska et al. 2012) and are produced during prophage induction.

Streptokinase, the most important prokaryotic plasminogen activator, is produced and secreted as a 47 kDa protein by many pathogenic streptococci belonging to different serogroups (Müller et al. 1989). Mechold et al. (1993) studied the genetic organization of the streptokinase gene in *Streptococcus equisimilis* H46A, and it appears that this structural gene is not subjected to horizontal transfer mediated by bacteriophages. However, the alleles of the streptokinase gene have a mosaic structure (Kapur et al. 1995), suggesting that they have been acquired through horizontal transfer, which, if it was not transduction, must be genetic transformation.

More than 37 strains of *S. pyogenes* have been fully sequenced, and it is currently clear that all of them are heavily lysogenized by different bacteriophages. Euler and colleagues recently developed a method for curing *S. pyogenes* from all the bacteriophages (Euler et al. 2016); they obtained a bacterial strain (CEM1ΔΦ) completely cured from all bacteriophage elements, which should be of great assistance to finally comprehend the role of bacteriophages in the *Streptococcaceae* family.

3.10 Listeria monocytogenes *Toxins*

Listeria monocytogenes is a Gram-positive, facultative anaerobic, nonspore-forming rod-shaped bacterium that is catalase positive and oxidase negative. It is motile at 20–25 °C, by means of peritrichous flagella, but not at 37 °C. It was originally described, by Murray and coworkers (1926), as *Bacterium monocytogenes* and later renamed as *Listeria monocytogenes* by Harvey in 1940, in honor of Joseph Lister. *L. monocytogenes* is normally transmitted by contaminated food, such as raw or pasteurized milk, cheese, ice cream, raw vegetables, raw poultry and other kind of meats, and by smoked fish. It can produce an invasive infection (where the bacterium shows a clear intracellular status) with production of septicemic syndrome (with an estimated mortality of 50%), meningoencephalitis (mortality 70%), and pneumonia and sometimes can be a cause of stillbirth in pregnant women. *L. monocytogenes* can move from cell to cell without requiring an extracellular phase, thus evading circulating antibodies; it achieves this by means of the nucleation of actin at the rear of the bacterium that "pushes" it into the neighboring cell (Tilney and Portnoy 1989). This microorganism produces a toxin (listeriolysin O), a hemolysin considered a true and crucial virulence factor (Cossart 1988), that is thiol-activated and cholesterol-dependent and has pore-forming activity. Listeriolysin O is encoded by the gene *hly*, which is part of a pathogenicity island called LIPI-1 (Vázquez-Boland et al. 2001).

L. monocytogenes is susceptible to several bacteriophages, and one of these phages is A511, a virus belonging to the *Myoviridae*, genus *Twortlikevirus* (Loessner and Scherer 1995; Adams and Carstens 2012) (Fig. 7). But the studies on this phage have been mainly focused on its use as a control agent for *L. monocytogenes*, rather than its role as mediator of HGT. A good example of these studies is Loessner's publication in 1996 and the fact that the FDA approved the use of bacteriophages as additives to meat and poultry products (Lang 2006). Sword and Pickett (1961) studied the occurrence of lysogeny in 123 strains of

L. monocytogenes, isolated from humans and animals in different parts of the world, identifying eight types of bacteriophage that lysed the bacterium. The aim of their study, however, was to use these viruses in phage-typing, since this technique, introduced by Craigie and Yen in 1938 for *Salmonella typhosa*, appeared to be a good tool for strain identification. As expected, Hodgson (2000) finally confirmed the occurrence of generalized transduction in *L. monocytogenes* that involved the participation of several bacteriophages, although the author could not prove the transduction of toxigenic genes into this species. However, Chen and Novick (2009) described bacteriophages with the ability to intergenerically (*S. aureus* to *L. monocytogenes*) transduce pathogenicity islands, hence creating bacterial strains capable of toxin production. This represents a completely new paradigm in the involvement of bacteriophages in HGT.

3.11 Helicobacter pylori *Toxins*

H. pylori (Marshall and Warren 1983) is a Gram-negative, non-sporulating, microaerophilic bacterium that is urease, catalase, hydrogenase, and oxidase positive. *H. pylori* is helix-shaped, making the bacterium capable of penetrating the stomach epithelium, and it is motile by means of two to six sheathed polar flagella. This species exhibits a remarkable ability to elaborate biofilms (Stark et al. 1999), as expected from a pathogen intimately associated with animal tissue. It can produce peptic ulcers or cancer in animal tissues, and appears to have derived from animal tissues its capacity to produce fucosylated O-antigen (Kusters et al. 2006). Pathogenic *H. pylori* strains contain a Cag pathogenicity island, spanning 40 kb and containing 27 genes which encode several effector proteins and one type IV secretion (T4S) system. The cytotoxin-associated *cag*A gene is important for virulence and is associated with the ability of the bacterium to cause ulcers (Broutet et al. 2001); this gene is believed to have been acquired by horizontal transfer from other bacteria (Tomb et al. 1997). As indicated by Zhang and coworkers in 2017, T4S systems are important in the sense that they play important roles in processes such as conjugative transfer of genetic material, the uptake and release of DNA, and the translocation of CagA protein (facilitated by CagX, a protein present in T4S) into gastric epithelial cells.

Another *H. pylori* virulence factor is the vacuolating cytotoxin A (VacA), a key toxin for the bacterium that causes massive swelling of the membranous compartments that comprise the late stages of the endocytic pathway (de Bernard et al. 1997).

Recently, Kyrillos et al. (2016) reported that *H. pylori* strains contain sequences ortholog to the bacteriophage φHP33 DNA that correlate with the presence of both virulence genes, thus further supporting the hypothesis that these genes were acquired via HGT (Fig. 7).

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Genomic Islands and the Evolution of Multidrug-Resistant Bacteria

Mario Juhas

Abstract The horizontal gene transfer is crucial for the evolution and adaptation of bacteria. An important part of the horizontal gene transfer is facilitated by the large, discreet DNA segments called genomic islands. Some genomic islands encode means of their own excision, self-transfer and integration into the chromosome, while others can be mobilized by other mobile genetic elements or are stably integrated into the chromosomes of the host bacteria. Genomic islands are involved in the dissemination of a wide variety of genes, including virulence and antibiotic-resistant genes. This review provides an update on the investigation of genomic islands with particular emphasis on their role in the evolution of multidrug-resistant bacteria.

Keywords Horizontal gene transfer · Genomic island · MDR bacteria · ICE · *Escherichia coli* · *Pseudomonas aeruginosa* · *Salmonella enterica* · *Proteus mirabilis* · *Acinetobacter baumannii* · *Staphylococcus aureus*

1 Horizontal Gene Transfer of Resistance Genomic Islands

The horizontal gene transfer is crucial for the evolution and adaptation of bacterial species (Soucy et al. 2015; Koonin 2016). An important part of the horizontal gene transfer is facilitated by the large, discreet DNA segments called genomic islands (GIs) (Carraro et al. 2017b; Juhas et al. 2009). Some GIs are nonmobile and firmly integrated into the chromosomes of the host bacteria. Alternatively, GIs such as SGI/PGI/AGI-like GIs found in *Salmonella enterica*, *Proteus mirabilis*, *Morganella morganii* and *Acinetobacter baumannii* described in this review can be mobilized by other mobile genetic elements that are present in the host genome (Siebor et al. 2016, 2018; Kiss et al. 2015; Murányi et al. 2016; Carraro et al. 2017a, b;

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de Curraize et al. 2018; Hawkey et al. 2018; Schultz et al. 2017). Finally, some GIs encode means of their own horizontal transfer into the new host cells. These mobile GIs include a large group dubbed integrative and conjugative elements (ICEs). Although ICEs are often found integrated into the host chromosome, they are capable of excision, transfer into the new host cells by conjugation and reintegration into the chromosome of the new host. ICEs known to be involved in the spread of antibiotic-resistant genes include ICEs found in *Haemophilus* spp. described previously or the recently identified *Pseudomonas aeruginosa* ICEs presented in this review (Juhas et al. 2007a, b, 2008, 2009; Carraro and Burrus 2014; Juhas 2015c; Botelho et al. 2018; Roy Chowdhury et al. 2016, 2017).

In addition to genes crucial for self-propagation, GIs can carry a broad spectrum of accessory open reading frames involved in the virulence (pathogenicity islands), symbiosis (symbiosis islands), metabolism (metabolic islands), fitness (fitness islands) or resistance to antimicrobials (resistance islands) (Juhas et al. 2009). As resistance GIs are capable of carrying large numbers of variable antibiotic-resistant genes, their acquisition can provide the host bacterium with the resistance to the whole array of antimicrobials. The process of the rapid evolution of the multidrug-resistant (MDR) bacteria facilitated by GIs has therefore been dubbed the evolution by "quantum leaps" (Groisman and Ochman 1996; Hacker and Carniel 2001). This review provides an update on the contribution of GIs to the evolution of MDR bacteria, with the particular emphasis on GI3, which contributed to the increased pathogenicity of the *Escherichia coli* O104:H4 2011 outbreak strain and the most recent analyses of SGI/PGI/AGI-like GIs in MDR *S. enterica*, *P. mirabilis*, *M. morganii* and *A. baumannii*, SCC*mec* in MDR *Staphylococcus aureus* and GIs involved in the evolution of MDR *P. aeruginosa*.

2 GI3 in MDR *Escherichia coli* O104:H4

The outbreak of the highly pathogenic *E. coli* strain O104:H4 in 2011 which started in Germany resulted in nearly 5000 cases of severe infections associated with bloody diarrhoea, nearly 1000 cases of haemolytic uremic syndrome and 82 deaths (Rasko et al. 2011). The increased pathogenicity of the *E. coli* O104:H4 2011 outbreak strain was attributed to the acquisition of a battery of mobile genetic elements (Grad et al. 2013). These included Shiga toxin 2 (*stx2*)-encoding prophage typically found in enterohaemorrhagic *E. coli* strains, which in combination with the increased aggregative adherence typical for the enteroaggregative *E. coli* led to the increased absorption of Shiga toxin in the infections caused by the *E. coli* O104:H4 outbreak strain (Juhas 2015a; Rasko et al. 2011).

Notably, the *E. coli* O104:H4 2011 outbreak strain was found to be resistant to multiple antibiotics. In addition to 90 kb IncI1 plasmid encoding the CTX-M-15 cephalosporinase, which was unique to the 2011 outbreak strain (Künne et al. 2012), comparative whole-genome analyses revealed the presence of the *E. coli* genomic island 3 (GI3) in the chromosomes of the enteroaggregative haemorrhagic *E. coli*

O104:H4 outbreak strains. GI3, considered to be the hotspot for microevolutionary genetic events in the *E. coli* O104:H4 strains, harbours multiple antibiotic-resistant genes, namely, $bla_{\text{TEM-1}}$, *dfrA7*, *strAB*, *sul2* and *tet(A)*A encoding resistance to ampicillin, trimethoprim, streptomycin, sulfamethoxazole and tetracycline, respectively (Roy Chowdhury et al. 2015). The resistance genes in GI3 are located in the complex antibiotic-resistant gene loci (CRL), which also include *ag43* encoding the virulence factor involved in the biofilm formation and *mer* genes encoding the resistance to mercury (Grad et al. 2013). Bioinformatics analysis revealed that the evolution of CRL in GI3 has been mediated by Tn*6029* family of transposons carrying the $bla_{\text{TEM-1}}$-*sul2-strA-strAB* gene cluster flanked by IS*26* (Roy Chowdhury et al. 2015).

The presence of the multiple antibiotic-resistant genes in the genome of *E. coli* O104:H4 contributed to the 2011 outbreak. In addition to limiting the available treatment options, the multiple antibiotic-encoding GI3 in combination with the IncI1 plasmid-encoded CTX-M-15 cephalosporinase played a key role in the ability of the *E. coli* O104:H4 2011 outbreak strain to outcompete the other commensal *E. coli* strains in the guts of patients that were treated with antibiotics (Bielaszewska et al. 2011; Juhas 2015a).

Interestingly, although found to be integrated into the chromosomes of the host *E. coli* O104:H4 strains, GI3 was previously shown to be mobile (Roy Chowdhury et al. 2015). To integrate into the chromosome, GI3 targets a 23 bp genomic sequence found in a broad spectrum of *Enterobacteriaceae*. This highlights the potential risk of GI3 being involved in the emergence of a number of other MDR *Enterobacteriaceae* in the future (Roy Chowdhury et al. 2015).

3 SGI/PGI/AGI-Like GIs in MDR *Salmonella enterica*, *Proteus mirabilis* and *Acinetobacter baumannii*

Salmonella genomic island 1 (SGI1, 42.4 kb), its variants and related GIs, such as SGI2, *Proteus* genomic island (PGI1) and *Acinetobacter* genomic island (AGI1) are frequently found in a broad spectrum of *Enterobacteriaceae* (Siebor et al. 2016; Soliman et al. 2017; Hamidian et al. 2015b, c). Although usually stably integrated into the host cell's chromosome, SGI1 can be mobilized by the helper plasmids of the incompatibility groups A and C (IncA and IncC) (Siebor et al. 2016). It was shown that mobilization of SGI1 is initiated by the helper plasmid-encoded master transcriptional activator complex AcaCD. AcaCD is crucial not only for the expression of genes involved in the excision of SGI1 from the chromosome but also for the expression of the three SGI1-encoded *tra* genes involved in the transfer of SGI1 into a new host cell (Kiss et al. 2015; Murányi et al. 2016). Recent studies showed that SGI1 can reshape the conjugative apparatus of the helper plasmids to promote its own transfer (Carraro et al. 2017a). Besides a conserved backbone comprised of 28 open reading frames involved in the life cycle, excision, transfer and reintegration

Fig. 1 Schematic representation of the SGI/PGI/AGI-like genomic islands. The figure shows the key features of the SGI/PGI/AGI-like genomic islands, which are involved in the evolution of MDR *Salmonella enterica*, *Proteus mirabilis*, *Acinetobacter baumannii* and *Morganella morganii*. SGI/PGI/AGI-like genomic islands are integrated into the chromosome in the 3′ end of the *trmE* gene and are flanked by the *attL* and *attR* attachment sites. In addition to the backbone region carrying open reading frames crucial for the life cycle (excision from the chromosome, transfer and integration into the new host's chromosome), SGI/PGI/AGI-like genomic islands also harbour MDR region encoding resistances to a number of different antibiotics, such as ampicillin (AMP), chloramphenicol (CHLO), sulfamethoxazole (SUL), streptomycin/spectinomycin (STR/SPE) and tetracycline (TET)

of SGI1, SGI1 harbours a complex MDR region conferring resistance to multiple antibiotics, including ampicillin, tetracycline, sulfamethoxazole, chloramphenicol/florfenicol and streptomycin/spectinomycin (de Curraize et al. 2018) (Fig. 1).

Proteus genomic island (PGI1) of 81.1 kb, found in *P. mirabilis*, has a gene backbone similar to that of SG1, in addition to the complex MDR region composed of transposons and IS elements and a number of antibiotic-resistant genes, including metallo-β-lactamase-resistant gene $bla_{\text{NDM-1}}$ and extended-spectrum-β-lactamase-resistant gene $bla_{\text{VEB-6}}$ (Siebor and Neuwirth 2014).

Recently, a novel resistance GI, GI*Pm1* of 55.8 kb, was identified in *P. mirabilis* inserted into the chromosome at the *trmE* site, as is typical for other GIs of this subfamily, such as SGI1 and PGI1. GI*Pm1* is composed of the gene backbone similar to GI found in *Enterobacter cloacae* DSM16690 and MDR region conferring resistance to multiple antibiotics (Siebor et al. 2018). The detection of the extra-chromosomal circular form GI*Pm1* suggests that GI*Pm1* is mobile; however, its mobilization is not facilitated by the IncA and IncC helper plasmids but by another, yet unknown mechanism (Siebor et al. 2018).

Recently, two new variants of SGI1 named SGI1-K7 (55.1 kb) and SGI1-*Pm*2CHAMA (53.6 kb) were identified in *P. mirabilis* whose MDR regions carried resistance genes never before identified in this subfamily of GIs, namely, $bla_{\text{CTX-M-15}}$ and $bla_{\text{CARB-2}}$ (de Curraize et al. 2018). Particularly alarming is the identification of $bla_{\text{CTX-M-15}}$ encoding an extended-spectrum-β-lactamase usually found in MDR *E. coli* clinical isolates among the open reading frames of SGI1-K7. Notably, CTX-M-15 was also found in the *E. coli* O104:H4 2011 outbreak strain (Künne et al. 2012; Rasko et al. 2011).

AGI1 with the gene backbone similar to SGI1, PGI1 and their variants was found in the chromosome of *A. baumannii*. AGI1 is integrated into the *A. baumannii* chromosome at the *trmE* site, similar to other GIs from this subfamily. Furthermore, AGI1 carries a MDR region harbouring genes bla_{PER}, *aadB*, *aadA13/2*, *aadA2*, *strAB* and *sul1* conferring resistance to cephalosporins, aminoglycosides and sulphonamides (Hamidian et al. 2015a). Besides AGI1, *A. baumannii* chromosome often harbours other types of resistant GIs, such as *A. baumannii*-resistant island (AbaR). These GIs, together with the plethora of other mobile genetic elements and

the high intrinsic resistance of *A. baumannii* to a number of clinically used antimicrobials, such as the second-generation cephalosporins, aminopenicillins and chloramphenicol, make MDR *A. baumannii* one of the main public threats nowadays (Pagano et al. 2016; Blackwell et al. 2015; Kim et al. 2017; Gallagher et al. 2017; Hawkey et al. 2018).

SGI1-like variant dubbed SGI1-L harbouring $bla_{\text{CARB-2}}$, *dfrA15*, *floR*, *sul1* and *tetA*(G) conferring resistance to amoxicillin, trimethoprim, phenicols, sulphonamides and tetracyclines has been recently identified in a MDR *Morganella morganii* subsp. *morganii* (Schultz et al. 2017).

While the gene backbone region of the SGI/PGI/AGI-like subfamily of GIs is relatively well conserved, their MDR region is very plastic as a result of microevolution events, which include IS element-mediated genome rearrangements and homologous recombination (de Curraize et al. 2018). Taken together with the ability to be mobilized by the helper plasmids, SGI1 and its variants and related GIs, such as PGI1 and AGI1, present a high risk for the dissemination of the clinically relevant antibiotic-resistant genes among other *Enterobacteriaceae* (de Curraize et al. 2018).

4 SCC*mec* in MDR *Staphylococcus aureus*

S. aureus is a highly versatile Gram-positive opportunistic pathogen causing a variety of diseases, ranging from minor skin infections to endocarditis, osteomyelitis, pneumonia and septicaemia. Methicillin-resistant *S. aureus* (MRSA) is considered to be among the main threats to human health nowadays (Juhas 2015a). Alarmingly, MRSA strains have in the meantime acquired resistances to a number of other antibiotics, including β-lactams, lincosamides and macrolides and more recently also vancomycin (Jani et al. 2017). Consequently, MDR MRSA strains are among the leading causes of morbidity and mortality and are among the most significant sources of both nosocomial and community-acquired infections (Anderson et al. 2014).

Resistance of *S. aureus* to methicillin was acquired via PBP2a-encoding gene *mecA* located on the staphylococcal chromosome cassette methicillin-resistant (SCC*mec*) island. SCC*mec* islands can be highly diverse ranging from 20 to 70 kb. Furthermore, SCC*mec* islands are classified into subtypes based on sequences of serine recombinases *ccrAB* and *ccrC* and regulators *mecI* and *mecR1* (Ray et al. 2016). Recombinases *ccrAB* and *ccrC* play an important role in the excision, circularization and site-specific integration of SCC*mec* into the *S. aureus* chromosome (Smyth et al. 2011; Ray et al. 2016).

The origin of *mecA* in *S. aureus* remains still unclear; however, bioinformatics analyses suggest that it has been acquired horizontally from other staphylococcal species, such as *Staphylococcus epidermidis*, *Staphylococcus fleuretti* or *Staphylococcus haemolyticus* (Juhas 2015a; Xue et al. 2017; Smyth et al. 2011; Ray et al. 2016; Wipf et al. 2015; Tsubakishita et al. 2010; Hosseinkhani et al. 2018). There are several lines of evidence for this claim. First, SCC*mec* subtype IV has been found in

the genome of *S. epidermidis* before it has been reported in *S. aureus* (Smyth et al. 2011). Second, the transfer of SCC*mec* island captured on staphylococcal conjugative plasmid into isogenic *S. aureus* and *S. epidermidis* has been demonstrated in vitro (Ray et al. 2016). Third, SCC*mec* elements have been identified in *S. epidermidis* carrying resistance genes against *β*-lactams, heavy metals and polyamines. It was shown that the resistance genes located on the *S. epidermidis* SCC*mec* elements originated from various bacteria, habitats and geographic regions (Xue et al. 2017). This highlights the role of *S. epidermidis* as a reservoir of resistance genes implicated in the evolution of MDR *S. aureus*. Fourth, *S. fleuretti* naturally harbours *mecA* in its chromosome and is therefore suspected to be the source of the *mecA* in the SCC*mec* island (Wipf et al. 2015; Tsubakishita et al. 2010). Fifth, homology searches showed the presence of the SCC*mec*-borne *ccrB*-encoding recombinase in the genomes of a number of other staphylococci (Fluit et al. 2013). Finally, highly diverse SCC*mec* islands were identified in the genome of *S. haemolyticus* recently, highlighting the role of *S. haemolyticus* in carrying the methicillin-resistant genes (Hosseinkhani et al. 2018).

Interestingly, the gene clustering approach has led to identification of a number of novel GIs which harbour a wide variety of resistance genes in the genome of *S. aureus*, recently. It has been suggested that these yet uncharacterized GIs can shed light on the evolution of MDR in *S. aureus* (Jani et al. 2017).

5 GIs in MDR *Pseudomonas aeruginosa*

P. aeruginosa is an opportunistic Gram-negative human pathogen frequently associated with the chronic nosocomial infections, particularly in immunocompromised individuals (those with AIDS, severe burns and cancer and cystic fibrosis patients) (Oliver et al. 2000; Azam and Khan 2018). The emergence and rapid spread of antibiotic-resistant *P. aeruginosa* led to the classification of the drug-resistant *P. aeruginosa* among the greatest threats to public health by WHO, CDC and ECDC (CDC 2013; ECDC 2015). *P. aeruginosa* infections are notoriously hard to eradicate due to the vast array of intrinsic, adaptive and acquired resistance mechanisms that confer resistances to a number of antimicrobials, including the last-generation carbapenems, which are usually used for the treatment of MDR bacteria (Azam and Khan 2018; Juhas et al. 2004, 2005; Wiehlmann et al. 2007; Juhas 2015b; Potron et al. 2015; Oliver et al. 2015).

Genes encoding resistance to carbapenems are often located on class 1 integrons; however, class 1 integrons are considered to be incapable of self-transfer. Thus for the transfer to the new host, class 1 integrons have to associate with other transferable elements, such as plasmids, transposons or GIs (Roy Chowdhury et al. 2016, 2017). Interestingly, class 1 integrons were found to be carried by the two globally dispersed GIs (GI1 and GI2) that are frequently found in *P. aeruginosa* multidrug-resistant clones ST235, ST253, ST111 and ST175. Both GI1 and GI2 harbour open reading frames conferring resistances to the entire range of antibiotics used to treat

P. aeruginosa infections, including carbapenems (Roy Chowdhury et al. 2016, 2017). Besides resistance to multiple antibiotics, *P. aeruginosa* GI1 and GI2 harbour genes implicated in their conjugative transfer to the new host cells and reintegration into the chromosome, thus suggesting their evolution from a novel, yet uncharacterized ICE. This study also highlights the role GIs play in the capture and dissemination of antibiotic-resistant genes and in the evolution of MDR *P. aeruginosa* (Roy Chowdhury et al. 2017).

Recent whole-genome sequencing analyses of the high-risk *P. aeruginosa* clone ST235 revealed the presence of a novel GI, ICE*Pae690* carrying $bla_{\text{GES-6}}$ carbapenemase gene (Botelho et al. 2018). $bla_{\text{GES-6}}$ was found on a class 1 integron In1076 located within ICE*Pae690*. Besides In1076 integron, ICE*Pae690* carried gene-encoding integrase, type IV secretion system, relaxase and type IV coupling protein involved in the excision, conjugation and reintegration of ICE*Pae690* and other genes encoding maintenance functions. ICE*Pae690* was integrated into the chromosome next to a tRNA^{Gly} gene and flanked by 16 bp ICE*clc*-like *attL* and *attR* sequences. The flanking *attL* and *attR* sequences are probably a result of recombination between the attachment sites *attB* and *attP* on the chromosome and ICE*Pae690*, respectively. This study also demonstrated that ICE*Pae690* is capable of the self-transfer by conjugation from the original *P. aeruginosa* clone ST235 to *P. aeruginosa* standard laboratory strain PAO1. It has been suggested that In1076 integron harbouring $bla_{\text{GES-6}}$ "hitch-hiked" ICE*Pae690* to promote its own propagation to other host cells through ICE-mediated conjugation (Botelho et al. 2018).

Recent whole-genome sequencing analyses led to the identification of a number of novel GIs (PAGIs) in the genomes of *P. aeruginosa*, namely, PAGI-13, PAGI-14, PAGI-15 and PAGI-16 carrying a broad spectrum of resistance genes, including $bla_{\text{IMP-6}}$, $bla_{\text{IMP-10}}$ and $bla_{\text{GES-24}}$. In PAGI-13, PAGI-14, PAGI-15 and PAGI-16, these resistance genes are located on the class 1 integrons and confer resistance to carbapenems (Hong et al. 2016; Silveira et al. 2016).

6 Conclusions

Work over the last years, in particular the recent bioinformatics and whole-genome sequencing analyses, highlighted the diversity of GIs and shed new light on the GI-mediated genome plasticity (Botelho et al. 2018).

It is now widely accepted that GIs play an important role in the evolution of a broad spectrum of bacterial species, including MDR bacteria, such as MDR *E. coli*, *S. enterica*, *P. mirabilis*, *M. morganii*, *A. baumannii* and *P. aeruginosa*, presented in this review but also in a number of other clinically relevant MDR pathogens. For instance, the master transcriptional activator complex AcaCD of the IncA/C helper plasmids originally found in the GIs of the SGI/PGI/AGI-like subfamily was shown to be involved in the mobilization of other unrelated resistance GIs, such as MGI*Vch*Hai6 of *Vibrio cholerae* which confers resistance to β-lactams, tetracycline,

sulfamethoxazole, trimethoprim, streptomycin/spectinomycin and chloramphenicol (Carraro et al. 2016).

Furthermore, recent analyses revealed that GIs are undergoing constant micro-evolution and continue to accumulate novel resistance genes within their sequences (e.g. by capturing other resistance gene-harbouring integrons and transposons) (Roy Chowdhury et al. 2016, 2017). This development is alarming particularly in connection with the ability of some GIs to self-transfer into the new host as the acquisition of such multi-resistance GIs can provide the host bacterium with the resistances to the whole array of antimicrobials.

Future studies will be required to elucidate the molecular mechanisms involved in the life cycle of some GIs, in particular the role the GIs-borne regulatory genes play in their propagation. Furthermore, additional analyses will be needed to elucidate the details of the process of acquisition of accessory genes, such as novel antibiotic-resistant genes, by GIs. Additional analyses are also required to assess the prevalence of the recently identified GIs, such as SGI/PGI/AGI-like GIs and their variants, PAGI-13, PAGI-14, PAGI-15, PAGI-16 and ICE*Pae690* in other bacterial species. This will contribute to the better understanding of GIs and their role in the spread of antimicrobial-resistant genes and the evolution of MDR bacteria.

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Horizontal Gene Transfer and Genome Evolution in the Phylum *Actinobacteria*

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Abstract *Actinobacteria* constitute one of the largest phyla in the bacterial domain, with many species known for their large genome sizes and high G + C content. Members of this phylum exhibit a wide range of morphological, physiological, and metabolic properties and are ubiquitously distributed in diverse ecological niches. The phylum includes pathogens, symbionts of plants and invertebrates, gastrointestinal commensals, and free-living bacteria. Many species are well known for their ability to produce a plethora of natural products relevant to agriculture, biotechnology, and medicine, including majority of naturally derived antibiotics widely used in clinics. In this chapter, we discuss recent advances that highlight the role of horizontal gene transfer (HGT) in the evolutionary history, genomic structure, and ecological diversity of *Actinobacteria*, with emphasis on HGT between species and between phyla. We highlight genome studies that describe how HGT has contributed to the success of promiscuous antibiotic producers as well as the pathogens that they target. We conclude with how methods in population genomics can be used to elucidate the impacts of selective pressures and environmental changes on rates and patterns of HGT in various *Actinobacteria* species and populations.

Keywords Horizontal gene transfer · Genome · Evolution · *Actinobacteria* · Biosynthetic gene clusters

1 Introduction

Actinobacteria have long been the major source of novel naturally derived antibiotics and analogs widely used today in clinical practice. The drug discovery revolution began in 1944, when the biochemist and soil microbiologist Selman Waksman described for the first time the isolation of streptomycin, the first effective treatment

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for tuberculosis, from the soil-dwelling *Actinobacteria Streptomyces griseus* (Schatz et al. 1944). Tuberculosis is a highly infectious disease caused by another actinobacterium *Mycobacterium tuberculosis*. Long considered the scourge of humanity, tuberculosis has, from ancient times, caused some of the deadliest epidemics ever seen in human history. Today, the disease remains a major public health threat, especially in Asia, where two million deaths are reported annually (Zumla et al. 2015). Waksman would go on to describe a variety of other valuable antibiotics produced by diverse *Streptomyces* species (Kutzner and Waksman 1959; Woodruff 2014). Since that time, enormous research effort has been directed toward this genus, and today more than half of all clinically useful bioactive compounds are derived from *Streptomyces*. While *Streptomyces* is the most well-known genus in the field of natural drug discovery, a diverse number of *Actinobacteria* have also been reported to be equally if not more prolific in producing compounds, enzymes, and other natural products relevant to medicine, agriculture, biotechnology, and energy production. Among these are members of the genera *Amycolatopsis*, *Micromonospora*, *Nocardia*, *Pseudonocardia*, *Saccharomonospora*, and *Salinispora* (Barka et al. 2015). Beyond antibiotics, multitude of compounds (known as secondary metabolites) derived from actinobacterial biosynthetic gene clusters (BGCs) have also been reported to be effective antifungals, antivirals, antitumors, anthelmintics, antiprotozoals, and immunosuppressants (Běhal 2000; Manivasagan et al. 2014).

The importance of *Actinobacteria* goes beyond the pharmaceutically relevant bioactive compounds they produce. In addition to the deadly *M. tuberculosis* and *Mycobacterium leprae* (causal agent of leprosy), many other species in this phylum cause devastating diseases in humans and animals (e.g., *Corynebacterium*, *Nocardia*, *Propionibacterium*, *Rhodococcus*, *Tropheryma*) (Barka et al. 2015). *Actinobacteria* are ubiquitously distributed in both aquatic and terrestrial ecosystems and play a critical role in ecosystem functions and biotic interactions. For example, *Actinobacteria* comprise the defensive mutualists of animals (e.g., *Pseudonocardia*, *Streptomyces*), gastrointestinal commensals (e.g., *Bifidobacterium*), soil inhabitants (e.g., *Micromonospora*, *Streptomyces*), marine dwellers (e.g., *Salinispora*), plant mutualists (e.g., nitrogen-fixing *Frankia*), and plant pathogens (e.g., scab-forming *Streptomyces*) (Ventura et al. 2007; Barka et al. 2015). Because many *Actinobacteria* species are able to degrade insoluble polymers such as cellulose and chitin, they also contribute significantly to the global carbon cycle through the breakdown of plant biomass (Anderson et al. 2012; Book et al. 2014).

The ecological, industrial, and biomedical importance of *Actinobacteria* cannot be overstated. However, the taxonomy of *Actinobacteria* has long been considered problematic, with species membership constantly being modified, reclassified, and improperly named (Embley and Stackebrandt 1994; Stackebrandt and Schumann 2006). This is especially true for *Streptomyces* in the early days of its discovery, when species were classified and named based on the antibiotics produced, rapidly escalating the number of species to over 3000 (Trejo 1970). The taxonomy of other genera of *Actinobacteria* has been historically difficult to resolve, with numerous incongruencies between phenotypic and genotypic groupings and named species (Embley and Stackebrandt 1994; Stackebrandt and Schumann 2006). With the

availability of genome sequences in the past two decades, it has become possible to reconstruct a more reliable and robust picture of the evolutionary history of *Actinobacteria* (Ventura et al. 2007; Nouioui et al. 2018), although majority of actinobacterial genomes sequenced so far belong to organisms relevant to human and veterinary medicine, biotechnology, and industry.

Genome-based studies of *Actinobacteria* reveal reticulate evolutionary processes due to horizontal gene transfer (HGT) and are likely to blur the boundaries between species (Ventura et al. 2007), producing metaspecies and fuzzy species with ambiguous species boundaries (Hanage et al. 2005). HGT can result in genetically mosaic genomes with genes having disparate origins which may be different from the history of the organism itself and therefore are difficult to classify (Gogarten et al. 2002; Bapteste and Boucher 2009; Corander et al. 2012). HGT is known to have shaped the evolutionary history and diversity of *Actinobacteria* (Fig. 1). Even in species long thought to be extremely clonal (e.g., *M. tuberculosis*), recent genome-wide studies indicate that they are not immune to HGT (Panda et al. 2018). Hence, we find that the ecological success of *Actinobacteria* in a wide variety of environments, from marine sediments and soil to the gut of eukaryotic hosts, has been shaped by their ability to gain and donate DNA. In this chapter, we highlight recent genome-based studies that explore their evolutionary history of the phylum *Actinobacteria* and the important role that HGT has played in generating their tremendous genetic, ecological, and biosynthetic diversity.

2 HGT in Free-Living, Antibiotic-Producing *Streptomyces* and *Salinispora*

Salinispora The marine-dwelling *Salinispora*, composed of three species *Salinispora arenicola*, *Salinispora tropica*, and *Salinispora pacifica*, is a rich source of structurally diverse natural products (Jensen et al. 2015). Although *Salinispora* spp. share >99% 16S rRNA gene sequence similarity (Jensen and Mafnas 2006) making species delineation is challenging, species-specific patterns of secondary metabolite production and gene distributions may likely contribute to speciation in the genus (Jensen et al. 2007; Freel et al. 2011; Ziemert et al. 2014). For example, rifamycins and saliniketals were produced exclusively by *S. arenicola* species and are the main secondary metabolites differentiating *S. arenicola* and *S. pacifica* (Bose et al. 2014).

Genome sequencing of *Salinispora* strains reveals that a large part of the genome is dedicated to BGCs and natural product assembly (Penn et al. 2009). For example, approximately 10% of the genome of *S. tropica* comprise a variety of polyketide synthases, nonribosomal peptide synthetases, and several hybrid clusters, with the majority being novel BGCs (Udwary et al. 2007). *S*pecies harbor an average of 14–18 polyketide and nonribosomal peptide BGCs per strain, but only <10% of the total predicted BGCs are conserved or thought to be shared by the common ancestor

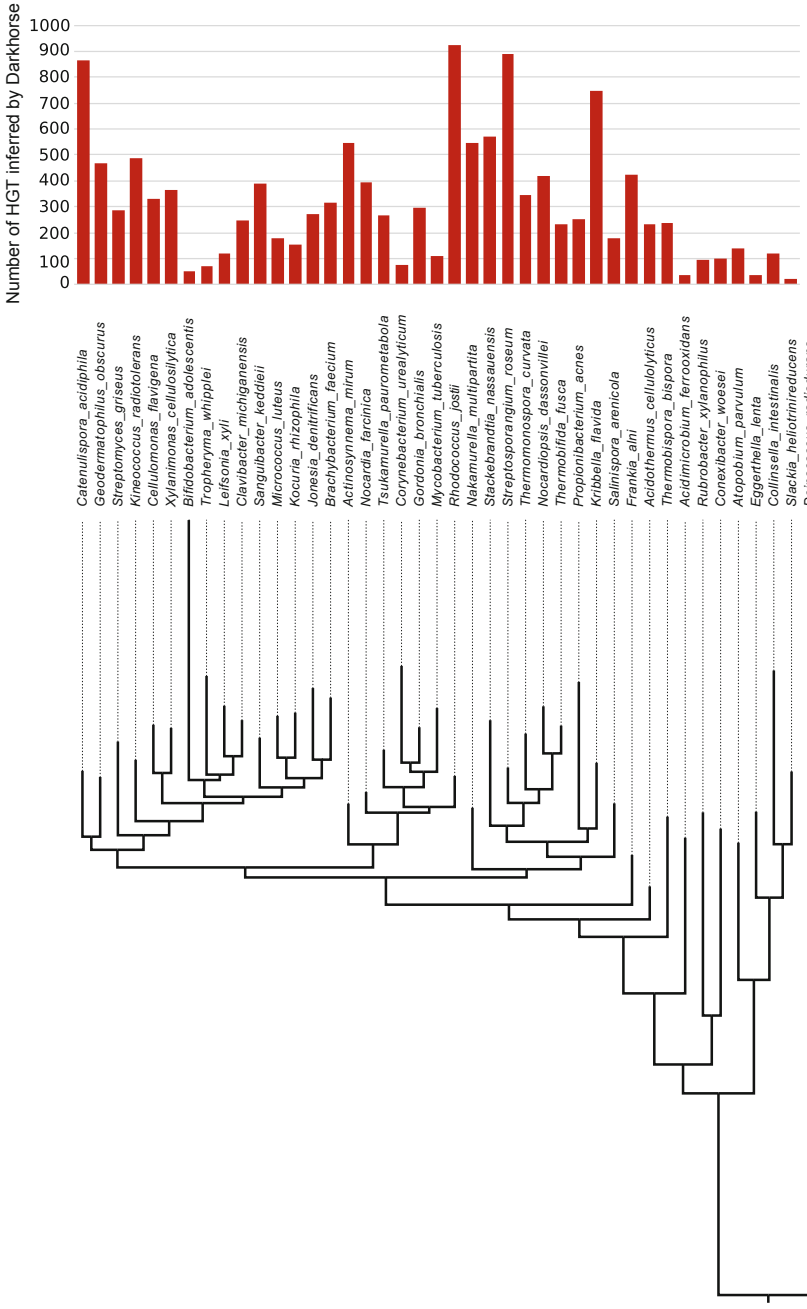

Fig. 1 Number of genes in *Actinobacteria* inferred to have experienced HGT. Left: Maximum likelihood phylogenetic tree of the 16S rRNA locus of representative *Actinobacteria* species. The phylogeny was built using PhyML (Guindon et al. 2010), with 100 bootstrap replicates, GTR nucleotide substitution model (Tavaré 1986) + I + G. Sequences were aligned using MUSCLE (Edgar 2004). *Deinococcus radiodurans* and *Thermus thermophilus* were used as outgroup taxa to root the tree. Right: HGT events were inferred using Darkhorse v.2 (Podell et al. 2008), which calculates a lineage probability index (LPI) based on BLAST scores and the phylogenetic distance of the match sequence from the query organism

of the genus (Ziemert et al. 2014). More than half of the BGCs are rare and are present in only one or two strains, suggesting that they were acquired relatively recently in the evolutionary history of *Salinispora* (Ziemert et al. 2014). Acquired BGCs are often incorporated into genomic islands that are frequently exchanged within and between *Salinispora* species and have evolved by gene gain, loss, and duplication followed by divergence (Udwary et al. 2007; Ziemert et al. 2014).

HGT of BGCs in *Salinispora* has greatly contributed to its tremendous chemical diversity and highlights a population-level strategy to secondary metabolite production. First, the same genomic island can encode one to five distinct BGCs depending on the strain and that the identity of a BGC within a specific island can vary dramatically between strains (Ziemert et al. 2014). While their exchange may be random, this diversity of BGC in the same chromosomal position may reflect a plug-and-play model of evolution that allows the strain to test the relative fitness effects of specialized metabolites that it produces (Letzel et al. 2017). Second, transposase-mediated HGT of BGCs can result in molecular diversification of a single class of small molecules as was observed in salinosporamide A and K in *S. tropica* and *S. pacifica*, respectively (Eustáquio et al. 2011; Freel et al. 2011). Third, functional redundancy resulting from HGT can also occur, as was observed in the BGC encoding for the peptidic siderophores called salinichelins, which were acquired in three independent HGT events throughout the evolutionary history of *Salinispora* (Bruns et al. 2018). The acquisition and retention of the salinichelin siderophores co-occurred with the loss of the more ancient desferrioxamine pathway. Lastly, the high number of strain-specific BGCs may be a population-level strategy that prevents the permanent loss of a BGC from the population by maintaining them in one or few strains. When the necessity arises (e.g., environmental change, competition with other organisms), these rare BGCs may be disseminated among other members of the population via recombination. Overall, the different ways by which *Salinispora* strains mix and match their BGCs suggest that the genus is able to maximize its population-level diversity and distribution of secondary metabolites while restricting the number of BGCs maintained within any individual genome (Ziemert et al. 2014). This may be a case of BGCs representing a shared genomic resource, built on the premise of the Black Queen Hypothesis wherein cells produce only a fraction of the common goods and rely on other cells to produce the others (Fullmer et al. 2015). BGC distribution, mobility, and structural rearrangement within and between *Salinispora* species can consequently have an immediate influence on fitness, on niche utilization, and consequently on bacterial diversification (Penn et al. 2009). This has been observed in *Salinispora* wherein BGCs exhibit high levels of species specificity and can be used to differentiate closely related but ecologically distinct populations (Penn et al. 2009).

Streptomyces *Streptomyces* constitutes a highly diverse bacterial group that is widely distributed in nature. In contrast to most bacteria, *Streptomyces* species are characterized by complex secondary metabolism and a fungal-like morphological differentiation that involves the formation of branching, filamentous vegetative growth, and aerial hyphae bearing long chains of reproductive spores (Flärdh and

Buttner 2009); hence they were originally misclassified as fungi. *Streptomyces* genomes are one of the largest in the bacterial domain, ranging from 8 to 11 Mb and are characterized by a large portion of accessory genes (Harrison and Studholme 2014). Genome analyses indicate that this remarkable genome structure has been shaped by widespread HGT not just with other *Streptomyces* lineages but also with other microbial phyla (Ventura et al. 2007; Zhou et al. 2012).

HGT of partial and entire BGCs is known to play a major role in generating the high number and variety of BGCs in *Streptomyces* genomes (Egan et al. 2001). In the marine-dwelling MAR4 lineage of *Streptomyces*, a total of 13 distinct kinds of hybrid isoprenoids (HIs) BGCs are recognized, each of which is predicted to encode the production of a distinct HI compounds (Gallagher and Jensen 2015). This chemical diversity has been postulated to have arisen as a result of the frequent HGT of ABBA prenyltransferases (PTases) in other *Streptomyces* lineages, which are enzymes responsible for the attachment of isoprene moieties to a variety of acceptor molecules, a key step in HI biosynthesis (Gallagher and Jensen 2015). Widespread duplication and HGT of PTases among unrelated BGCs may also explain the relatively high number of ABBA PTase homologs in all of the MAR4 genomes, which average five copies per strain, compared to other *Streptomyces* species which carry one copy per strain (Gallagher and Jensen 2015). Recipients of *Streptomyces* DNA are not limited to other *Actinobacteria* genera. For example, the antibiotic bicyclomycin, the only naturally derived antibiotic that selectively inhibits the transcription termination factor Rho, is synthesized by *Streptomyces cinnamoneus* (Miyoshi et al. 1972; Skordalakes et al. 2005). HGT of intact BGC encoding bicyclomycin is facilitated by mobile genetic elements, and transfer can span across phylogenetically distant taxa within *Actinobacteria* as well as numerous members of *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria* (Vior et al. 2018). Other *Streptomyces*-derived BGCs have also been reported to have had a history of HGT, such as those that encode the antibiotics streptomycin (Tolba et al. 2002), foxicin (Greule et al. 2017), antimycin (Joynt and Seipke 2018), and granaticin (Deng et al. 2011), as well as toxins such as coronafacoyl phytotoxins (Bown et al. 2017) and tunicamycin (Sechler et al. 2017).

Strain-level variation in genomic and BGC content has only recently been recognized and highlights the importance of how recent HGT events can impact recent evolutionary divergence among closely related strains. Multiple *Streptomyces* species with identical 16S rRNA gene sequences and considered the same species can exhibit distinct secondary metabolomes (Antony-Babu et al. 2017). In *Streptomyces albus*, a promiscuous producer of numerous clinically relevant compounds, a total of 48 BGCs were identified in a mere 7 strains (Seipke 2015). Of these, 18 specify the core BGC of the species, 14 are auxiliary BGCs, and 16 are strain-specific (Seipke 2015). Similar results were also reported in two sister-taxa of *Streptomyces* inhabiting distinct geographic ranges demarcated by latitude. Variation in BGC content and consequently divergence between them are reported to have been driven primarily by both recent HGT and gene loss events (Choudoir et al. 2018).

The rate in which genes are horizontally acquired and retained in *Streptomyces* lineages may not necessarily reflect widespread and rampant HGT and instead may have been influenced by the length of time that has occurred since the origins of the genus. This was demonstrated by McDonald and Currie who used a molecular clock and showed that HGT is rare in *Streptomyces*, with an estimated average of 10 genes per million years that have been horizontally acquired and retained (McDonald and Currie 2017). The authors argue that the high number of HGT events in large bacterial genomic datasets may be due to the long periods of time that separate sampled strains (McDonald and Currie 2017). Even with few housekeeping genes, often used in multilocus sequence analyses (MLSA), similar results have been reported. The fixation of ancestrally recombined genes in *Streptomyces* can result in an indefinitely large number of contemporary lineages that have inherited "horizontally acquired" genes through vertical descent (Andam et al. 2016). However, observed low rates of HGT may only be limited to core genes, but BGCs and other accessory genes are likely to exhibit recent recombination more frequently.

The unique chromosomal structure in *Streptomyces* genomes is likely to explain the large pan-genomes and frequent HGT in the genus. *Streptomyces* genomes consist of a large linear chromosome, with a conserved central region flanked by highly variable arms characterized by large DNA rearrangements (Bentley et al. 2002; Ikeda et al. 2003). Double-strand break repair and nonhomologous end joining (NHEJ) in the chromosome can result in major chromosome structure alteration and promote HGT (Hoff et al. 2018). Moreover, widespread interspecies HGT can be mediated by the pool of highly diverse actinophages (Sharaf et al. 2017). Some of these actinophages have remarkably mosaic genome structure, suggesting frequent exchange with other phages. For example, the actinophages of *Streptomyces flavovirens* Sf1 and Sf3 share 100% identity in conserved regions interspersed between highly variable regions with high variability (with sequence similarity ranging from 23.9 to 87.5%) (Sharaf et al. 2017). Lastly, *Streptomyces* also utilize a unique conjugative DNA transfer mechanism directed by the enzyme TraB, a plasmid-encoded DNA-translocase that transfers a double-stranded DNA-molecule to the recipient (Thoma and Muth 2015) [in contrast, other conjugation machineries transfer only single-stranded DNA (Grohmann et al. 2018)]. A multi-protein complex then spreads the transferred plasmid to neighboring mycelial compartments of the recipient, resulting in the rapid dissemination of the donor DNA (Thoma et al. 2015).

3 HGT in the Pathogens *Mycobacterium*, *Rhodococcus*, and *Corynebacterium*

Mycobacterium The genus *Mycobacterium* comprises more than 180 species of which about 15 deadly pathogens are commonly encountered in humans and animals (Gupta et al. 2018). The extent of HGT and its impacts on the evolution of

Mycobacterium remain controversial (Godfroid et al. 2018), particularly so in the human-restricted *M. tuberculosis* which have long been considered clonal, immune to HGT, and evolving only by random genetic drift and selection (Hershberg et al. 2008; Gagneux 2018). However, recent genome-wide studies of different *Mycobacterium* species indicate that *Mycobacterium* genomes had undergone many episodes of intra- and interspecies transfer of genes from taxonomically diverse sources (Boritsch et al. 2016; Panda et al. 2018).

Horizontally acquired genes have been reported to have played a major role in the pathogenesis of *Mycobacterium* species. In a genome-wide analysis of HGT in *Mycobacterium* using the program T-REX which infers probable HGTs based on the statistical reconciliation of gene and species trees (Boc et al. 2012), genes acquired from outside of the genus have been reported to be integral in the emergence of its antibiotic resistance, virulence, and other metabolic pathways (Panda et al. 2018). The major donor clades include other genera in the phylum *Actinobacteria* (*Rhodococcus*, *Gordonia*, *Segniliparus*, *Streptomyces*, and *Tsukamurella*) with some genes also donated by *Proteobacteria*. *Mycobacterium* phages Sbash and Whirlwind (*Siphoviridae*) also appear to be frequent partners (Panda et al. 2018). However, there is increasing evidence that HGT has been more frequent early in the evolution of the genus, and perhaps this might explain the long-held view that HGT is rare in *Mycobacterium*. An ancient HGT event has been reported to have contributed to the ability of *M. tuberculosis* to persist for long periods of time in lung hypoxic lesions, which is a major factor in the high burden of latent tuberculosis (Levillain et al. 2017). The ancient HGT event involves the gene cluster for the synthesis of the molybdenum cofactor, which allows an organism to respire nitrate and to persist in a dormant state under hypoxia, a stress condition encountered in lung tuberculosis lesions (Williams et al. 2014). This HGT occurred after the divergence from environmental non-tuberculous mycobacteria but before the clonal expansion of the *M. tuberculosis* clonal complex, with the most likely donor being closely related to plasmids of the betaproteobacterium *Burkholderia vietnamensis* (Levillain et al. 2017). Ancient genomic islands also persist and continue to be mobilized by conjugative plasmids and phages among mycobacteria species (Reva et al. 2015).

Interspecies HGT within the genus appears to be common in many other species of *Mycobacterium*. For example, *Mycobacterium abscessus* is an emerging, multidrug-resistant, non-tuberculous mycobacterium responsible for a wide spectrum of infections (skin and soft tissue diseases, central nervous system infections, bacteremia, and ocular infections). It is composed of three subspecies: *abscessus*, *massiliense*, and *bolletii.* Studies show that while HGT through homologous recombination is prevalent within the species, the rate of DNA acquisition is asymmetrical among the three subspecies (Sapriel et al. 2016). Genome sequences of hybrid strains indicate that horizontally acquired large genomic blocks with sizes up to 93 Kb are distributed throughout their genomes and are likely acquired through punctuated massive HGT events (Sapriel et al. 2016). What is more worrisome is that these genomically hybrid strains are significantly associated with macrolide resistance and with cystic fibrosis patients with lung infections or chronic

colonization (Sapriel et al. 2016). In *Mycobacterium avium* subsp. *hominissuis*, genomic islands carrying genes associated with virulence, antibiotic resistance, and fitness are highly variable and are shared among different *Mycobacterium* species (Sanchini et al. 2016). There is also evidence of HGT in the genomes of other subspecies of *M. avium* (*avium*, *silvaticum*, and *paratuberculosis*) (Uchiya et al. 2017). Even core housekeeping genes have also been reported to have been transferred between *Mycobacterium* species, as in the case of a recombinant *rpoB* in some *Mycobacterium yongonense* strains gained from *Mycobacterium parascrofulaceum* (Kim et al. 2017a) and *rpoB* in some *M. massiliense* strains acquired from *Mycobacterium abscessus* (Kim et al. 2017b).

Genome-wide mosaicism in *Mycobacterium* created through HGT within and between species is likely mediated by distributive conjugal transfer (DCT), a newly described form of chromosomal conjugation that is not driven by conjugative plasmids and the origin of transfer (*oriT*) sequence as in other conjugation processes (Gray et al. 2013). Through chromosomal transfer, DCT results in a mosaic transconjugant structure, similar to the products of meiosis in eukaryotes (Gray et al. 2013). Genome sequencing of transconjugants of *Mycobacterium smegmatis* reveal that up to a quarter of the genome, distributed over 30 segments with sizes ranging from 50 bp to 225 Kb, has been derived horizontally (Derbyshire and Gray 2014). DCT has also been observed in another species, *Mycobacterium canettii*, a close relative of *M. tuberculosis* that has been isolated on rare occasions from cases of tuberculosis in East Africa (Mortimer and Pepperell 2014). While more data is needed to explain the mechanism of transfer, it appears to involve the co-transfer of multiple chromosome segments into the recipient after fragmentation in the donor cell before transfer, followed by recombination in the recipient chromosome to generate a mosaic pattern (Derbyshire and Gray 2014).

Rhodococcus Rhodococci are aerobic and nonmotile soil organisms that can be found in a variety of environments such as soil, water, and insect guts (Bell et al. 1998). While most species are free-living and saprophytic, two are known to be pathogenic. *Rhodococcus equi* is a pulmonary pathogen of young horses and AIDS patients (von Bargen and Haas 2009; Topino et al. 2010), while *Rhodococcus fascians* causes leafy gall syndrome in more than 120 plant species representing both monocots and dicots, herbaceous and woody plants (Putnam and Miller 2007).

Conjugative plasmids are essential in the virulence and host adaptation of pathogenic *Rhodococcus* species. In *R. equi*, conjugative plasmids confer the ability to survive and replicate inside host macrophages (von Bargen and Haas 2009). Three types of plasmids have been identified: the circular equine-associated pVAPA and porcine-associated pVAPB and the linear bovine-associated pVAPN. The vap pathogenicity island (PAI) in the plasmids consists of multiple genes encoding virulence-associated proteins called vaps (Letek et al. 2008; MacArthur et al. 2017). pVAPN is a 120-kb invertron-like linear replicon unrelated to the two other plasmids and has been reported to have originated via HGT of the vap PAI to a linear replicon (Valero-Rello et al. 2015). Invertrons are mobile genetic elements composed of DNA with inverted terminal repeats at both ends, which can vary from 10 to

1000 bases (Sakaguchi 1990). Although highly diverse, the distribution of the three types of plasmids is strongly correlated with the animal host but can be exchanged and hence can bring about corresponding host shifts (Anastasi et al. 2016; MacArthur et al. 2017). It is also notable that the pathogenicity of *R. equi* evolved through the recruitment or co-option of core actinobacterial traits, following the horizontal acquisition of key host-adaptive genes (Letek et al. 2010). Gene co-option (also known as preadaptation or exaptation) involves rapid adaptive changes by taking traits that have evolved for one purpose and using them in a new context with new roles (True and Carroll 2002).

The linear plasmid pFiD188 is essential to the virulence of the plant pathogen *R. fascians* (Francis et al. 2012). Although majority of genes in the *R. fascians* chromosome appear to have not experienced frequent HGT, acquisition of only four pFiD188-encoded functions (secondary metabolism [*att*], gene transcription [*fasR*], cytokinin biosynthesis [*fasD*], and cytokinin activation [*fasF*]) is sufficient for *Rhodococcus* to infect plants (Creason et al. 2014). This is because the *fas* operon encodes the enzymatic machinery for the production of cytokinins, which cause hormonal imbalance in plants, resulting in abnormal growth (Pertry et al. 2009). Hence, plasmid-mediated HGT can rapidly convert nonpathogenic *R. fascians* strains into phytopathogenic variants.

Corynebacterium *Corynebacterium* species are members of the normal human microflora, often isolated from the skin, mucous membranes, and gastrointestinal tract (Bernard 2012; Sangal and Hoskisson 2016). However, they are now increasingly being recognized as causing opportunistic diseases particularly among individuals who are immunocompromised, have prosthetic devices, or have stayed in hospitals or nursing homes for long periods of time (Funke et al. 1997). HGT mediated by PAI and mobile elements is known to play an important role in the virulence and antibiotic resistance of pathogenic *Corynebacterium* species.

The most clinically significant species is *Corynebacterium diphtheriae*, the causal agent of diphtheria, a highly infectious disease of the throat and upper airways, through the production of a potent bacteriophage-encoded exotoxin diphtheria toxin (*tox*) (Holmes 2000). The *tox* gene and another major virulence factor (pilus), which play important roles in adhesion of *C. diphtheriae* to different host tissues (Mandlik et al. 2007), are found in genomic islands that are mobilizable between strains. Even more alarming is that the number of pilus gene clusters present in a strain and the variation introduced by gene gain or loss significantly correlate with variation in adhesive and invasive properties in *C. diphtheriae* strains (Sangal and Hoskisson 2016). Moreover, genes that encode resistance against common antibiotics can also be rapidly disseminated in the bacterial population. For example, in the antibiotic-resistant *C. diphtheriae* BH8, the genes that encode chloramphenicol, sulfonamides, and tetracyclines are carried in a gene region flanked by insertion sequences and can be horizontally acquired via transpositional integration (Trost et al. 2012).

HGT has also been reported in other pathogenic *Corynebacterium* species. In *Corynebacterium striatum* isolated from immunocompromised patients with underlying respiratory diseases, genome analysis reveals resistance genes mobilized by

IS3504 and ISCg9a-like insertion sequences (Nudel et al. 2018). Moreover, the presence of resistance genes flanked by unpaired insertion sequences suggests that a single recombination event could rapidly disseminate resistance to antibiotics aminoglycosides, macrolides, lincosamides, and tetracyclines in the bacterial population (Nudel et al. 2018). In another example, *Corynebacterium pseudotuberculosis* is a facultative intracellular pathogen and causes several infectious and contagious chronic diseases in goat, sheep, buffalo, and horses (Baraúna et al. 2017). The major toxin of *C. pseudotuberculosis* is phospholipase D encoded by the *pld* gene and is carried by a PAI PiCp1 (McKean et al. 2007; Ruiz et al. 2011). Fifteen other PiCp types, which contain several virulence factors such as genes that encode for fimbrial subunits, adhesion factors, iron uptake, and secreted toxins (Ruiz et al. 2011), are known to be present and are shared within and among the different *C. pseudotuberculosis* biovars (Baraúna et al. 2017; Viana et al. 2017).

4 *Actinobacteria* as HGT Donors in Microbial Communities

Actinobacteria are ubiquitous in different habitats and are major players in the functioning of many microbial communities. The abundance of diverse *Actinobacteria* and their ability to mobilize genetic material suggest that they can facilitate the rapid and widespread dissemination of antibiotic resistance genes across multiple taxa (D'Costa et al. 2006). This is because antibiotic resistance genes are often encoded on the same BGCs that encode the synthesis of antibiotics and other bioactive compounds. Antibiotic-producing bacteria carry resistance genes as a self-protecting mechanism toward the antibiotics they produce (Hopwood 2007) or to modulate their own signaling activity (Goh et al. 2002; Sengupta et al. 2013). Hence, it has long been postulated that *Actinobacteria* can transfer their BGC-associated resistance genes to other taxa (Benveniste and Davies 1973), but only after advances in metagenomic sequencing do we find significant and widespread evidence of this.

HGT between *Actinobacteria* and other microbial taxa in a community highlights the role of this phylum as a major reservoir of antibiotic resistance genes that can be widely disseminated to other phyla, including clinically relevant pathogens. Multidrug-resistant soil-dwelling bacteria that harbor resistance cassettes against five classes of antibiotics (beta-lactams, aminoglycosides, amphenicols, sulfonamides, and tetracyclines) exhibit perfect nucleotide identity to genes from diverse human pathogens, such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Salmonella enterica* (Forsberg et al. 2012). Transfer of resistance genes is facilitated by integrases and transposases adjacent to the resistance genes (Forsberg et al. 2012). Other mechanisms of transfer may be prevalent in soil communities. For example, the transient nature of broad host range plasmids provides a direct, rapid link in transferring DNA between Gram-negative (*Proteobacteria*) and Gram-positive bacteria (*Actinobacteria* and *Firmicutes*) in soil communities (Klümper et al. 2015). Lastly, unidentified or undiscovered taxa that act as intermediates can

facilitate an indirect transfer of resistance genes between *Actinobacteria* and other phyla but remain invisible to current HGT detection methods. We also have to recognize that these resistance genes have had a long history of diversification that began long before the era of clinical antibiotics (D'Costa et al. 2011). It is likely that HGT events in the soil may have been more prevalent in the past than it is today. In soil bacteria inhabiting pristine and remote Antarctic surface soils, a total of 177 naturally occurring genes conferring resistance to antibiotics were reported to have originated from antibiotic-producing bacteria including *Actinobacteria*. These resistance genes were subsequently disseminated through ancient HGT events and have since been vertically inherited over many generations in the recipient lineages (Van Goethem et al. 2018).

While HGT is expected to occur more frequently between more closely related taxa (Andam and Gogarten 2011; Bolotin and Hershberg 2017), a possible mechanism that can mediate inter-phyla transfer of resistance genes has been recently proposed between *Actinobacteria* and *Proteobacteria* (Jiang et al. 2017). Called carry-back, this mechanism involves the conjugative transfer of a carrier sequence from *Proteobacteria* to *Actinobacteria*, followed by transposon-mediated recombination of the carrier sequence with the resistance gene in the *Actinobacteria*. This results in the formation of a sandwich structure of actinobacterial DNA flanked by proteobacterial DNA. Upon the death of the *Actinobacteria* cell, the sandwich-structured DNA is released and taken up by neighboring *Proteobacteria* that integrates the full resistance-transposon sequence into its own DNA. The carry-back mechanism has been demonstrated to have caused the transfer of two genes conferring resistance against chloramphenicol and lincomycin from *Streptomyces* to pathogenic *Proteobacteria* such as *Pseudomonas*, *Klebsiella*, and *Enterobacter* (Jiang et al. 2017). How prevalent this mechanism is across different *Actinobacteria* taxa, or between *Actinobacteria* and other phyla besides *Proteobacteria*, remains to be explored.

5 HGT and Genomic Heterogeneity Within Species

Microbial species are remarkably heterogeneous. They may be clonal, but microbial populations are often composed of multiple co-circulating lineages distinguished by phenotypic and genetic differences (Sánchez-Romero and Casadesús 2014; Davis and Isberg 2016; Takhaveev and Heinemann 2018). Such tremendous genomic heterogeneity leads to the pan-genome, defined as the totality of all unique gene families found in a group of genomes, often many times larger than a single genome (Medini et al. 2005). This variation in gene content can drive species divergence (Papke et al. 2007; Youngblut et al. 2015), metabolic diversity and versatility (Silby et al. 2011), and symbiotic relationships (De Maayer et al. 2014). Results from the few population-level studies of *Actinobacteria* reveal enormous inter-strain genetic and phenotypic variation (Doroghazi and Buckley 2014; Antony-Babu et al. 2017;

Garcia et al. 2018; Neuenschwander et al. 2018), but the evolutionary and ecological factors that drive this variation remain poorly understood.

Gene content variability among strains of the same species is largely attributed to both differential gene loss and HGT (Snel et al. 2002; McInerney et al. 2017; Hall et al. 2017; Bobay and Ochman 2018). The power of microbial population genomics, involving sequencing of hundreds and even thousands of closely related isolates of the same species, has revealed an astonishing level of genomic diversity both in terms of allelic differences and gene content variation (Whitaker and Banfield 2006; Vinatzer et al. 2014; Sheppard et al. 2018). Strain-level genomic heterogeneity can also provide valuable insights into how rates and patterns of HGT are impacted by selective pressures due to recent environmental changes such as climate change, host switching, and clinical interventions. While population genomics has been more commonly used to explore pathogen populations with implications for transmission studies, treatment regimens, and new drug target development (Wilson 2012), in *Actinobacteria*, such analyses have been widely used only in *M. tuberculosis* (Lee et al. 2015; Comas et al. 2015; Stucki et al. 2016; Otchere et al. 2018). However, population genomics is seldom employed in studies of other pathogenic species of *Actinobacteria* and environmental populations. *Actinobacteria* species offers an excellent model to explore within-species heterogeneity in a variety of environments, whether they be within a single eukaryotic host or between host species, in cryptic ecological niches, and across large geographical distances.

6 Conclusions and Future Outlook

We have only discerned a small fraction of the mechanisms, impacts, and barriers of HGT in *Actinobacteria* species. There is much yet to be discovered, and one promising approach to address these topics is population genomics. The ability of *Actinobacteria* to donate and gain DNA has tremendously influenced its survival in a variety of environments since its ancient origin. Recent comparative studies reveal that the genomic repertoires of actinobacterial species and populations are in constant flux as a result of widespread HGT, changing in response to the local environment, clinical interventions, and microbial neighbors. *Actinobacteria* use a variety of mechanisms to acquire DNA from their environment and from other cells, from mobile genetic elements to double-stranded DNA conjugation and phages. Acquired DNA can be mixed and matched in BGCs to generate diverse chemical inventory that a species can potentially produce, while resistance and virulence genes can be transferred to create deadly pathogens. In microbial communities, *Actinobacteria* are important HGT donors, disseminating genes with potential selective advantage in recipient lineages.

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Photobacterium damselae: How Horizontal Gene Transfer Shaped Two Different Pathogenic Lifestyles in a Marine Bacterium

Carlos R. Osorio

Abstract *Photobacterium damselae* is a marine pathogenic bacterium that includes two distinct subspecies, *piscicida* and *damselae*. The subspecies *damselae* is considered a non-clonal and generalist pathogen for a variety of marine animals and also for humans, whereas subsp. *piscicida* is a more specialized pathogen and only infects fish. Interestingly, most of their known virulence factors are encoded within mobile genetic elements, which include plasmids and pathogenicity islands. Highly virulent subsp. *damselae* isolates harbor the large conjugative plasmid pPHDD1 that encodes two potent cytotoxins, damselysin (Dly) and phobalysin P (PhlyP), whereas a third hemolysin phobalysin C (PhlyC) is encoded in chromosome I within a highly variable region that resembles a hot spot for recombination of horizontally acquired DNA. In addition, a chromosomal pathogenicity island encoding a vibrioferrin siderophore synthesis and uptake system is a feature of many subsp. *damselae* strains, and a large virulence plasmid that encodes a type III secretion system has been described in the type strain of this subspecies. Regarding the subspecies *piscicida*, many isolates harbor a small (pPHDP10) and a large plasmid (pPHDP70) that encode the apoptotic toxin AIP56 and the siderophore piscibactin for host iron scavenging, respectively. Moreover, subsp. *piscicida* genomes have undergone an expansion of insertion sequence (IS) elements that caused a massive gene decay, a process likely fueled by its host-dependent lifestyle. The repertoire of horizontally acquired DNA within the species is completed with a number of antibiotic resistance plasmids and integrative and conjugative elements (ICEs) that exhibit geographical distribution patterns. Collectively, horizontal gene transfer can be considered as a major driving force that shaped the distinct pathogenic strategies of each subspecies of *P. damselae*.

Keywords *Photobacterium damselae* · Damselysin · Hemolysin · AIP56 · Plasmid · Siderophore · Pathogenicity island

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1 Introduction

The marine bacterium *Photobacterium damselae* currently includes two subspecies, namely, subsp. *damselae* and subsp. *piscicida*. In order to understand this story, it is pertinent to go through the recent taxonomical changes undergone by these microorganisms.

In 1963, natural populations of white perch (*Morone americana*) and striped bass (*Morone saxatilis*) in the Chesapeake Bay (Maryland, USA) were seriously affected by an unknown bacterium, which was initially included within the genus *Pasteurella* with no specific designation (Snieszko et al. 1964). Later, the name *Pasteurella piscicida* was proposed (Janssen and Surgalla 1968). This name remained in use for decades, even though it was the only marine representative of this genus and despite the numerous phenotypical inconsistencies between *P. piscicida* and the rest of *Pasteurella* species. On the basis of analyses of 16S rRNA gene sequences and of DNA-DNA hybridization data, it was proposed its reassignation to the genus *Photobacterium* as *P. damselae* subsp. *piscicida* (Gauthier et al. 1995). This pathogen causes high financial losses in cultures of marine fish of importance in aquaculture including seriola, sea bream, sea bass, and sole, among others (Kusuda and Salati 1993; Toranzo et al. 1991; Magariños et al. 1996, 2003). Nowadays, *P. damselae* subsp. *piscicida* is one of the most devastating and widely distributed pathogens of cultured marine fish, causing a disease known as photobacteriosis, pasteurellosis or pseudotuberculosis (Toranzo et al. 2005).

The sibling subspecies, *P. damselae* subsp. *damselae*, was isolated for the first time in 1971 as an "unnamed marine *Vibrio*" causing infection in humans (Morris et al. 1982). This same organism was isolated a few years later from skin ulcers in natural populations of damselfish (*Chromis punctipinnis*) in California, being dubbed *Vibrio damsela* (Love et al. 1981). Genetic and phenotypic studies suggested that *V. damsela* was more related to species of the genus *Photobacterium* than of the genus *Vibrio*, and the name *Photobacterium damsela* was coined (Smith et al. 1991). At this point, the studies of Gauthier et al. (1995) demonstrated that *Photobacterium damsela* was closely related to *Pasteurella piscicida*, and therefore these two organisms were assigned to the same species, *P. damselae*, with category of subspecies (Gauthier et al. 1995). *P. damselae* subsp. *damselae* is not only considered an important pathogen in marine fish aquaculture but is capable of causing disease in other marine animals as crustaceans, mollusks, and cetaceans and also in humans (Rivas et al. 2013a).

The taxonomic placement of these two microorganisms within the same species epithet was not free of controversy for years. It is true that the similarity at the 16S gene sequence between the two subspecies is virtually 100% (Osorio et al. 1999), and they show a high percentage of DNA-DNA relatedness (Gauthier et al. 1995). However, a number of phenotypical traits showed to be differential between subsp. *piscicida* and subsp. *damselae*. Biological properties and capabilities positive only for subsp. *damselae* include, among others, growth at 37 °C, swimming motility, nitrate reduction, hemolysis on sheep blood agar, urease, production of gas from

glucose and maltose, serological diversity, and the ability to infect other animal hosts in addition to fish (Fouz et al. 1992; Magariños et al. 1992; Thyssen et al. 1998; Botella et al. 2002).

Thus, the observation of these phenotypical differences suggested that subsp. *piscicida* had undergone a generalized loss of metabolic, biochemical and cellular features with respect to subsp. *damselae*. The recent elucidation of the genome sequences of a few strains for each subspecies is indeed pointing at this idea. On the light of a recent study, *P. damselae* subsp. *piscicida* genome has undergone a massive proliferation of insertion sequence (IS) elements that caused a large number of pseudogenes and gene deletions. In consequence, subsp. *piscicida* has experienced the loss of many biological functions (Balado et al. 2017a). This process of massive gene decay is suggested to be intimately linked to an adaptation to a host-dependent lifestyle in this subspecies. In vitro studies reported that subsp. *piscicida* promotes its internalization into fish cells and according to this it might partially behave as a facultative intracellular pathogen (Elkamel and Thune 2003; Elkamel et al. 2003). However, confirmation of the existence of an intracellular stage during in vivo infection has not been provided so far. It is known that *P. damselae* subsp. *piscicida* is unable to survive in seawater microcosms in a culturable state for longer than 4–5 days (Janssen and Surgalla 1968; Toranzo et al. 1982), and so far there are no studies reporting the isolation of *P. damselae* subsp. *piscicida* directly from seawater. A number of recent studies have demonstrated that subsp. *piscicida* induces apoptosis of fish macrophages and neutrophils that results in lysis of these cell types by post-apoptotic necrosis (do Vale et al. 2005).

In contrast, the subsp. *damselae* has been isolated from seawater, seaweeds, seafood, and apparently healthy animals (Buck et al. 2006; Lozano-León et al. 2003; Chiu et al. 2013; Grimes et al. 1985; Serracca et al. 2011; Nonaka et al. 2012), and it is believed that it can behave as a free-living bacterium with the ability to cause opportunistic infections in animals under favorable conditions. Indeed, subsp. *damselae* strains can survive in seawater microcosms for long periods of time, maintaining not only their culturability but also their infectivity for fish (Fouz et al. 1998). Subsp. *damselae* pathogenesis in fish is not due to apoptosis induction in host cells, and this subspecies relies on the production of a variety of cytolysins in order to cause fish disease (Osorio et al. 2018).

This wide divergence in the lifestyles of the two *P. damselae* subspecies can be understood under the light of the acquisition of different virulence factors by horizontal gene transfer in each subspecies and also by the massive proliferation of insertion sequences in subsp. *piscicida* in particular. In the following points, we will describe the role of mobile genetic elements in the process of shaping two distinct pathogenic and living strategies for each of the two subspecies of *P. damselae*.

2 *P. damselae* subsp. *damselae*: A Generalist Pathogen with High Genetic Diversity

Recent studies on *P. damselae* subsp. *damselae* isolates from geographically related disease outbreaks reported a high genetic diversity among the isolates (Terceti et al. 2016, 2018). On the light of these studies, subsp. *damselae* is considered a multiclonal pathogen. In addition, each isolate contains specific gene combinations in the chromosomal regions encoding the synthesis of cell envelope polysaccharides (Osorio et al. 2018; Terceti et al. 2018). Outbreaks in fish farms can be caused by heterogeneous populations, which include highly virulent strains and also strains of low virulence (Pedersen et al. 1997; Terceti et al. 2018). Indeed, two main sets of isolates can be distinguished within *P. damselae* subsp. *damselae*, on the one hand, the isolates exhibiting strong hemolytic activity and high virulence and, on the other hand, the isolates with weak hemolytic activity and lower virulence degree.

Comparative genome analysis has revealed the presence of large numbers of unique genes to each strain. Within the strain-specific DNA fraction, an important contribution of prophage DNA sequences was found (Vences et al. 2017; Terceti et al. 2018). Notably, plasmid DNA constitutes a major contributor of the genetic differences among subsp. *damselae* strains. The type strain contains a large plasmid of ca. 203 kb dubbed pPHDD203 that encodes a type III secretion system (Vences et al. 2017). This system has not been functionally characterized so far, but it is known that pPHDD203 plasmid is not widespread in the subspecies since to date it has been found uniquely in the type strain CIP102761, and proved to be absent in a large number of isolates analysed (Vences et al. 2017; Terceti et al. 2018). As detailed below, other mobile genetic elements show a widespread presence in subsp. *damselae* strains and encode some of the most characteristic virulence factors of this marine bacterium.

2.1 *Emergence of Highly Virulent and Highly Cytotoxic Lineages of* P. damselae *subsp.* damselae *Following Acquisition of a Conjugative Virulence Plasmid*

Early studies revealed that some *P. damselae* subsp. *damselae* isolates produced high amounts of a cytolytic toxin, and such isolates were also more pathogenic for mice than isolates that did not produce the toxin (Kreger 1984). Subsequent characterization of this toxin revealed that it is a phospholipase D active against sphingomyelin, and was later dubbed damselysin (Dly), encoded by *dly* gene (Kothary and Kreger 1985; Kreger et al. 1987; Cutter and Kreger 1990). Currently, it is known that damselysin toxin is encoded within a virulence plasmid dubbed pPHDD1 (Rivas et al. 2011). The size of this plasmid in the type strain CIP102761 has been established in 149 kb, whereas in strain RM-71 this plasmid has 153 kb and contains additional genes absent in the type strain. The RM-71 plasmid consists of

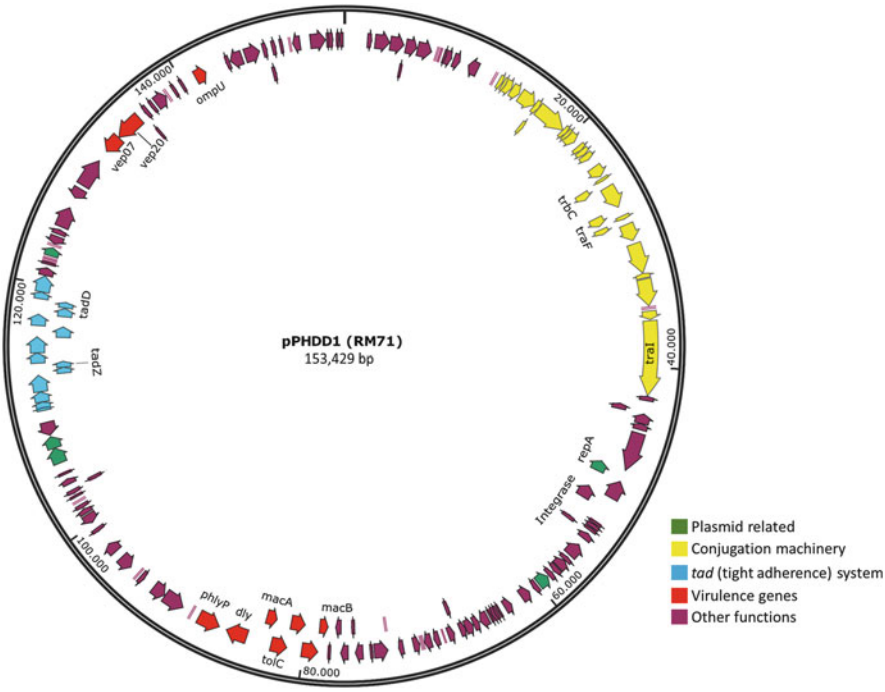

Fig. 1 Map of pPHDD1 virulence plasmid of *P. damselae* subsp. *damselae* strain RM-71 (GenBank Acc number NC_014653). Specific modules and genes are highlighted and represented with different color codes

153,429 bp and encodes 172 predicted proteins (Rivas et al. 2011) (Fig. 1). At least five modules have been distinguished within the pPHDD1 sequence, which include a replication module, a partitioning module, a *tad* (tight adherence) module, a conjugation module and a hemolysin/cytotoxin module. The replication module includes a *repB* gene plus a putative iteron region that contains three directed tandem repeats of the 9-mer TAAGATCTA. A PCR-amplified 2.6-kb sequence that included *repB* and the putative iterons was proved to be capable of independent replication in *Escherichia coli* when ligated to a kanamycin resistance marker, suggesting that such plasmid region contains the pPHDD1 replication origin (Rivas et al. 2011). The *tad* module comprises genes predicted to encode the biogenesis of Flp-type pili which might be involved in adhesion to biotic and abiotic surfaces. The conjugation module includes a set of *tra* genes encoding the proteins of a type IV secretion system. Indeed, it was reported that a marked version of pPHDD1 could be conjugally transferred from a RM-71 donor cell to a *P. damselae* subsp. *piscicida* recipient cell, and hemolytic activity was observed in the transconjugants (Rivas et al. 2011).

Notably, in addition to damselysin toxin, the hemolysin/cytotoxin module of pPHDD1 encodes a pore-forming toxin with hemolytic activity which was initially dubbed $HlyA_{pl}$ and subsequently renamed as phobalysin P (PhlyP) (Rivas et al.

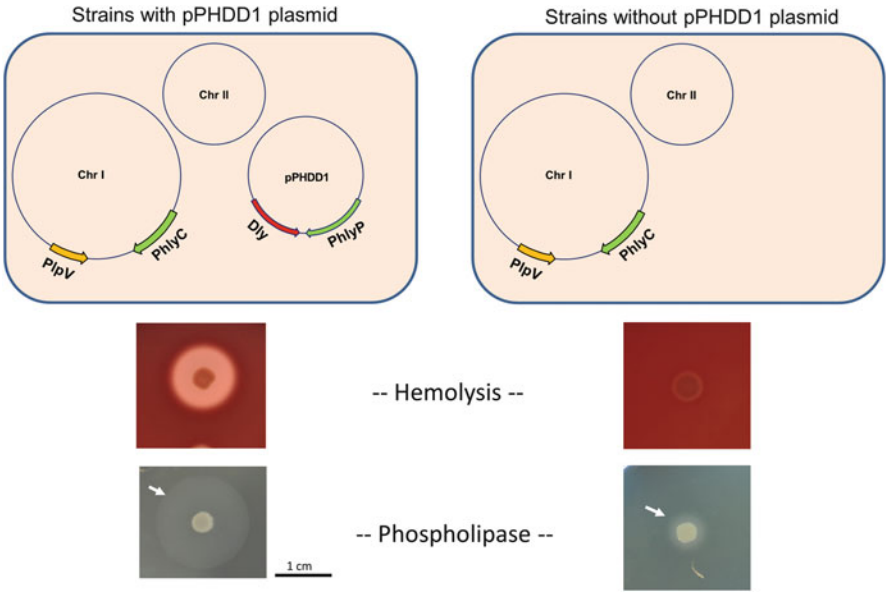

Fig. 2 Schematic representation of the two main categories of *P. damselae* subsp. *damselae* strains. Strains that harbor the virulence plasmid pPHDD1 produce the four cytotoxins Dly, PhlyP, PhlyC, and PlpV (left panel), whereas plasmidless strains only produce the chromosome-I-encoded cytotoxins PhlyC and PlpV (right panel). This differential production of hemolysins correlates with the different phenotypes of hemolysis on sheep blood agar plates and of phospholipid degradation. White arrows denote the diameter of the degradative halo of phospholipids in agar plate assays

2011, 2013b, 2015a). Currently, it is recognized that Dly and PhlyP cytotoxins cooperate in a synergistic manner to produce cell damage (Rivas et al. 2013b, 2015a). The hemolysin module of pPHDD1 also comprises a set of five genes which encode a TolC protein and AcrAB, plus two additional proteins. TolC and AcrAB conform a tripartite multidrug and toxin secretion efflux pump. This system might have a role in efflux of toxic substances, as well as in the secretion of virulence factors, since TolC protein is part of the type I secretion system. Secretion of Dly and PhlyP occurs via the chromosomally encoded type II secretion system (Rivas et al. 2015b).

Presence of pPHDD1 confers a strong hemolytic phenotype to *P. damselae* subsp. *damselae* strains, as well as a great ability to degrade phospholipids (Fig. 2). In addition, pPHDD1 plasmid confers a high cytotoxicity activity to *P. damselae* subsp. *damselae* cells (Rivas et al. 2015a) and also a higher virulence degree compared to plasmidless isolates (Terceti et al. 2016; Vences et al. 2017). A recent study unveiled the presence of novel potential virulence factors encoded within pPHDD1, and include a putative serum resistance Vep07-like protein, an OmpU adhesin and a transferrin receptor Vep20-like (Matanza and Osorio 2018)

(Fig. 1). Thus, the contribution of pPHDD1 plasmid to the emergence of a highly virulent lineage of *P. damselae* subsp. *damselae* could be even higher than expected.

The origin of pPHDD1 plasmid is so far unknown. Some sequence stretches of this plasmid bear similarity to plasmids described in other *Vibrionaceae* species (Rivas et al. 2011). However, the closest homologues of damselysin toxin are found exclusively in some members of the *Pseudoalteromonadaceae* and *Aeromonadaceae* families (Osorio et al. 2018). Therefore, considering the results of sequence comparisons of pPHDD1 with the DNA sequences available to date in public databases, the genetic structure of pPHDD1 plasmid can be considered as something unique to *P. damselae* subsp. *damselae*.

2.2 *Chromosome-Borne Cytotoxin Genes Are Encoded Within Potential Hot Spots for Gene Acquisition in* P. damselae *subsp.* damselae

As described above, acquisition of the virulence plasmid pPHDD1 has contributed to the emergence of highly virulent and cytotoxic lineages within *P. damselae* subsp. *damselae*. However, plasmidless strains are virulent for fish species and toxic for fish and human cell lines, an observation that fuelled research on the virulence factors produced by this set of strains. Interestingly, it was demonstrated that almost all the *P. damselae* subsp. *damselae* strains, regardless of whether they contain pPHDD1 plasmid or not, harbor a chromosome I-borne gene, $hlyA_{ch}$, that encodes a pore-forming toxin dubbed PhlyC (phobalysin C) (Rivas et al. 2013b). This toxin is 92% identical to its plasmid-encoded homologue PhlyP and is responsible for the weak hemolytic activity produced by plasmidless strains (Fig. 2). It also contributes to a large extent to virulence for fish and to toxicity for fish and human cell lines (Vences et al. 2017). Plasmidless strains do not produce damselysin phospholipase, but they still exhibit some degree of phospholipase activity which is attributable to the chromosome-encoded PlpV phospholipase (Vences et al. 2017) (Fig. 2).

Interestingly, the $hlyA_{ch}$ gene encoding PhlyC is located within a highly variable region of chromosome I in this subspecies. The gene diversity is so high, that each one of the 13 strains in which this region has been studied in detail, contains a unique gene repertoire (Rivas et al. 2014; Osorio et al. 2018; Terceti et al. 2018). The analysis of this genomic region uncovered the presence of features related to acquisition of foreign DNA, as integrases, insertion sequence elements, and tRNA genes, as well as tandem repeats (Fig. 3).

Phylogenetic analyses of the plasmid and chromosomal versions of phobalysin toxin suggested that $hlyA_{ch}$ (chromosome) and $hlyA_{pl}$ (plasmid) genes originated from a gene duplication event and started to diverge within *P. damselae* subsp. *damselae*. In support of this hypothesis, it was found that PhlyP and PhlyC sequences show a high degree of similarity between them as compared to their homologues in other *Vibrio* and *Photobacterium* species (Rivas et al. 2014). Of note,

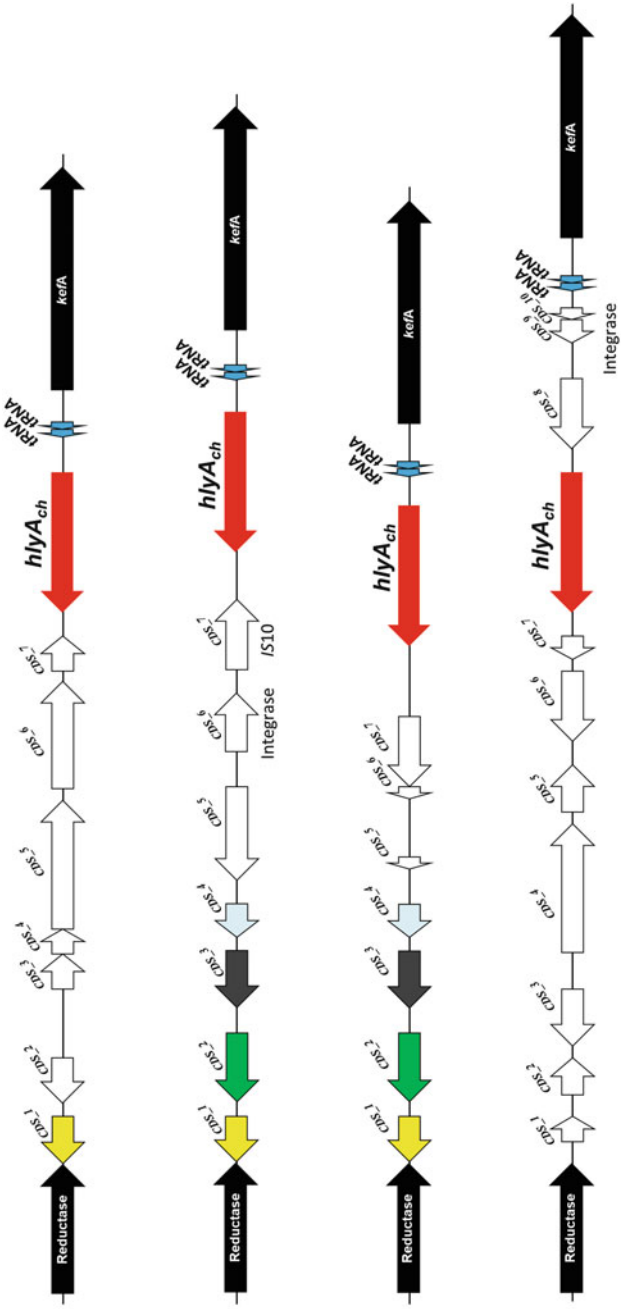

Fig. 3 Schematic representation of the diversity of gene repertoires found in the genomic context of *hlyA*$_{ch}$ gene that encodes phobalysin C (PhlyC) cytotoxin, in four selected *P. damselae* subsp. *damselae* strains. Two conserved flanking genes are depicted as black arrows (reductase gene and *kefA* gene). Arrows with the same color are shared by more than one strain, and white arrows denote ORFs unique to a single strain. As many as 26 unique genes were found in this variable genome region among the 4 shown genomes. Genes related to the acquisition of DNA by horizontal gene transfer, which include tRNA genes, integrase genes, and IS*10* elements are highlighted (modified from Terceti et al. 2018)

the sibling subspecies *P. damselae* subsp. *piscicida* lacks homologues of the PhlyP and PhlyC toxins.

2.3 *Siderophore Vibrioferrin Genes Are Part of a Horizontally Acquired Pathogenicity Island in* P. damselae *subsp.* damselae *Isolates*

Pathogenic bacteria utilize a variety of mechanisms to obtain iron from the host. A widespread mechanism for iron acquisition in many bacterial pathogens is based on the biosynthesis of siderophores, high-affinity iron chelators that are secreted by the bacterium and further transported back into the cell as Fe-siderophore complexes (Crosa 1989). Early studies have reported that virulence of *P. damselae* subsp. *damselae* for fish and mice was increased when an iron excess was provided, and the production of siderophores of the hydroxamate type was observed (Fouz et al. 1994, 1997). Further studies demonstrated that some strains of this pathogen produce the siderophore vibrioferrin (Balado et al. 2017b), and a proteomic study unveiled the production of iron-regulated proteins similar to those of vibrioferrin synthesis and transport in other *Vibrio* species (Puentes et al. 2017). Interestingly, production of vibrioferrin and presence of the vibrioferrin biosynthetic gene *pvsD* were features restricted to a fraction of the *P. damselae* subsp. *damselae* strains (Balado et al. 2017b), an observation that suggested that the vibrioferrin system genes might be encoded within a mobile DNA element. Indeed, a recent study unveiled the draft genome sequence of the *P. damselae* subsp. *damselae* strain 940804–1/1 positive for the vibrioferrin synthesis and transport genes, and it was shown that these genes were included within a large piece of DNA that was absent from other *P. damselae* subsp. *damselae* genomes and had features of a pathogenicity island (Terceti et al. 2018). In addition, the comparison between the genome context of the type strain CIP102761, which is negative for the vibrioferrin gene cluster, and the sequence of the contig of strain 940804–1/1 containing the vibrioferrin genes, clearly suggests that the vibrioferrin siderophore system of *P. damselae* subsp. *damselae* is encoded within a pathogenicity island acquired by horizontal gene transfer, and it has been inserted within a tRNA-Ser gene in chromosome II. The homologue of this tRNA-Ser gene in the genome sequence of the type strain CIP102761 has been annotated with locus tag VDA_RS15290 and is located between the two ORFs VDA_000334 and VDA_000335 (Fig. 4).

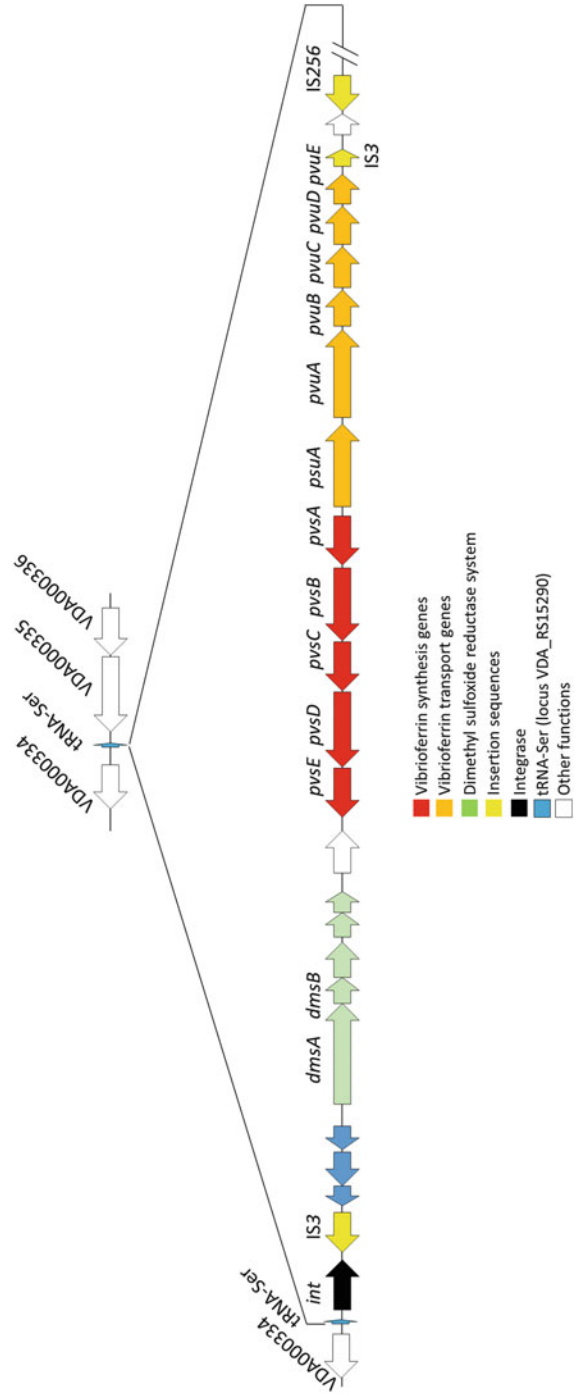

Fig. 4 Partial gene map of the putative pathogenicity island (PAI) that encodes siderophore vibrioferrin biosynthesis and utilization functions in *P. damselae* subsp. *damselae* strain 940804–1/1 (GenBank Accession number PVXF01000047.1). This PAI is inserted within a tRNA-Ser gene that is located between ORFs VDA000334 and VDA000335 according to the annotation of the complete genome sequence of the type strain CIP102761

2.4 *Large Conjugative Plasmids Provide Antibiotic Resistance to subsp.* damselae *Isolates*

The use of antibiotics in aquaculture facilities is believed to promote the maintenance and the spread of mobile genetic elements carrying antimicrobial resistance determinants (Cabello et al. 2013). Several studies have reported antimicrobial resistance profiles in *P. damselae* subsp. *damselae* isolated from disease outbreaks in fish farms, which suggested that strains of this subspecies might encode antimicrobial resistance functions (Fouz et al. 1992; Pedersen et al. 2009; Chiu et al. 2013). However, the complete genome sequence of the subsp. *damselae* type strain CIP102761 did not reveal the presence of antimicrobial resistance plasmids. In addition, analysis of the draft genome sequences of seven subsp. *damselae* isolates from different European locations also failed to reveal the presence of resistance determinants carried within plasmids or other mobile elements (Vences et al. 2017; Terceti et al. 2018). These observations suggested that presence of antimicrobial resistance genes in this subspecies may follow a geographical pattern. In support of this hypothesis, a recent study reported the description of a large conjugative plasmid, pAQU1, in a subsp. *damselae* strain isolated from seawater in an aquaculture site in Japan (Nonaka et al. 2012). pAQU1 has 204,052 bp and encodes seven drug resistance genes: $bla_{\text{CARB-9}}$-like, *floR*, *mph*(A)-like, *mef*(A)-like, *sul2*, *tet*(M), and *tet*(B). This plasmid demonstrated to be stably maintained in *P. damselae* subsp. *damselae* even with low antibiotic selection pressure (Bien et al. 2015). Subsequent studies reported the existence of pAQU1-like plasmids in subsp. *damselae* and also in different *Vibrio* species isolated in Japan and in China (Nonaka et al. 2014, 2015; Li et al. 2017). Therefore, the presence of pAQU1-like plasmids in subsp. *damselae* strains from geographical areas other than Asia still awaits investigation.

3 *P. damselae* subsp. *piscicida*: A Specialized Fish Pathogen That Has Experienced a Massive Gene Decay and Generalized Insertion Sequence Proliferation

The above described genetic heterogeneity of *P. damselae* subsp. *damselae* populations contrasts dramatically with the genetic similarity observed between subsp. *piscicida* isolates from close geographical areas. *P. damselae* subsp. *piscicida* is considered as serologically homogeneous, and no serotypes are distinguished (Kusuda and Fukuda 1980; Magariños et al. 1992). As an example of this genetic similarity, the genomes of two subsp. *piscicida* isolates from distantly located geographical points in the Iberian Peninsula demonstrated to be 98% identical (Balado et al. 2017a). However, there is evidence of the existence of two major chromosomal configurations within this subspecies, which correspond to the European isolates on the one hand, and to the Asian and American isolates on the other hand. This same study unveiled that the *P. damselae* subsp. *piscicida* genomes

have experienced a massive proliferation of insertion sequence (IS) elements (Balado et al. 2017a). It has been estimated that IS elements account for 12–17% of the total genome of two analysed strains isolated from the Iberian Peninsula, which represents an IS density of approximately 0.15 elements per kb, and constitutes one of the highest IS density values ever reported for a bacterial pathogen. A total of 15 different IS elements and transposons were found in the genome of DI21, one of the best studied subsp. *piscicida* isolates. Of note, two IS elements were predominant, not only in the two chromosomes but also in the plasmids. An IS*1* element (IS*Pda1*) was found in >400 copies, and a second element of the IS*91*-family (IS*Pda2*) occurred in >150 copies (Balado et al. 2017a).

A comparative sequence analysis between subsp. *piscicida* and subsp. *damselae* genomes demonstrated that the massive number of IS elements in subsp. *piscicida* has caused a large number of pseudogenes and gene deletions. In consequence, subsp. *piscicida* strains have experienced the loss of a large number of biological functions in comparison to subsp. *damselae*. This process of massive reduction in gene functions is believed to be intimately linked to an adaptation of subsp. *piscicida* to a host-dependent lifestyle. Of note, pseudogene formation and gene deletions in subsp. *piscicida* genomes have been particularly promoted within the small chromosome (Chr II), where extensive gene rearrangements and DNA sequence losses have occurred in comparison to the Chr II of subsp. *damselae* (Fig. 5). This finding is in agreement with the current hypothesis that small chromosomes of species of the family *Vibrionaceae* have a more flexible gene content than the larger chromosome Chr I (Harrison et al. 2010; Cooper et al. 2010).

The virulence plasmids and pathogenicity islands discovered in subsp. *damselae* strains have not been found in the subsp. *piscicida* genomes studied so far. Thus, it is clear that the pathogenic strategy of subsp. *piscicida* against fish cells does not rely on the production of membrane perturbing hemolysins as Dly, PhlyP, and PhlyC. Rather, as detailed below, subsp. *piscicida* cells harbor a small virulence plasmid that enables the bacterium to induce apoptosis of fish cells.

3.1 A Small Plasmid Encodes One of the Virulence Hallmarks of subsp. piscicida*: The Apoptosis-Inducing Toxin AIP56*

Experimental infection of sea bass (*Dicentrarchus labrax*) by intraperitoneal injection of *Photobacterium damselae* subsp. *piscicida* was initially observed to result in the induction of apoptosis of peritoneal neutrophils and macrophages, which appeared to constitute a novel and powerful pathogenic strategy (do Vale et al. 2003). Subsequent studies revealed that the apoptotic process was mediated by a novel apoptosis inducing protein of 56 kDa dubbed AIP56, secreted in high amounts by virulent strains (do Vale et al. 2005). Aip56 is a metalloprotease A-B exotoxin,

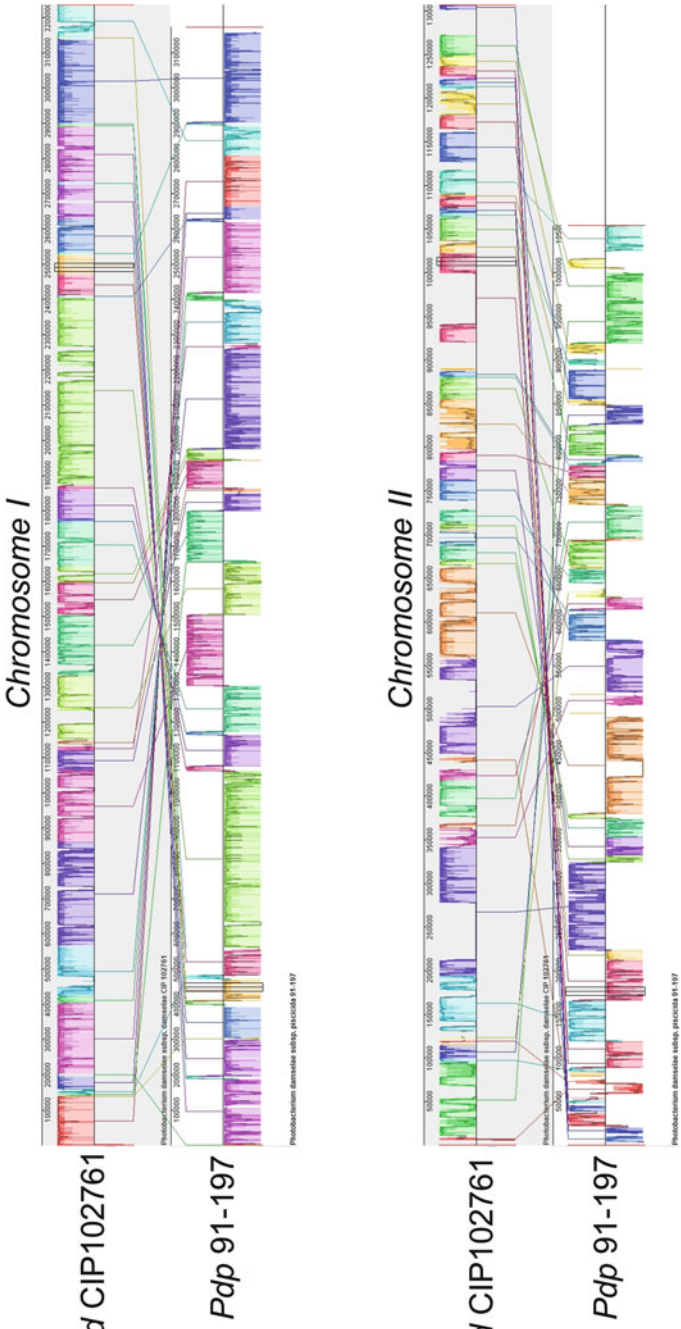

Fig. 5 Comparative analysis of genome structure between *P. damselae* subsp. *damselae* type strain CIP102761 (GenBank Accession number ADBS01000000) and *P. damselae* subsp. *piscicida* 91–197 (GenBank Accession numbers AP018045 to AP018048). The synteny regions are represented with the same color blocks. Blocks above the central line denote that the aligned regions are in the forward orientation relative to the CIP102761 genome, whereas blocks below the central line denote regions that align in the reverse complement orientation. Inverted and translocated regions are connected by lines. Note that chromosome II of subsp. *piscicida* has undergone a massive reorganization and loss of DNA sequences in comparison to subsp. *damselae*

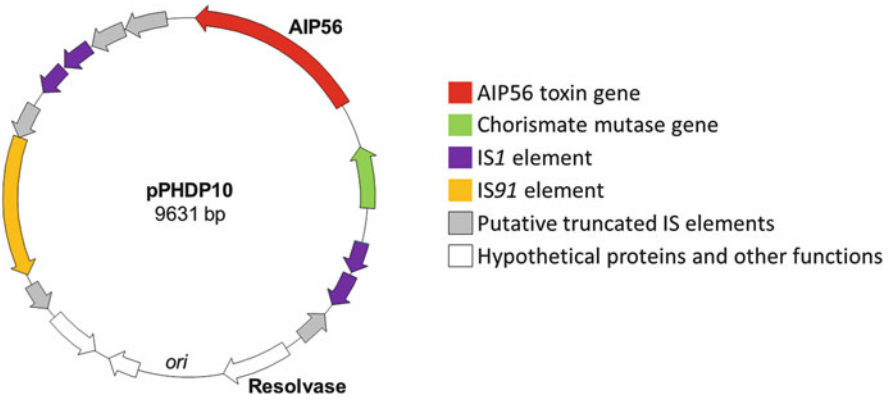

Fig. 6 Gene map of the pPHDP10 virulence plasmid of *P. damselae* subsp. *piscicida* strain MT1415 (GenBank Accession number DQ069059). Major features of this plasmid include the apoptosis inducing toxin AIP56, and a chorismate mutase of yet uncharacterized function. Note that a great percentage of plasmid sequence corresponds to insertion sequence elements

that acts by cleaving NF-κB (Silva et al. 2013; Pereira et al. 2014), and is secreted by the type II secretion system (do Vale et al. 2017).

Interestingly, the gene encoding AIP56 is located within a small plasmid of 9631 bp that was dubbed pPHDP10 (do Vale et al. 2005). pPHDP10 contains, in addition to *aip56* gene, open reading frames (ORFs) coding for several transposases, a resolvase, and a chorismate mutase (Fig. 6). Notably, pPHDP10 belongs to a recently characterized, novel family of plasmids dubbed MRB, which stands for *m*arine *R*NA-*b*ased (Le Roux et al. 2011). This is a family of plasmids so far only reported in members of the family *Vibrionaceae*, characterized by the feature that their *ori* regions do not encode a Rep protein. Instead of this, MRB-family plasmids *ori* regions encode two partially complementary RNAs that govern plasmid replication and incompatibility (Li et al. 2010; Le Roux et al. 2011). The two RNAs are transcribed from opposite strands. The smaller RNA, dubbed RNA I (68 nt) works as a negative regulator and is fully complementary to the larger RNA II (Le Roux et al. 2011).

pPHDP10 is therefore a small plasmid that lacks evident *cis*- and *trans*-acting sequences for conjugative transfer. However, a study reported conjugation-mediated transmission of the *aip56* gene and neighboring sequences via formation of a cointegrate between pPHDP10 and a co-resident integrating and conjugative element, ICE*Pda*Spa1 (Osorio et al. 2008). Evidence suggested that pPHDP10 is capable of promoting its integration into other DNA molecules, likely facilitated by the number of transposase genes that contribute to a large proportion of the pPHDP10 sequence. Hence, a precursor of pPHDP10 might have gained access to subsp. *piscicida* cells by a similar mechanism of conjugation forming a cointegrate with a mobilizable or a self-transmissible element. The AIP56 gene is present in all virulent subsp. *piscicida* strains tested so far, and is known to be absent in two non-virulent strains analysed (do Vale et al. 2005). The origin of pPHDP10 plasmid

and of the *aip56* gene in particular is uncertain, and no closely related homologues of *aip56* gene have been reported so far in bacteria other than *P. damselae* subsp. *piscicida*. Interestingly, a recent study provided evidence that AIP56-like putative toxins are encoded by strains of the marine bacterium *Vibrio campbellii* as well as by insect pathogenic bacteria (Silva et al. 2010).

3.2 *Siderophore Piscibactin Is Encoded Within a Plasmid-Borne Pathogenicity Island that Resembles the* Yersinia *High-Pathogenicity Island*

Some *P. damselae* subsp. *piscicida* strains produce a phenolate siderophore dubbed piscibactin which is structurally similar, but not identical, to yersiniabactin produced by pathogenic *Yersinia* species (Souto et al. 2012). Notably, the genes responsible for the biosynthesis and transport of piscibactin are clustered in a pathogenicity island-like structure (Osorio et al. 2006) which is part of a mobilizable plasmid of ca. 70 kb dubbed pPHDP70 (Osorio et al. 2015). The best studied pPHDP70 molecule is derived from strain DI21, which consists of 68,686 bp and encodes 53 open reading frames (Fig. 7). The G+C content of pPHDP70 varies from 21% to 54%, suggesting that the plasmid is a mosaic composed of genes from different sources. In support of this hypothesis, different parts of the plasmid show closest homologues to a diverse taxonomy of species. As an example, genes of a putative chaperone usher pathway show similarity to *Edwardsiella ictaluri*, whereas *repA* and *parAB* genes, putatively involved in plasmid replication and maintenance, are almost identical to genes reported in the multidrug resistance plasmid pAQU1 recently described in a Japanese isolate of *P. damselae* subsp. *damselae* (Nonaka et al. 2012). In addition, pPHDP70 sequence includes three different insertion sequence elements, IS*1*, IS*3*, and IS*91*. The largest element, IS*91*, occurs in six copies and represents 10% of the total plasmid sequence, whereas IS*1* (with two ORFs, *tnpA* and *tnpB*) and IS*3* occur in double and single copy, respectively. Interestingly, IS*1* and IS*91* elements also occur in the small virulence plasmid pPHDP10 (Fig. 8a), suggesting that these elements are undergoing an expansion into subsp. *piscicida* replicons since, as described above, these two elements also occur in a multicopy fashion in the subsp. *piscicida* chromosomes. Interestingly, the IS elements present in pPHDP70 did not cause any evident disruption of coding sequences, which suggest that selective pressure has maintained plasmid genes functional.

Undoubtedly, the most notable feature of pPHDP70 is the presence of a pathogenicity island that includes 14 genes (dubbed *irp* genes, which stands for iron-regulated proteins) related to the synthesis and transport of siderophore piscibactin (Osorio et al. 2006, 2015; Souto et al. 2012) (Fig. 7). These 14 genes encode a putative outer membrane receptor FrpA, a putative ABC transporter, two transcriptional regulators AraC1 and AraC2, and the biosynthetic genes for piscibactin

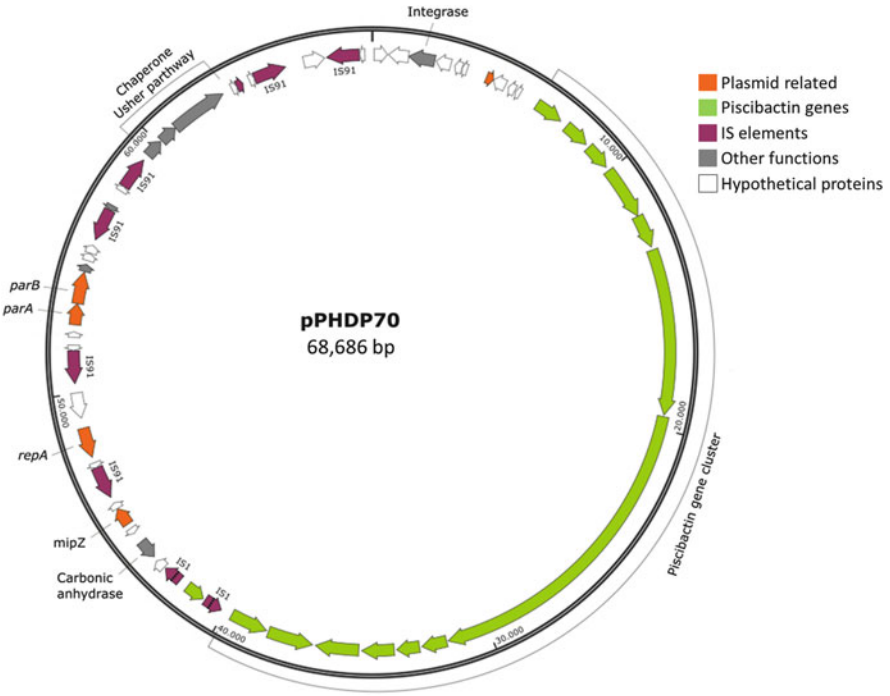

Fig. 7 Gene map of pPHDP70 plasmid encoding the functions for the synthesis and utilization of siderophore piscibactin in *P. damselae* subsp. *piscicida* strain DI21 (GenBank Accession number KP100338)

production. Piscibactin is a phenolate siderophore whose biosynthesis is carried out by a non-ribosomal peptide synthetase-based mechanism (Souto et al. 2012). There is supporting evidence that all the biosynthetic functions for piscibactin production are encoded within the pPHDP70-borne pathogenicity island that includes the 14 *irp* genes. In this regard, pPHDP70 was found to confer piscibactin biosynthesis and utilization following its conjugative transfer into a recipient *Vibrio alginolyticus* strain (Osorio et al. 2015). It is pertinent to say that pPHDP70 lacks genes for conjugation machinery. However, it can be transferred at low frequencies into recipient strains. Such transfer is believed to be facilitated by the coresident plasmid pPHDP60, a self-transmissible plasmid that has been reported in some *P. damselae* subsp. *piscicida* strains (Balado et al. 2013a).

Database searches have revealed that clusters homologous to the piscibactin pathogenicity island are also present in human and marine animal pathogenic vibrios, including *Vibrio cholerae*, *V. mimicus*, *V. ordalii*, *V. anguillarum*, and *V. coralliilyticus*, among others (Osorio et al. 2015) (Fig. 8b). It is notable that a similar cluster is also present in strains of the non-pathogenic marine bacterium *Photobacterium phosphoreum*. However, one of the most striking aspects of the piscibactin pathogenicity island is its resemblance to the *Yersinia* high-pathogenicity

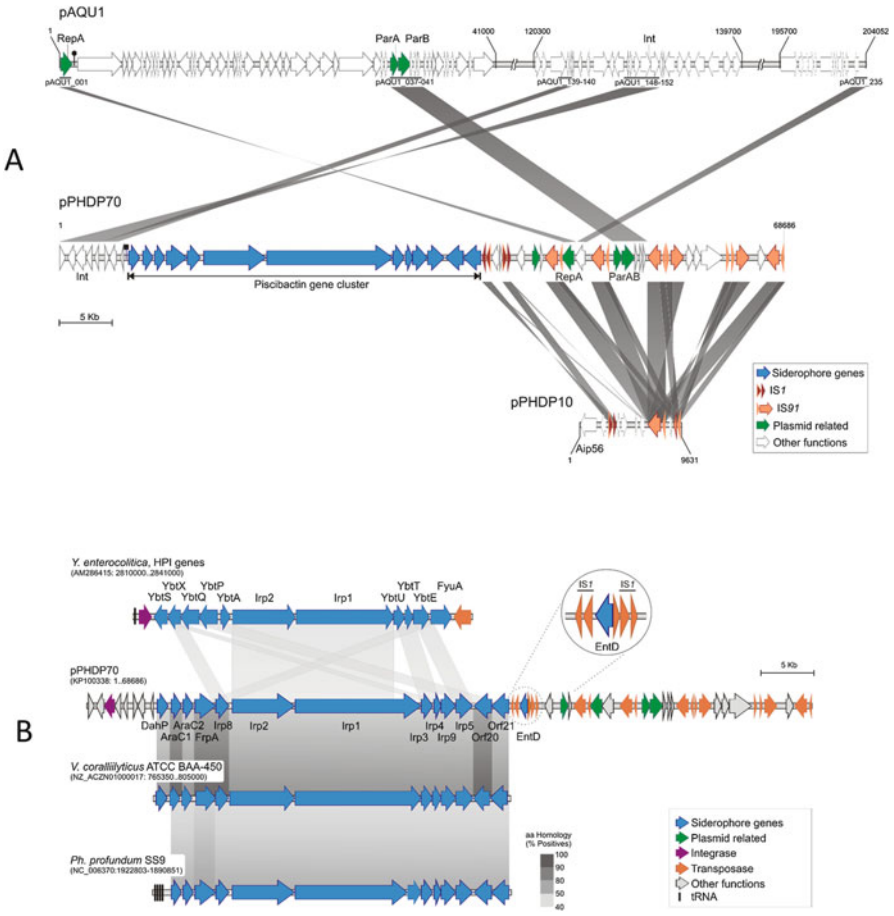

Fig. 8 (**a**) Graphical representation and comparative analysis of pPHDP70 and pPHDP10 virulence plasmids of *P. damselae* subsp. *piscicida*, and their sequence similarities with regions of the multidrug resistance plasmid pAQU1 reported in *P. damselae* subsp. *damselae*. Genes encoding transposases, plasmid-related functions, and the piscibactin gene cluster are highlighted. Homologous genes shared between plasmids are connected by gray bands. pPHDP70 shares plasmid backbone genes with pAQU1 and insertion sequences with pPHDP10. (**b**) Comparative analysis of pPHDP70 plasmid with homologous gene clusters in the *Yersinia* high-pathogenicity island (HPI) and in marine bacteria of the family *Vibrionaceae*. Of note, the pPHDP70 piscibactin cluster is plasmid borne, whereas the other homologous clusters are chromosomally encoded (reproduced from Osorio et al. 2015)

island (HPI), a chromosome-borne island that encodes functions for the biosynthesis and utilization of the siderophore yersiniabactin in pathogenic *Yersinia* species (Carniel et al. 1996). This constitutes an interesting example of the similarities of pathogenicity islands encoding virulence factors, between terrestrial and marine animal pathogens. This suggests that horizontal gene flow can surmount barriers

between terrestrial and aquatic ecosystems. In addition, the integration of the piscibactin pathogenicity island into a plasmid molecule is believed to have contributed to an enhanced capability for its dissemination by conjugation to other bacterial species. Indeed, the *P. damselae* subsp. *piscicida irp* gene cluster constituted the first report of a HPI-like cluster that was plasmid-borne, since all the homologous clusters are locked into the chromosomes (Osorio et al. 2015).

P. damselae subsp. *piscicida* strains cured of pPHDP70 plasmid showed reduced virulence for fish. However, the piscibactin biosynthesis system is not a hallmark of the subspecies at all. Indeed, pPHDP70 occurs within a fraction of *P. damselae* subsp. *piscicida* strains, and so far it has been detected only in strains of this subspecies from European countries as well as from countries of the Mediterranean basin, whereas the strains from Asia and America tested to date lack this plasmid (Osorio et al. 2015). These findings opened the question of which is the type of siderophore produced by the pathogenic subsp. *piscicida* strains that do not harbor pPHDP70 plasmid. Recent studies suggested that these strains might secrete endogenous citrate as iron scavenger, a mechanism that is widespread in the subsp. *damselae* strains, and therefore it might constitute an ancestral trait for subspecies *damselae* and for subspecies *piscicida* (Balado et al. 2017b).

3.3 Multidrug Resistance Plasmids and Integrative and Conjugative Elements (ICEs) in subsp. piscicida

Early studies reported the presence of transferable resistance plasmids in *P. damselae* subsp. *piscicida* strains (Kim and Aoki 1993, 1994, 1996a, b; Morii et al. 2003). Later, the complete sequences of large conjugative, multidrug-resistance plasmids were determined in strains of this subspecies isolated from Asia and North America (Kim et al. 2008; del Castillo et al. 2013). Notably, one of these plasmids dubbed pP9014, is a IncP-1 plasmid that showed similarity to other plasmids isolated from soil and also from human clinical specimens (del Castillo et al. 2013). However, this plasmid has not been detected in subsp. *piscicida* strains from other locations of the globe tested so far, and it is restricted to Japanese isolates. Indeed, the genome sequencing of two subsp. *piscicida* strains from Spain revealed the lack of antimicrobial resistance plasmids (Balado et al. 2017a).

Interestingly, one subsp. *piscicida* strain isolated from cultured sole (*Solea senegalensis*) in Spain contributed the first report of a fish pathogen-derived integrative and conjugative element (ICE) which was dubbed ICE*Pda*Spa1 (Juiz-Rio et al. 2005; Osorio et al. 2008). ICEs are self-transmissible elements that transfer between bacterial species by conjugation and then integrate into the host chromosome. ICE*Pda*Spa1 is an element of the SXT/R391 family, a group of elements which are widespread in *Vibrio cholerae* populations as well as in other Gram negative bacteria, of marine or terrestrial origin (Burrus et al. 2006). This family of ICEs disseminates antibiotic resistance genes among bacteria (Rodríguez-Blanco

et al. 2012) and may also provide resistance to bacteriophages via ICE-encoded restriction-modification systems (Balado et al. 2013b). Notably, ICEs of the SXT/R391 family can drive the transmission of mobilizable islands from donor to recipient bacterial cells (Daccord et al. 2010). ICE*Pda*Spa1 confers tetracycline resistance to *P. damselae* subsp. *piscicida*, and has been found to promote the transfer of the virulence plasmid pPHDP10 (encoding the apoptotic toxin AIP56) via formation of a cointegrate (Osorio et al. 2008).

4 Conclusions and Perspectives

Mobile genetic elements have shaped the evolution of the two subspecies of *Photobacterium damselae* (Fig. 9). A main factor in the subspecies diversification is the expansion of IS elements in subsp. *piscicida*, a process that caused a massive gene decay and deletions of large DNA stretches. As a consequence, subsp. *piscicida* cells lack many biological functions which are otherwise functional in subsp. *damselae* cells. As an example, the inability of subsp. *piscicida* to swim with a flagellum correlates with the inactivation in this subspecies of several genes for flagellum biosynthesis (Balado et al. 2017a). Concomitant to the gene decay process, subsp. *piscicida* may have adapted to some degree of dependence with its fish host. To what extent subsp. *piscicida* is a host-dependent pathogen, still awaits further investigation. Along this process, subsp. *piscicida* cells have likely gained a series of plasmids which have shaped its pathobiology. Of note is the acquisition of the small virulence plasmid pPHDP10 encoding the apoptosis-inducing toxin AIP56, which is widespread in isolates from different parts of the world. More geographically restricted is pPHDP70 plasmid encoding a siderophore piscibactin system for iron uptake, which is believed to be restricted to isolates from Europe, both in the Atlantic and Mediterranean basins.

It cannot be ruled out the possibility that some of the virulence plasmids acquired by subsp. *piscicida* constituted the entry port for IS elements that eventually expanded within the two chromosomes. In support of this hypothesis, IS*PdaSpa1* element (IS*1* family) has only been found in subsp. *piscicida* strains, but not in subsp. *damselae* (Balado et al. 2017a), and also occurs within the two virulence plasmids pPHDP10 and pPHDP70. Since the small plasmid pPHDP10 does not show geographical restriction, it might be hypothesized that IS*PdaSpa1* element gained access to a cell of the incipient subsp. *piscicida* lineage via acquisition of a pPHDP10-like plasmid. Loss of this plasmid in some subsp. *piscicida* lineages might have subsequently occurred, aided by the putative lack of addiction systems in this plasmid.

Subsp. *damselae* has not undergone a process of gene decay, and it is a facultative pathogen capable of thriving in a free-swimming planktonic state. Still, horizontal gene transfer has clearly shaped its pathobiology. At some stage in the subspeciation process, subsp. *damselae* gained a pPHDD1-like virulence plasmid. The strong cytotoxicity of Dly and PhlyP toxins has undoubtedly contributed to the rise of

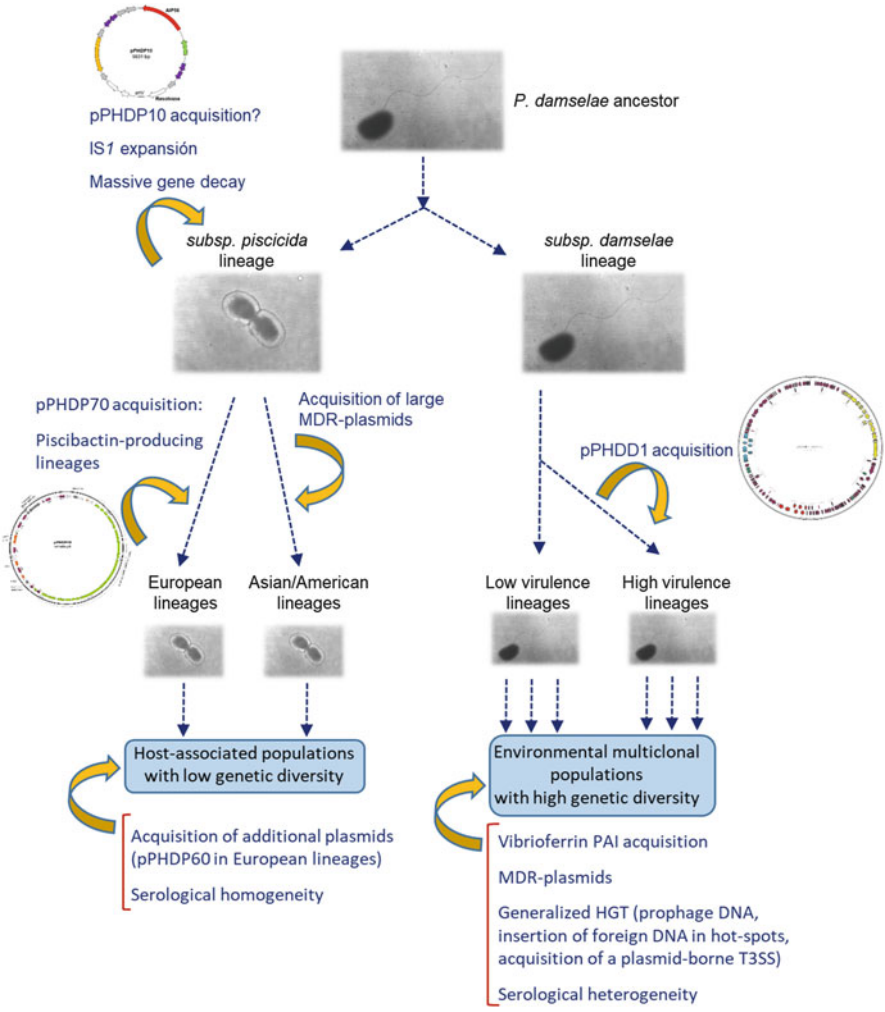

Fig. 9 Proposed evolutionary model for the subspeciation process within the species *P. damselae*, highlighting the major roles of DNA acquisition by horizontal gene transfer

subsp. *damselae* lineages with high virulence potential. Whether acquisition of this plasmid occurred in one or in multiple events in the subspecies is so far unknown. Also intriguing is the location of the chromosomal version of the PhlyC hemolysin gene within a highly variable DNA region. The high similarity between plasmid (PhlyP) and chromosomal (PhlyC) counterparts of the phobalysins suggests that these two gene versions might have evolved within the subspecies, either from an ancestral copy in the *P. damselae* ancestor, or upon acquisition of an ancestral copy by horizontal gene transfer from a related *Vibrio* or *Photobacterium* species.

Acquisition of novel virulence traits via horizontal transfer of plasmids has provided a mechanism for subspecies diversification within the species *P. damselae*. This transfer appears to account for much of the pathobiological differences between subsp. *piscicida* and *damselae*. Undoubtedly, the study of these two subspecies and of their process of diversification constitutes a fantastic biological model for the study of bacterial evolution.

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Part II
Horizontal Gene Transfer Between Bacteria and Animals, Plants, Amoeba and Fungi

Horizontal Gene Transfer in Metazoa: Examples and Methods

Yuki Yoshida, Reuben W. Nowell, Kazuharu Arakawa, and Mark Blaxter

Abstract Horizontal gene transfer (HGT) is now widely accepted as an indispensable mechanism in the evolution of microbes, but its contribution in metazoans still raises controversies. This is partly due to the methodologies used for the comprehensive prediction of HGT candidates from genomic information and also because the specific pathways that allow the incorporation of foreign DNA in the germline cells and subsequently to their chromosomes remain elusive. Here, we review the methods for HGT detection and examples of HGT events in two metazoan groups, bdelloid rotifers and tardigrades. Both groups are parthenogenetic and are capable of surviving desiccation (anhydrobiosis), and the roles of these features in promoting HGT, and of HGT loci to these phenomena, are discussed.

Keywords Horizontal gene transfer · Alien Index · HGT Index · Bdelloid rotifer · Tardigrade

1 Introduction

The evolutionary history of life is commonly represented as a tree-like process, with species separating and diversifying through repeated, nested bifurcations. However, it is increasingly becoming clear that the evolution of life also includes reticulation events—the exchange of genetic material across extended phylogenetic distances. In bacteria, horizontal exchange of genetic material between strains and species is common, via transducing phage, conjugation and direct transformation by exogenous

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DNA. This can drive rapid adaptation, for example, in resistance to anthropogenic antibiotic challenge. In eukaryotes, horizontal transfer of genetic material that does not conform to the bifurcating tree-like behaviour of the bulk of the genome takes two major forms. One is introgression or hybridisation, where one species acquires genetic material from another, usually closely related, via sexual reproduction between individuals from distinct lineages. In some cases, the event of hybridisation results in a lineage that is reproductively isolated and can thus form a new hybrid species.

The other form of reticulate evolution is horizontal gene transfer (HGT). We reserve the term HGT for transfer of genetic material into a eukaryotic nuclear genome from a distantly related donor through non-sexual mechanisms. HGT into eukaryotic genomes can be from other eukaryotes, from bacteria or from viruses. The relative importance of HGT in eukaryote evolution has been enthusiastically discussed for decades, and interest has grown since the beginning of the genome era (Salzberg et al. 2001; Stanhope et al. 2001). The emergence of high-throughput sequencing technology has enabled sequencing of numerous eukaryotes, and comparative genomics analyses have identified sequences that are likely to have originated by horizontal transfer in many taxa.

While HGT simply describes transfer of DNA from a donor to a recipient through non-sexual mechanisms, it is important to distinguish simple transfer of DNA and the incorporation of functional genes into the recipient genome. In eukaryotes, where gene expression is the tightly regulated outcome of complex transcriptional control, it is likely that most HGT events are neutral or mildly deleterious to the recipient (Blaxter 2007).

The mechanisms of HGT into eukaryotic genomes are unclear, though several possibilities exist. HGT may be part of the life strategy of pathogens and parasites. The tumour-inducing plasmid of *Agrobacterium rhizogenes* is transferred to the host, where it causes neoplastic diseases [crown gall and hairy roots; reviewed in (Nester et al. 1984)]. Viruses can transfer genes into the human genome (Pett and Coleman 2007; Sung et al. 2012; Crosbie et al. 2013), and insertions of viral origin are common in sequenced eukaryotic genomes. However, these HGTs largely occur in somatic cells, causing disease in the recipient, and will not be inherited by the next generation. Most eukaryotes have effective genome surveillance systems that prevent, or at least minimise, the activities of elements in their germ lines, reducing the likelihood of HGT acquisition from these sources.

Endogenous double-strand break repair mechanisms can result in the insertion of DNA fragments into genomes, irrespective of their source or possible function. When these breaks are repaired in cells that go on to contribute to the next generation, the HGT event is transmitted and can become part of the core genome of the species. As an example, this neutral or non-functional HGT process has resulted in the insertion of many copies of mitochondrial genome fragments into the human and other genomes. These fragments, called nuclear-mitochondrial transfers (NUMTs), are not functional. NUMTs are not expressed (at any significant level) and do not contribute to the biology of the nuclear genome other than as bulk noncoding DNA. Once inserted, they evolve neutrally, incorporating “disabling” mutations (nonsynonymous substitutions, insertions and deletions) and have no adaptive significance. The high frequency of NUMTs suggests that these transfers arise because of the (ubiquitous) presence of

the mitochondrion in germline cells. Similar abundances of chloroplast DNA transfers into photosynthetic lineage genomes and of vertically transmitted endosymbiont DNA into arthropod and nematode genomes (Dunning Hotopp et al. 2007) are other examples of non-functional HGTs that arise because of the proximity of the donor genome to the germline (Blaxter 2007).

HGTs can acquire functional roles in their new host genome. Gene expression in (most) eukaryotes is complex and requires that a gene has the correct chromatin marks, has effective enhancer and promoter elements, (usually) contains spliceosomal introns and has the correct transcriptional termination and polyadenylation signals. To generate protein products at high levels, the transcript should also utilise a codon set optimised for the cellular environment. A horizontally transferred DNA segment may contain a gene that is intact based on its sequence, but because it lacks any one of these components, is not expressed and is not functional. In the case of HGT from bacteria or archaea, therefore, a gene will have to acquire enhancer and promoter elements, acquire spliceosomal introns and acquire transcription termination and poly (A) addition signals in order to be expressed. To be expressed at high levels, its codon usage will have to be ameliorated to match that of the new host genome (Blaxter 2007). HGT events into a eukaryote nucleus from other eukaryotes may result in the acquisition of loci that already carry transcriptional control signals and introns from their originating genome. Whether these function in the new host genome environment will depend on the phylogenetic relatedness of the donor and recipient taxa, but it can be imagined that functional integration might be easier. Thus, functional HGTs should display features of integration not only into the genome of the host but also into the molecular biological and physiological systems of the host.

Functional HGT loci that have their origins through HGT can have a wide range of enzymatic and biological roles. Husnik and McCutcheon (2018) offered a broad classification of the functions of horizontally transferred genes based on their contributions to the recipient cell. Maintenance HGTs complement endogenous functions, allowing endogenous gene loss, while innovative HGTs convey new phenotypes to the host cell and enable adaptation to novel factors. For example, a major set of maintenance HGTs involved transfer to the nucleus of genes from the endosymbiotic, alphaproteobacterial ancestor of mitochondria. These loci still play roles in mitochondrial function. Similar ancient transfers occurred from ancestral plastid and chloroplast genomes in photosynthetic lineages and of nucleomorph genes after secondary symbioses (Spang et al. 2015; Ku et al. 2015a, b; Archibald 2015; Koonin 2015; Pittis and Gabaldón 2016; Zaremba-Niedzwiedzka et al. 2017). The loss of a gene in the host can be compensated by HGTs from endosymbionts and other sources, as observed in both insects and amoeba (Nikoh et al. 2010; Husnik and McCutcheon 2016; Nowack et al. 2016).

Innovative HGTs have contributed to phenotypic diversity. Herbivorous animals in particular have acquired enzymes for digestion of plant cell wall and other complex carbohydrates from bacterial (and fungal) donors (Ricard et al. 2006; Richards et al. 2011; Schuster and Sommer 2012; Boschetti et al. 2012; Savory et al. 2015; Wybouw et al. 2016). To supplement restricted plant-derived diets, herbivores have acquired loci that drive synthesis of essential nutrients (Alsmark

et al. 2013; Husnik et al. 2013). Some herbivores have acquired enzymes derived from bacterial metabolism to detoxify toxic plant compounds (Daimon et al. 2008; Acuña et al. 2012; Wybouw et al. 2014, 2016). In return, plants have exploited bacterial genes to protect themselves from herbivores. For example, *Epichloë* fungi, which are endophytic in grass, produce a toxin from a likely HGT locus which kills larvae of herbivorous insects (Daborn et al. 2002; Ambrose et al. 2014). Several examples of functional HGT involve acquisition of bactericidal effectors (amidases and lysozymes) (Dunning Hotopp and Estes 2014). HGTs also contribute to extremotolerance in various eukaryotes (Raymond and Kim 2012; Blanc et al. 2012; Schönknecht et al. 2013; Eyres et al. 2015; Mock et al. 2017; Harding et al. 2017).

Although the advent of whole genome sequence data has accelerated the detection of HGTs in eukaryotic genomes, their discovery, validation and biological significances remain controversial (Danchin 2016). In this chapter, we describe the patterns of HGT found in genomes from two animal phyla: bdelloid rotifers, whose genomes contain many functional HGTs, and hypsibiid tardigrades, where an initial claim of high numbers of HGTs was found to be elevated due to technical errors (Fig. 1). We review HGT detection methods and suggest solutions to the problems of robust and credible HGT detection in genome projects.

2 HGT in Extremotolerant Tardigrades

Tardigrades are a phylum of meiofaunal ecdysozoan animals found almost ubiquitously in marine, freshwater and limno-terrestrial sedimentary environments. Many freshwater and terrestrial tardigrades are cryptobionts, capable of entering anhydrobiosis and/or cryobiosis (Keilin 1959; Guidetti et al. 2011). Genome sequences are currently available for three species of Tardigrada, all from the class Eutardigrada: *Ramazzottius varieornatus* (Yamaguchi et al. 2012; Tanaka et al. 2015; Hashimoto et al. 2016), *Hypsibius exemplaris* (Yoshida et al. 2017) and *Milnesium tardigradum* (Bemm et al. 2017). All three species are parthenogenetic, freshwater or terrestrial taxa. Analyses of their genomes identified tardigrade-specific gene families that are likely to contribute to their extremotolerant abilities (Yamaguchi et al. 2012; Tanaka et al. 2015; Hashimoto et al. 2016).

The initial publication of a draft of the *H. exemplaris* genome (Boothby et al. 2015) reported an extremely high number of loci that apparently were acquired by this species by HGT from a wide range of bacterial, protistan and other eukaryotic donors. The authors compared the best similarity scores obtained for each protein-coding locus in metazoan versus bacterial and non-metazoan databases and proposed HGT status for any where the bacterial match was much better than any match to an animal sequence. One sixth (17.5%) of the predicted protein-coding genes were reported as having sequence similarity signatures of HGT, exceeding the rates previously known in any other metazoan (including rotifers, see below). It was suggested that the tardigrades' cryptobiotic lifestyle somehow drove the high

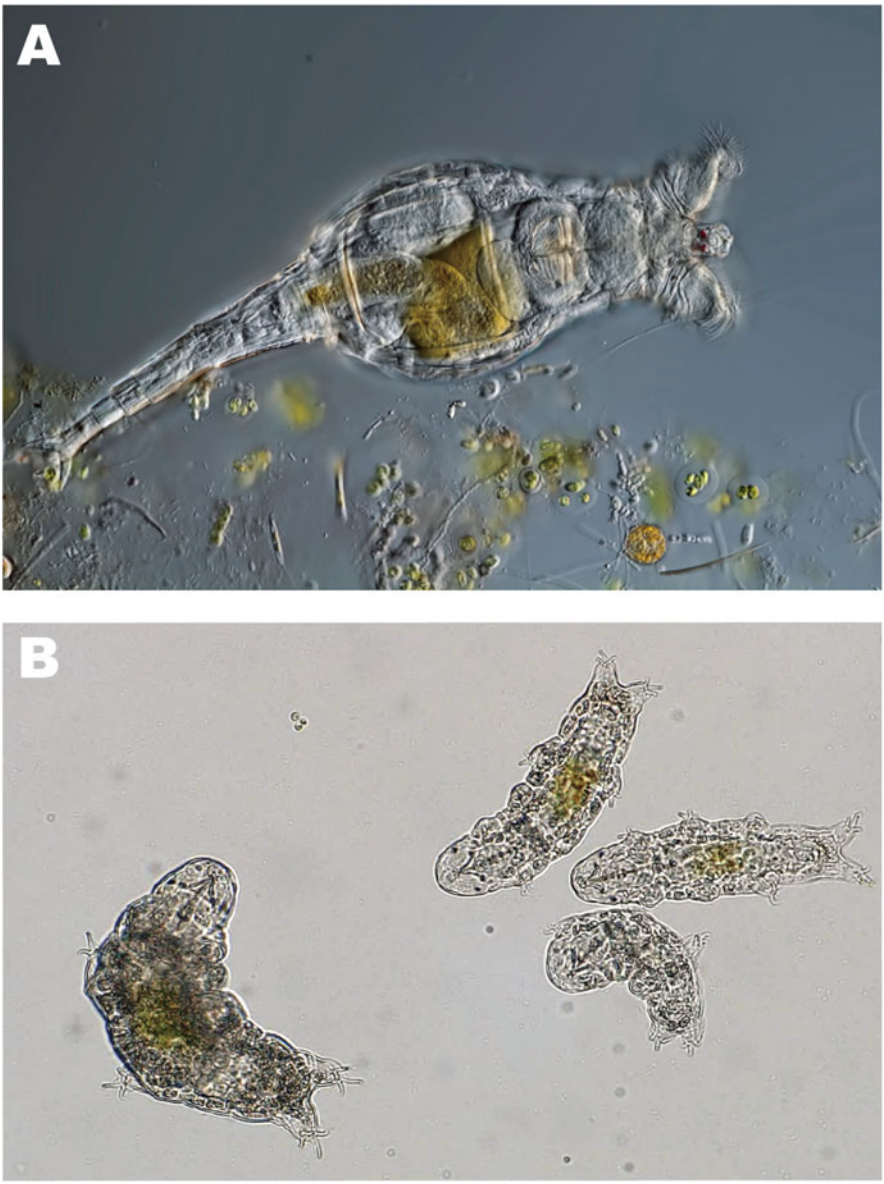

Fig. 1 Meiofaunal animals analysed for HGT. (**a**) The rotifer *Rotaria macrura* (light micrograph; body length of specimen 1 mm). (**b**) The tardigrade *Hypsibius exemplaris* (light micrograph, body length of specimen 600 nm)

frequency of HGT, which in turn allowed the animal to acquire additional loci that played adaptive roles in cryptobiosis, a virtuous circle.

However, this claim was almost immediately refuted by multiple independent analyses that found a high level of bacterial contamination in the assembly and raw data. The genome assembly reported was over 200 Mb, while previous independent estimates of genome size using Feulgen densitometry and flow cytometry had suggested the genome was 70–100 Mb (Gabriel et al. 2007; Koutsovoulos et al. 2016). It was possible to identify near-complete bacterial genomes (from *Chitinophaga* species, likely commensals living in or on the tardigrade cuticle, and several others) in the assembly and to exclude other assembled contigs because of biologically unlikely coverage and sequence similarity (Delmont and Eren 2016; Koutsovoulos et al. 2016; Bemm et al. 2016). After applying rigorous filtering of contaminants from the published draft, many possible HGT candidates remained. Koutsovoulos et al. (2016) performed phylogenetic analysis of each HGT candidate, excluding any where the initial assessment based on sequence similarity alone was countered by phylogenetic signal of animal origin, or of low confidence in any placement. They identified only 0.2% of the genes as strong HGT candidates and an additional ~1.5% as possible candidates. Two groups independently sequenced *H. exemplaris*, from the same source population (Koutsovoulos et al. 2016; Arakawa 2016; Yoshida et al. 2017), and generated more contiguous drafts with much smaller genome spans. Arakawa developed a novel ultralow input sequencing protocol to sequence the genome of a cleaned single individual of *H. exemplaris*, thoroughly inspected to be free of bacterial contamination, and obtained sequence data nearly free of any contamination (Arakawa 2016; Arakawa et al. 2016). By mapping these data to the published draft, nearly one third (31.7%) of the draft contigs, including the longest 11 contigs, were identified as contaminant. The majority of HGT candidates were predicted from these contaminants. In response to the above series of rebuttals, the lead authors argued that the 2.5–4.6% of putative HGT candidates remaining after exclusion of bacterial genomes were still high in comparison to other metazoan genomes and that their assertion that HGT and cryptobiosis were linked in the *H. dujardini* genome could stand (Boothby and Goldstein 2016).

We therefore re-sequenced the genome of *H. exemplaris* with single molecule real- time sequencing long reads and filtering short and long reads with the single specimen ultralow input sequencing approach (Yoshida et al. 2018) and assembled these data using heterozygosity-aware protocols into a highly contiguous genome having just the expected size (104 Mb) (Yoshida et al. 2017). Hashimoto et al. reported the high-quality genome sequence of another tardigrade in the superfamily Hypsibioidea, *Ramazzottius varieornatus*, with a span of 54 Mb and scaffold N50 length of 4.74 Mb. The genomes of *H. exemplaris* and *R. varieornatus* were annotated using transcriptomic data from developmental stages and multiple conditions including anhydrobiosis in order to have comprehensive coverage of expressed loci. Putative HGT candidates were identified from these gene sets and further validated through phylogenetic analysis. These analyses were consistent with the reinvestigation of the original draft genome data, with only 0.7% of all protein

coding genes being credible candidates. The upper bound, less credible set of possible candidates in *H. dujardini* included only 1.8%. In *R. varieornatus*, the less credible set amounted to only 1.2%, despite this species being much more desiccation tolerant.

The hypothesis that tardigrades have an extraordinary level of acquisition of functional genes through HGT and that this acquisition is related to DNA damage repair induced by anhydrobiosis thus does not hold, but is there no relationship between anhydrobiosis and HGT? Within our credible HGT candidate set, we have identified at least two instances where horizontally acquired genes are likely to contribute to the mechanisms of anhydrobiosis.

In *R. varieornatus*, Hashimoto and colleagues first reported that all four catalase genes are likely to derive from HGT (Hashimoto et al. 2016). Catalases decompose hydrogen peroxide and are important in cellular defence from oxidative damage. Metazoan catalases (called clade III catalases) are typically 500 amino acids in length and have two domains: a catalase domain and a catalase-related domain. All four catalases in *R. varieornatus* contained an additional glutamine amidotransferase-like domain, making them more similar to bacterial clade II catalases. No typical clade III metazoan catalases were identified. Clade II catalases are known to tolerate higher temperatures and be more resistant to protein denaturants than metazoan clade III catalases (Switala et al. 1999). These catalases of *H. exemplaris* were also clade II (Yoshida et al. 2017), and clade II catalases were found in the draft genome of *M. tardigradum* (Bemm et al. 2017). These tardigrade clade II catalases may thus contribute to resistance during anhydrobiosis. *M. tardigradum* belongs to the order Apochela, while *R. varieornatus* and *H. exemplaris* are in Parachela (within the class Eutardigrada). This suggests that an original horizontal transfer event may have occurred within or before the common ancestor of Eutardigrada. Molecular dating estimated the separation of these two orders to be between 433 and 474 My, suggesting a very ancient origin of these genes (Regier et al. 2005).

Another HGT candidate with functions related to anhydrobiosis are tardigrade trehalose-6-phosphate synthase (TPS) genes. The non-reducing sugar trehalose is accumulated in extremely high concentrations in a large number of anhydrobiotes, and it has been shown to be absolutely required for survival during anhydrobiosis in *Caenorhabditis elegans* (Erkut et al. 2011; Crowe 2014). However, trehalose has been found to be absent or only detected at very low levels (less than 3% of dry weight) in tardigrades (Hengherr et al. 2008). In line with these findings, the genomes of *H. exemplaris* and *M. tardigradum* lacked TPS. However, a locus encoding a protein with linked TPS and trehalose-6-phosphate phosphatase (TPP) domains was present in *R. varieornatus*. This tardigrade gene clustered within a clade of bacterial TPS loci in phylogenetic analyses. The genes in this bacterial clade predominantly derived from species in Chitinophagaceae (phylum Bacteroidetes). We note that precisely this group, the Chitinophagaceae, were major contaminants of the Boothby et al. draft *H. exemplaris* assembly, but this locus passes stringent tests for true HGT: it has spliceosomal introns and expression in poly(A) RNA. Many species belonging to the family Macrobiotidae display elevated amounts of trehalose

during anhydrobiosis, implying that these disaccharides must be synthesised by these tardigrades (Jönsson and Persson 2010). Further analysis covering a wider diversity of species in phylum Tardigrada will be necessary to elucidate whether such patchy conservation of TPS is the result of multiple gene loss events (i.e. HGT occurred only once, more than 400 Ma) or whether multiple HGT events occurred after the separation of families within Eutardigrada (i.e. ca. 100 Ma).

3 Lessons from the Tardigrades

The unravelling of the claim of extensive HGT in tardigrades, and its association with cryptobiosis, highlighted the importance of very careful assessment of claims of HGT. HGT is a rare event, and, as with all science, extraordinary claims require extraordinary evidence. Methods for detection of putative HGTs from a genome are inherently predictive, and predictive methods should always be assessed in terms of the possibilities of false positives. This is particularly the case with genomes newly assembled from previously unexplored regions of the tree of life, and for species where functional genomic data such as transcriptomic datasets, are sparse.

We explored the contribution of the comprehensiveness and maturity of annotation to the level of likely false positives in putative HGT detection by comparing predicted HGT proportions between well-annotated, high-quality gene sets and ab initio annotations (using the 'industry standard' Augustus pipeline) of a set of non-vertebrate metazoan genomes obtained from Ensembl (Fig. 2). Uncurated ab initio gene predictions resulted in extremely high predicted HGT proportions. For example, the well-studied genome of *Drosophila melanogaster* was predicted to contain ~4% putative HGTs. In every case the mature gene predictions contained many fewer potential HGT candidates. Therefore, the reality of HGT predictions must be conditioned by the understanding that such predictions contain false positives, especially when the assembly and annotations are in the early stages of curation. After thorough validation, the level of HGT was very limited in tardigrades, in keeping with proportions found in other metazoans (Fig. 2). The overprediction of HGTs in all uncurated ab initio gene sets suggests that these artefacts are produced because the gene finding systems are undertrained. Sadly, many erroneous predictions gather support from sequences already present in the public databases which are themselves the products of undertrained gene finding. This snowball of annotation error is currently unavoidable when using the bulk databases provided by the International Nucleotide Sequence Database Consortium nodes (GenBank, ENA, DDBJ) but can be minimised by only using well-validated data as evidence in gene finding (e.g. the UniProt protein database or the set of reference genomes curated by Ensembl or NCBI). These findings emphasise the need for validation of HGT candidates using multiple criteria.

In particular we note that precise application of a series of technical and informatic procedures is critical to ensuring that erroneous claims of HGT do not arise and that claims must be strongly evidenced before being accepted as valid. At the

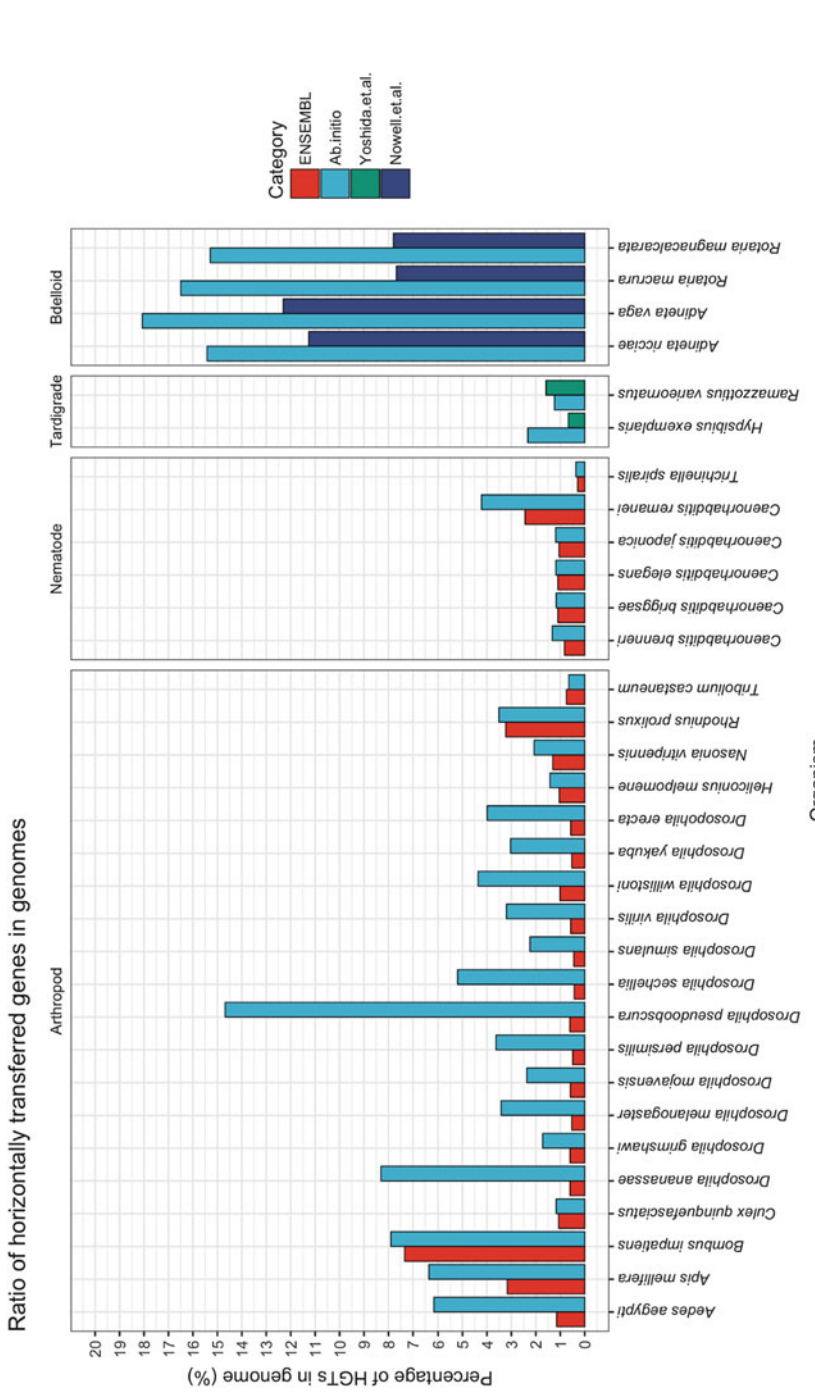

Fig. 2 HGT proportions in metazoan genomes. The proportions of genes identified as being likely HGT candidates were calculated using data taken from Ensembl Genomes for a range of non-vertebrate species, along with de novo assemblies of tardigrades and bdelloid rotifers. Additionally, ab initio gene predictions for the same Ensembl genomes were conducted using the Augustus toolkit, and HGT proportions were calculated. The validated HGT proportions for tardigrades and bdelloids were taken from Yoshida et al. (2017) and Nowell et al. (2018)

outset of a genomics program, it is essential that samples of the target organism are cleaned as far as is possible of contaminants, to minimise contribution to the initial raw data from gut or endophytic microbiomes, adherent organisms, host- or food-derived material and DNA from contamination during processing. While the 'holobiome' of a target species is of interest (i.e. it could be interesting to know the composition and likely physiology of its microbiome), a primary genome sequencing project needs to focus on specific target organism(s). The requirement for technical cleaning of samples before DNA extraction is most important, and most difficult, for specimens sourced from the wild.

Secondly, it is important to pay close attention during the assembly process to the signatures associated with contamination. Most contaminants can be robustly and rationally filtered at this stage because of their different genomic molarity compared to the target species, their unique sequence properties (bulk GC content or pattern of k-mer content) and their differential presence in independent samples.

Lastly, it is important to include several tools and approaches to detect and verify putative HGT events. These approaches should include sequence similarity and phylogenetics assessments of the putative HGT locus, accepting strong evidence only when the analyses are unequivocally in support. In addition, functional HGT should be supported by evidence of integration into the host genome, acquisition of a gene structure compatible with expression in the new location and the presence of polyadenylated transcripts derived from the HGT locus. The presence of DNA with phylogenetic evidence of origin through horizontal transfer but lacking any supporting functional evidence would suggest that the fragment is a non-functional insertion. It is important to note that the presence of a non-functional insertion cannot be argued to be a gene waiting to evolve a function or retained by the host for some future purpose. Evolution does not look forward, and a non-functional piece of DNA is not taken up and stored in the hope of future usefulness. Acquisition is random, not directed, and insertions are only selected once useful.

4 High Levels of HGT in Bdelloid Rotifers

While the precise levels and evolutionary impacts of HGT in eukaryotic evolution continue to be debated, it is already clear that the proportion of genes with signatures of HGT is usually low. Most maturely annotated metazoan genomes examined thus far have 1% or less of protein coding genes with likely origin through HGT. An exception to this finding are the bdelloid rotifers (Fig. 1).

These tiny lophotrochozoans are abundant in a variety of limno-terrestrial and freshwater habitats, including permanent and temporary ponds, leaf litter and moss. In addition to analyses of their roles in ecosystem function, bdelloids are useful models for the study of two fundamental biological processes. Firstly, they have gained fame as ancient asexuals, as neither males nor meiosis have been observed in over 500 described species, despite centuries of observation, while molecular and fossil evidence suggest that bdelloids have been evolving without sex for tens of millions

of years (Welch et al. 2009). The persistence and diversification of the asexual bdelloids are at odds with theory that suggests that asexual lineages should be short lived. Secondly, many bdelloid species are remarkably tolerant of desiccation and can survive extended periods of extreme water loss via anhydrobiosis (Ricci 1998; Ricci and Covino 2005). In this they resemble the tardigrades, but the two groups are likely to have evolved their cryptobiotic abilities independently, perhaps in association to miniaturisation.

Early explorations of bdelloid sequence data found that their genomes carried a remarkably high proportion of genes with signatures of HGT from non-metazoan origins (Gladyshev et al. 2008). These putative HGT loci showed sequence similarity to a variety of bacteria, fungi and plants and were concentrated in sub-telomeric regions of the bdelloid genome. Subsequent work by Boschetti and colleagues using first expressed sequence tags (ESTs) (Boschetti et al. 2011) and then transcriptome data (Boschetti et al. 2012) from the bdelloid *Adineta ricciae* estimated that 8–9% of protein-coding genes were of foreign (non-metazoan) origin. These genes participated in a range of biochemical and metabolic functions. A similar proportion of putative HGT loci was reported for the first whole genome sequence for a bdelloid, from the closely related species *Adineta vaga* (Flot et al. 2013). Subsequent comparative analyses of additional transcriptomes (Eyres et al. 2015) and whole genomes (Nowell et al. 2018) from multiple divergent bdelloid species with different ecological characteristics have also found similar levels of putative HGT into the expressed genome.

The finding that a substantial proportion of bdelloid genes are of foreign origin, and have presumably arrived in the bdelloid genome via HGT, is thus robust. The proportion of HGT identified is consistent across numerous studies from independent research groups, using different data types and multiple species, and have been estimated using methods that are fully cognizant of the potential pitfalls in HGT prediction discussed above. This remarkable pattern appears to be unique to bdelloids, as no other metazoan investigated with rigour has levels of HGT approaching the ~10% consistently reported for rotifers. Why are bdelloid rotifers so different from other Metazoa in their levels of HGT? Several hypotheses have been proposed, most of which adduce their ancient asexuality, lack of typical meiosis and cryptobiotic abilities to suggest either some adaptive role for the ability to take up and utilise foreign DNA in the absence of normal sexual genetic exchange or HGT as an inevitable by-product of processes that have evolved to maintain the integrity of the nuclear genome under pressure of damage induced by cryptobiotic stressors. The field is now exploring a suite of questions to test these hypotheses. Do bdelloids take up foreign DNA more readily than other taxa, and do these HGTs get functionalised more rapidly? Are there components of the mechanisms that bdelloids use to maintain genome integrity, such as double-strand break (DSB) repair or gene conversion, that predispose them to HGT? The elucidation of these problems is important not only for understanding the bdelloids, which are fascinating in themselves, but also for defining the evolutionary forces that shape the genomes of all animals.

One way to test these hypotheses is to ask how particular characteristics of bdelloid ecology or evolution may have resulted in higher levels of foreign genes, in particular the long-term lack of sex and recombination and anhydrobiosis. Previous work has placed emphasis firmly on anhydrobiosis (Gladyshev et al. 2008; Boschetti et al. 2012; Flot et al. 2013), proposing a link between HGT and the repair of DSB damage in DNA induced by UV radiation while animals are desiccated (Gladyshev et al. 2008; Hespeels et al. 2014). Dehydration does cause DSB damage, and this must be repaired on rehydration for the animals to survive. The model suggests that fragments of exogenous DNA from bacteria, fungi and other organisms present in the environment or consumed as food particles might be occasionally incorporated into the host genome during the DSB repair. This model predicts a positive correlation between the frequency of desiccation and the number of foreign genes present in the genome and also an elevated level of HGT in other anhydrobiotic taxa. Some of the discussion of the extraordinary levels of apparent HGT in the initial draft genome of the tardigrade *H. exemplaris* invoked just this putative mechanism.

However, comparative analyses of desiccating and non-desiccating species of bdelloid, and other anhydrobiotic taxa, cast doubt on the role of desiccation in HGT. For example, some bdelloid species live in permanent aquatic environments and have lost the ability to survive desiccation. These taxa show levels of HGT that are comparable to anhydrobiotic species (Eyres et al. 2015; Nowell et al. 2018). An analysis of HGT using transcriptome data in both desiccating and non-desiccating species from the genus *Rotaria* did suggest a small increase in the cryptobiotic species (13% vs. 10%) but also found several HGT candidates that were unique to the non-cryptobiotic species (Eyres et al. 2015). These loci suggest that acquisition of loci by HGT does not require cryptobiotic stress or cryptobiotic DSB repair mechanisms. Evidence from other anhydrobiotic taxa is also mixed. Few foreign genes have been detected in the tardigrades *R. varieornatus* and *H. exemplaris* (Yoshida et al. 2017) or the chironomid midge, *P. vanderplanki* (Gusev et al. 2014). Tardigrade DNA may be protected by tardigrade-specific nuclear proteins during anhydrobiosis and does not experience high levels of DSBs (Hashimoto et al. 2016). In *P. vanderplanki*, however, there is evidence of extensive DNA damage (Gusev et al. 2010) similar to that found in *A. vaga* (Hespeels et al. 2014). Overall, the lack of extensive HGT in both systems does not support a hypothesised link between HGT and anhydrobiosis.

Could long-term asexuality provide a better explanation for the high levels of HGT in bdelloids? There has been speculation that horizontal transfers between rotifers and other organisms might be a side-effect of a mechanism of maintenance of genomic integrity under asexuality. Thus rotifer-to-rotifer "horizontal transfer" might operate to recombine alleles among individuals, in lieu of more conventional forms of sex. However, a study that claimed to find evidence of between-rotifer transfers (Debortoli et al. 2016) was subsequently shown to be the result of accidental cross-contamination between samples (Wilson et al. 2018). There is thus no compelling evidence for frequent HGT to be a substitute for sex in bdelloids.

It is important to note that the overall rate of HGT in bdelloids is low in absolute terms, on the order of about 10 gains per million years (Eyres et al. 2015). Perhaps it is not the rate of foreign import that is unusually elevated in bdelloids but the rate at which such genes are subsequently retained (Nowell et al. 2018). Bdelloids might experience an equivalent level of primary transfers as other meiofauna living in similar ecological and environmental conditions, but these foreign DNA fragments are rapidly eliminated from non-bdelloid genomes because of the selective pressures imposed by both sex (meiosis) itself and by recombination more generally. In this model, HGT events are exceedingly rare occurrences in all taxa but have accumulated to relatively high levels in bdelloids over the evolutionary timescales during which recombination has been absent because of the inefficiency of elimination in the absence of sex.

Long-term asexuality could increase the rate of retention of newly inserted DNA in two ways. First, the distribution of fitness effects for HGT events in asexuals may be shifted such that foreign genes inserted into an asexual genome are on average less deleterious relative to the same events in a sexual species. This might arise because asexual transfers do not affect cellular processes and/or genomic structures that are crucial to normal meiosis, which would otherwise be highly deleterious or lethal. We note that HGT candidates are not randomly distributed in bdelloid genomes but are concentrated in sub-telomeric regions (Gladyshev et al. 2008). This distribution of HGT loci resembles the partitioning of novel (largely non-HGT) genes and the majority of non-genic repeats to chromosomal arms in *Caenorhabditis* nematodes and may reflect the roles of these regions as testbeds for novel function. This insertion pattern of HGT loci resembles the patterns of insertion of mobile genetic elements (transposons, retrotransposons and related elements) in many species. Telomeres themselves play a crucial role in meiosis, particularly in the pairing of homologous chromosomes during meiotic prophase (Siderakis and Tarsounas 2007; Lee et al. 2012). It is possible that insertions into bdelloid telomeres are tolerated because purifying selection acting on telomere function in meiosis is either weakened or removed entirely. Insertions into gene-rich, non-telomeric regions are more likely to disrupt cellular functions and be deleterious regardless of the mode of reproduction.

Second, the lack of recombination in asexuals reduces the power of selection to remove transfers, even if they are initially mildly deleterious (i.e. Hill–Robertson interference). The reduced effectiveness of selection in asexuals has already been shown for point mutations in a number of sexual–asexual comparisons, including in bdelloids (Barraclough et al. 2007) and *Timema* stick insects (Bast et al. 2018). Similarly, newly inserted foreign DNA might be able to persist longer in asexuals than in sexuals, providing sufficient time for amelioration. Even if asexuals are not subject to higher rates of initial incorporation of foreign DNA, they may realise higher rates of standing functional HGT simply because of suppressed recombination and reduced effectiveness of selection. Countering this is the fact that most asexual lineages are short lived in evolutionary terms, and so accumulation of HGTs will not in practice be realised. That such a high proportion of genes encoded in

bdelloids are of non-metazoan origin is perhaps the most convincing evidence for ancient asexuality.

It is still tempting to seek an association between cryptobiosis, ancient asexuality and HGT in the bdelloids. Could they be linked, with HGT promoted by DNA damage during cryptobiotic episodes bringing in genetic novelty otherwise unavailable to bdelloids? The non-directed nature of uptake and the long time required for amelioration of each gene argue against this hope. It may be that other mechanisms that permit longevity of the bdelloid asexual lineage have as a by-product affected rates of functional integration of foreign DNA. One candidate for such mechanisms might be high rates of gene conversion between alleles and homologues that serves to repair mutations and also results in amelioration of HGT fragments. These ideas require careful scrutiny and extensive testing and are the subject of ongoing research into bdelloids.

In prokaryotes the contributions of HGT to evolution, adaptation and differentiation in prokaryotes are richly documented and uncontroversial (Polz et al. 2013; Vos et al. 2015). However, in eukaryotes, and Metazoa in particular, functional HGT is much rarer, and the realised rate of HGT is orders of magnitude lower than in bacteria [reviewed in Husnik and McCutcheon (2018)]. Functional HGT is an exceedingly rare event and for it to impact adaptation appears to be the exception rather than the rule. In this regard, the bdelloids may provide the single exception. Much remains to be elucidated regarding the evolutionary causes and consequences of HGT in bdelloid rotifers. Future work must consider all forms of (potential) recombination within a population genetics framework, measuring and modelling rates of DNA uptake, patterns of amelioration and, especially, the differences within and between individual lineages. It may be that bdelloids represent an edge case among metazoans and that the high level of HGT found here is a product of their unusual life history and is therefore unrepresentative of eukaryotes more generally. Thus, understanding what drives HGT in bdelloids will improve our understanding of the overall contribution of ongoing HGT to eukaryotic evolution.

5 Bioinformatic Approaches to the Prediction of HGT

Cataloguing the specific loci involved in functional HGT in Metazoa, and thus measuring rates and patterns of HGT, depends sensitively on the methods used to identify HGT candidates in genomes and transcriptomes. These methods have developed rapidly in the last decade as more and more complete metazoan and eukaryote genomes have been sequenced, providing essential background in which to embed both analytic strategies and improve the credibility of predictions.

In bacteria, recently transferred genomic fragments can be distinguished from their new host genome by a range of metrics, including GC content and additional k-mer spectra, amongst which tetranucleotide content appears most informative, CG skew in relation to the surrounding genome, and codon usage pattern. In addition, sequence similarity and phylogenetic analyses comparing the candidate HGT locus

to the genomes of other, potential donor species can be used to show phylogenetic incongruence compared to the bulk genome. Less emphasis is place in bacterial HGT analysis on more subtle but essential components of amelioration, such as acquisition of promoter elements optimally functional in the new host cell. Bacteria have very large effective population sizes, and thus amelioration at the level of GC content, k-mer spectra, CG skew and codon usage can be relatively rapid. The enduring signal of (relatively) ancient HGT is phylogenetic evidence of incongruence. An array of methods have been developed for HGT detection in bacteria (Zaneveld et al. 2008).

Tools developed for bacterial metagenomics and detection of HGT in bacteria applications have been very effectively used in contamination detection in eukaryotic genome assemblies (including the draft genome of the tardigrade *H. exemplaris*) and can aid HGT detection (Eren et al. 2015; Clasen et al. 2018). The EuGI toolkit, developed from a bacterial HGT detection program, uses tetranucleotide spectral composition to identify extended regions that are likely to derive from HGT events (Clasen et al. 2018). By identifying long (~100 kb) segments of assembled genomes that have tetranucleotide spectra that differ from that found in the local genomic region, and from the genome as a whole, EuGI was able to identify recent HGT events in protist, fungal and non-vertebrate genomes. Tetranucleotide frequency spectra are also used by the Anvi'o toolkit (Eren et al. 2015) to build complex discriminators to separate segments that derive from distinct genomic sources and can identify recent HGTs. We note that tetranucleotide spectra perform poorly on short sequences and are also likely to fail to identify older, ameliorated HGTs.

In eukaryotes, functional integration of an HGT from a bacterial source is more difficult than simple acquisition of foreign DNA, as it requires acquisition of (in most cases) spliceosomal introns, mRNA initiation and polyadenylation signals and integration into the more complex transcriptional landscape of its host. Horizontal acquisition from other eukaryotes requires less amelioration. It is important to note that, for example, different eukaryotic groups can have very different spliceosomal intron systems and thus that donor introns may not be recognised by the recipient machinery. The lower effective population sizes of most eukaryotes mean the amelioration processes must be slower than is observed in bacteria. A phylogenetic incongruity signal will remain, and this is the primary source of evidence for HGT in eukaryotes. Evidence of amelioration—features of the putative HGT that closely resemble those of the host—are instead largely used to exclude contamination and to support functional integration into host systems. The process of sexual reproduction and the permeability of species barriers also mean that hybridisation between species, and thus introgression of genes from a donor to a recipient, can occur. These processes will result in sets of genes that have phylogenetic histories that differ from the species' history and could be classified as HGTs. As noted above, we reserve the term HGT for a locus that has been acquired through asexual mechanisms, but the distinction between HGTs and introgressed genes is necessarily fuzzy: how deeply separated do two hybridising taxa have to be to generate observed loci with phylogenies indistinguishable from true HGTs? Can we tell whether a gene was acquired through ancient sexual reproduction rather than non-sexual uptake?

Table 1 Methods for detection of HGT

Sequence similarity based				
Method	Reference	Input	Similarity search tool, database and match threshold	Algorithm and threshold
Alienness (Alien Index)	Rancurel et al. (2017)	Predicted proteins	BLASTp (blastcl3); nr GenBank; 1.0E-03, no filtering for low complexity regions	AI = ln(best Metazoa E-value + 1e−200) − ln (best non-Metazoa E-value + 1e−200) AI > 14 ‖ 26
HGT Index	Boschetti et al. (2012)	CDS (transcript assembly)	BLASTx; UniProt KB KW-0181 (complete proteomes); 1.00E-05	$hU = h_{nonmet} - h_{met}$ $hU \geq 30$
Class C	Crisp et al. (2015)	CDS (transcript assembly)	BLASTx; UniProt KB KW-0181 (complete proteomes); 1.00E-05	$hU = h_{nonmet} - h_{met}$ $hU \geq 30$ && non-metazoan bitscore > 100
Integrated pipeline[a]	Koutsovoulos et al. (2016); Nowell et al. (2018)	(Either of above)	(Either of the above)	Sum of bitscores wins; putative HGT must have support for 'nonmet' designation from a given proportion of the rest of the hits (default ≥ 90%)

Sequence composition based			
Method	Reference	Input	Algorithms and threshold
EuGI	Clasen et al. (2018)	Genome and database of 'genomic islands'	1: OUP (oligonucleotide usage pattern of segment; tetranucleotide spectrum [$k = 4$]) 2: D (rank order distance between two OUP spectra) 3: PS (pattern skew; D calculated for forward and reverse strands of the same segment) 4: RV (relative variance of segment normalised by GC content of segment) 5: GRV (relative variance of segment normalised by GC content of whole genome) A genome segment is classified as a HGT island when D > 1.5, PS < 55 and RV/GRV > 1.5

[a]See https://github.com/reubwn/hgt

The identification of HGTs in bdelloid rotifers and tardigrades described above (Yoshida et al. 2017, 2018; Nowell et al. 2018) was based on initial candidate determination by sequence similarity followed by validation by phylogenetics, gene structure analysis and expression. The initial candidate lists were built by sequence similarity searches against taxonomically annotated sequence databases, identifying sequences that were much more similar to bacterial (or other potential donor) proteins than to any eukaryote or metazoan sequence. This HGT Index or Alien Index approach is the most commonly deployed tool for HGT identification (Table 1).

The Alien Index was introduced for the identification of HGTs in bdelloid rotifers (Gladyshev et al. 2008) and uses the Expect (E) values for matches from database similarity searches as its core metric. E-values are the expectation that a match of the given quality (length, match score) would be found in a database of a particular size and base or amino acid composition. In this method, translated coding sequences (CDS) from the target genome are used as queries in a BLAST search (protein–protein, i.e. BLASTp) against the NCBI GenBank non-redundant protein (nr) database. High-scoring matches with an E-value cut off of less than 1e-3 are retained and filtered to remove matches to proteins from the target species' taxonomic group (e.g. Rotifera or the bdelloid searches), proteins with incomplete taxonomy and very short overlaps (less than 100 amino acids in length). For each target sequence, up to 1000 hits are grouped based on their taxonomic origin (Bacteria, Fungi, Plantae, Metazoa and other Eukaryotes including protists) and the lowest E-value recorded. If no hits are found for the particular taxonomic group, the E-value is set to 1. The lowest E-value from each taxonomic group is used to calculate the Alien Index (AI):

$$\begin{aligned} \mathrm{AI}_{\mathrm{Metazoa:NonMetazoa}} = & \log(\text{E-value}_{\mathrm{Metazoa}} + \text{1e-200}) \\ & - \log(\text{E-value}_{\mathrm{NonMetazoa}} + \text{1e-200}) \end{aligned}$$

The $\mathrm{AI}_{\mathrm{Metazoa:NonMetazoa}}$ is positive if the target sequence has greater similarity to non-metazoan sequences. Rather obviously, E-values are contingent on the search strategy (e.g. initial k-mer or word length match value, whether simple sequence is masked and the scoring matrix used in evaluation) and on the size and composition of the database searched. The approach usually employs default program values (e.g. for BLASTp a word size of 6, masking of simple sequence off, BLOSUM62 matrix for scoring). For the rotifer data (Gladyshev et al. 2008), the AI varied between +460 and −460. Gladyshev and colleagues suggested that an $\mathrm{AI} \geq 45$ was a good indicator of foreign origin, while targets with $0 < \mathrm{AI} < 45$ were indeterminate (Gladyshev et al. 2008). The Alien Index has been deployed widely, including in Alienness, a taxonomy aware web application for rapid detection of HGT candidates (Rancurel et al. 2017). In Alienness, Rancurel and colleagues recommended an $\mathrm{AI} > 14$ threshold for minimizing false positives and $\mathrm{AI} > 26$ for predicting candidates that are more likely to produce phylogenies with higher support for HGT.

The HGT Index approach was first implemented for analysis of the transcriptome assembly of the bdelloid rotifer *Adineta ricciae* (Boschetti et al. 2012). A gene set (in the case of *A. ricciae* a transcriptome assembly) is used as a query in a translated sequence similarity search (i.e. BLASTx) against custom, taxon-specific subsets of the UniProtKB database of genic sequences (e.g. Metazoa, Plantae, Fungi, Eubacteria, Archaea and other Eukaryotes). Sequences from organisms belonging to the same phylum as the target organism are excluded from these taxon-specific databases. To simplify the search, only UniProtKB sequences from complete

proteomes were used. High-scoring matches with an E-value cut off of less than 1e-5 are retained. The HGT Index (hU) is calculated from the following formula:

$$hU_{\text{Metazoa:Non-metazoa}} = H_{\text{Non-metazoa}} - H_{\text{Metazoa}}$$

where $H_{\text{Non-metazoa}}$ and H_{Metazoa} are the bitscores of the best hit against the non-metazoan and metazoan databases. While the hU approach thus avoids some of the contingent issues that arise from the simple use of E-values, it is still dependent on the specific search strategy used. Again, program defaults are standardly deployed. While target sequences that have $hU > 0$ are more likely to have a non-metazoan origin, Boschetti and colleagues suggest using $hU \geq 30$ as a filter. Using this HGT Index approach, 9.7% of the *A. riccae* transcriptome was identified to be of foreign origin (Boschetti et al. 2012). Among the 2792 candidates, 1884 had no significant metazoan match. Phylogenetic assessment of the remaining 908 candidates confirmed 887 as strong candidates for HGT into the *A. ricciae* genome. Using the same approach, strong HGT candidates were also identified in the rotifer *Brachionus plicatilis*, the nematode *Caenorhabditis elegans* and the fly *Drosophila melanogaster* (Boschetti et al. 2012).

The HGT Index approach was refined by Crisp et al. (2015). They proposed qualification of HGT candidates into Class C (those with $hU \geq 30$ and best non-metazoan bitscore $\geq$100), Class B (class C loci that are members of orthologue groups obtained using Markov linkage clustering where the metazoan proteins in the cluster have an average $hU \geq 30$) and Class A (Class B proteins where their best metazoan BLASTp bitscore is $<$100 and their ortholog group contains no genes with a best metazoan bitscore $\geq$100). Class A HGTs represent a minimum estimate of strongly supported HGTs for a target species. The application of these thresholds identified an average of 68 Class A genes in *Caenorhabditis* species' genomes, 4 in *Drosophila* species' genomes and an average of 32 genes in several primate species' genomes. However, as with the initial hypothesis of over 200 HGTs in the human genome (Lander et al. 2001; Salzberg et al. 2001), these assessments have been challenged (Salzberg 2017) (see below).

The draft genome from the tardigrade *H. dujardini* was submitted to public databases, and the proteins predicted from the contaminating *Chitinophaga* proteins thus are present in public databases annotated as metazoan in origin. Submission of data with erroneous taxonomic attribution is not confined to this assembly project. This contamination of the public databases has the dual negative effect of obscuring contamination (future projects discovering *Chitinophaga* sequences may be misled into believing them reliably metazoan) and confounding efforts to identify HGT. We have proposed an additional filter, which we here call Consensus Hit Support (CHS), for the AI and hU methods to improve confidence (Koutsovoulos et al. 2016; Nowell et al. 2018). Both AI and hU are calculated using only the 'best' hits from Metazoa and non-Metazoa (i.e. those with the lowest E-value or highest bitscore) and can mislead if the top-ranked subject sequences are themselves taxonomically misclassified. The addition of a CHS filter mitigates this problem by requiring that any HGT candidate identified as non-metazoan based on similarity to the top-ranked

hit should be supported by a specified proportion of the rest of the hits. Essentially, CHS asks whether there is a consensus in the taxonomic annotation derived from the top-ranked hit. The CHS filter makes HGT candidate identification less prone to errors associated with contamination issues in either the subject genome or the database itself, as information from all hits is considered.

AI and hU approaches can only suggest candidate HGT loci. We have suggested that additional criteria should be assessed to support claims of HGT (Koutsovoulos et al. 2016; Nowell et al. 2018). Phylogenetic analyses are essential, as marginal differences in alignment scores in similarity searches can translate into significant AI or hU scores. Potential homologues of HGT candidates should be gathered by an iterative search process such as phi-BLAST, where the search is widened based on the first rounds of matches to discover additional sequences that are likely to be members of the same homology group, halting when searches converge on a stable set of likely homologues. Psi-BLAST searches that fail to converge can also identify initial matches that are due to chance similarities or sequence biases and thus offer poor support for HGT status. Multiple sequence alignments should be inspected to affirm credibility of the alignment, asking if all the presumed homologues overlap with each other, or are only linked by their independent overlaps with the initial query sequence. A phylogenetic analysis of the target locus alongside all its best-matching putative homologues (from both source and recipient taxa) can reveal that there is no strong phylogenetic signal in the sequence or that the index approach has elevated a marginal difference to categorical significance.

6 All that Glitters Is Not Gold: HGTs as Hypotheses

> The argument for lateral gene transfer is essentially a statistical one . . . As with all statistical arguments, great care needs to be exercised to confirm assumptions and explore alternative hypotheses. In cases where equally if not more plausible mechanisms exist, extraordinary events such as horizontal gene transfer do not provide the best explanation. (Salzberg et al. 2001)

> (T)he evolutionary relationships among proteins cannot be concluded solely from the ranking of database hits in homology searches (for example BLAST reports). This is not a new conceptual point . . . but one that seems to have been overlooked in this instance. Phylogenetic analysis must be a central component of any protein family or genome annotation effort. (Stanhope et al. 2001)

Extraordinary claims require extraordinary evidence, and HGT should only be accepted when other, more likely explanations have been exhausted. Thus, reports that the human genome had 223 HGT candidates (Lander et al. 2001), with 113 having homologues only in bacteria and vertebrates, were immediately rebutted through statistical and phylogenetic analyses (Salzberg et al. 2001). Only ~40 lower-quality candidates were left unrefuted. Detailed phylogenetic analysis of 28 of the proposed HGT candidates found that the majority were present in more anciently derived eukaryotes, rejecting HGT origin of these genes (Stanhope et al. 2001). Salzburg

(2017) similarly reanalysed and rejected 32 of the human genome Class A HGTs predicted by Crisp et al. (2015).

HGT detection through bioinformatics is predictive only, and all predictive methods result in both false negatives (missed HGTs) and false positives. The overprediction of HGT in initial ab initio gene predictions from new genome assemblies (see 'Lessons from the Tardigrades' above) is sobering. Even the current state-of-the-art gene predictors have base-level sensitivities in the range of 93–99% and specificities of 80–92% (Coghlan et al. 2008) in model organism genomes, when compared to highly validated gene sets. Errors in gene prediction are directly reflected as false positives in HGT detection (Fig. 2). While we found that curated metazoan genomes from Ensembl and our reanalysed tardigrade genomes still had around 1% HGT, this does not immediately imply that all metazoan genomes contain 1% HGT. We suggest that these numbers indicate that at the worst, our methods still contain up to 1% of false positives. These candidates must be validated.

Even where loci have a very strong AI or hU Index and phylogenetic support, we recommend using independent data for validation. Does the locus have strong evidence of incorporation into the recipient metazoan genome? Does it have spliceosomal introns? Is it physically linked in the genome assembly to other expressed loci of unproblematic metazoan origin? Is it present in all haplotypes of the recipient species or in related species? Is the locus a functional HGT or a dead-on-arrival acquisition of foreign DNA? Is the locus expressed in the recipient species at significant levels? HGT is undoubtedly real and has been an important process in organismal evolution. However, uncritical reporting of what are, in the end, low-confidence HGT candidates obscures the true rate and significance of horizontal transfer.

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Horizontal Gene Transfer Between *Wolbachia* and Animals

Trinidad de Miguel, Oude Zhu, and Tomás G. Villa

Abstract The present chapter is focused on the occurrence of horizontal gene transfer events from the endocellular bacteria *Wolbachia* to invertebrates and the implications of this phenomenon in the hosts' reproductive behaviour, speciation, and acquisition of new abilities.

Keywords *Wolbachia* · Horizontal gene transfer · HGT

1 Introduction

The exchange of genetic material is a well-recognized mechanism for phenotypic innovation and niche adaptation, which allows bacteria to acquire novel features such as pathogenicity, antibiotic resistance, and different metabolic properties (Bushman 2002). While horizontal gene transfer (HGT) among prokaryote has been extensively studied and appears to be a major event, even among distantly phylogenetic-related species, the integration of prokaryotic DNA fragments into eukaryotic organisms has been regarded as a rare event with unclear functional and evolutionary consequences (Sieber et al. 2017), with the only exception of chloroplasts and mitochondria DNA transfer to the nuclear eukaryotic genome of the host cell (Boucher et al. 2003).

During the last decades, some studies have demonstrated the occurrence of HGT events from the endocellular bacteria *Wolbachia* to its invertebrate hosts, and the consequences of this genetic exchange are being studied.

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2 The Genus *Wolbachia*

Members of the genus *Wolbachia* are gram-negative bacteria from the order Rickettsiales, which infect many species of arthropods and also some nematodes. Estimations suggest that up to 52% of insect species are infected with strains of *Wolbachia* (Weinert et al. 2015). These heritable bacteria establish complex interactions with their hosts, being in some cases parasitic and in some others mutualistic (Siozios et al. 2018).

One of the main particularities of this genus, and the reason why it is being studied and used as a biocontrol agent, is the ability to maximize their spread by manipulating the reproductive capabilities of its hosts by one of these four mechanisms: male killing (Hurst et al. 2000), feminization (Rousset et al. 1992), parthenogenesis (Stouthamer et al. 1999), or cytoplasmic incompatibility (Yen and Barr 1971).

As an obligate intracellular bacterium colonizing the germline of its hosts, it is inherited from mother to offspring, and it has been proved not to be transmitted to humans or other vertebrates (Carvalho and Moreira 2017). Since the discovery that *Wolbachia* prevents the transmission of some arboviruses when the vector *Aedes aegypti* is infected with both the bacteria and the virus (Hedges et al. 2008), intentionally *Wolbachia*-infected mosquitoes have been released in Australia in order to try to establish stable colonies of *Wolbachia*-infected *Ae. aegypti* unable to transmit dengue. The experiment involved first the successful artificial infection with *Wolbachia* of a mosquitoes' population (McMeniman et al. 2009), since *Ae. aegypti* is not a natural host for this bacterium, and then their release to the field with no further intervention. Five years after the release nearly 100% of the mosquitoes' population in the area harbored the bacterial infection, due to *Wolbachia*'s reproductive manipulation of its host by cytoplasmic incompatibility (Hoffmann et al. 2014). And nowadays, this population continues to block dengue transmission in Australia (Carvalho and Moreira 2017). In spite of this success, a recent study reports the higher prevalence of insect-specific flaviviruses in *Wolbachia*-infected mosquitoes if compared to the *Wolbachia*-free ones (Amuzu et al. 2018). Although these insect-specific flaviviruses are not of medical importance, this fact could bring into question the safety of the method. Since the *Wolbachia*-mediated viral inhibition seems not to be universal for flaviviruses, the concern of this technique could be the emergence of a new virus serotype able to evade the protection offered by *Wolbachia*. Nonetheless, following the excellent results in Australia, this method is also being used in Brazil, Vietnam, Indonesia, and Colombia against dengue, zika, and chikungunya viruses (Carvalho and Moreira 2017).

3 Mechanisms for Prokaryote-Eukaryote Gene Transfer

The evolutionary and functional significance of HGT between prokaryote and eukaryote has always been subject to controversial (Danchin 2016) and is regarded as unusual, except for the HGT from chloroplasts and mitochondria to their

eukaryotic hosts. It is assumed that these organelles of endosymbiotic origin (Margulis 1970) have experienced a drastic reduction of their genome size as a consequence of HGT to the nucleus of the host cell (Martin et al. 1998; Gray et al. 1999). And interestingly, intracellular obligate parasitic bacteria have got reduced genomes in comparison with their free-life counterparts, which may be indicative of a similar process.

But, how can a prokaryotic cell come in contact with a eukaryotic one and be able to transfer part of its genetic material to it? Unlike the processes of genetic material exchange between prokaryotes, which have been extensively studied and are well understood, we still do not have a clear answer for the mechanisms underlying prokaryotic-eukaryotic HGT. One proposed model assumes that both cells would come in intimate contact by phagocytosis. It would explain the evidence of prokaryotic genes found in phagocytic unicellular eukaryotes such as *Trichomonas* or *Entamoeba* (De Konig et al. 2000; Field et al. 2000). But, what about pluricellular organisms like metazoan species, in which the germ line cells are separated from the somatic cells? Although some authors suggest that the bacteria-animal gene transfer in somatic cells are of importance because they can induce mutations leading to cancer or autoimmune diseases (Robinson et al. 2013), in the case the HGT event would take place in a eukaryotic somatic cell, the genetic material acquired would not be transmitted to the next generation and would be lost. Hence, the integration of a gene in the germline is the key to its successful fixation at the level of population.

The genus *Wolbachia*, as explained above, is an obligate intracellular parasite of invertebrates, maternally inherited by transovarian transmission. The intimate contact of this bacterium with the germline of its hosts makes this association a perfect candidate to prokaryote-eukaryote HGT.

4 First Reports on HGT from *Wolbachia* to Invertebrates

The first report on HGT from *Wolbachia* to invertebrates was written after the studies of Kondo et al. (2002) with the adzuki bean beetle *Callosobruchus chinensis* and the unusual behavior of infection with a specific lineage of this bacterium. These authors identified three different lineages of *Wolbachia* infecting the adzuki bean beetle. Due to the inability to culture those endosymbionts outside the insect host, the identification was based on the sequence analysis of the *Wolbachia* surface proteins (wsp). Surprisingly, one of the three wsp, named wBruAus, could neither cause cytoplasmic incompatibility in the host nor be eliminated from the offspring of beetles after treatment with antibiotics. These facts suggested the possibility that wBruAus might not have a microbial entity. Besides, protein wBruAus showed an inheritance pattern linked to sex, females containing twice more amount of protein than males, this suggesting a possible linkage to chromosome X. The authors concluded wBruAus is a genome fragment of *Wolbachia*, which has been transferred to *Callosobruchus chinensis'* X chromosome, being this the first report on such a

HGT. In spite of the relatively big size of the gene fragment (>1% of the whole bacterial genome), no functional genes were found in it.

Since then, many research articles have reported cases of HGT between *Wolbachia* and invertebrates.

A study using several insect and nematode species found *Wolbachia* DNA fragments of nearly the entire bacterial genome size inserted in the host genomes (Hotopp et al. 2007). These authors defend that prokaryote to eukaryote gene transfers must be much more common and important as previously thought. And they alert about the fact that during the eukaryote sequencing projects, bacterial sequences are routinely excluded assuming they come from a contamination. This fact hinders the possibility to detect recent bacterial HGT to eukaryotes.

5 The Role of *Wolbachia* in Animal Speciation

The influence of *Wolbachia* infections in speciation of invertebrates has been described in several studies. In the spider mite *Panonychus mori*, the reproductive incompatibility between infected and uninfected individuals led to a genetic divergence among populations (Gotoh et al. 2005). And the presence of *Wolbachia* and other endosymbionts in arthropods has shaped mitochondrial DNA evolution to the extent of some authors advising against the use of mtDNA differentiation to define taxonomic units (Hurst and Jiggins 2005).

And the role of HGT from *Wolbachia* to its hosts in speciation has also been reported. A study on the *Wolbachia* of filarial nematode worms (Fenn et al. 2006) describes a fragment of bacterial DNA inserted in the genome of its nematode host *Onchocerca volvulus*, which causes disease in humans. The authors provide evidence that this event predated the divergence between two species: the human parasitic *O. volvulus* and the cattle parasitic *O. ochengi*. This report, which is the second on HGT evidence between *Wolbachia* and invertebrates, points out how important those facts can be to the history of evolution and biodiversity in animals.

6 Functional Genes Coming from HGT Between *Wolbachia* and Animals

The first reports on HGT between *Wolbachia* and invertebrates failed to find functional genes of prokaryotic origin in the host cells that could explain the evolutionary meaning of such events. Nevertheless, some years before the first reports, Keen and Roberts (1998) postulated an interesting hypothesis following the discovery of cellulases and other plant degrading enzymes in plant parasitic nematodes. Before that time, scientists assumed the absence of such enzymes in nematodes and attributed the plant colonization to mechanical disrupture of the cell

walls using stylets or spears. Those authors suggested for the first time the possible prokaryotic origin of such genes coming from a HGT between a microbe in the digestive system of a non-parasitic nematode and its predator, turning it into a parasitic one. And the evidences did not take long to show up. At the beginning, many successive discoveries succeeded to confirm this idea: the nematode cellulases discovered were significantly similar to that from bacteria. But as the sequencing techniques improved and the databases expanded the HGT hypothesis began to fall apart, since the nematode cellulases no longer constituted a monophyletic group with the bacterial cellulases (Mitreva et al. 2009). This is the reason why it was necessary to find evidence of gene transfer to prove this hypothesis. And this came in 2010 when Danchin et al. described how nematodes have acquired at least six different types of bacterial genes encoding plant wall degrading proteins followed by extensive gene duplication, this promoting plant parasitism ability. The phylogenetic analyses made by those authors can only be explained by the HGT hypothesis, the prokaryotic genes coming from the plant pathogen *Ralstonia solanacearum.* Similar findings proved the role of HGT in the evolutionary transition toward plant parasitism in fungi and arthropods (Wybouw et al. 2016). But, why did previous phylogenetic analyses of cellulases fail to prove the HGT hypothesis? As the databases expanded it was evident that many unrelated organisms, not only bacteria, harbored proteins with significant similarity to nematode cellulases. Is it then possible to be facing a case of HGT from bacteria to several unrelated taxons? Independent transmission of homologous DNA to different domains of life seems to be extremely rare and have no functional and ecological explanation. Nevertheless, it has been described that a lysozyme from bacterial origin has been transferred to plants, insects, fungi, and Archaea (Metcalf et al. 2014), probably being used by the host with antibacterial purposes. This would indicate that, although HGT events among domains have been regarded at the beginning as extremely rare and with no evolutionary significance, they must be continually happening and shaping the tree of life.

If the functionality of the genes coming from *Ralstonia* could be proved, it is likely to think it would be easier to find functional genes coming from *Wolbachia*, whose evidence of HGT to its hosts has been largely demonstrated (Wu et al. 2013). As described above, it is more likely for such a genetic transfer to be fixed in the population if we consider an endosymbiont colonizing the germline instead of a piece of food in the gut. Studies with the mosquitoes *Aedes albopictus* and *Ae aegypti* had suggested the functionality of the proteins acquired via HGT from *Wolbachia* due to the high transcription levels (Klasson et al. 2009; Hou et al. 2014) but no function could be attributed to them. The first report on functional genes from symbiotic bacteria to animals (prior to the nematodal cellulases report) involved a bacterium closely related to the extant *Wolbachia* and the pea aphid *Acyrthosiphon pisum* (Nikoh and Nakabachi 2009). Aphids and other insects possess bacteriocytes, specialized adipocytes containing obligate mutualistic bacteria such as the genus *Buchnera aphidicola*. These bacteria are necessary to their hosts to obtain essential amino acids and other chemicals. Moreover, *Buchnera* has lost many genes that are essential for bacterial life, becoming thus an obligate endosymbiont. Nikoh et al.

found evidence indicating that aphids could have acquired functional genes from bacteria related to the genus *Wolbachia* via HGT, and that those genes are being used to maintain *Buchnera* inside the bacteriocyte, where they are highly expressed.

In recent years, several examples of functional genes transferred from *Wolbachia* to animals have been described. A research with the human filarial parasite *Brugia malayi* describes for the first time the transfer of a functional gene (an essential ferrochelatase) from the *Wolbachia* endosymbiont to its nematode host (Wu et al. 2013). Members of the Nematoda lack the ability to synthesize heme, but *B. malayi* has acquired the enzyme of the last step in the heme biosynthetic pathway and this enzyme has evolved within the metazoan organism into a typical eukaryotic gene, presenting introns and exons.

Another interesting study on the *f* element of the isopod *Armadillidium vulgare* contributes largely to our understanding of the evolutionary meaning of HGT between prokaryotes and eukaryotes (Cordaux and Gilbert 2017). The *f* element is a nuclear *Wolbachia* insert involved in female sex determination in the terrestrial isopod *A. vulgare*. This DNA fragment, which accounts for about the entire genome, comes from a feminizing *Wolbachia* and has been inserted by micro-homology-mediated recombination in a relatively recent event. The functionality of the *f* element is demonstrated by the phenotypic feminization of genetic males, although at a lower ratio than the feminization mediated by the infection with *Wolbachia*.

7 Current Perspectives

The genus *Wolbachia* is an exciting organism with much importance in several areas of the biological research such as symbiosis, sex determination, or HGT and with proven potential in the control of arboviruses' transmission.

The fact that it is an obligated endosymbiotic bacterium living in the germline of its animal hosts makes it an ideal candidate to study phenomena of HGT between prokaryotes and eukaryotes, since the transferred DNA will be inherited by the offspring resulting in a fixation at the population level. Although some decades ago such HGT events were considered rare and the transferred fragments were thought not to have evolutionary significance or functionality, many studies report the occurrence of these facts and the role they play in speciation and acquisition of new abilities by the eukaryotic recipients. As explained above, some animals have acquired a whole set of enzymes from bacteria that enable them to degrade the plant cell wall. Others have acquired this way the ability to produce toxins with antibacterial properties or to maintain into its cells mutualistic bacteria lacking essential genes. Some organisms have developed new metabolic possibilities or have altered their reproductive behavior, deriving in a divergence among populations or even speciation. The understanding of how these HGT processes affect the biology of the eukaryotic species has even led to suggest the possibility to design pest eradication strategies based on the control of the genes involved (Ioannidis et al. 2014; Chou et al. 2015).

In the last years, an increasing number of studies are showing that HGT from bacteria to eukaryotes, far from being a negligible event, is a driving force for ecological transition and evolution of many invertebrates. And *Wolbachia*, due to its ecological and reproductive features, is outlined as a promising candidate to be the donor of important genes in the biology of nematodes and arthropods, still to be studied.

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Horizontal Gene Transfer in Obligate Parasites

J. M. Ageitos, M. Viñas, and T. G. Villa

Abstract Parasitism entails a tight interaction between host, parasites, and the host's commensal organisms; this derives into a coevolution process that in turn represents an extreme parasite specialization, associated with reductive evolution and streamlining. Horizontal gene transfer (HGT), as the asexual transfer of genetic material between or among distantly related species, may play an important role in host-parasite relations. HGT is especially important in the prokaryotic genome evolution; however, HGT is also present in eukaryotic genomes, for instance, the exchange of genetic sequences with organelles, endosymbiotic microorganisms, or even parasite genomes, and the host nucleus. Although parasitic symbiosis is classically defined as an arms race between host defenses and parasites, it has been identified the expression of exogenous parasitic genes in the host that provide selective advantages. Notwithstanding, the main part of HGT events in parasites takes place between them and commensal organisms, enabling selective advantages for the parasites. In this chapter, we will discuss some interesting cases of HGT in parasites that affect and belong to different kingdoms and the importance of this process in host-parasite coevolution.

Keywords Horizontal gene transfer · Obligate parasites · *Wolbachia* · *Chlamydia* · *Microsporidia* · Parasitic plants

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1 Introduction

The importance of horizontal gene transfer [HGT, or lateral gene transfer (LGT)] events have outlined from genomic and metagenomic analyses, being more numerous than the previously observed in studies of pathogenic bacteria. It was found that the exchange of genetic information has played a role in the evolution of numerous organisms, including parasitic ones and their host (Lena and Walker 2009). HGT can be explained from symbiotic relations, including the parasitism, as well from pathogenic events, such as viral infection and phagocytosis (Wijayawardena et al. 2013). HGT events include transmission to endosymbiotic genes of mitochondria and chloroplasts, although the presence of these genes is controversial, since the foreseeable microbial origin of these organelles, however, cannot explain the presence of bacterial genes in eukaryotes, suggesting that this event has been present during the evolution of eukaryotic organisms (Huang 2013). The permanence of these elements in the genomes is indeed interesting, since they are subjected to natural selection. The term genetic parasites is applied to transposons, plasmids, and viruses. Their persistence can be explained by their selfish nature, the occurrence of duplication in the genomes (Iranzo and Koonin 2018), and the impossibility of their elimination (Iranzo et al. 2016). Genetic parasites are considered an important factor in the genomes variation, promoting the translocation of genomic sequences and, therefore, in the evolution of organisms (Zhang et al. 2014b); in this way, even mobile genetic elements cannot be considered as authentic parasites themselves; they simply promote the HGT (Soucy et al. 2015).

Identification of HGT events is usually based on comparative genomics and molecular phylogenetic analyses. The basic local alignment search tool (BLAST) from the National Center for Biotechnology Information (NCBI) (Sayers et al. 2012) is widely used for the initial homology comparison and sequence search. Phylogenic conflicts appear as sequence homology, DNA, or proteins, between phylogenetically unrelated organisms and by phylogenetic incongruences, sequences that do not match with the rest if the genome and the phylogeny obtained by other methods, such as 16S sRNA analyses. When these sequences do not appear in phylogenetically related organisms and appear with a patchy distribution, they are usually considered as HGT candidates. The source of sequences is usually obtained from public databases, as well as sequencing projects. Some researchers use the direct amplification of the genes of interest by polymerase chain reaction (PCR) or study the gene expression (mRNA) by quantitative reverse transcription PCR (qRT-PCR). In addition to gene homology, parametric analyses (Becq et al. 2010) are based on the preferential codon usage, oligonucleotide frequencies, or CG content, in order to identify the origin of the sequences; however, as the time pass after the HGT event, it tends to homogenize the composition with the recipient DNA, by a process termed amelioration (Drezen et al. 2017). Other methods include proteomic tools for identification of proteins and peptides and the comparison of the sequences from described in related species (Adato et al. 2015).

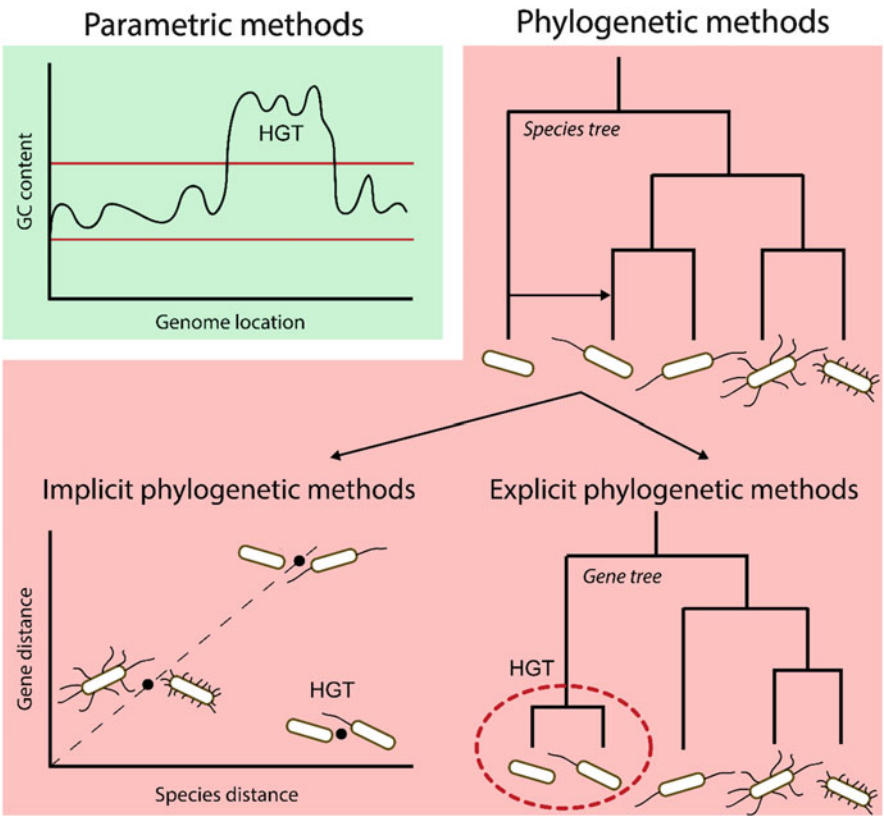

Fig. 1 Conceptual overview of methods to infer HGT. Image modified from Ravenhall et al. (2015) under Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0/)

Phylogenetic studies are performed with different approaches (Fig. 1), including maximum likelihood, maximum parsimony, neighbor joining, Bayesian inference, or Fitch-Margoliash methods. Frequently, researchers calculate the evolutionary distance employing different methods in order to perform phylogeny trees and compare topologies. It is important to notice that even phylogenetic incongruences are indicators of HGT; there are required additional evidences for HGT events identification. However, different methods tend to infer different HGT events (Ravenhall et al. 2015), which requires the employment of specific strategies such as the use of combined (Schönknecht et al. 2014) or complex methods (Huson and Bryant 2006) or other new strategies (Zamani-Dahaj et al. 2016; Rancurel et al. 2017; Kim et al. 2018).

Misidentification of HGT events may occur by methodological and/or analytical artifacts, such as the presence of an unidentified common ancestor, sequence conservation, misidentification of genes, loss of genes in related species, lack of comparable sequences (insufficient taxon sampling), cloning/sequencing errors, or

even database contamination (Huson and Bryant 2006; Beiko and Ragan 2009; Schönknecht et al. 2014; Wijayawardena et al. 2015).

In the case of parasites, additional problems can appear by sample contamination (Deitsch et al. 2001), since sometimes it is difficult to ensure the purity of host and parasite as independent sources, as the case of uncultivable or intracellular parasites as well as intracellular organelles. All these concerns produce that some plausible candidates for HGT events were manually discarded if the phylogeny difference is excessively high. In the same way, main part of the studies only compared related taxa, being ignored events of HGT that could occur if a more inclusive study were performed.

Here we are in front of vicious circle; in so far as the HGT remains as an "improbable event" that must be probed without doubt, real events could be underestimate (Schönknecht et al. 2014), while, if we do not consider independent lines of evidence and alternative scenarios for phylogenetic incongruences (Huang 2013), and the employed methodology remains prone to imprecise identification (Wijayawardena et al. 2015), unambiguous identification of HGT will remain as a challenge.

Obligate parasites have suffered extreme reduction of their genomic information in the process of host adaptation; therefore, HGT could be studied more clearly. In the same way, intimate contact with their host and microorganisms sharing ecological niche allows the occurrence of HGT, since the acquisition of functionalities is subjected to a different evolutive pressure than in other organisms. In the case of *horizontal gene transfer in obligate parasites*, we face a unique opportunity for unraveling the mechanism of HGT and evolution, through discussing several notorious and interesting cases of HGT in parasites and their hosts, in order to illustrate the importance of this process in host-parasite coevolution.

2 HGT in Parasitic Bacteria

It is well-accepted that bacteria are prone to HGT by several reasons, since, although the acquisition of new sequences may be deleterious to the recipient, the high ratio of multiplication of bacteria ensures that transferring genes providing an advantage to the host became predominant in the population, while deleterious insertions disappear, in the same way that deleterious mutations are lost (Thomas and Nielsen 2005). However, bacteria are usually defined as mutualists, commensals, and pathogens, but rarely as parasites. Only among obligate intracellular bacteria (α-proteobacteria such as *Wolbachia*, *Chlamydia*, and *Rickettsia*) a real parasitic relationship with the host can be considered, but the dividing lines between mutualistic-parasitic-pathogenic are not as clear-cut as it might appear. Even if it is considered that HGT in intracellular bacteria is infrequent, it is described a transference of ATP/ADP translocase of *Arabidopsis* plastid to proteobacteria, with homologs in *Rickettsia* and *Chlamydia* (Wolf et al. 1999) and other α-, β-, γ-, and δ-proteobacteria (Ku et al. 2015).

Wolbachia (specifically treated in another chapter of this book) is a genus of obligate intracellular α-proteobacteria transmitted cytoplasmically by maternal line that widely affects arthropods and nematodes. The relationship between *Wolbachia* and its host varies from reproductive parasitism to mutualistic symbioses (Wu et al. 2004), and it is considered as a candidate for future mutualistic association, similarly to the mitochondria (Weeks et al. 2007; Margulis 2009). *Wolbachia* infects the reproductive cells, manipulating the host reproduction (Werren et al. 2008) by cytoplasmic incompatibility (Alam et al. 2011), parthenogenesis (in hymenopteran wasps), male killing (insects), or feminization (crustacean) (Bordenstein and Reznikoff 2005; Klasson et al. 2009). This intimate contact with gametes during development could facilitate the HGT, and it is possibly the best documented. In the case of the fly *Drosophila ananassae*, it was transferred nearly the entire genome of the parasite to the host, and although *Wolbachia* infection is maternally transmitted, the insert genome is paternally inherited (Hotopp et al. 2007). It has been reported that at least 28 genes of *Wolbachia* are transcribed in *D. ananassae*, although their function is, so far, unclear (Werren et al. 2008). It was also detected the presence of two extensive *Wolbachia* insertions in the genome of a Tsetse fly (*Glossina morsitans morsitans*), covering the 52 and 48% of *Wolbachia* genome. The inserted genes are related with parasite-host interaction, but authors conclude they might not be expressed (Brelsfoard et al. 2014). In the case of functional insertions, in 2009 two different research groups reported the identification of *Wolbachia* insertions in the mosquitos *Aedes aegypti* and *Anopheles gambiae* (Klasson et al. 2009; Woolfit et al. 2009). Salivary gland surface (SGS) proteins are involved as receptors for *Plasmodium*, and those genes have been horizontally transferred with *Wolbachia*, although the directionality of the HGT is not clear. Woolfit and collaborators (Woolfit et al. 2009) supported the theory of host-to-*Wolbachia* HGT, while Klasson and collaborators considered the *Wolbachia*-to-host the most plausible direction (Klasson et al. 2009). Curiously, *Wolbachia* also parasitizes nematode parasites, being implicated in several HGT events with them, for the sake of clarity; they will be discussed in the section regarding the "parasite/host."

Chlamydiae are a phylum of Gram-negative, obligate intracellular α-proteobacteria, described as symbionts, or pathogens. Moore and Ouellette proposed that the complex cell cycle of some *Chlamydia* functions as parasites (Fig. 2), since after the formation of chlamydial inclusion, the host cell remains healthy and only absorption of nutrients takes place, until parasite emerges and kills the host (Moore and Ouellette 2014). A recent study has identified several HGT candidates in the *Chlamydia* genera, being the main part occurred between species of the genera but also with organism outside the phylum *Chlamydiae*. HGT intra phylum are related with cell signaling, metabolism, and storage, while HGT inter phylum are mainly related with metabolism and information storage and processing (Kim et al. 2018).

From a different phylum (putative bacterial phylum TM6), *Babela massiliensis* is a mandatory endoparasite of the amoeba *Acanthamoeba castellanii*. *B. massiliensis* genomic sequence differs from the type member of TM6, presenting sequences with high homology with other bacteria and eukaryotes, suggesting extensive HGT

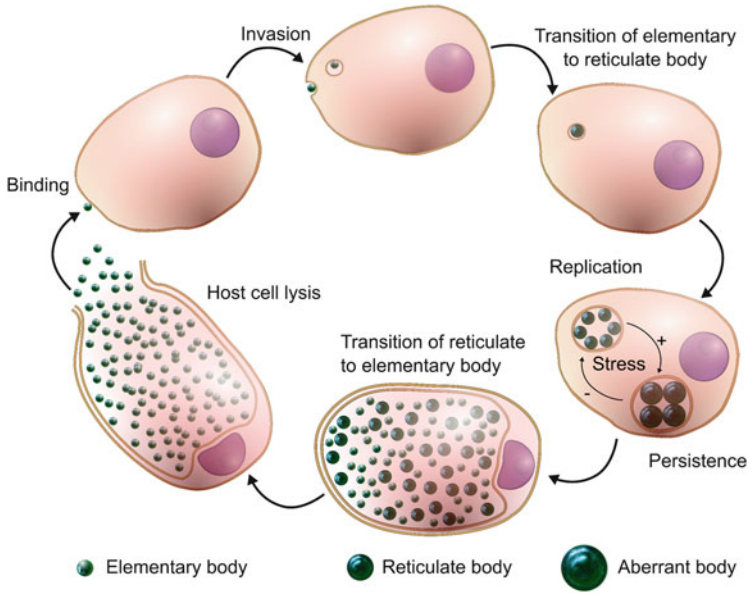

Fig. 2 Life cycle of *Chlamydia* sp. Scheme based on an image of Elwell et al. (2016)

events, probably with other sympatric microorganism inside the amoeba host (Pagnier et al. 2015). Eukaryotic genes include multiple ankyrin repeat-containing proteins, as well as other proteins related with protein-protein interactions.

3 HGT in Parasitic Fungi

The members of the fungal genus *Pyrenophora* are parasite/pathogen microorganisms affecting crop plants, such as wheat (*Triticum aestivum*). This genus is an example of recent HGT, in the late 1970s, the avirulent *P. tritici-repentis* has acquired a 11 kb region encoding the toxin ToxA by HGT from *Phaeosphaeria nodorum*, a major fungal pathogen of wheat (Mehrabi et al. 2011). But this genus has also underwent HGT from other origin, such as functional enzymes from bacteria (xylanase, beta-galactosidase, cyanophycinase, succinylglutamate desuccinylase/aspartoacylase, *N*-acetylglucosaminyltransferase, and UDP-glucosyltransferase, among others), and possibly plants (a leucine-rich repeat protein) (Sun et al. 2013). This is an example of how HGT events can induce an evolutionary advantage by means of accelerated evolution and adaptation of new enzymatic pathways.

Microsporidia is a group of obligate intracellular fungal parasites of animal cells. The genome of these microorganisms is extremely simplified and lacks conventional mitochondria, they also own prokaryote characteristics. These microorganisms replicate inside of animal cells, inhibiting cell apoptosis and controlling the cellular

machinery (Selman and Corradi 2011; Huang 2018). *Microsporidia* is a phylum of fungi prone to HGT events, since as intracellular pathogens, some of them use the host nuclei for their development, increasing the possibility of gene exchange (Corradi 2015). In addition, *Microsporidia* genomes are usually small and simple, representing a good example of genome reduction in parasitic organisms, with a coding capacity of only ~2000 genes, in *Encephalitozoon* sp., with what acquisition of new enzymes, or the recovery of functions, can be stabilized in a simpler way. Due to the extreme genome reduction, some microsporidian are unable of synthetizing nucleotides de novo, depending on scavenging the host, and several of these genes were identified as HTG (Alexander et al. 2016). The content of genes from foreign origin varies between the 0.34 and 2.11% of genes, depending of the specie (Lukeš and Husník 2018). One example are the ADP/ATP translocases present in *Microsporidia*, which have the same origin that the ones in a coinfecting bacteria, *Chlamydia* (Richards et al. 2003). Other examples of HTG from bacteria in *Microsporidia* are catalase (Fast et al. 2003), CAP-domain-containing protein, endomembrane metalloprotease, folic acid synthase pathway, glycosyl transferase, GTP cyclohydrolase I, photolyase, or superoxide dismutase (Selman and Corradi 2011; Corradi 2015; Alexander et al. 2016).

Nosema bombycis is a microsporidian parasite of the domesticated *Bombyx mori* (silkworm) that produces notorious losses in silk industry. Pan et al. (2013) found that this microorganism has been subjected to multiple HGT events, with duplicated genes from different origins, such as bacteria, Archaea, virus, and several transposable elements from it host, *B. mori*. Some of the duplicated genes are related with the cytotoxic metabolic pathway, and they were subjected to positive selection, indicating that HGT could increase the pathogenic ability of the parasite. The plasticity of microsporidian parasites in the acquisition of new functions is reflected in the incorporation of a homologous of septin 7, a transmembrane protein, by *Ordospora colligata* from its host, the arthropod *Daphnia magna*. Septins can help the attachment of *O. colligata* to N-cadherin-like surface receptors of the epithelial cells of its host, inducing the endocytosis and facilitating the cell invasion (Corradi 2015; Pombert et al. 2015).

Encephalitozoon romaleae is a non-hyphal parasitic fungus of isolated from the grasshopper (*Romalea microptera*) (Johny et al. 2009). The genes acquired by HGT are related with the salvage of purines and folate synthesis, critical steps for the DNA synthesis (Pombert et al. 2012), from γ-proteobacteria (Alexander et al. 2016), and a purine nucleotide phosphorylase from animal origin (Selman et al. 2011). *Encephalitozoon cuniculi* (Fig. 3) parasitizes animals, including human, and it has found HGT of ATP transporter genes from *Chlamydia*. ATP transporters are located in *E. cuniculi* membrane, as well as in the mitosome [a microsporidian remnant mitochondrion, (Tsaousis et al. 2008)], and are employed to obtain ATP from the host cell. The HGT could be explained in the fact that both intracellular parasites can coexist in the host cells, as it was observed in the co-infection with *Chlamydia trachomatis* (Lee et al. 2009). An example of acquisition of new or recovering functionalities is the location of an ATP/ADP translocase in the surface of the mitosomes of *E. cuniculi*. This translocase co-located with the mitochondrial heat-

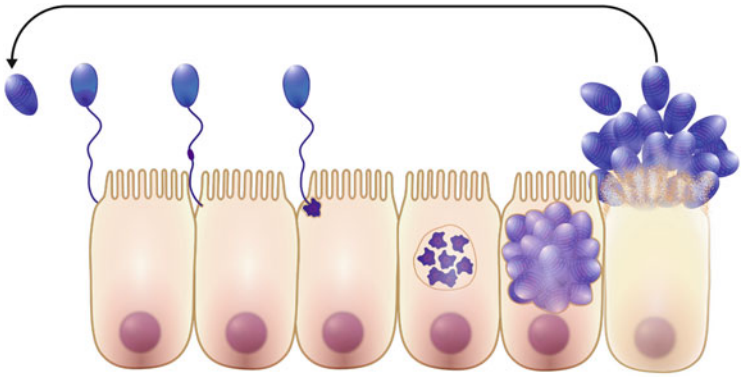

Fig. 3 Life cycle of *Encephalitozoon* sp. Spore infects the host cell by the extruded polar tubule. Spore injects the nucleus and sporoplasm into the host cell. Inside the cell the sporoplasm replicates, in some genera inside of a membrane termed parasitophorous vacuole (Lee et al. 2009; Bohne et al. 2011). Sporoplasm continues replicating and forming spores by consuming host ATP (Lukeš and Husník 2018). Host cell became a bag of spores, and eventually the membrane became disrupted; released spores can infect new cells. Image based on the described by Wasson and Peper (2000)

shock protein 70, a chaperone which requires ATP for its function. The location of the translocase in the mitosome allows the transport of ATP inside the organelle, providing new functions to the parasite (Tsaousis et al. 2008).

Batrachochytrium dendrobatidis is a chytrid fungus that produces chytridiomycosis in Amphibians. The study of the genome of *B. dendrobatidis* has shown the possibility of multiple HGT genes: 3 genes from plants, 19 bacterial genes, and 1 from oomycetes. Those genes are involved in metabolic pathways, niche adaptation, stress response, and defense (Sun et al. 2016a). The significance of HGT genes is reflected in the presence of duplications, some of them participate cooperatively in the same pathways, and multi-copies, for instance, the phosphate-responsive 1 family protein from plants is presented in 9 copies.

4 HGT in Parasitic Protists

Blastocystis spp. is a eukaryotic parasite widely present in human intestines, and it is related with diarrheal processes. Eme and collaborators have found that the ~2.5% of its genes were acquired by HGT from prokaryote and eukaryote organisms (Eme et al. 2017). Those acquired genes are related with the adaptation to the gut environment, such as pH homeostasis and oxygen-stress resistance, or with the metabolism of nitrogen, amino acids, and carbohydrates. This is another example of HGT that improves the adaptability of the parasite to its host, and, for instance, different subtypes of *Blastocystis* differ in the nature of HGT events and in the copy number of the acquired genes.

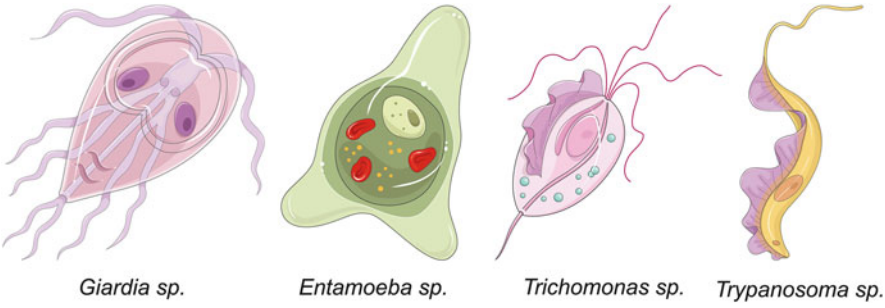

Fig. 4 Schematic representation of several parasitic protist discussed in this chapter. Protist illustrations were reused from Servier Medical Art (https://smart.servier.com/) under the Creative Commons Attribution 3.0 (https://creativecommons.org/licenses/by/3.0/)

Giardia lamblia (synonyms, *Giardia intestinalis* and *Giardia duodenalis*) is a flagellated protist parasite (Fig. 3) of humans and other animals that produces one of the most common parasitic infections (Giardiasis) (Ankarklev et al. 2010). *G. lamblia*, *Entamoeba histolytica* and *Trichomonas vaginalis* are amitochondriate protists, which means they lack a proper mitochondria. Although *G. lamblia* contains mitochondria-related organelles termed mitosomes, but unlike mitochondria, they do not contain DNA (Tovar et al. 2003; Martincová et al. 2015). It was reported that *G. lamblia* has several ferredoxins similar to the ones present in some gram-negative bacteria and organelles of eukaryotes, suggesting their origin in a HGT event (Nixon et al. 2002). Those findings were previous to the proper description of mitosomes, which only contains 21 protein types related with iron-sulfur cluster biosynthesis (Martincová et al. 2015). Nixon and collaborators also described other enzymes, such as the alcohol dehydrogenase 3, NADH oxidase, and nitroreductases, with an HGT origin from anaerobic prokaryotes, present also in *Entamoeba histolytica* (Nixon et al. 2002).

E. histolytica (Fig. 4) is a parasite of humans and other primates, producing the second cause of death by parasitosis in humans (Stanley 2003). Several studies have ranged from 8 (Romero et al. 2016) to 16 (Alsmark et al. 2009) different HGT events related with proteobacterial genes. The association of *E. histolytica* with bacteria that share ecological relationship during pathogenesis play a critical role in HGT; however, the found genes were not related with virulence of antibacterial resistance but with metabolically pathways, since some of them are estimated to occur 253 million years ago, having influence in the speciation of *E. histolytica*. For instance, the iron-sulfur cluster genes of *E. histolytica* were acquired by HGT from epsilon proteobacteria and are homologous to the ones in *Helicobacter pylori* and *Campylobacter jejuni* (Van Der Giezen et al. 2004).

Trichomonas vaginalis is a human protozoan parasite (Fig. 4) (Alsmark et al. 2013) and the causative agent of trichomoniasis (Schwebke and Burgess 2004). Alsmark and collaborators (Alsmark et al. 2009) found that HGT has played an important role in *T. vaginalis* evolution, affecting several metabolic pathways,

estimating 152 events, from which only the 2% are functional (Gluck-Thaler and Slot 2015). One of this acquired genes is *N*-acetylneuraminate lyase (de Koning et al. 2000), an enzyme from γ-proteobacteria that could provide nutritional advantages (Wijayawardena et al. 2013). Another HGT is a long insertion of 27 clustered genes from the bacterium *Peptoniphilus harei* (Strese et al. 2014) which could provide multiple synthetic functions to *T. vaginalis*.

Cryptosporidium parvum is an apicomplexan parasite that produces cryptosporidiosis in children and immunosuppressed persons, such as patients with acquired immune deficiency syndrome. This microorganism owns multiple HGT from different origin, such as algal and eubacterial origin (Huang et al. 2004a). These genes have improved the adaptation of the parasite to the anaerobic environment of the intestine, providing resistance to drugs and possibly evading the host immune response (Sateriale and Striepen 2016).

Plasmodium falciparum (Fig. 4) is one of the etiological agents of malaria, with a 99.7% of estimated malaria cases in Africa; malaria is a major factor in the selection of the human genome (Kwiatkowski 2005), causing 435,000 deaths in the year 2017 (WHO 2018). *P. falciparum* has acquired epigenetic machinery by HGT from animals (Histone H4 Lysine 20 modifier) and conceivably from algal endosymbionts (H3K36 methyltransferase). This machinery is related with the ability of the parasite in pathways of invasion, cytoadhering, and immune evasion (Kishore et al. 2013). *P. falciparum* also present plantlike genes in its genome (Huang et al. 2004b) and peroxiredoxin, an antioxidant enzyme, from a prokaryotic origin (Djuika et al. 2015). It has been reported that a related species, *Plasmodium vivax*, has been subjected to HGT from humans (Bar 2011), but it is still controversial, since previous studies supporting the same information have been shown to be artifacts (Deitsch et al. 2001), and so far, there have not been reported any additional information validating that finding. Another controversial study was published in 2010; Hecht and collaborators reported HGT from parasite-host for *Trypanosoma cruzi* (Fig. 4) to humans (Hecht et al. 2010). The insertion of the minicircle sequence of the ethological agent of Chagas disease into its host genome was certainly astonishing, especially because its size (30 nm) is in the limit of the nuclear pore complex (9–40 nm) (Elmer et al. 2013). A later independent study showed that this putative HGT event between *T. cruzi* and human did not be sustainable, concluding that those results could be due to technical artifacts (Wijayawardena et al. 2015). This fact emphasizes the importance of using different methodologies to confirm HGT events. Alsmark and collaborators performed an analysis of HGT in two groups of protist parasites, mucosal parasites (*G. lamblia*, *T. vaginalis*, and *E. histolytica*) and insect-transmitted blood parasites (*P. falciparum, P. vivax*, *P. yoelii yoelii*, *T. brucei*, and *T. cruzi*). In the case of insect-transmitted blood parasites, it was found 33 HGT events, most of them from *Proteobacteria* donor origin (22), while in the mucosal parasites the number of HGT events increases to 88 (Alsmark et al. 2013). Interestingly, mucosal parasites had similar HGT than insect-transmitted blood parasites but also multiple sequences from Bacteroidetes and Firmicutes, the predominant lineages in gut microbiota (Ley et al. 2008). Similar results were found in other rumen ciliates (Ricard et al. 2006), indicating that the

genes transferred by HGT are an adaptation to an anaerobic environment, rich in carbohydrates, and specific function for colonization, such as β-galactosidase for mucus degradation (Alsmark et al. 2013).

5 HGT in Parasitic Plants

Even all parasites are prone to HGT, due to their intimate contact with the hosts, parasitic plants are considered as a model for the study of HGT. The high ratio of HGT events in parasitic plants can be explained due to their haustorial feeding (Fig. 5).

The haustorium (Fig. 5) is an adaptation of parasitic plants that allowed the transition from autotrophism to heterotrophism; this invasive organ allows the intracellular exchange, where water, nutrients, and genetic material are transferred, and it is also found in biotrophic fungal parasites of plants (Yoshida et al. 2016). It has been found that through these connections, the transport of genetic material, gene silencing signals, virus, and even organelles is possible (Dörr and Kollmann

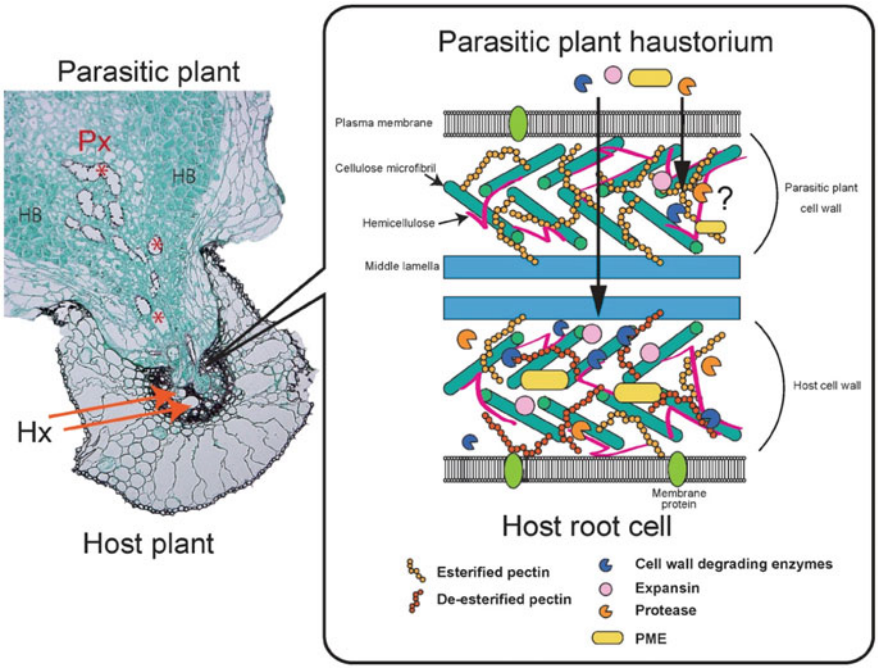

Fig. 5 Parasitic plant interactions with a host plant. Micrography of the plant parasite *Striga* and scheme of interaction of parasite plant and its host. Parasite xylem cells (Px), host xylem cells (Hx), hyaline bodies (HB), pectin methylesterase (PME). Image taken from Mitsumasu et al. (2015) under Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0/)

1995; Birschwilks et al. 2006; Tomilov et al. 2008; Schneider et al. 2018). The HGT between host and parasite mitochondria can represent up to 40% of the mitochondrial genomes in the family Rafflesiaceae (Xi et al. 2013; Davis and Xi 2015; Delavault et al. 2017). The majority of mitochondrial HGT events, the gene transfer occurs between host to parasite plants, and interestingly those HGT-acquired genes are preferentially expressed in the haustorium (Yang et al. 2016). Yang et al. performed a conscientious study on HGT in parasitic plants, identifying 46 different HGT events from different plants that confer to parasite plants functions such as defense against infection, insect toxins, or transcription-related enzymes (Yang et al. 2016). Schneider and collaborators (Schneider et al. 2018) found several genes of *Galium*-plastid origin in the mitochondrial genome of *Aphyllon epigalium*, a parasitic plant of *Galium* L. in the western USA (Colwell et al. 2017). Another example of plasmid-derived HGT in mitochondria is the holoparasite *Lophophytum mirabile*, with at least five genes from a host origin, the order Santalales (Gandini and Sanchez-Puerta 2017). Also, it has been recently described the transmission of mitochondrial genes from a host (*Artemisia*) to parasite in the family Orobanchaceae (*Orobanche coerulescens*) (Kwolek et al. 2017). Although host-to-parasite is the most common HGT event, it was also found the contrary several mitochondrial genes were transferred from the parasitic genus *Cuscuta* to their host *Plantago* (Mower et al. 2010). This type of HGT events can improve the mitochondrial genetic diversity in plants. Another interesting example are several mitochondrial genes of the fern *Botrychium virginianum* that are originally from root-parasitic Loranthaceae (Davis et al. 2005). Perhaps the most extreme case of mitochondrial HGT is the root holoparasite *Orobanche californica*, in which mitochondrion has a 93% sequence homology with the ones in its host, *Capsicum annuum* (QASEM 2009; Gandini and Sanchez-Puerta 2017). In addition to mitochondria, the host-to-parasite HGT events occurs in other organelle, for instance, Li et al. (2013) described the transfer between chloroplasts of *Haloxylon ammodendron* and the genome of its parasite *Cistanche deserticola*. In the case of Holoparasites (plant obligate parasites), the genome of their chloroplasts has been drastically reduced, losing part of the genes related with the photosynthesis. In the case of *C. deserticola*, HGT seems to have restored some functions by acquiring two copies of the gene *rpo*C2 (DNA-dependent RNA polymerase) from *H. ammodendron*, a plant adapted to the desert and osmotic and salt stress (Li et al. 2013).

Nuclear HGT between host and parasite has been described in the RIKEN Plant Science Center by Yoshida et al. (2010). The plant parasite *Striga hermonthica* (Del.) Benth. has acquired the gene *ShContig9483* from sorghum by mRNA-mediated process; however, the function of this gene is unknown. Xi et al. (2012) have found that the parasitic plant *Rafflesia cantleyi* Solms-Laubach has several dozens of actively transcribed genes from its obligate host *Tetrastigma rafflesiae* Miq. Those genes could provide an increase in the fitness of the parasite, maximizing resource extraction as well as reducing the response of the host (Yang et al. 2016). Another example is the acquisition of strictosidine synthase genes of the family Brassicaceae by the root-parasitic plant *Orobanche aegyptiaca* and the shoot-parasitic plant *Cuscuta australis*. Authors have found the expression of the

strictosidine synthase-like genes in several steps of the development of the parasitic plants (Zhang et al. 2014a). In a similar manner, an acyltransferase gene, from the family Fabaceae, was present in parasitic plants of the genus *Cuscuta* (*C. pentagona* and *C. australis*). This enzyme is related with the anthocyanin modification, suggesting that the HGT event can improve the biotic and abiotic resistance of the parasites (Sun et al. 2016b).

Despite of the numerous and well-documented HGT between parasitic plants and their hosts, several studies have been questioned or demonstrated to be erroneous, mainly originated from sample contamination, highly conserved sequences or unintentional misinterpretation of the results (Renner and Bellot 2012).

6 HGT in Parasitic Nematodes

Globodera pallida is a plant-parasitic nematode that produces major crop losses, especially in potato. *G. pallida* has acquired several genes by HGT along the time from rhizobial bacteria, and several of these genes are active invertases (Glycosyl Hydrolase Family 32) expressed in the digestive system of the nematode, allowing to the parasite to feed from host sucrose (Danchin et al. 2016). Improving of the parasitism ability mediated by HGT is not infrequent, expansin-like proteins, pectate lyases and cellulases from exogenous origin have been identified in the genome of plant-parasitic nematodes (Danchin et al. 2010). For instance, the nematode *Meloidogyne incognita* has a polygalacturonate gene (GH28), generally absent from animals, with high similarity with the enzyme of *Ralstonia solanacearum*, a bacterial plant pathogen; this wall-degrading enzyme increases the plant parasitism ability of *M. incognita*, as well as other 46 genes probably acquired by HGT (Cotton et al. 2014; Danchin et al. 2016).

Strongyloides venezuelensis is a parasitic nematode of brown rats (*Rattus norvegicus*) (Viney and Kikuchi 2017) that has acquired by HGT a synthetic pathway which makes it stand out from other parasitic nematodes. Nematodes are natural heme auxotrophs, which means that they are not able to synthetize the heme group. The genome of *S. venezuelensis* contains a functional gene of ferrochelatase from α-proteobacteria, an enzyme responsible of the last step of heme biosynthesis and iron chelation (Nagayasu et al. 2013). Another nematode with a similar HGT event is the human parasite *Brugia malayi*, one of the causative agents of the lymphatic filariasis. This filarial nematode also acquired a functional enzyme by HGT, ferrochelatase, from a bacterial origin (Wu et al. 2013). Since other members of Nematoda are unable to synthetize heme, this HGT event provides to these organisms a critical advantage as compared with other blood-feeding parasites. On the other hand, *B. malayi* has suffered multiple HGT with its symbiont *Wolbachia* (>4.5% of *Wolbachia* genome), some of them related with murein metabolism (Lena and Walker 2009; Ioannidis et al. 2013). The importance of *Wolbachia*-mediated HGT is that genes from *Wolbachia* were found in another *Wolbachia*-free filarial parasites, *Onchocerca flexuosa* and *Acanthocheilonema viteae* (McNulty

et al. 2010). McNulty and collaborators found at least three proteins, ABC transport-related, present in genome. These findings support the *Wolbachia*-mediated HGT in an ancestor of *Onchocerca* sp. and *Brugia* sp. (McNulty et al. 2012). Interestingly, the HGT of *Wolbachia* genes could compensate the lack of *Wolbachia* itself, which can be considered as an obligate mutualistic symbiont for filarial nematodes. In the same way, the current treatments against filarial infections, using antibiotics against *Wolbachia*, may select endosymbiont loss and enrichment in filaria with HGT from *Wolbachia* (Dunning Hotopp 2011). It has been reported that parasitic Nematoda can also acquire genetic material from their host, for instance, it has been found that the dog hookworm (*Ancylostoma caninum*) has in its genome a DNA transposon termed *bandit* and it is related with *Hsmar1* transposon from humans, suggesting a HGT origin (Laha et al. 2007).

7 HGT in Parasitic Trematodes?

The genus *Schistosoma* belongs to the parasitic Trematoda with a complex reproductive cycle, which includes several sequential hosts, producing schistosomiasis in the definitive vertebrate host, including humans (Wijayawardena et al. 2013). This genus is a controversial hot spot in the study of HGT, because, although multiple transfer of parasite-to-host and host-to-parasite sequences have been proposed (Huang et al. 2017), many of them have been refused or proved to be contamination-driven (Wijayawardena et al. 2015). Reports of the evidence of HGT between *Schistosoma* and salmonids HGT (Melamed et al. 2004) and the "no evidence" (Grunau and Boissier 2010), albumin in *Schistosoma mansoni* (Williams et al. 2006) or mammalian albumin contamination (DeMarco et al. 2007), 13 cases of HGT critically analyzed Wijayawardena and collaborators that turned out to be caused by technical artifacts or contaminations (Wijayawardena et al. 2015) showed that we must be cautious with HGT claims, especially when controversial or sensationalist reports come to light.

8 HGT in Parasitic Insects

Even the difference between parasitism and parasitoidism is sufficiently clear for the authors, it is also true that some parasites eventually reach to kill the host and biological cell cycle of parasitoids cannot be completed without their host, therefore, in this section fill be focused in HGT in parasitoid insects.

Cotesia congregata is a parasitic wasp that, similarly to other Hymenoptera, has associated symbiotic viruses, the bracoviruses. These viruses are produced in the wasp ovaries and are injected into the host jointly with wasp eggs. Bracoviruses has originated HGT with Hymenoptera from at least 100 million years, but interestingly, HGT events also occur in the wasp's host. Gasmi et al. (2015) found multiple

Bracovirus sequences in the lepidopteran genomes, such as monarch butterfly (*Danaus plexippus*), silkworm (*Bombyx mori*), or small mottled willow moth (*Spodoptera exigua*). Those acquired genes have been domesticated by the host and are expressed, providing protection against the viral pathogen baculovirus. The genome of the parasitoid *wasp Nasonia vitripennis* encodes several proteins bearing ankyrin repeat, typical from Poxviruses; however, the study of the sequences has shown that they have been acquired from *Wolbachia* origin (Werren et al. 2010; Dunning Hotopp 2011).

9 Final Remarks

In this chapter, we have revised multiple reports that show how HGT can provide evolutive advantages to the parasites in the acclimatization to the host. The acquisition and recovery of metabolic pathways improve the fitness of the parasites, by increasing their evolutive plasticity. Extreme specialization of parasites could become an important disadvantage, since their life history and fecundity depend entirely on the host; however, it is possible to find parasites in nearly all living organisms. It is also observed how the same group of parasites can affect distantly related host, both phylogenetically or ecologically. Therefore, it is conceivable that the high number of HGT play an important role in this disparity. On the one hand, as more parasitic genomes become unraveled, the mechanisms of their evolution will be logically clarified. Also, the low number of parasite sequences available in the databases hinders HGT identification, since limited sequencing projects of parasitic organisms are available. Nevertheless, although parasites show a high ratio of HGT, the number of studied organisms is still limited, representing a small fraction of the vast number of known parasites.

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Association Between Horizontal Gene Transfer and Adaptation of Gastric Human Pathogen *Helicobacter pylori* to the Host

Surekha Challa and Nageswara Rao Reddy Neelapu

Abstract *Helicobacter pylori* exhibit remarkable survival even in the vulnerable environments such as acidic, peristalsis, phagocytosis and oxidative stress. These stresses on the pathogen in the host induce damage of DNA in the pathogen. *H. pylori* acquired the ability to survive DNA damage by transformation-mediated recombination DNA repair. This repair mechanism helps the pathogen in successfully infecting the host. While many pathogens are competent for transformation only in certain environmental conditions such as starvation, *H. pylori* is competent throughout the growth. *H. pylori* may acquire the genetic material from the surrounding environment and contribute to evolution and genetic diversity. The mechanism in acquiring genetic material is 'horizontal gene transfer', the major contributing factor in the development of bacterial diversity. Horizontal gene transfer may help the pathogen *H. pylori* in acquiring antigenic determinants, genes of antibiotic resistance and virulence factors from other organisms to alter and influence pathogenicity. In this chapter, we review and discuss the association between horizontal gene transfer and adaptation of gastric human pathogen *H. pylori* to the host.

Keywords Antibiotics resistance · Evolution · Horizontal gene transfer · *H. pylori* · Macro-diversity · Multidrug resistance · Nickel-binding proteins · Nickel transporter genes

1 Introduction

Helicobacter pylori was discovered in human stomach, dental plaque, oral lesions, saliva, tonsil and adenoid tissue. *H. pylori* was known for causing gastrointestinal disorders like gastritis, ulcers and gastric cancer (Neelapu et al. 2014; Neelapu

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2018). Sometimes *H. pylori* may trigger some other diseases like otitis, sinusitis, phyrangitis, laryngitis and glossitis (Kurtaran et al. 2008). Microorganisms survive in nature either as individuals or in a community known as biofilm (Challa et al. 2018). *H. pylori* uses biofilm lifestyle to survive in unfavourable environmental conditions such as pH, antibiotics, immune defences, disinfectants, nutritional changes and high temperatures (Challa and Neelapu 2018). Biofilm provides a strong platform for interaction and communication among the individuals present in the colony (Mohana Sheela et al. 2018; Neelapu et al. 2018). Till date research to prevent bacterial infections involved identification of drug targets, drugs (Neelapu et al. 2013, 2015, 2016; Neelapu and Pavani 2013; Nammi et al. 2016, 2017), vaccines (Pasupuleti et al. 2017) and antibiofilm agents (Challa and Neelapu 2018). This review discusses how bacterium *H. pylori* acquire traits via horizontal gene transfer (HGT) and adapt to the particular niche.

2 Role of HGT and Mechanisms of *H. pylori* Adaptation to the Host

The "selective pressures on the invading *H. pylori* bacteria would expose it to environment (e.g., exposure to antibiotics and changes in gastric pH or mucosal defences) and host factors (e.g., specific and nonspecific defence mechanisms) (Ferrero and Jenks 2001). These pressures are harmful damaging DNA of *H. pylori* and sometimes may also prevent the colonization of *H. pylori* strain (O'Rourke et al. 2003). *H. pylori* are competent enough to pick DNA from the surroundings either from other *H. pylori* strains, or from other bacteria in the gut of the host (via HGT) or sometimes even in from the host (Fig. 1). Then *H. pylori* use acquired transformation-mediated recombination DNA repair for successful infection of the pathogen" (Dorer et al. 2010). This transformation is helping the bacteria to adapt itself in the gastric niche of the host (Schuster et al. 2008). Literature also reports changes in the genomic material of *H. pylori* when transmitted between individuals of the host. Burst of mutations will be induced when exposed to selective pressures mentioned above (O'Rourke et al. 2003). These bacteria (*H. pylori*) harbour genes which are affected and/or not mutated changing the surface components of bacteria (Linz et al. 2013). This becomes disadvantage to the pathogen, where it is indirectly recognized by the host. During evolution some of the genes will be deleted and some genes will be imported via HGT from the already adapted bacteria which are coexisting in the gut of the host altering the surface components (Linz et al. 2013). This importation helps the bacteria to shape its genome and adapt to the host of the genome (Schuster et al. 2008; Eppinger et al. 2006). This demonstrates the role of HGT in shaping the genome of bacteria to adapt it to the new host.

HGT, the "key evolutionary force", transferred genetic material between genomes and thereby shape the genome of bacteria. This helped many bacteria to

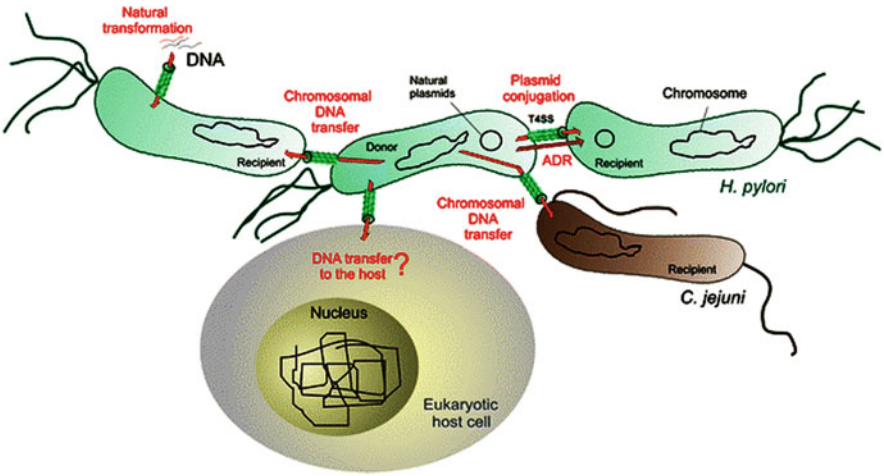

Fig. 1 *H. pylori* are competent enough to pick DNA from the surroundings either from other *H. pylori* strains or from other bacteria (*Campylobacter jejuni*) in the gut of the host (via HGT) or sometimes even from the host (Source: Fernandez-Gonzalez and Backert 2014)

gain genes and selectively provided advantages to the bacteria (Fernandez-Gonzalez and Backert 2014; Garcia-Aljaro et al. 2017). Literature reports that adaptation of *H. pylori* to the gastric niche (Vinella et al. 2015; Fischer et al. 2016), micro- and macrodiversity in *H. pylori* (Alm et al. 1999; Hofreuter and Haas 2002) and antibiotic resistance in *H. pylori* (Von Wintersdorff et al. 2016; Lood et al. 2017) are due to HGT. This section discusses in detail (a) HGT of nickel-binding proteins, nickel transporter genes and their role; (b) macrodiversity in *H. pylori* and HGT; and (c) antibiotic resistance in *H. pylori* and HGT. This section further discusses how HGT has shaped the genome of *H. pylori* in due course of evolution.

2.1 HGT of Nickel-Binding Proteins, Nickel Transporters Genes and Their Role

H. pylori utilizes specific enzymes or Ni proteins like urease and [NiFe] hydrogenase for colonization of gastric tract in humans (Fischer et al. 2016). The pH in the stomach is acidic and urease (Ni protein) of *H. pylori* helps in changing/converting the acidic pH in the stomach to neutral pH. Urease needs a cofactor nickel to convert urea into CO_2 and NH_3 (Neelapu et al. 2014; Fischer et al. 2016). These compounds are used by the bacteria to maintain the pH in the bacterium cytoplasm near to neutral. [NiFe] hydrogenase (Ni protein) is another enzyme where a bacterium utilizes molecular hydrogen as a source of energy (Fischer et al. 2016). Nickel is scarcely or meagrely available in the human body. So, *H. pylori* requires nickel transporter genes for acquisition of nickel and colonization of *H. pylori*. Thus,

"...acquisition of nickel transporters and Ni-binding proteins by gastric *Helicobacter* species was a key event for the emergence of one of the most successful bacterial pathogens, *H. pylori*..." (Vinella et al. 2015; Fischer et al. 2016). Transporters (NixA, NiuBDE, NikABCDE and NikZOppBCDE), Ni-dependent enzymes (urease, hydrogenase) or Ni-binding proteins (Hpn and Hpn-2) were reported in all *Helicobacter* species (Vinella et al. 2015; Fischer et al. 2016).

2.1.1 HGT of Nickel-Binding Proteins Histidine-Rich Proteins

Histidine-rich proteins, Hpn and Hpn-2, are known to protect gastric *Helicobacter* species against nickel overload. They also accumulate intracellular nickel and store this nickel indirectly helping them to colonize the stomach of the host. Vinella et al. (2015) revealed that histidine-rich proteins (Hpn) emerged in the last common ancestor (LCA) of gastric *Helicobacter* species. Hpn and hpn-2 genes are specific to the gastric *Helicobacter* species and are not in enterohepatic species (Vinella et al. 2015). Hpn plays a major role in the protection of *H. pylori* against nickel overload and participates in the accumulation of intracellular nickel storage, while Hpn-2 is not required for both these functions (Fig. 2) (Vinella et al. 2015). Hpn interacts with

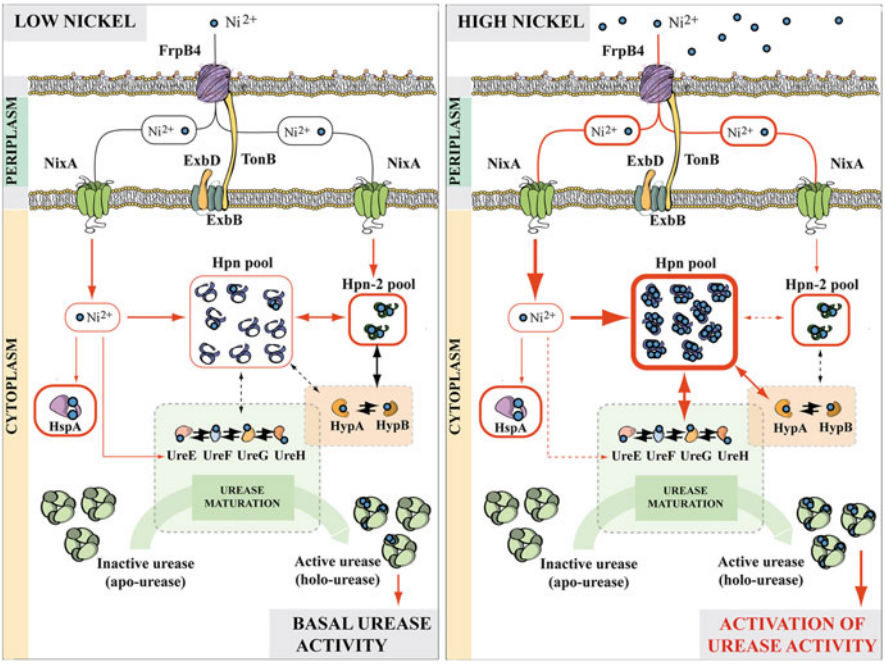

Fig. 2 Role of Hpn and Hpn-2 in nickel trafficking, protection against nickel overload, urease activity and colonization of the host stomach (Source: Vinella et al. 2015)

the UreA urease subunit, while Hpn and Hpn-2 interact with the HypAB hydrogenase maturation proteins (Fig. 2) (Vinella et al. 2015). Hpn and Hpn-2 are essential for colonization of gastric *Helicobacter* species in the host stomach (Vinella et al. 2015). Vinella et al. (2015) proved that *hpn* and *hpn* mutants of *H. pylori* were not able to colonize the stomach in the mouse model, whereas *hpn* and *hpn* mutants of *H. pylori* when complemented with wild genes were able to establish and colonize in the mouse model (Fig. 2). This allowed the *Helicobacter* gastric species to thrive in the stomach by protecting them against nickel overload, participating in the accumulation of intracellular nickel storage and colonization of the host stomach. Thus acquisition of Ni-binding proteins (Hpn and Hpn-2) via HGT followed by a ". . .decisive evolutionary event allowed the bacteria to adapt the human stomach a niche that no other bacterium colonized and helped in the emergence of *Helicobacter* species . . . ."

2.1.2 HGT of Nickel Transporters Genes

Emergence of Ni-binding proteins (Hpn and Hpn-2) in gastric *Helicobacter* species was further supported by HGT of nickel transporter genes NixA and NiuBDE. Gastric *Helicobacter* species were able to pick up nickel-binding proteins and nickel transporter genes via HGT and adapted itself to the gastric niche. Fischer et al. (2016) revealed that LCA of gastric *Helicobacter* species and *H. pylori* acquired Ni-binding proteins and nickel transporter genes via HGT to survive in the stomach (Fig. 3). The successful acquisition of nickel transporters genes NixA and NiuBDE via HGT allowed the bacteria to utilize nickel transporter genes for urease activity (nickel-dependent urease activity) by a decisive evolutionary event. This evolutionary event can be considered as a significant change in the genome of gastric *Helicobacter* species allowing the bacteria to adapt the human stomach a niche that no other bacterium colonized and helped in the emergence of *Helicobacter* species.

The key role of nickel transporter genes and Ni-binding proteins shows that nickel plays a very important role in the colonization of *H. pylori*. Campanale et al. (2014) carried out a pilot study by supplementing *H. pylori*-infected patients with the nickel-free diet for 1 month and found that the nickel-free diet was able to enhance the efficiency of eradication therapy. This study recommends nickel-free diets for those patients who are infected with *H. pylori*, and further clinical trial studies are also required to prove the safety of the diet.

2.2 *Macrodiversity in* H. pylori *and HGT*

Macrodiversity between *H. pylori* strains is due to intragenomic rearrangements like deletion, inversion, or translocation (Alm et al. 1999). *H. pylori* possess insertion sequences like IS605 and IS606 and several plasticity zones in strains like Hp26695

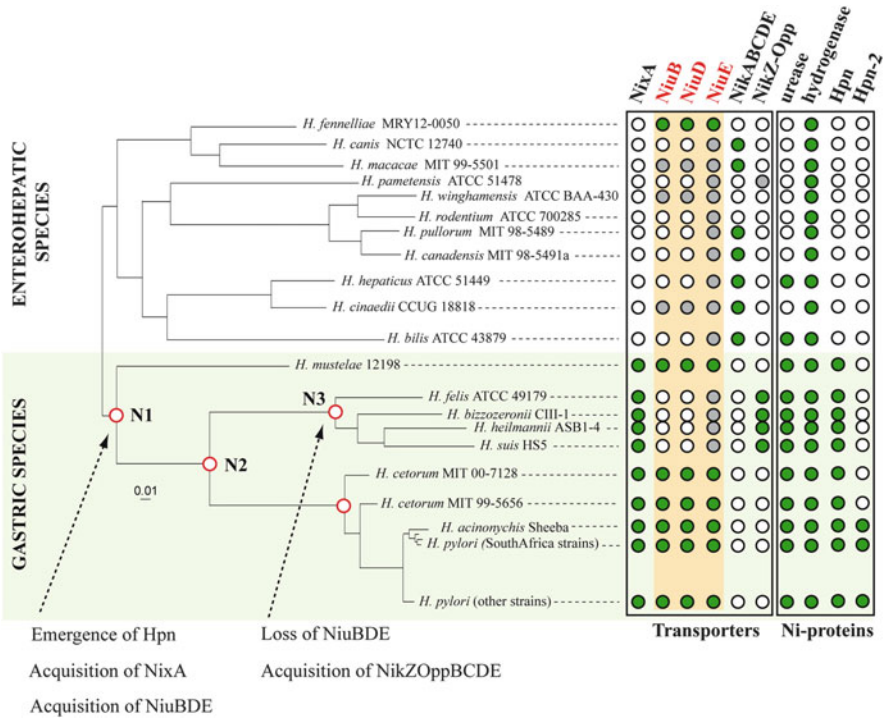

Fig. 3 Distribution, phylogeny and evolutionary history on acquisition of nickel transporter genes by gastric *Helicobacter* species (Source: Fischer et al. 2016)

and HpJ99. Plasticity zones are not limited to these *H. pylori* strains, but were also present and reported in other strains of *H. pylori*. These plasticity zones differ in GC content when compared to chromosomal GC content. For example, Hp26695 contains five plasticity zones with GC contents of 33% (zone 1), 35% (zone 2), 33% (zone 3), 43% (zone 4) and 33% (zone 5), which differ from the chromosomal GC content of 39% (Tomb et al. 1997). These plasticity zones in *H. pylori* might have been received via horizontal gene transfer. Conjugation and natural transformation are the mechanisms of HGT identified in *H. pylori*.

Nedenskov-Sorensen et al. (1990) first described the natural transformation in *H. pylori*, and several genes were identified in *H. pylori* which are acquired via transformation process (Schmitt et al. 1995; Hofreuter et al. 1998; Ando et al. 1999; Smeets et al. 2000). Natural transformation in *H. pylori* is mediated by type IV secretion system (Hofreuter et al. 2001). *H. pylori* encodes four T4SSs including cagPAI (mediates injection of CagA protein and induces proinflammatory signalling), comB (system involved in the uptake of DNA from the environment) and tfs3 and tfs4 genes (role not yet known). *H. pylori* also contain diverse genetic modules ". . .due to the modular structure, plasmids might either pick up chromosomal genes of *H. pylori* or integrate sequence modules from foreign plasmids, which are taken

up by the bacteria during its natural transformation competence (gene shuffling) leading to macrodiversity among *H. pylori* strains and rapid generation of substrains (Hofreuter and Haas 2002)...".

2.3 *Antibiotic Resistance in* H. pylori *and HGT*

H. pylori has developed antibiotic resistance to proton-pump inhibitors, clarithromycin, metronidazole, macrolide, amoxicillin, levofloxacin, etc., or combinations of them (Savarino et al. 1997; Bardhan et al. 2001; Torres et al. 2001; Osaki et al. 2006; Zullo et al. 2007; Ndip et al. 2008; Boyanova et al. 2009; Gao et al. 2010; Sun et al. 2010; Wüppenhorst et al. 2011; Bolor-Erdene et al. 2017; Lee et al. 2018). Multidrug resistance (MDR) or antibiotic resistance in *H. pylori* can be eradicated by identifying new or alternative drug targets, developing new drug combinations (Neelapu et al. 2013, 2015, 2016; Neelapu and Pavani 2013; Nammi et al. 2016, 2017; Pasupuleti et al. 2017) and using Chinese herbs (Huang et al. 2015). The new drug combinations developed for *H. pylori* in view of MDR are levofloxacin or moxifloxacin (novel class of fluoroquinolones) with amoxicillin, rifabutin and furazolidone. The Chinese herbs, namely, emodin, baicalin, schizandrin and berberine, can also be used to treat MDR in *H. pylori* (Huang et al. 2015).

Literature reports interspecies and intraspecies gene transfer of metronidazole and clarithromycin resistance between *Helicobacter* species (Table 1). Pot et al. (2001) proved interspecies transfer of antibiotic resistance genes between *H. pylori* and *Helicobacter acinonychis*. To prove these Kusters and group demonstrated that

Table 1 Antibiotic resistance genes metronidazole and clarithromycin transferred to *H. pylori* and *Helicobacter acinonychis* via HGT

S. no	Recipient strain	Donor DNA	Antibiotic resistance
1	*H. pylori* 26,695	*H. acinonychis* NCTC12686 MtzR	Metronidazole
2	*H. pylori* 26,695	*H. acinonychis* Sheeba MtzR	Metronidazole
3	*H. pylori* J99	*H. acinonychis* NCTC12686 MtzR	Metronidazole
4	*H. pylori* J99	*H. acinonychis* Sheeba MtzR	Metronidazole
5	*H. acinonychis* Sheeba MtzS	*H. pylori* 1061 MtzR	Metronidazole
6	*H. acinonychis* Sheeba MtzS	*H. pylori* NCTC11637	Metronidazole
7	*H. acinonychis* Sheeba MtzS	*H. pylori* pRdxA	Metronidazole
8	*H. acinonychis* Sheeba MtzS	*H. pylori* 1061 MtzR/ClaR	Clarithromycin
9	*H. acinonychis* Sheeba MtzS	23S rDNA PCR product of 1061 MtzR/ClaR	Clarithromycin
10	*H. acinonychis* NCTC12686 MtzS	*H. pylori* 1061 MtzR	Metronidazole
11	*H. acinonychis* NCTC12686 MtzS	*H. pylori* NCTC11637	Metronidazole
12	*H. acinonychis* NCTC12686 MtzS	*H. pylori* 1061 MtzR/ClaR	Clarithromycin

Source: Pot et al. (2001)

". . .*H. acinonychis* is competent for natural transformation and *H. pylori* can acquire antibiotic resistance by uptake of DNA (HGT) from other *Helicobacter* species and vice versa. . . ." (Pot et al. 2001). Pot et al. (2001) isolated DNA from *H. acinonychis* isolate NCTC12686 (NCTC12686 MtzR) and *H. acinonychis* isolate Sheeba (Sheeba MtzR) metronidazole-resistant strains. This isolated DNA was used for natural transformation of two metronidazole-sensitive *H. pylori* as per the standard protocol of Wang et al. (1993). Upon transformation metronidazole-resistant transformants were obtained for both *H. pylori* strains. Similarly, *H. acinonychis* strains were readily transformed to clarithromycin resistance strains by uptake of PCR product via natural transformation. The above two experiments demonstrate that bacterium like *H. pylori* can acquire antibiotic resistance genes like metronidazole and clarithromycin via HGT contributing to the antibiotic resistance of the pathogen *H. pylori*. This also shows that *H. pylori* naturally has a way to successfully infect the host even in the presence of harmful antibiotics.

3 Conclusion

Helicobacter pylori survives even in the vulnerable environments such as acidic, peristalsis, phagocytosis and oxidative stress. These stresses induce damage in pathogen DNA and *H. pylori* had acquired the ability to survive DNA damage by transformation-mediated recombination DNA repair. *H. pylori* is competent throughout the growth which may help in acquiring the genetic material via HGT from the surrounding environment and contribute to evolution and genetic diversity especially macro-diversity. *H. pylori* has acquired nickel-binding proteins (Hpn and Hpn-2) and nickel transporter genes (NixA and NiuBDE) via HGT which helped the pathogen to establish itself as gastric species during the course of evolution. This further helped the pathogen *H. pylori* to adapt itself and survive in the gastric niche. *H. pylori* also has the capability to acquire genes of antibiotic resistance (metronidazole and clarithromycin) in addition to antigenic determinants and virulence factors via HGT from other organisms to alter and influence pathogenicity. This review clearly reveals the role of horizontal gene transfer in gastric human pathogen *H. pylori* to adapt itself to the host.

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Authors Contribution CS and NNR initiated the review, participated in writing and revised the manuscript.

Conflict of Interest The authors declare that there is no potential conflict of interest.

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The *Rhizobiaceae* Bacteria Transferring Genes to Higher Plants

Martha-Helena Ramírez-Bahena, Alvaro Peix, and Encarna Velázquez

Abstract The family *Rhizobiaceae* includes several bacterial genera able to induce root or stem nodules, which can be beneficial for the plant, or hypertrophies, such as tumours, which cause plant damage. The members from genus *Agrobacterium* are well known by their ability to transfer genes to different plants originating tumours, and this feature has been biotechnologically exploited to produce transgenic plants. Nevertheless, the taxonomy and phylogeny of this genus has been confusing in the last decades after its reclassification into the genus *Rhizobium*. The presence of the telomerase-coding gene *telA* is a unique characteristic of the *Agrobacterium* clade, and it has been recently recovered as a separate genus. However, some tumour-inducing strains remain classified within genus *Rhizobium*, and some other species have been reclassified into genus *Allorhizobium*. The phylogenies of the virulence genes harboured by pTi plasmids inside or outside T-DNA are different as well as the symptoms induced in plants. In this chapter we revise the evolution of the taxonomy of tumorigenic species from family *Rhizobiaceae* over time, their interactions with different plants, the implications of horizontal gene transfer (HGT) in plant evolution and their use to obtain transgenic plants.

Keywords *Rhizobiaceae* · *Agrobacterium* · *Rhizobium* · *Allorhizobium* · HGT · Plants · Tumours · Hairy roots · Transgenic plants · Plant evolution

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1 Introduction

Genetic information can be transferred between different organisms by horizontal (or lateral) gene transfer (HGT) and the set of genes from an organism that can be transferred to other organisms is called mobiloma (Siefert 2009). HGT is particularly frequent between prokaryotes especially when they coexist in the same habitat, being currently recognized as a major evolutionary force (Lacroix and Citovsky 2016). Among *Rhizobiaceae* members, whose plant interaction abilities are linked to autoconjugative plasmids, chromids or genomic islands, HGT is a common event (Andrews et al. 2018). Also, to family *Rhizobiaceae* belong the most known bacteria able to transfer DNA (T-DNA) to plants, which constitute a mechanism of pathogenicity having been exploited for long to obtain transgenic plants (Gelvin 2017). Classically, *Rhizobiaceae* strains able to transfer T-DNA to plants were classified into genus *Agrobacterium*; nevertheless, currently there are strains classified into other genera, such as *Rhizobium* and *Allorhizobium*. Since the genes involved in HGT to plants are harboured in plasmids which can be transferred among species or genera, strains belonging to different genera can carry similar T-DNA regions. This implies that the genes harboured in pathogenicity plasmids cannot be used for taxonomic purposes. Therefore, currently, core genes harboured in chromosomes are used for the classification and identification of *Rhizobiaceae* species and genera and accessory genes harboured in mobile elements are used to study the phylogenies of virulence and T-DNA genes.

In this chapter we review the historical evolution of the taxonomy of bacteria belonging to the family *Rhizobiaceae* able to transfer genes to plants, the type of genes harboured by these bacteria and the relation to both taxonomic classification and the symptoms induced in plants, as well as the implications of HGT in plant evolution and their use to obtain transgenic plants.

2 The Taxonomic Evolution of *Rhizobiaceae* Bacteria Transferring Genes to Plants

The plant galls and the legume nodules were already described in the seventeenth century by Malpighi in his book *Anatomia Plantarum* published in 1679 (Malpighi 1679). Nevertheless, the bacteria responsible for both symptoms could not be isolated until the nineteenth century. The first isolation of a bacterium causing galls in young grapevines was reported by Fidriano Cavara (1897a, b), and Smith and Townsend (1907) proposed the name *Bacterium tumefaciens* for the bacteria isolated from different plant galls. The first isolation of a bacterium inducing nodules in faba bean was reported by Martinus Willem Beijerinck (1888) who named it *Bacillus radicicola* which was later reclassified as *Rhizobium leguminosarum* (Frank 1889).

The species *R. leguminosarum* was included in the family *Rhizobiaceae* created by Conn (1938), and years later this author also included in this family the new genus *Agrobacterium*, which encompassed the plant tumour-inducing species *Agrobacterium tumefaciens* and *Agrobacterium rubi*, the hairy root-inducing species *Agrobacterium rhizogenes* and the non-pathogenic species *Agrobacterium radiobacter* (Conn 1942). All these species names were included in the validation lists of Skerman et al. (1980) where *A. tumefaciens* was recorded as the type species of genus *Agrobacterium*.

When the first edition of the Bergey's Manual of Bacteriology was published in 1984, the most relevant changes in *Rhizobium* and *Agrobacterium* were the definition of biovars within some species of both genera. Three biovars were defined within *R. leguminosarum* (Jordan 1984), two biovars within *A. radiobacter* and three biovars within *A. tumefaciens*, presenting the species *A. rhizogenes* having the same characteristics that of the biovar 2 of this species (Kersters and De Ley 1984). Later, the biovar 3 of *A. tumefaciens* was assigned to a new species named *Agrobacterium vitis* on the basis of phenotypic characteristics and DNA-DNA hybridization (Ophel and Kerr 1990).

The most relevant change in the family *Rhizobiaceae* takes place several years after Woese's proposal (Woese 1987), when the *rrs* gene analysis was used for the reclassification of complete genus *Agrobacterium* into genus *Rhizobium* taking into account that some species of both genera are closely related and that the symptoms produced by these genera in plants are linked to the presence of different plasmids (Young et al. 2001). For example, the type strain of *A. rhizogenes*, which carry a plasmid involved in hairy roots inducing, is phylogenetically close to *R. tropici*, a species carrying symbiotic plasmids (Young et al. 2001).

Although the reclassification of the complete genus *Agrobacterium* into genus *Rhizobium* was controversial (Farrand et al. 2003; Young et al. 2003), as it was officially performed in the International Journal of Systematic Bacteriology (IJSEM), the species *Agrobacterium larrymoorei* (Bouzar and Jones 2001) was also reclassified into genus *Rhizobium* (Young 2004). After this date, only the species described outside IJSEM were named *Agrobacterium*, such as *Agrobacterium albertimagni* (Salmassi et al. 2002) and *Agrobacterium fabrum* (Lassalle et al. 2011), and those described in IJSEM as *Rhizobium*, such as *Rhizobium pusense* (Panday et al. 2011), *Rhizobium nepotum* (Puławska et al. 2012a) and *Rhizobium skierniewicense* (Puławska et al. 2012b).

One of these species, *A. fabrum*, was proposed after the genomic analysis of *Agrobacterium* strains from different species, which were distributed into genomic groups (genomovars) G1 to G9, G11, two unnamed genomic groups and G13 (Mougel et al. 2002; Costechareyre et al. 2009), and correspond to G8 (Lassalle et al. 2011). The G4 includes the type strains of *A. tumefaciens* and *A. radiobacter*, the G11 that of *A. rubi*, the G1 that of *A. rhizogenes* and the two unnamed groups include several strains of *A. larrymoorei* and *A. vitis* (Mougel et al. 2002). Later it was shown that the G2 includes *R. pusense* (Panday et al. 2011) and the new genomovar G14 includes the species *R. nepotum* (Puławska et al. 2012a). The remaining genomic groups contain strains isolated from different sources, and they have not been assigned to date to any *Agrobacterium* species.

The taxonomic status of *Agrobacterium* species changed again in 2015 when Mousavi et al. performed a revision of the family *Rhizobiaceae* and reclassified several of its species in different genera, one of them *Agrobacterium*, which remains a valid name without need of recover it, to which the species *R. pusense*, *R. nepotum* and *R. skierniewicense* were transferred as new combinations (Mousavi et al. 2015). These authors also transferred the species *Rhizobium vitis* (formerly *A. vitis*) to the old genus *Allorhizobium*, which also remains valid, as a new combination, and the species *Rhizobium rhizogenes* was maintained within the genus *Rhizobium* (Mousavi et al. 2015).

From year 2015 ahead, several new species of genus *Agrobacterium* isolated from plant tumours have been described (Table 1), such as *Agrobacterium arsenijevicii* (Kuzmanović et al. 2015) and *Agrobacterium rosae* (Kuzmanović

Table 1 Species currently included in the genus *Agrobacterium*

Species	Source of isolation	Pathogenicity symptoms	References
Genus *Agrobacterium*			
A. tumefaciens	*Malus* sp.	Tumours	Smith and Townsend (1907), Conn (1942)
A. rubi	*Rubus* sp.	Tumours	Hildebrand (1940), Starr and Weiss (1943)
A. larrymoorei	*Ficus benjamina*	Tumours	Bouzar and Jones (2001)
A. albertimagni	*Potamogeton pectinatus*	Not tested	Salmassi et al. (2002)
A. fabrum	*Prunus cerasus*	Tumours	Lassalle et al. (2011)
A. pusense	Rhizosphere of *Cicer arietinum*	Not tested	Panday et al. (2011), Mousavi et al. (2015)
A. nepotum	*Prunus*, *Vitis* and *Rubus*	Tumours	Puławska et al. (2012a), Mousavi et al. (2015)
A. skierniewicense	*Chrysanthemum*, *Prunus*	Tumours	Puławska et al. (2012b), Mousavi et al. (2015)
A. arsenijevicii	*Prunus* and *Rubus*	Tumours	Kuzmanović et al. (2015)
A. deltaense	*Sesbania cannabina* nodules	Not tested	Yan et al. (2017a)
A. salinitolerans	*Sesbania cannabina* nodules	Not tested	Yan et al. (2017b)
A. bohemicum	*Papaver somniferum*	No pathogenic	Zahradník et al. (2018)
A. rosae	*Rosa x hybrida*	Tumours	Kuzmanović et al. (2018b)
Genus *Allorhizobium*			
A. vitis	*Vitis vinifera*	Tumours	Ophel and Kerr (1990)
Genus *Rhizobium*			
R. rhizogenes	*Malus* sp.	Hairy roots	Riker et al. (1930), Conn (1942), Mousavi et al. (2015)
R. tumorigenes	*Rubus* sp.	Tumours	Kuzmanović et al. (2018a)
Genus *Parahizobium*			
P. polonicum	*Rubus* sp.	No pathogenic	Puławska et al. (2016)

et al. 2018b), but also some new species have been isolated from legume nodules, such as *Agrobacterium deltaense* (Yan et al. 2017a) and *Agrobacterium salinitolerans* (Yan et al. 2017b) or from other sources, such as *Agrobacterium bohemicum* (Zahradník et al. 2018). Moreover, a new species of genus *Rhizobium*, *Rhizobium tumorigenes*, which is able to produce tumours in different plants, was recently isolated from cane gall tumours on thornless blackberry (Kuzmanović et al. 2018a), and a non-pathogenic species isolated from *Prunus* tumours, *Pararhizobium polonicum*, has been classified within the genus *Pararhizobium* (Puławska et al. 2016).

3 Chromids in Family *Rhizobiaceae*

During the 1970s and 1980s, there was a great development in electrophoretic techniques since the first separation techniques of large DNA molecules were developed (Fangman 1978) and the keys of their electrophoretic mobility were explained (Lerman and Frisch 1982; Lumpkin and Zimm 1982). Thanks to the work of many scientists using *Saccharomyces cerevisiae* as a model (Mikus and Petes 1982; Carle and Olson 1984) and integrating new variants to conventional electrophoresis they became very quickly in highly resolutive techniques (Schwartz and Cantor 1984).

One of these techniques is known as pulsed-field gel electrophoresis (PFGE) (Schwartz and Cantor 1984) and more than 20 years later, due to its versatility and resolution power, it was still considered the "gold standard" for evaluating epidemiological relationships in most bacteria of clinical importance (Goering 2010). Even nowadays, despite the emergence of Whole Genome Sequencing (WGS) as an alternative in the field of the molecular epidemiological investigation (Lytsy et al. 2017), first of all, PFGE provides a very visual sense of global chromosome monitoring.

Using this technique Ferdows and Barbour (1989) examined the genome of *Borrelia burgdorferi*, the causative agent of Lyme disease revealing the existence of linear chromosomes in bacteria and breaking the paradigm that the chromosome structure of prokaryotes is constituted by a single circular chromosome that can be sometimes accompanied by extrachromosomal elements. Lin et al. (1993) revealed the presence of a linear chromosome also in *Streptomyces lividans*, and later it has been observed that the chromosomal architecture varies depending on the genera of Actinobacteria (Kirby 2011).

The chromosomal architecture was also studied in other bacteria as occurred in the case of the strain *A. tumefaciens* C58 whose genome was analysed with PFGE revealing the existence of four replicons, two of them large, of 3000 and 2100 kb, a 450-kb cryptic plasmid and the 200-kb Ti plasmid. Genes essential for the metabolism of this strain were detected in the larger replicons, which suggested their chromosomal nature. Using PFGE and X-rays to linearize the DNA, it was suggested

that the 3 Mb chromosome is circular whereas the 2.1 Mb chromosome is linear (Allardet-Servent et al. 1993).

The exhaustive analysis carried out on a large number of sequenced genomes currently available showed that all these extrachromosomal elements that had been considered as "secondary chromosomes" possess a set of characteristic properties that make them different from the primary chromosomes, besides that few of their genes are shared by distant species, although they harboured some essential genes for the bacterial metabolism. The fact that these "secondary chromosomes" have plasmid-type replication systems led Harrison et al. (2010) to propose the new term chromid, which represents a single class of elements that have a consistent set of properties different to those from both chromosomes and plasmids. They have characteristics typical of the chromosomes but also other typical of plasmids and carry both core and auxiliary genes (Harrison et al. 2010), but they could no longer be called chromosomes, so the idea of the existence of a single chromosome in bacteria is once again valid.

Thus, chromids are defined based on three main characteristics, which make them different from both chromosomes and plasmids. They have plasmid-type maintenance and replication systems, their nucleotide composition is close to that of the chromosome and they carry core genes that are found on the chromosomes in other species. Recently chromids have been divided into two types based on their specific plasmidic replication origin, belonging to either the iterons or repABC type (Fournes et al. 2018). To date the chromids have been found in a minority of bacteria, and their distribution seems to be linked to the phylogeny of the bacterial group where they are detected, as occurs in the case of the genus *Agrobacterium*, in which the presence of a linear chromid distinguishes it from other genera from family *Rhizobiaceae* (Ramírez-Bahena et al. 2014).

The maintenance of linear replicons in the bacterial cell, such as in the eukaryote, requires specific systems to protect the ends of the replicons (telomeres) against the activity of the nucleases, and to completely replicate the telomeres. It has been observed that in *Borrelia* spp. (Casjens 1999), *A. tumefaciens* C58 (Huang et al. 2012) and several phages (Ravin 2003), the telomeres form covalently closed hairpin loops presenting an uninterrupted DNA chain to the replication machinery. Replication through hairpin telomeres produces inverted repeat circular dimers, and the replicated intermediate is a substrate for a DNA breakage and an enzyme that releases the linear strands (Chaconas et al. 2001). This enzyme is a telomere resolvase (ResT) in *Borrelia* spp., a protelomerase TelN in the linear phage N15 and a protelomerase TelA in *A. tumefaciens* C58 (Ravin 2003; Slater et al. 2009; Huang et al. 2012).

The presence of linear chromids in *Agrobacterium* was verified using a variation of the PFGE technique (Ramírez-Bahena et al. 2012) that allowed the visualization of chromids in strains in which observation was not possible due to easy degradation of this genetic material. Ramírez-Bahena et al. (2014) showed the co-occurrence of a protelomerase and a linear chromosome in *Agrobacterium*. These authors detected the *telA* gene in all *Agrobacterium* strains that harbour a linear replicon, but not in other strains confirming the relationship between *TelA* and the presence of linear

replicons. But still, there is the question of whether the acquisition of *telA* is a unique event for a clade characterized by the presence of a linear chromid. This issue was addressed by investigating the evolution, radiation and maintenance of the linear chromosomal structure between the members of the *Agrobacterium* clade and the neighbouring genera (Ramírez-Bahena et al. 2014).

In the case of the family *Rhizobiaceae* the acquisition of a chromid can be considered a relevant characteristic because to date the *telA* gene is only found in species from the genus *Agrobacterium*. Within this family, apparently, a single event of acquiring a *telA* gene by an ancestor allowed the linearization of chromid and the maintenance of linear geometry in the progeny. Observing the great diversity of species of the genus *Agrobacterium* (defined on the basis of this characteristic) it highlights the evolutionary success associated with this unusual genome geometry and questions the real nature of the advantages that it confers (Ramírez-Bahena et al. 2014).

4 Large Plasmids in Family *Rhizobiaceae*

The plant tumours originated at the base of the stems and roots of plants are known as crown galls, and, as was previously mentioned, the first bacterium responsible for these tumours was named *A. tumefaciens*. Nevertheless, the mechanism by which this pathogen induced these tumours was discovered many years later because although many plants are susceptible to produce tumours after the inoculation with *A. tumefaciens*, they are only found naturally on woody plants (Kado 2010). As Kado widely explained in his review of 2014, initially three possibilities were managed as the cause of plant tumours, the production of chemical irritants, the production of phytohormones (auxines) or the production of a tumour-inducing principle, which initiate and promote the tumour growth, by *A. tumefaciens* (Kado 2014).

These three possible causes were deeply studied until the 1960s decade of the past century when several authors demonstrated that the induction of the crown gall involved the transference of genetic material from the bacteria to the plant cells (reviewed by Kado 2014). Kerr (1969) observed that the oncogenicity can be transferred among strains of *A. tumefaciens* inoculated in the same plant; however, the mechanism of this transference remained unknown until the 1970s decade when several authors demonstrated that the oncogenicity can be transferred from pathogenic strains to non-pathogenic ones (Kerr 1971; Hamilton and Chopan 1975; Van Larebeke et al. 1975) and Hamilton and Fall (1971) observed that pathogenic strains lost their virulence after several sub-cultures at high temperature (36 °C) or treating them with acridine orange (Lin and Kado 1977), indicating that the oncogenicity is linked to a plasmid, whose lost implies that of the virulence.

Although large and small plasmids were found in virulent and avirulent strains of *Agrobacterium* (Merlo and Nester 1977; Sheikholeslam et al. 1978), Chilton et al. (1977) found traces of plasmidic DNA from *A. tumefaciens* in crown galls. After the

results of these and other researchers in the 1970s decade, it was established that a large plasmid named pTi confers virulence to *A. tumefaciens* which represented the first case of HGT between two domains, Bacteria to Eukarya (Kado 2009). Later, in the 1980s decade, the existence of HGT to plants was also shown in the case of strains from the species *A. rhizogenes* which harbours a plasmid named pRi involved in the hairy roots induction (Chilton et al. 1982).

Also in the 1970s decade, it was proposed a relation between the presence of plasmids and the legume nodulation ability by *Rhizobium* because, as occurred in the case of *Agrobacterium*, the treatment with acridine orange leads to a loss of the nodulation ability (Higashi 1967; Dunican and Cannon 1971; Nuti et al. 1977; Żurkowski and Lorkiewicz 1979). In the case of *Rhizobium* it was also showed the transfer of nodulation ability from a nodulating strain to a non-nodulating mutant of *R. leguminosarum* associated with the transfer of a plasmid (Brewin et al. 1980). As in the case of *Agrobacterium*, after several decades of work, it was established that the genes involved in the legumes nodulation ability are located in large plasmids and the term pSym was proposed by Hooykaas et al. (1981) who reported that the pSym of *R. trifolii* determines host specificity and also controls other crucial steps in nodule formation and nitrogen fixation on clover. Nevertheless, in the case of rhizobia they do not need the existence of wounds in the plants to infect them as occurred in the case of *Agrobacterium* and the pSym is not transferred to the plant because the complete cells are introduced in the plant cells through a complex and highly regulated process (Schultze and Kondorosi 1998).

5 *Rhizobiaceae* Plasmids Involved in HGT to Plants

The first discovered plasmid encoding genes transferred from bacteria to plants was the pTi plasmid of *A. tumefaciens* (Zaenen et al. 1974), which is responsible for the crown gall tumours formation (Van Larebeke et al. 1974; Watson et al. 1975). These plasmids are conjugative and harbour genes related to the use of the unusual amino acids octopine and nopaline which are involved in the transference of this plasmid (Genetello et al. 1977). The tumour-inducing strains can carry octopine plasmids, as occurs with the type strain of *A. tumefaciens* (Koekman et al. 1979), or nopaline ones, as occurs in the case of *A. fabrum* C58 (Depicker et al. 1980). Both octopine and nopaline pTi plasmids contain a region named T-DNA which is transferred to the plant cell being covalently linked to the nuclear DNA (Chilton et al. 1980; Willmitzer et al. 1980; Bevan and Chilton 1982; Chilton et al. 1982).

The opine and nopaline synthases are encoded by genes located inside the T-DNA region (Depicker et al. 1982; Barker et al. 1983; Bevan et al. 1983; Joos et al. 1983), which also harboured genes involved in auxin and cytokinin synthesis (Akiyoshi et al. 1984; Van Onckelen et al. 1986). Nevertheless, other section of the pTi, located outside T-DNA, and named *vir* region is also required for virulence and it contains six operons organized into a regulon in the following order *vir*A, B, G, C, D and E (Rogowsky et al. 1990; Schrammeijer et al. 2000; Hattori et al. 2001). From

them, the *virB* operon proteins, required for oncogenesis, are associated with the membrane of *A. tumefaciens* forming a type of transport system belonging to the type IV of secretion system (Christie 1997; Christie et al. 2005) whose structural features have a high degree of conservation among them (Cascales and Christie 2003; Zechner et al. 2012).

The strains able to induce hairy roots from *A. rhizogenes* also belong to two types, the agropine strains which elicit roots containing agropine, mannopine, mannopinic acid, and agropinic acid and the mannopine strains eliciting roots containing only mannopine, mannopinic acid and agropinic acid (Petit et al. 1983). These strains carry two types of plasmids named pRi (Moore et al. 1979; White and Nester 1980), which also contains a T-DNA region with genes able to induce hairy root phenotype (Chilton et al. 1982; Spano et al. 1982; De Paolis et al. 1985; White et al. 1985; Cardarelli et al. 1987). Two regions, named TL-RNA and TR-DNA, were stably integrated in the plant genome (White et al. 1985), and they are present in agropine-type pRi plasmids which can induce root proliferation by two independent transformation mechanisms, one of them due to auxin biosynthesis related to TR-RNA and another equivalent to that induced by mannopine pRi plasmids related to the TL-RNA (Vilaine and Casse-Delbart 1987). The pRi plasmid also harboured *vir* genes outside T-DNA region involved in the virulence of *A. rhizogenes*, commonly included in the six transcriptional units *vir*A, B, C, D, E and G with nearly identical organization to that of *A. tumefaciens* (Liang et al. 1998; Moriguchi et al. 2001). In the case of *A. rhizogenes*, the carboxy terminus of GALLS protein contains a type IV secretion signal suggesting that it may be also transported to plant cells by the type IV secretion system (Hodges et al. 2006, 2009).

6 *Rhizobiaceae* Species, Plasmids and Plant Symptoms

At the time when the first species of genus *Agrobacterium* were described, it was common in Microbiology to link the genus and species concept with the symptoms originated in higher organisms. In this way the species of this genus producing different plant symptoms were classified into different species, for example *A. tumefaciens* and *A. rhizogenes*, whose type strains were able to produce tumours and hairy roots, respectively (Jordan and Allen 1974). When it was demonstrated that these abilities are related to different plasmid types, the pathogenicity of *Agrobacterium* strains has become secondary, since it was evident that the taxonomy cannot be based on extrachromosomal background. The application of this principle was the argument for the reclassification of genus *Agrobacterium* into genus *Rhizobium* in which also the ability to nodulate different legumes was no longer a criterion for species definition (Young et al. 2001). In this way, for more than a decade, the genus *Rhizobium* contained symbiotic and pathogenic species for plants which carry different plasmids. However, the only supporting point for this reclassification really was the phylogenetic position of the type strain of *A. rhizogenes*, which in 16S rRNA gene phylogenies was closer related to *Rhizobium* species than to *Agrobacterium* ones (Fig. 1).

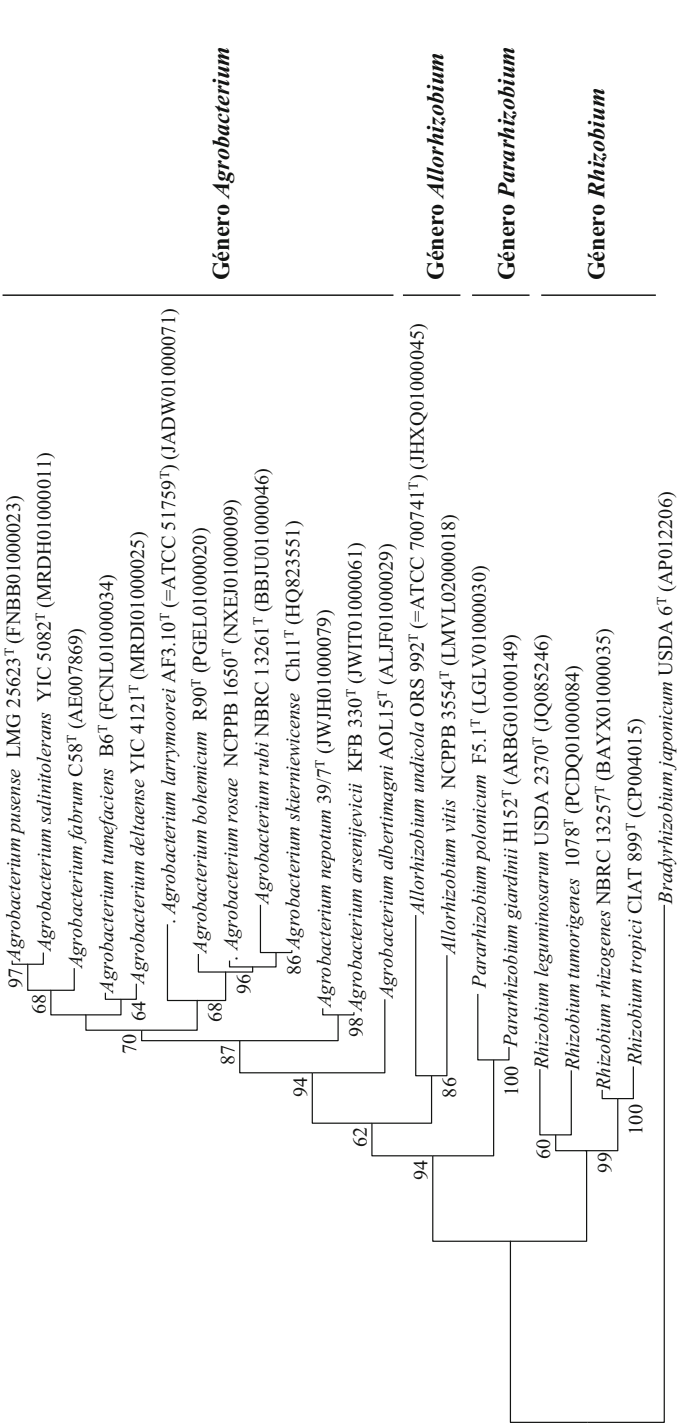

Fig. 1 Neighbour-joining phylogenetic tree based on the analysis of the complete sequences of the 16S rRNA gene showing the phylogenetic relationships of species from family *Rhizobiaceae* belonging to genus *Agrobacterium*, *Rhizobium*, *Allorhizobium* and *Pararhizobium*. Distance was calculated by the Kimura's two-parameter model. The significance of each branch is indicated by a bootstrap value (in percentage) calculated for 1000 subsets (only values higher than 50% are indicated). Bar, 1 substitution per 100 nucleotide positions

The species *A. rhizogenes* has revealed itself as a complex species that confused taxonomists for decades because the plant hypertrophies originated by the type strain of this species are linked to a pRi plasmid which has many regions similar to those found in the pTi of *A. tumefaciens* (Jouanin 1984) and the hairy roots were always considered harmful symptoms as the tumours produced by this species, whereas the legume nodules produced by *Rhizobium* are beneficial for these plants (Velázquez et al. 2017).

The analysis of housekeeping genes later confirmed that the type strain of the species *A. rhizogenes* belonged to the genus *Rhizobium* being closely related to the type strain of *R. tropici* (Velázquez et al. 2010), but the type strain of *A. rhizogenes* carry a pRi, whereas that of *R. tropici* carry a pSym. Currently, the species *A. rhizogenes* also contains non-pathogenic strains, such as K84, and some strains able to induce plant tumours, as occurs with the strains 163C and AKE10, which carry a pTi instead a pRi (Velázquez et al. 2010). For many years *R. rhizogenes* was the only species able to produce hairy roots or plant tumours which really belongs to genus *Rhizobium*; nevertheless, very recently other species named *Rhizobium tumorigenes* has been described by Kuzmanović et al. (2018a). This species, which was isolated from cane gall tumours on thornless blackberry (*Rubus* sp.), carries a pTi plasmid and is able to produce tumours in sunflower seedlings and kalanchoe plants (Kuzmanović et al. 2018a).

From the reclassification of several *Rhizobiaceae* genera by Mousavi et al. (2015), another classic rhizobial genus also contains tumourigenic species as occurs in the case of genus *Allorhizobium* whose species *Allorhizobium vitis* encompasses strains carrying octopine or nopaline pTi plasmids which originate tumours on grapevines (*Vitis vinifera*) and sunflowers (Ophel and Kerr 1990). Also, the new genus *Pararhizobium* proposed by Mousavi et al. (2015) contains a species, *Pararhizobium polonicum*, isolated from crown galls (Puławska et al. 2016). This species harboured genes similar to those found in other *Agrobacterium* species and carry a plasmid that does not contain the T-DNA region, although it harboured genes responsible for nopaline catabolism almost identical to those that were found on nopaline-type pTiC58 (Puławska et al. 2016).

The remaining species originating plant tumours belong to the genus *Agrobacterium* and carry different pTi plasmids. The type species of this genus is *A. tumefaciens* (Tindall 2014) and the type strain of this species is able to produce tumours on different plants such as tobacco, sunflowers and *Rubus*, but not on grapevines (Ophel and Kerr 1990). The type strain of the species *A. rubi* only formed tumours on *Rubus* (Ophel and Kerr 1990), the type strain of *A. larrymoorei* on *Ficus benjamina* (Bouzar and Jones 2001), the type strain of *A. rosae* on tomato and carrot disks (Kuzmanović et al. 2018b) and the strains of *A. skierniewicense* in chrysanthemum and *Prunus* (Puławska et al. 2012b). In the case of the species *A. nepotum*, one strain which is not the type strain of this species, was able to produce tumours in sunflowers (Puławska et al. 2012a). In the case of *A. arsenijevicii*, genes harboured in the pTi plasmid have been detected by PCR (Kuzmanović et al. 2015).

Other described species are non-pathogenic, as also occurs with the type strain of the old non-pathogenic species *A. radiobacter* (Tindall 2014), such as *A. bohemicum*

whose strains do not contain pTi or pRi plasmids after the whole sequence analysis, visualization of plasmids in agarose gels and amplification of virulence regions by PCR (Zahradník et al. 2018). Pathogenicity experiments have not been carried out in the case of species isolated from sources other than tumours such as *A. pusense* (Panday et al. 2011), *A. albertimagni* (Salmassi et al. 2002), *A. deltaense* (Yan et al. 2017a) and *A. salinitolerans* (Yan et al. 2017b).

7 *Rhizobiaceae* HGT Role in Plant Evolution and Obtention of Transgenic Plants

The T-DNA transfer to the plants from *Agrobacterium* strains that inhabit in natural environments commonly leads to the occurrence of different plant diseases such as tumours or hairy roots. However, the natural transfer to plants of *Agrobacterium* genes that can be vertically inherited has become evident after the analysis of genomes from different plants such as *Nicotiana*, *Linaria* and *Ipomoea* (Quispe-Huamanquispe et al. 2017). At the end of the past century, White et al. (1983) detected in *Nicotiana glauca* genome a region homologous to some regions of T-DNA (cT-DNA) from *Agrobacterium* and Horsch et al. (1984) showed that a kanamycine resistance gene was inherited by the progeny of *Nicotiana plumbaginifolia* cells transformed by a strain of *A. tumefaciens* in whose T-DNA this gene was introduced. More recently Intrieri and Buiatti (2001) found different genes of this bacterium in the genomes of 15 species of *Nicotiana* and concretely the *rol* genes phylogeny seem to be related with the *Nicotiana* evolution. This was confirmed analysing *Nicotiana tomentosiformis* and ancestral tobacco species whose genome contains four cT-DNAs derived from *Agrobacterium* strains (Chen et al. 2014), one of them highly expressed in roots of some tobacco cultivars which could be selected by nature or by tobacco growers (Chen et al. 2016). In the case of *Linaria* the *rolC* gene is the most conserved within cT-RNA genes, although none of them are expressed in the plant tissues (Matveeva et al. 2012). These authors reported that the species of *Agrobacterium* that transfers the genome of *Ipomoaea* contains two regions of *Agrobacterium*, *IbT*-DNA1 and *IbT*-DNA2, which in this case are expressed at detectable levels in the plants and are inheritable by vertical transmission (Kyndt et al. 2015). These authors reported that *A. rhizogenes* is the donor of the genes, but this species is currently named *Rhizobium rhizogenes* and therefore probably the genus *Agrobacterium* is not the main responsible of the permanent HGT to plants since most of T-DNA genes detected in plants to date come from a species of genus *Rhizobium*.

All these studies evidenced the presence of bacterial genes from T-DNA region of *Agrobacterium*/*Rhizobium* in plant genomes; nevertheless, to have effects in plant evolution the external genes should be inherited by the offspring, they must be integrated in the cell genome not being lost during successive cell division, the transformed cells must enter the germen line, and the integrated sequences must be

maintained during the evolution and, finally, the existence of phenotypes related to the presence of these genes (Quispe-Huamanquispe et al. 2017). They are expressed in low amounts in some plants but they do not develop hairy roots or tumours (Chen et al. 2014; Kyndt et al. 2015). It is not clear how the plants have precluded the expression of the T-DNA after its insertion in their genomes and several hypotheses have been considered, such as the insertion in a transcriptionally inactive region of the plant genome or to gene silencing after the insertion in transcriptionally active regions (Kyndt et al. 2015; Chen et al. 2016). In the case of the *IbT*-DNA genes and *Ipomoea batata,* it has been speculated that these genes have transferred an adaptative advantage to the plant (Kyndt et al. 2015). Nevertheless, the results obtained to date are preliminary, and further studies must be carried out to understand the role of HGT in plant evolution.

The ability of T-DNA to integrate into the plant genome has been also exploited for more than 30 years to obtain transgenic plants. At the beginning of the 1980s decade of the past century, Fraley et al. (1983) inserted bacterial genes conferring resistance to aminoglycoside antibiotics into a pTi of *A. tumefaciens* which was introduced in petunia and tobacco plants. In the same year, it was reported that the T-DNA region is flanked by identical direct repeated sequences in a nopaline pTi (Yadav et al. 1982), and 2 years later it was reported the existence of directly repeated 25 bp T-DNA border sequences in the nopaline pTi of strain C58 (Wang et al. 1984). These findings allowed during the 1980s decade the designing of different vectors to transform higher plants and rapid methodologies for this purpose (Herrera-Estrella et al. 1983; Murai et al. 1983; Bevan 1984; Deblaere et al. 1985; Fraley et al. 1985; Sheerman and Bevan 1988) that have continued later in order to replace the native T-DNA of *Agrobacterium* strains by sets of foreign genes to obtain transgenic plants (revised by Gelvin 2003, 2017).

The advances obtained in the 1980s decade allow the obtaining of transgenic plants, and at the end of the 1990s decade, more than 9000 field trials in the USA with different transgenic plants had already been carried out (Snow and Morán-Palma 1997). The main transgenic plants cultivated worldwide are soybean, maize, cotton and canola (James 2003). These transgenic crops can be resistant to insects or herbicides (Prado et al. 2014; Tabashnik and Carrière 2017; Schütte et al. 2017) and in 2017 the amount of hectares cultivated with these crops reached the 189.8 millions worldwide against the 160 million hectares cultivated in year 2011 (Clive 2011; ISAAA 2017). These data pointed out that the interest for the cultivation of transgenic plants is increasing in the last years despite the drawbacks that can have the use of GMO (Genetic Modified Organisms) for the man and the environment (Domingo 2016; Pellegrino et al. 2018).

The transformation methods based on *Agrobacterium* have been applied to several plants along the time, including legumes, cereals, vegetables, tropical plants, turf grasses, root plants, nuts and fruits, oil plants, woody plants, industrial plants and medicinal plants (Abiri et al. 2016). In the last years, strategies to improve the efficacy of transformation of monocotyledons, which have a low integration rate of *Agrobacterium* genes, have been developed (Anand et al. 2018), and many works focused on the transformation of medicinal plants in order to analyse its effect on the

secondary metabolites production (Abiri et al. 2016; Banerjee 2018). The enormous amount of works aiming the design of new methodologies and the obtaining of new transgenic plants in the last years highlights the interest in continuing to use the technology based on *Agrobacterium* for plant engineering.

8 Conclusions and Future Perspectives

Although within the family *Rhizobiaceae* several genera contain species able to transfer genes to plants, most of them belong to the genus *Agrobacterium*, which currently contains several species able to induce tumours in different plants and some ones whose pathogenicity has not been shown. The number of *Agrobacterium* species described has increased considerably in the current decade thanks to the new molecular tools, particularly the analysis of complete genomes. Since there are several genomovars still not assigned to an *Agrobacterium* species, it is expected that its number will continue to grow in the coming years and that more species of other genera able to induce plant tumours are discovered. The advances in genomic techniques also will allow to deepen in the knowledge of the HGT from *Rhizobiaceae* to plants and its involvement in the plant evolution as well as to design new methodologies using *Agrobacterium* as a tool for the obtaining of transgenic plants.

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Role of Horizontal Gene Transfer in Evolution of the Plant Genome

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Abstract Horizontal gene transfer (HGT) transmits genetic material across species via more than ten possible mechanisms in nature. HGT is well studied, and accumulated evidence shows that HGT has contributed to transfer of genetic material and the evolution of the plant genome. This evolution in the plant genome aids plants in adapting to changing environment conditions. In this chapter we focus on the mechanism of contacts between two species, vectors, and acquisition of genetic elements, and also discuss the possible impacts on the evolution of plants.

Keywords Horizontal gene transfer · Genome evolution · Adaptation · Plant genome · Genetic material · Donor · Recipient

1 Horizontal Gene Transfer: A Universal Phenomenon

Horizontal gene transfer (HGT) is widely identified as an important and significant tool in the evolution of the genome in eukaryotes (Babić et al. 2008). HGT is basically the process of transmitting genetic material within and among species of microbes and eukaryotes. HGT enables expanding the limitations of the pool of genes across species, thereby enhancing the genotypes and phenotypes of plants. HGT is often observed between organisms that are either in intimate relationship among themselves (mutualistic relationship) or have occasional cell–cell contacts between plants (parasitic relationship) (Bock 2010). HGT can also be termed as "the non-genealogical genetic material transmission within organisms" (Goldenfeld and

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Woese 2007). HGT assists with transferring genes and its functions from the host to the recipient and thus imparts evolutionary traits. Transfer of genes is, mostly, a parental phenomenon, that is, from parents to offspring. But in the case of HGT, genetic material is transferred/transmitted from the donors to hosts (recipients) that are evolutionarily and phylogenetically distant in relationship.

HGT is an universal phenomenon widely observed among bacteria, virus, fungi, and eukaryotes (Bansal and Meyer 2002). Earlier, HGT was reported as a frequent phenomenon in lower organisms (McGinty et al. 2011; Rolland et al. 2009; Hotopp 2011). Recently, HGT showed an upward trend in eukaryotes as numerous genome sequences are available. Within the fungal kingdom, HGT was observed among *Aspergillus* and *Podospora*; *Stagonospora* and *Pyrenophora*; and *Fusarium* and *Alternaria* (Slot and Rokas 2011; de Wit et al. 2011). In the animal kingdom, this phenomenon is observed between insects, nematodes, amoebae, and asexual animals (Fournier et al. 2009; Hotopp 2011; Scholl and Bird 2011; Mayer et al. 2011). Several reports exist on the transmission of genetic material from viruses, arthropods, fungi, nematodes, and protozoa to plants via HGT (Liu et al. 2010; Richardson and Palmer 2006). There are also reports on inter-kingdom transfers between fungus and aphids (Moran and Jarvik 2010), humans and bacteria (Anderson and Seifert 2011), and plants and silkworm (*Bombyx mori*) (Zhu et al. 2011).

Among the eukaryotes, unicellular eukaryotes generally experience maximum HGT because they can often engulf their prey, releasing the DNA near the nucleus, since they lack a sequestered germline that acts as a barrier among animals; and the apical meristems acts similarly in plants (Keeling and Palmer 2001; Andersson et al. 2003; Richards et al. 2003; Andersson 2005). Most of the foreign genetic material detected in the protists was acquired from donor bacteria (Doolittle 1998). HGT frequency in unicellular eukaryotes ranges from a single gene to several dozen genes, accounting for less than 1% of the genome (Andersson 2005). A virus/viroid HGT is rarely found in higher eukaryotes (animals, fungi, and plants) because bacteria are donors (Garcia-Vallve et al. 2000; Rosewich and Kistler 2000; Screen and Leger 2000; Intrieri and Buiatti 2001; Veronico et al. 2001; Watts et al. 2001; Wolf and Koonin 2001; Kondo et al. 2002; Zardoya et al. 2002; Hall et al. 2005).

2 Horizontal Gene Transfer in Plants and Plant Genome Evolution

HGT occurs as a result of frequent direct intimate contact in the midst of recipient and donor, by methods such as parasitism, symbiosis, epiphytism, endophytism, and grafting. Apart from these methods, gene transfer can also be accomplished with a vector bridge system similar to pollen, fungi, bacteria, viruses, viroids, plasmids, transposons, and insects. Figure 1 highlights various modes by which HGT takes place in plants. Among these, transposon-mediated HGT is considered as the most significant tool in generating genomic variation leading to evolution in plants (Gao et al. 2014). Reports suggest that plants exhibited germline transposable elements

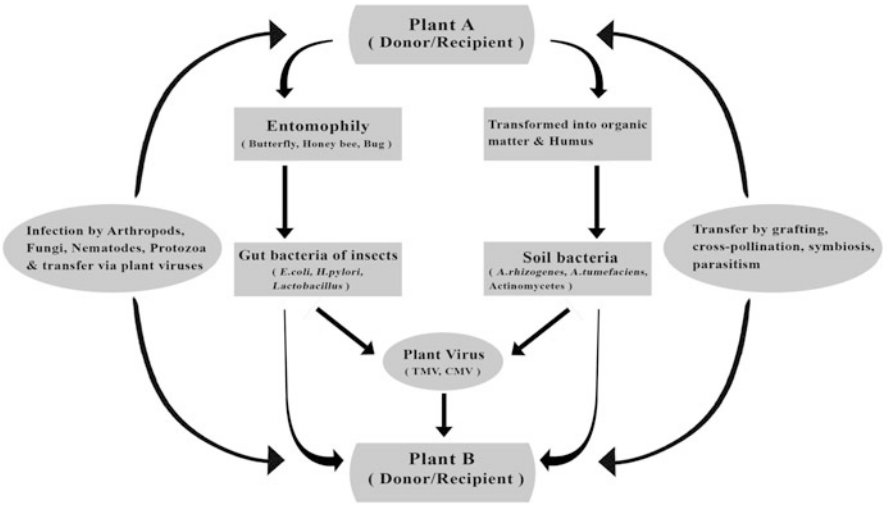

Fig. 1 Diverse modes of horizontal gene transfer (HGT) by other organisms in plants

that moved horizontally on numerous occasions. But, HGT occurrence is still relatively low when compared to lower eukaryotes and prokaryotes (Kidwell and Lisch 2001; Feschotte and Wessler 2002; Diao et al. 2005).

Earlier, HGT was considered as a rare and insignificant event in plants, except for symbiotic plants, but HGT has greatly contributed to the evolution of higher plants (Klasson et al. 2009; Yoshida et al. 2010). However, recent studies related to invertebrates and plants suggested the presence of prokaryotic genetic materials. Evolutionary studies reveal that all multicellular eukaryotes evolved from prokaryotes, also further corroborating the presence of ancient HGT in plants (Danchin et al. 2010; Moran and Jarvik 2010; Chirstin et al. 2012; Ni et al. 2012; Huang and Gogarten 2006). For example, early land plants facing acute/intense UV irradiation on the Earth surface might have had damaged DNA that hampered the normal cell cycle. Further, plants might have inherited these genes to repair the damaged DNA and survive. Evidence of DNA repair genes in land plants ascertains HGT occurred from bacteria to land plants in ancient times (Davidsen et al. 2004).

Land plants might have evolved from the aquatic to terrestrial landscape by acquiring traits through HGT. These traits allowed the plants not only to sustain abiotic stresses such as desiccation, nutrient limitations, heat, and cold, but also to survive against herbivory and microbial infections. Basically, the genes for these traits fall into gene classes such as plant defense and stress tolerance (Alcázar et al. 2010). The subtilase gene family present in plants shares identical homologues with the bacterial genome. It is proved that the sequences of these genes do not share homology with fungi or animals (Schaller et al. 2012). Phylogenetic analysis of the subtilase gene family in plants indicated that these genes might have undergone quick gene duplication after a unique HGT occurrence from bacteria. These subtilase genes aided plants by degrading protein in seeds and fruits, initiating lateral roots,

and forming stomatal patterns. Subtilases also helped the plants in water conduction, as well as in protection against desiccation and microbial infections in land plants. Similarly, genes related to polyamines were acquired via HGT. Polyamines, key compounds in plants, act in many processes such as stress response, signal transduction, cell proliferation, translational processes, cell differentiation, and structure development (Alcázar et al. 2010; Kusano et al. 2008; Neuteboom et al. 1999; Tanaka et al. 2001).

The genetic exchange partners of plants include both prokaryotes and eukaryotes from various kingdoms. At the same time, plants belong to both sides of the coin: that is, they act as both the donor and the recipient of horizontally mobilized genes. The bacterium *Agrobacterium* has donated functional genes by transfer of *Ti*-plasmid into the host plant genus *Nicotiana*, thus exemplifying a natural HGT mechanism (Intrieri and Buiatti 2001; Suzuki et al. 2002). Various other instances of bacterium to plant HGT include acquisition of aqua-glyceroporins from Eubacteria (1200 million years ago) (Zardoya et al. 2002) and glutathione biosynthesis genes from an α-proteobacterium (Copley and Dhillon 2002). The combined effect of transferred genes mostly depends on the genes that accumulate in a species. If the gene that is acquired by an organism is found to be beneficial, it is retained by the plant in subsequent generations. For example, 96 genes from 53 families found in *Physcomitrella patensa* showed sequence homology with seed/land plants and prokaryotes. Numerous gene transfers were also found from the bacterial kingdom to the Plantae in the course of evolution (Huang and Gogarten 2008; Price et al. 2012). These findings indicate that HGT is a dynamic process that leads to gradual accumulation of genes in the host plant system over a period of time. This phenomenon in plants is evidence of the fact that HGT is much wider and more continuous process than it has been hypothesized. HGT is also found to contribute diversely to various metabolic pathways, the development and regulation of higher land plants, and their chemical outputs. Various genes of bacterial families responsible for carbohydrate metabolism were found to have been transferred to the land plants via HGT. These genes are responsible for starch production, breakdown of cellulose, and seed fertilization, etc.

Certain evolutionary models also presume that weakly protected or completely unprotected zygotes in their life cycle are highly prone to HGT. It is evident that plants exhibit independent reduced gametophytes with acquired traits. Such traits are generally seen in nonvascular and vascular plants without seeds. Thus, it can be inferred that HGT through pollen grains from distantly related plants may have been deposited on the stigma of other plants, leading to the transfer of foreign genes to transform the zygote and the young embryo (Christin et al. 2012).

2.1 Importance of HGT in Plant Evolution

Evidence and reports are in favor of HGT as one of the possible methods for developing phylogenetic divergence in plants. These reports reveal that the plants

acquire genes via various HGT pathways (Gogarten et al. 2002; Lerat et al. 2005; Andersson 2005). Studies also reveal various possible bidirectional pathways involving HGT, not only between two groups but also within the groups, such as Archea–Eukaryotes, Bacteria–Eukaryotes, Eukaryotes–Bacteria, and even within Eukaryotes (Andersson et al. 2003; Watkins and Gray 2006; Guljamow et al. 2007; Nedelcu et al. 2008). The importance of HGT in plants and its impact on plant evolution depends on the number of genes that are acquired by the plant via HGT in the course of time. However, HGT will exert limited impact if only a few genes are transferred. In contrast to its role in prokaryotic evolution, in plant evolution HGT is considered to be less significant. One of the major factors for this hypothesis is that very few plant genomes have been completely sequenced and studied as compared with prokaryotes. This makes it difficult to determine the actual number of acquired genes over the course of time and their effect on the plant. However, the importance of HGT as a significant evolutionary force in plants cannot be underestimated with recent studies. These studies revealed the acquisition of novel genes from ancestors with mitochondrial and plastid origins (Hotopp et al. 2007). Seehausen (2004) showed that hybridization of animal and plant genomes is caused by HGT and also showed the immense impact on the evolution of both species.

Plant evolution is highly affected by the genes acquired from bacteria, fungi, and other eukaryotic genomes (Martin et al. 1993; Huang and Gogarten 2008). There have been attempts to fit in HGT with the neo-Darwinian principle of evolution. However, the literature is limited to genetic exchange between or within organisms in a single generation (Arber 2008). Once HGT is completed, the fate of transferred genes within the population is ascertained by natural selection, which is more of a Lamarckian principle (Goldenfeld and Woese 2007). Feder (2007) suggested that the existing genes change functions by modifying a single nucleotide. But, to add new genes with their functions, HGT uses other modes of gene transfers such as genome duplication and hybridization. These factors together account for the evolutionary changes that are often observed and challenge the neo-Darwinian principle (O'Malley and Boucher 2005; Koonin 2009).

2.2 Transferred Genes and Their Fate in Plants

Genes that are transferred via HGT are identified to be responsible for plant-specific metabolic and physiological functions. Genes responsible for carbohydrate metabolism (starch biosynthesis and cellulose degradation), and pollen and seed germination, etc., are from the bacterial genome. Similarly, the plant subtilase gene family is from a bacterial lineage (Schaller et al. 2012). Yue et al. (2012) have identified that genes responsible for plant polyamines and hormone synthesis are from bacteria and other organisms. Another such example of successful HGT in plants is regarding two gene families that encode for acyl-activating enzyme 18 (AAE18) and YUCCA flavin monooxygenase (YUC_3) enzymes. These gene families are also obtained from

organisms such as Planctomycetes, Verrucomicrobia, and the CFB group of bacteria (Cheng et al. 2006; Wiszniewski et al. 2009). Similar evidence shows that horizontally transferred genes have a major role in the evolution and sustenance of land plants in the changing environment. The golden rule for determining the successful expression of the transferred genes is by studying the phenotypic traits related to the genes. Changes in the phenotypes of *Nicotiana* and *Ipomea batatas* from incorporation of rol genes from *Agrobacterium* led to induction of the hairy root system in both plants. The genes majorly responsible for the hairy root system are the rolA, rolB, and rolC suite genes (Matveeva et al. 2012; Kyndt et al. 2015; Maurel et al. 1991). The changes in the phenotype of the plant might vary and can result from multiple factors including the transferred genes (Quispe-Huamanquispe et al. 2017).

2.3 HGT in Plant Evolutionary Scenario

Stable incorporation and expression of foreign gene in the recipient plant must fulfill four major criteria (Huang 2013; Lacroix and Citovsky 2016):

- Transfer into the recipient cell and integration with its genome.
- The transferred DNA should be stable enough and not lost during the recipient cell division process.
- The transformed recipient cell must become a part of its own germline.
- The integrated genome, if proved to be beneficial for the recipient, must be able to preserve itself in the course of evolution.

It is generally presumed that plants with unguarded or weakly protected zygotes are more vulnerable to HGT, as is often seen in nonvascular and seedless vascular plants. In such plants HGT occurs via pollen grains from even distantly related plants. Then, transfer to the DNA in the foreign pollen transforms the zygote and thus the young embryo (Christin et al. 2012).

Land plants show massive structural diversity and complexity, which can be attributed to the ancient genes acquired through HGT from various lineages. These events impart to land plants the capacity to synthesize various biochemical products as an end product of their metabolic pathways to carry out complex functions. The most important class of genes that are acquired by the plants via HGT are subtilase and the polyamine-synthesizing gene family (Neuteboom et al. 1999; Tanaka et al. 2001; Zhao et al. 2000; Alcázar et al. 2010; Kusano et al. 2008). These acquired traits acted in the growth and development of the plants by imparting tolerance against both biotic and abiotic stresses. Primitive land plants faced intense UV stress from the atmosphere, which must have caused DNA damage. However, evolutionary studies suggest the uptake of bacterial DNA repair-related gene families as are found in modern land plants. These genes must have helped the plants to survive the intense atmospheric stresses (Davidsen et al. 2004). These findings suggest predominantly that HGT is a driving process that leads to incorporation, accumulation, and expression of acquired genes in the recipient plants during the course of evolution.

These studies also proved that primitive gene transfer is a rather more recurrent phenomenon than it was thought to be. The combined effects of these genes rely greatly on the number of genes that are acquired by the plant in the course of evolution, depending on the usefulness of the genes to the plant (Huang and Gogarten 2008; Price et al. 2012).

Although we are still at a very early stage of understanding the intricate details of gene transfer across the plant kingdom, rendering them various complex functionalities, it is evident that the gene transfer networks should be analyzed to understand the actual evolutionary process. This stage can be achieved by genotype cluster analysis of the plant genomes from an evolutionary dimension. It was also observed that these horizontally transferred genomes among and within lineages are highly dependent on the habitat and ecology of the donor and recipient concerned. The actual mechanism by which this event occurs can further be determined by analyzing the cluster. This analysis requires taking into consideration the combined effect of genetic recombination and the rate of either positive or negative selection. The use of techniques such as CRISPR (clustered regularly interspaced short palindromic repeats) or whole-genome polymerase chain reaction (PCR) will shed more light on plant evolution.

3 Different Mechanisms of Horizontal Gene Transfer in Plants

The mechanism of HGT is broadly differentiated into three categories based on the type of genetic material involved: organular transfers, germline DNA transfers, or RNA transfers. Thyssen et al. (2012) reported various past instances of HGT from the first category, organular transfers (gene transfers from/to organelles). Organelles were responsible for HGT in tobacco varieties *Nicotiana sylvestris* and *Nicotiana tabacum* when both plants were grafted. It was also recorded that sexually incompatible plants such as oak and birch showed organelle-based HGT (Stegemann et al. 2012). Apart from these, organelle-to-nucleus HGT is also reported between mitochondria and nucleus among single plant species of *Arabidopsis* (Archibald and Richards 2010; Bonen 2006). Wang et al. (2012) also reported a chloroplast–nucleus transfer in tobacco.

Among several reports on the second category of HGT, DNA transfers, *Agrobacterium* is known to actively transfer nuclear DNA to the host. *Agrobacterium* transfers its T-DNA into the plant cell and subsequently transmits these traits to future generations via plant cell division (Neelapu et al. 2018). The T-DNA of *Agrobacterium* is used for HGT to some tobacco plants, and is assumed to be a mikimospine strain of *Agrobacterium rhizogenes* (Matveeva and Lutova 2014). In plants, however, HGT is a much more complicated process: it occurs either as a direct pathway or a vector-mediated pathway. Direct plant–plant DNA transfer uses several methods such as grafting, symbiosis, parasitism, pathogenesis, epiphytism,

and endophytism, involving direct contact between the partners participating in HGT (Yoshida et al. 2010; Stegemann and Bock 2009; Bukhalid et al. 2002). Vector-mediated gene transfer uses pollen, fungi, bacteria, viruses, transposons, insects, etc. as vectors not involving any direct contact between HGT partners (Capy et al. 2008; Trobridge 2009; Danchin et al. 2010). The third category type of gene transfer is RNA transfer, transfers of RNAs from viruses to host. Genes from RNA viruses were identified in the plant genetic material as evidence of RNA transfers via HGT. Double-stranded RNA polymerase genes from RNA viruses such as totivirus and partitivirus were reported in the plant genome (Liu et al. 2010). Prokaryotes use such methods as transformation, transduction, and conjugation for HGT of genetic material.

3.1 DNA Transfer Between Plastids and Nuclear Genomes

Other than the nucleus, plant DNA is also found in the mitochondria and chloroplasts. This organelle DNA in plants provides three distinct modes of intracellular HGT: HGT from mitochondria to nucleus, HGT from chloroplast to nucleus, and HGT from mitochondria to chloroplast. These HGTs are also bidirectional. It is postulated that a plastid has become a part of the plant cell by endosymbiosis, and the genetic material by means of HGT has become beneficial to the plants during the endosymbiotic period (Burger and Lang 2003; Andersson 2005; Thyssen et al. 2012). The exchange of genetic sequence between plastids and nucleus is often seen as a common phenomenon that enables the host nuclear genome in the cell to control the organular genetic activity. It was reported that these types of HGTs induced stress tolerance in both *Oryza sativa* and *Arabidopsis* spp. (Cullis et al. 2008).

A similar genetic exchange is seen between the two organelles (mitochondria and chloroplast) as envisaged with the nucleus. The mitochondrial genome is believed to be highly adaptive toward HGT when compared to nuclear and other organular genomes in plants (Woodson and Chory 2008; Archibald and Richards 2010). HGT has rendered the mitochondrial genes both native as well as foreign. For instance, there is evidence that 13.4% of the mitochondrial genome is homologous with the nuclear genome in rice (Notsu et al. 2002). At the same time, the genome of *Arabidopsis* mitochondria contains sequences that include 16 foreign plastid genes, 41 gene sequences from nuclear or retro-transposons, and 2 fungal or viral gene fragments (Marienfeld et al. 1999). The organelle–nucleus HGT process and vice versa is a continuing evolutionary event that contributes greatly to plant evolution. Earlier this was considered to be a fundamental process of endosymbiotic events (Giulotto et al. 2010; Timmis et al. 2004). These reports suggest that most of the genes, with their functions from ancestral organelles, might have been gradually incorporated into the plant genetic material (Brennicke et al. 1993; Bonen 2006; Thyssen et al. 2012).

3.2 Direct Plant-to-Plant HGT

Direct or intimate contact between two plants results in direct HGT between plants (Bock 2010). The method involves techniques such as grafting or parasitism wherein the plant acts both as donor and as host of HGTs. Grafting is a very important method in direct HGT between plants, involving cell-to-cell contact between two distinct plant varieties. During grafting, the rootstock and scion of the plants come in direct contact with each other, followed by development of various competent calli at the site of grafting. Competent cells/calli which are generated from the union of the rootstock and scion enable transfer of native genome and/or organellar DNA fragments and/or mRNA and/or the plastids. Transfer of genetic material from one plant to another takes place through the plasmodesmata or vesicular transport channels (Mower et al. 2010; Archibald and Richards 2010; Feschotte et al. 2010; Liang et al. 2012; Roney et al. 2007; Bock 2010). This transfer of genes results in integration of the transferred genes onto the nuclear or organellar genome of either the scion plant or the rootstock plant. This process enables the transfer of huge amounts of genetic traits even among plants distantly related with one another (Bock 2010; Stegemann et al. 2012; Thyssen et al. 2012). These genetic materials, after incorporation into the host plant, give rise to chimeric genes, but the expression of the acquired traits in the subsequent generations of the host plants is still questionable. Direct HGT is believed to be responsible for transfer of genes and transposable elements, and sometimes the whole chloroplast genome between different species that are sexually active (Stegemann et al. 2012; Stegemann and Bock 2009).

The second method for direct gene transfer between plants is a parasitic relationship of the host plant with its donors (bacteria, fungi, insects, or even other parasitic plants). Transfer of genetic material takes place via the plasmodesmata channels of the host plant and acts as a cell-to-cell communication pathway (Lucas et al. 2009; Zambryski and Crawford 2000). It is documented that HGT exists between 4000 parasitic plants and their host plants (Davis et al. 2005; Yoshida et al. 2010; Mower et al. 2004; Davis and Wurdack 2004). Parasitic plants contain a specialized organ such as the haustoria found in *Cuscuta* sp. for insertion of genetic material into the vascular bundles of the host plants. This penetrative organ of the parasite plant establishes a permanent contact with the host plant, resulting in its connection to the plasmodesmata of the surface cells. This contact facilitates the exchange of large amounts of genetic material between the two plants via the apoptotic cells of each in a mutualistic trend. It is also documented that mRNA transfer is facilitated in HGT, thus enabling the transfer of functions of one plant to become incorporated into the other (Roney et al. 2007). One such recorded instance is a nuclear monocot gene transferred into the genome of the parasitic plant *Striga hermonthica* (Yoshida et al. 2010). Phylogenetic evidence about host–parasite HGT is found between plants of the order Malpighiales and its parasite of the Rafflesiaceae family (endophytic parasite) (Davis and Wurdack 2004). Evidence of HGT between two nonflowering plants of the family Orobanchaceae was reported. A plastid gene from *Orobanche* (broomrape) was horizontally transferred to a closely related plant of the genus

Phelipanche (Park et al. 2007). Even after these reports of direct HGTs between plants; there is still lack of substantial proof regarding successful incorporation of foreign genetic material in the host as it may have been discarded during the cell division in the host plant. On successful integration, however, these foreign genes do facilitate the expression of integrated genes and bring about a notable change in the characteristics of the plant during the course of evolution.

3.3 Vector-Mediated HGT Between Plants and Other Genomes

HGT in plants occurs between plant and other genomes mediated by various other possible vector systems such as viruses, bacteria (*Agrobacterium* spp., *Escherichia coli*), insects, fungi, pollen, nematodes, and transposable elements (Broothaerts et al. 2005; Fortune et al. 2008; Bergthorsson et al. 2003; Mayer et al. 2011). The genes acquired via HGT are listed in Table 1.

3.3.1 HGT in Plants Via Viruses

The transfer of genetic material from host to virus is also quite common (Liu et al. 2010). HGT is often seen with retroviruses and a few DNA viruses; and HGT also occurs between virus and plant genome. HGT includes transfer of genetic information from fungi, bacteria, and various higher eukaryotic genomes to plants via viruses. Viruses such as tobacco mosaic virus (TMV), cucumber mosaic virus (CMV), and *Pithovirus* are extremely potent vectors for the transfer of genetic information. These viruses have high potential to capture genes from the host genome and transfer these to the recipient genome. This mechanism of the virus allows capturing the host genome, which eventually becomes integrated into their own genome. Later, when the same virus infects other organisms, for example, a plant parasite/insect, this enables HGT from one species to another. Various virus-mediated pathways of HGT are proposed in plants: nematode–virus–plant, nematode–virus–insect–plant, insect–virus–plant, plant–virus–aphid–plant, etc. (Ogata et al. 2007).

3.3.2 HGT in Plants Via Bacteria

Bacteria such as *Agrobacterium tumefaciens*, *Agrobacterium rhizogenes*, and *Escherichia coli* commonly possess a vector system similar to that of plasmids that is widely used for HGT in plants. These bacteria are widely used as a tool for developing transgenics by transferring genes from other plant species to the bacterial genome. There is evidence for transfer of naked DNA from wilted transplastomic

Table 1 List of candidate genes transferred via horizontal gene transfer (HGT) in plants

Accession number	Transferred gene	Donor	Recipient	Gene function	No. of amino acids in protein	Gene location	Reference
KPQ15043	*GCN5-related N-actyltransferase*	β,γ-Probacteria	*Oryza sativa*	Arginine biosynthesis	78	Cytosol	Peng et al. (2016)
XP_001702190	*Glycyl-tRNA synthetase*	Bacteria	*Oryza sativa, Arabidopsis thaliana*	Translation	695	Plastid/ mitochondria	Freist et al. (1996)
AAB04021	*Dihydrodipicolinate synthase (dapA)*	γ-Probacteria	*Coix lacryma-jobi*	Lysine biosynthesis	377	Plastid	Dante et al. (1999)
ABF98201	*ThiC family protein*	Bacteria	*Oryza sativa japonica*	Thiamine biosynthesis	639	Plastid	Buell et al. (2005)
AAC49669	*Polyribonucleotide phosphorylase (PNPase)*	*Chlamydomonas reinhardtii*	*Spinacia oleracea* (spinach)	RNA degradation	822	Plastid	Hayes et al. (1996)
CAA44054	*ATP/ADP translocase*	Chlamydiae	*Solanum tuberosum*	ATP/ADP transport	386	Mitochondria	Emmermann et al. (1991)
AAC49624	*MGDG synthase*	*Escherichia coli, Bacillus subtilis*	*Cucumis sativus* (cucumber)	Lipid biosynthesis	525	Plastid	Shimojima et al. (1997)
XP_003564370	*rps2*	*Brachypodium distachyon*	Actinidia	Disease resistance	910	Mitochondria	Bergthorsson et al. (2003)
YP_006666125	*atp1*	Eudicot	*Malus domestica*	ATP synthesis	510	Mitochondria	Goremykin et al. (2012)
NP_188714	*Topoisomerase 6 subunit B*	Crenarchaea	*Arabidopsis thaliana*	Protein binding	670	Cytosol	Salanoubat et al. (2000)
AAA33897	*Alpha amylase*	*Bacillus* sp.	*Oryza sativa*	Carbohydrate metabolism	438	Plastid	Mitsui et al. (1996)
AGB96853	*Sodium hydrogen antiporter*	Unknown	*Harrisia earlei*	Ion transport	14	Plastid	Franck et al. (2013)

(continued)

Table 1 (continued)

Accession number	Transferred gene	Donor	Recipient	Gene function	No. of amino acids in protein	Gene location	Reference
NP_001140902	*3-Dehydroquinate synthase*	β,γ-Probacteria	*Zea mays*	Amino acid biosynthesis	440	Plastid	Schnable et al. (2009)
AAA33134	*Aspartate aminotransferase(AAT)*	*Escherichia coli*	*Daucus carota*	Nitrogen and carbon metabolism	405	Plastid	Turano et al. (1992)
ABY56788	*Uroporphyrinogen-III synthase*	Bacteria	*Hordeum vulgare*	Porphyrin binding	299	Plastid	Ayliffe et al. (2009)
EMS46157	*Leucyl-tRNA synthetase*	Bacteria	*Triticum urartu*	Translation	919	Plastid/ mitochondria	Ling et al. (2013)
PWZ31609	*Diaminopimelate decarboxylase (lysA)*	Bacteria	*Zea mays*	Lysine biosynthesis	486	Plastid	Sun et al. (2018)
OVA00275	*Semialdehyde dehydrogenase*	μ-Probacteria	*Macleaya cordata*	Amino acid metabolism	379	Cytosol	Liu et al. (2017)
OVA18201	*HAD-superfamily hydrolase*	*Trichuris trichiura, E. coli*	*Macleaya cordata*	Cold stress response	387	Unknown	Liu et al. (2017)
CAO78893	*Allantoate amido hydrolase*	Bacteria	*Glycine max, A. thaliana*	Purine degradation	483	Unknown	Werner et al. (2008)
AYM55281	*Secreted in xylem (SIX gene)*	*Fusarium oxysporum*	*Musa* sp.	Promotes virulence, effector protein	140	Cytosol	Czislowski et al. (2018)
APP91304	*Secreted in xylem (SIX gene)*	*Fusarium oxysporum*	*Solanum lycopersicum*	Promotes virulence, effector protein	79	Root	Schmidt et al. (2013)

tobacco plants to the bacterial genome (Pontiroli et al. 2009). These bacteria can then transform a new plant species using a bacterial transformation method or other transgenic techniques (Schlüter et al. 1995; Broothaerts et al. 2005; Richards et al. 2006). HGT via *Agrobacterium* T-DNA requires a specific criterion to be fulfilled before the acquired gene is transferred to the host plant. The gene is structurally modified in context to its expression system. The structural modification avoids rapid cell division inside the plant, which can otherwise be highly detrimental to the host (Quispe-Huamanquispe et al. 2017). The probable reason for structural modification is that the repertoire of the donor species (bacteria) and the recipient host (plant) have comprehensibly widened in the course of evolution (Lacroix and Citovsky 2018).

3.3.3 HGT in Plants Via Fungi

Fungi, similar to bacteria and viruses, have the ability to infect other organisms and enable HGT between the host and recipient. Mycoviruses (viruses infecting fungi) can infect plant fungal pathogens or endophytic fungi that are in physical relationship or a close association with plants. Thus, mycoviruses have a chance for HGT from fungi to plants. Recently, the stable replication and integration of the genome of two viruses, *Penicillium aurantiogriseum totivirus I* and *P. aurantiogriseum partiti virus I*, in the hybrid protoplast obtained from crossing *Nicotiana benthamiana* and *N. tabacum* was documented (Nerva et al. 2017). There is also evidence for HGT via fungi in *Oryza sativa*, where genes from the fungus are transferred to a plant (Richards et al. 2009). The co-infection of plant pathogen *Rhizoctonia solani* by the cucumber mosaic virus (CMV) from potato along with *N. benthamiana* suggests a possibility and evidence for virus transmitting genetic material across the living kingdom (Andika et al. 2017). In reverse, in HGT involving transfer of genome from plant to fungi, the acquired genetic material is transferred to another plant species from the transformed fungi (Roossinck 2019). This possible HGT pathway involves the plant–fungus–insects–plant mode, wherein the insects can be either aphids or nematodes. The shikimate pathway in many land plants is responsible for biosynthesis of aromatic compounds such as alkaloids and carbohydrate metabolism. It is believed that this pathway is integrated via HGT from symbiotic fungi (Richards et al. 2006; Gribaldo et al. 2009).

3.3.4 HGT in Plants Via Insects

Another mode of HGT observed and reported in plants is via insects. Insects act as a vector for HGT, and the most common example for insect vector HGT is bees. Bees not only carry pollen from one plant to another but also induce HGTs between related, and even between distantly related plant species. Bees also might carry bacteria in their gut, which might be carrying genomes of other species. These bacteria may promote HGT either by transferring their own genome or the acquired

genomes from other species when coming in contact with a host plant (Mohr and Tebbe 2007). Other instances involve evidence for the presence of a cellulase gene in nematodes such as *Brugia*, *Meloidogyne*, *Bursaphelenchus*, and *Pristionchus*. This gene is homologous to the genes present in bacteria, fungi, plants, and other nematode species. Similarly, carotenoids, the colored pigments mainly produced by plants, fungi, and other microbes, are utilized by herbivores for either control of oxidation and/or light detection. But, ironically, the carotenoid biosynthesis genes that have been detected in pea aphids bear a resemblance to fungal carotenoid genes. These genes help them in combating natural enemies by displaying a red–green color polymorphism. This observation indicates the initiation of HGT following a plant–fungi–aphid pathway (Moran and Jarvik 2010). These nematodes can then transfer the acquired genes to other species of plants. The limiting factor in this method, however, is that there is a lack of evidence regarding the functionality and evolutionary efficacy of these inherited genes and also regarding the mechanism which favors such processes (Mayer et al. 2011).

3.3.5 HGT in Plants Via Transposons

Transposable elements (TEs) have a characteristic attribute to muster, multiply, and amalgamate into plant genomic DNA (Diao et al. 2011; Novick et al. 2010; Roulin et al. 2009; Sormacheva et al. 2012; Capy et al. 2008). These TEs or 'jumping genes' are basically fragments of DNA that inherit the capacity to jump from one chromosome to another. There are two basic types of TEs, based on their transposition mechanism. The class I TE, also known as retrotransposons, requires a RNA intermediate for their transposition. They follow the copy–paste module wherein they are reverse transcribed before integration into the recipient genome, enabling them to multiply and increase their copy genome to be active. The class II TEs, also known as transposons, follow a cut-and-paste mechanism. Rather than being reverse transcribed, these detach from the native chromosomal locus and are reintegrated into another locus (Kazazian 2004; Serrato-Capuchina and Matute 2018). Both classes of TEs act as free molecules in their native plant cell before transposition: this actually increases their chances of merging into any vector genome and being transposed to other genomes. Some TEs have an envelope-like coding domain which makes them structurally similar to retroviruses (Piskurek and Okada 2007; Laten et al. 2003; Fortune et al. 2008). HGT is reported in *Oryza* sp. for LTR retrotransposons RIRE1 (Roulin et al. 2008). The other instance of HGT is μ-like transposons that are transferred between *Setaria* (millet) and rice (Diao et al. 2005, 2011). The frequency of transfer of retrotransposons is found to be higher than that of transposons (Silva et al. 2004).

HGT via transposons or TEs is one of the most important modes for transfer of genetic information between plant genomes. HGTs via TEs is commonly known as HTT. HTT actually permits these elements to be incorporated into the native/host genome, where TEs multiply and escape before the anti-TE mechanism of the host genome is activated. This action enables them to rapidly carry out HGT between the

plants (Wallau et al. 2012; Dotto et al. 2015; El Baidouri et al. 2014; Peccoud et al. 2017; Schaack et al. 2010; Panaud 2016). Another HTT process involves transfer of the TEs into the viral genome, which transfers the acquired genome upon infection of the host. This stage is followed by activation of TE and their subsequent integration into the recipient genome (Capy et al. 2008). Various instances of transposon-mediated HGT have been reported wherein the genomic DNA sequences were captured and transduced into the recipient plant genome.

4 Conclusion

HGT is considered as a defining force affecting the course of evolution in plants. Conventional information suggested that HGT is frequent in prokaryotes or unicellular eukaryotes, but a rare event in plants. HGT was rare in plants because of resistance in the plant germline and the apical meristems (Goremykin et al. 2008; Koulintchenko et al. 2003; Zhu et al. 2011). However, with the advent of molecular techniques and bioinformatic methods, contrasting findings were reported. Extensive studies about HGT confirm that plants have acquired and successfully integrated gene families from different sources such as bacteria, fungi, viruses, nematodes, and other plants. Several studies reported the presence of viral and bacterial genes in the mitochondrial genome, and also reported transfer of these genes in the mitochondrial genome to the plant nuclear genome via the apical meristem path. Other similar observations established the fact that neither apical meristems nor germline can be a significant obstacle for HGT if the ecological conditions are maintained (Yoshida et al. 2010; Christin et al. 2012; Moran and Jarvik 2010; Ni et al. 2012). The studies revealed that gene transfer is evident across all major land plant species including mosses, ferns, and flowering plants. Studies also revealed that HGT is essential for the land plants as various specific functionalities are acquired by a plant in the course of evolution to adapt to the changing environment. Evidence for HGT was identified and confirmed by whole-genome studies and transcriptome-level analysis. Extensive research with improved techniques is being implemented in genomic analysis to provide more insights into the evolution of plants with respect to HGT (Maumus et al. 2014; Richards et al. 2009; Won and Renner 2003; Yoshida et al. 2010; Yue et al. 2014). HGT involves the transfer of a gene or group of genes in a preferential/selective fashion at a given point in time, and the genes then aid in the adaptation and evolution of the plants. To accurately analyze HGT among species, uniform criteria such as wide-scale phylogenetic analysis and whole-genome analysis are needed (Schönknecht et al. 2014). Based on the data, a deeper understanding of the evolutionary advantages acquired by the plants over the course of time can be generated, thus paving the path for designing bioengineered plants or other higher eukaryotes.

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Fungal Horizontal Gene Transfer: A History Beyond the Phylogenetic Kingdoms

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Abstract Horizontal gene transfer (HGT) has been traditionally assumed as limited in eukaryotes in contrast with its high frequency observed in prokaryotes. Although this event remains controversial in eukaryotic cells, evidences suggest that HGT is a relevant mechanism in eukaryotic genome development, as an evolutionary shortcut, triggering the appearance of new capabilities in the involved organisms. The best well-known transfer comes from endosymbionts to the host nuclear genome, often called endosymbiotic gene transfer, which represents an intracellular transference. Moreover, due to fungal inherent characteristics, as chitin cell walls and osmotrophic feeding, these organisms are considered as the most recalcitrant to allow gene transfer to them. However, the exponential number of genome sequences available on the databases has boosted the number of described gene transference events. Thus, the detection of the transferred genes, as well as their putative mechanisms of transference, is under study as a trending topic. Initially, genes from bacteria to fungi were the first detected candidates. Secondly, some complete bacterial metabolic pathways have been detected as transferred. Nowadays, the transference has been noticed from fungi to fungi or even from fungi to other eukaryotic organisms as aphids and plants or endosymbionts. This fact is opening the skyline to new unexpected gene or cluster movements.

Keywords Fungi · Yeast · Aphids · Spider · Plant · HGT · Horizontal gene transfer

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1 Introduction

Horizontal gene transfer (HGT, also called as lateral gene transfer) is the mechanism through which genetic information moves across normal mating barriers between different kinds of more or less distantly related living organisms and, thus, stands in distinction to the transmission of genetic information from parent to offspring. HGT is considered a powerful driving force on the evolution of living beings, in both prokaryotes and eukaryotes. Because of the genomic modification due to HGT, one organism can acquire novel functional genes rapidly from other organisms. These newly incorporated genes could allow: (i) a fast adaptation of the recipients to a changing environment or new ecological niches (Gogarten and Townsend 2005; Mitreva et al. 2009; Richards et al. 2011a), (ii) acquisition of novel functions (Pennisi 2004; Gogarten and Townsend 2005), (iii) metabolic networks expansions (Pál et al. 2005) and (iv) speciation, triggering the evolution of these recipient strains (Lawrence 1999).

The efforts for depicting a "universal tree of life" for ribosome owner organisms rooting in LUCA (Last Universal Common Ancestor) and establishing a relationship between all the living organisms are based on a continuum on the evolution of species through the heredity of genetic information and its expression constrained only by the adaptive changes triggered by the environment. However, HGT has been so frequent in the history of life that some classic concepts as "tree of life" or LUCA fade away themselves in support of the network of life concept (Doolittle 1999a; Ragan et al. 2009; Soucy et al. 2015).

High-throughput genomes sequencing has extremely increased the knowledge about genetic changes of the living beings in all the scale of evolution. Surprisingly, not all these new data fit a straightforward vertical-descent model. An amazing number of gene trees do not fit with accepted organisms' relationships or, at least, show topology discordances. Thus, many genes present patterns across genomes and taxa that cannot be explained by means of vertical genetic transmission or gene loss. Sometimes, they exhibit compositional features (e.g. GC content, different codon usage, different nucleotide frequencies) distinct from the surrounding genome, being characteristic of more-distant taxa (Médigue et al. 1991; Lawrence and Ochman 1997; Karlin et al. 1998; Ochman and Bergthorsson 1998; Moszer et al. 1999; Garcia-Vallvé et al. 2000; Medrano-Soto et al. 2004). Thus, the simplest explanation has been that there is an exchange of genetic material (and concomitantly, a stable integration of these genetic elements in the receptors) among different strains, species and also kingdoms (Doolittle 1999b; Eisen 2000).

1.1 HGT in Eukaryotes

Although HGT has been mainly identified in prokaryotic genomes (Koonin et al. 2001; Dagan et al. 2008; Treangen and Rocha 2011; Kloesges et al. 2011), phylogenomic analyses of eukaryotic genomes have demonstrated that several

distantly related microbial eukaryotes have usually acquired metabolic genes from prokaryotes at an appreciable rate (Andersson et al. 2003, 2007; Huang et al. 2004; Loftus et al. 2005; Eichinger et al. 2005; Hall et al. 2005; Ricard et al. 2006; Gladyshev et al. 2008; Bowler et al. 2008). Thus, this flux of genes to protists suggests an important role in their genome evolution. Besides, HGT from prokaryotes has also been identified in multicellular eukaryotic genomes (Scholl et al. 2003; Richardson and Palmer 2007; Dunning Hotopp et al. 2007; Richards et al. 2009; Yue et al. 2012). However, the documented examples of HGT in eukaryotes suggest that this evolutionary mechanism is more prominent in microorganisms, both prokaryotic and eukaryotic.

It has to be considered that the HGT screening procedures mostly used to date have been designed to detect putative gene transfers from prokaryotes to eukaryotes, which are easier to detect and justify than transfer between eukaryotes. Thus, the majority of the reported gene transfer processes describe inter-domain events in the direction to eukaryotes (Andersson et al. 2003, 2007; Loftus et al. 2005; Eichinger et al. 2005; Andersson 2005; Ricard et al. 2006; Dunning Hotopp et al. 2007). However, the increasing number of genomic currently data available from diverse eukaryotic organisms have shown intra-domain gene exchange (Graham et al. 2008; Danchin et al. 2010; Yue et al. 2012; Li et al. 2014).

An additional point of interest is the existence of specific barriers to HGT through eukaryotes as recipient organisms, for example, the main structure of the eukaryotic cell, with the particular disposition of the genome compartmentalized in a nucleus as a tightly packaged chromatin. Besides, there are incompatibilities in their genome and differences in the transcriptional, splicing and transductional traits among potential donors (whether they are prokaryotes or eukaryotes) and recipient eukaryotes (Keeling and Palmer 2008; Richards et al. 2011b). The big size of eukaryotic genomes with complex gene or protein interactions organized as interactomic networks (Szklarczyk et al. 2015, 2018) could also contribute to hinder HGT in these organisms.

The gene silencing of acquired foreign DNA through a number of molecular mechanisms could be achieved, including RNA-directed cytosine methylation in the DNA in plants (Matzke and Mosher 2014) and of H3K9 in the germ line of *Drosophila* (Yu et al. 2015). There are other co-transcriptional alternatives involving RNA processing and control quality for alien DNA silencing (Zaratiegui et al. 2011; Zofall et al. 2012; Dumesic et al. 2013; Ugolini and Halic 2018). Alternatively, the transferred DNA could also be physically eliminated as, for example, during chromatin diminution in ciliates, foraminifera and some animals (Coyne et al. 2012; Wang et al. 2017).

On the other side, a tendency driven by studies from prokaryotes suggests that genes involved in some highly conserved metabolic functions as replication, translation and transcription are laterally transferred less often than genes encoding energetic or adaptive metabolic functions (Nakamura et al. 2004). In base to that, the complexity hypothesis proposes that these replicative/transcriptional/translational genes should not be affected by HGT, being the specific markers that would proportionate the phylogenies of species that reflect the true evolutionary relationships of the species under study (Jain et al. 1999; Wellner et al. 2007; Cohen et al. 2011).

In contrast, the scientific literature presents some reports detailing the presence of chimeric rDNA genes in fungi that could evidence the transfer of ribosomal genes between them (Kauserud et al. 2007). However, this kind of event seems not to be advantageous for the receptor strains, since an orthologue gene displaces or chimerizes through recombination the original gene and this new gene has to function in a new foreign context (Bull et al. 2007).

Although a general mainstream assumption over HGT proposes that each studied gene has undergone HGT independently of any other gene (Dagan et al. 2008; Marcet-Houben and Gabaldón 2010). Numerous observations of multiple genes transferred together have been detailed. In that case, the synteny and genetic linkage between putatively transferred genes should be considered.

1.2 Mechanisms of Horizontal Gene Transfer in Fungi

Based on different phylogenomic studies, it has been estimated that 0.1–2.8% of genes on a typical fungal genome have been affected by HGT (Marcet-Houben and Gabaldón 2010; Wisecaver et al. 2014). Besides, up to 5% of clustered biosynthetic genes in fungi have arisen through HGT (Gojković et al. 2004; Hall et al. 2005; Wisecaver et al. 2014).

Different selfish genetic element transferences in fungi have been detailed that could act as a vehicle for gene transference in fungus. These selfish genetic elements include (i) mycoviruses (van Diepeningen et al. 1998), (ii) plasmids (Kempken 1995), (iii) group I introns (Gonzalez et al. 1998) and (iv) transposons (Belbahri et al. 2008). Besides, the transference of bacterial conjugative plasmids from bacteria to *Saccharomyces cerevisiae* has been observed (Heinemann and Sprague 1989). Recently, the development of a conjugal gene transfer system (Trans-Kingdom Conjugation) from *Escherichia coli* to *S. cerevisiae* has been performed (Moriguchi et al. 2013). However, in *S. cerevisiae*, a mechanism dedicated to DNA uptake has not been documented, although under specific artificial laboratory conditions, genetic transformation of this species with plasmid DNA has been obtained (Nevoigt et al. 2000). In a similar way, *Agrobacterium tumefaciens*-Ti-DNA-mediated transformation has been documented, under laboratory controlled conditions, in several microbial species, such as *S. cerevisiae* (Bundock et al. 1995), *Agaricus bisporus* (de Groot et al. 1998; Chen et al. 2000), *Aspergillus awamori* (de Groot et al. 1998), *Trichoderma* spp. (Cardoza et al. 2006; Zhong et al. 2007; Malmierca et al. 2013), *Monascus purpureus* (Campoy et al. 2003), *Rhizopus oryzae* (Michielse et al. 2004), *Penicillium digitatum* (Wang and Li 2008), *Umbilicaria muehlenbergii* (Park et al. 2013), *Botrytis cinerea* (Cheng et al. 2018) or *Claviceps paspali* (Kozák et al. 2018). Unfortunately, the molecular pathways underlying HGT in fungi remain to be fully explained.

In addition to the considerations previously observed hindering HGT in eukaryotes, other restrictions to HGT representing barriers against these events, mainly in distantly related species, have been described: (i) nuclear envelope, (ii) the storage of DNA in chromatin, (iii) RNA interference systems, (iv) separate reproductive cell

lines, (v) gene promoter specificity, (vi) incompatibility of intron splicing systems and (vii) alternative gene codes (Jaramillo et al. 2015). Thus, the vegetative incompatibility mechanisms limit cell fusion and heterokaryon formation with genetically different individuals (Glass et al. 2000; Daskalov et al. 2017). In contrast, the fungi suffering hyphal anastomoses between heterokaryon incompatible species, cytoplasmic or nuclear DNA exchange could not be completely prevented (Hoekstra 1994; Walton 2000).

Furthermore, fungi genome defence mechanisms could contribute to hinder HGT to fungi. The mechanisms include repeat-induced point mutations, pre-meiotically induced methylation, meiotic silencing by unpaired DNA, sex-induced silencing, somatic co-suppression, and several co-transcriptional RNA surveillance processes (Rossignol and Faugeron 1994; Hane et al. 2015; Gladyshev 2017). However, a constant flux of genes from bacteria to fungi has been described (Jaramillo et al. 2015) (Fig. 1).

1.3 HGT Transfer from Bacteria to Fungi

Different scenarios should be examined involving HGT in fungi: (i) HGT in which fungi are the receptors/hosts acquiring genes from other organisms (both prokaryotes or eukaryotes) and (ii) HGT events in which fungi are the origin of the xenologue DNA, which is transferred to other organisms. Thus, among interkingdom gene transfers, the transference of bacterial DNA to fungi has been widely documented, where yeasts have been the most commonly described event. In example, in *S. cerevisiae*, enzymes involved in the biotin biosynthesis are encoded by six genes, and two of them (*BIO3* and *BIO4*) seem to have been recently acquired through HGT from a bacterial source. *BIO3* and *BIO4* encode two adjacent steps of the biotin biosynthesis, 7,8-diamino pelargonic acid synthetase and dethiobiotin synthetase, respectively. In other eukaryotes, these two activities appear to be performed by a single protein. Phylogenetic analysis of these genes suggests that they were acquired individually from different prokaryotic donors, *BIO3* from gamma-proteobacteria and *BIO4* from alpha-proteobacteria (Hall and Dietrich 2007). Moreover, additional genes involved in scavenging of nutrients such as alkyl-aryl sulphatase *BDS1*, dihydroorotate dehydrogenase *URA1*, methionine gamma-lyase, nucleoside-sugar epimerase or acetyl-transferase YJL218W have also been acquired from a bacterial source in this yeast (Hall et al. 2005; Hall and Dietrich 2007).

The yeast *Candida parapsilosis* presents two bacterial genes from HGT. One encodes a putative proline racemase, and the second belongs to the phenazine F (PhzF) superfamily. Intriguingly, a synteny analysis suggested that *C. parapsilosis* has lost the endogenous fungal form of PhzF, and subsequently reacquired it from a proteobacterial source. Other *Candida* species, *Clavispora lusitaniae*, and *Pichia guilliermondii* also contain a fungal PhzF homolog, although originated from an ancient HGT event, from a member of the proteobacteria. There is evidence that *Schizosaccharomyces pombe* and Basidiomycotina also obtained a PhzF homolog

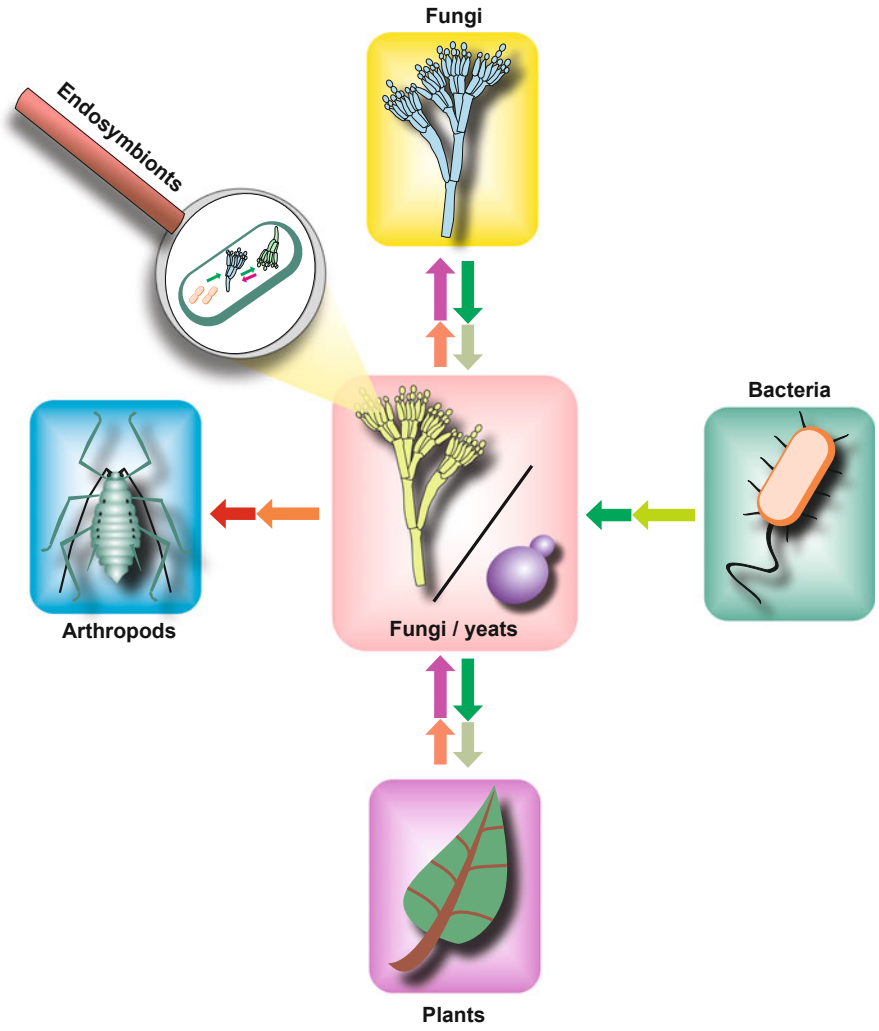

Fig. 1 Schematic summary of the HGT events described along the text, which have yeast or fungi as gene receptors/donors. Thus, transference between fungi species, bacteria and fungi, plants and fungi, fungi and arthropods or fungal endosymbionts are presented

through HGT. Phylogenetic analysis also infers that there were independent transfers of bacterial proline racemases into members of the Pezizomycotina and protists (Fitzpatrick et al. 2008; Naranjo-Ortíz et al. 2016). Similar studies also describe the putative case of HGT involving a bacterial alanine racemase in *S. pombe* (Uo et al. 2001).

One of the largest HGT events in yeasts was described by Dujon and co-workers (2004) in *Yarrowia lipolytica*, where nine genes from *Y. lipolytica* (encoding three hypothetical proteins, two Yee/YedE family proteins, two putative acyltransferases,

an adenylate kinase and a D-aminopeptidase) were the target of HGT. Besides, four genes from *Kluyveromyces lactis* (a conserved glyoxalase domain containing protein, an alcohol dehydrogenase, an hypothetical protein of unknown function, and a putative regulatory protein), as well as one gene from *Debaryomyces hansenii* (encoding a YdhR precursor), were described as horizontally transferred from bacteria by the same authors (Dujon et al. 2004). Besides, detoxification genes in *Y. lipolytica* and *Rizopus oryzae* seem to be the result of two independent acquisitions of a bacterial arsenate reductase encoding gene from bacteria that might have provided them with the ability to detoxify arsenics (Marcet-Houben and Gabaldón 2010).

Other fungal species, out of yeast, have also obtained genes from bacteria through horizontal transference. Thus, several Class III alcohol dehydrogenase and flavohemoglobin genes of Ascomycota come from proteobacteria (Andersson et al. 2003). Moreover, the transference of histidine kinase genes from *Streptomyces coelicolor* to *Candida*, *Neurospora* and *Fusarium* has been proposed (Brinkman et al. 2001). In fungi, a recent transfer of a bacterial catalase encoding gene has been described in the microsporidian pathogen *Nosema locustae* (Fast et al. 2003). Similar transfers to the Dothideomycetes *Stagonospora nodorum* and *Mycosphaerella fijiensis* and the Leotiomycetes *Botrytis cinerea* have been suggested (Marcet-Houben and Gabaldón 2010).

Genome analyses from fungal species are extending the number of proposed HGT events. Hence, several species of the *Colletotrichum* genus (*Colletotrichum graminicola, Colletotrichum higginsianum* and *Colletotrichum gloeosporioides*) have genes from the bacterial origin (Jaramillo et al. 2015). Five of these genes, those encoding an arginosuccinate lyase, glutathionylspermidine synthase, an oligoxyloglucan reducting-end-specific cellobiohydrolase, a glucarate dehydratase and a serine endopeptidase S1, are found in the three genomes. The presence of an ortholog gene coding a hydroxyacyl-CoA dehydrogenase from bacterial origin in the genomes of *C. higginsianum* and *C. gloeosporioides* but absent in *C. graminicola* genome has been determined. In the same sense, genomes from *C. graminicola* and *C. higginsianum* share a gene from the bacterial origin coding a succinyl-diaminopimelate desuccinylase; in the genomes of *C. graminicola* and *C. gloeosporioides* appear three genes for a L-asparaginase, an acetyl-CoA synthetase and a 2-deoxy-D-gluconate 3-dehydrogenase that are absent in *C. higginsianum*. These genes could be involved in carbohydrate metabolism, amino acid metabolism or secondary metabolism or are secreted degrading enzymes, and several of them are expressed during plant infection by these fungi (Jaramillo et al. 2015).

In addition to metabolic genes, other genes involved in cell wall biosynthesis have been detected as the result of HGT, although the function for these enzymes in fungi is unclear, but a possible role in the bacterial cell walls degradation has been speculated. Thus, the first three enzymes of the bacterial peptidoglycan biosynthesis pathway, MurA, MurB and MurC, have been identified in *Aspergillus terreus, Aspergillus oryzae* and *Aspergillus flavus,* though only *murA* and *murB* are present in *Aspergillus fumigatus* and *Neosartorya fischeri* (Marcet-Houben and Gabaldón 2010).

1.4 HGT Transfer Between Fungi

Transference of genes between different fungi has been also documented (Fig. 1). Most of the well-supported HGT involving fungal donors and recipients reported so far involves entire metabolic pathways related with (i) the production of toxins and related compounds, (ii) the assimilation of nutrients and (iii) the obtaining of new metabolic capacities (e.g. detoxification). Thus, genes coding a complete sterigmatocystin biosynthetic pathway in *Podospora anserina* have been acquired from *Aspergillus* (Slot and Rokas 2011). Besides, the locus *ASP3* from *S. cerevisiae*, containing four identical genes encoding cell wall-associated L-asparaginase II, arose in this yeast via HGT from *Wickerhamomyces anomalus* (or a close relative) (League et al. 2012). In the genome of *S. cerevisiae* EC1118, three regions (38 kb in chromosome VI, 17 kb in chromosome XIV and 65 kb in chromosome XV) have been proposed as HGT transmitted as a result of inter-fungal HGT. However, only the origin of the 17 kb in chromosome XIV seems to have a clear origin from a *Zygosaccharomyces*-like species (Novo et al. 2009). Moreover, the recent transfer of a 65-kb genomic region between *Torulaspora microellipsoides* and *S. cerevisiae* wine yeasts or closely related strains has been also documented (Marsit et al. 2015).

Another proposed gene that has been disseminated by HGT is *FSY1*, a gene encoding a specific high-affinity fructose/H^+ symporter. This has been proposed as originated in *Pezizomycotina*, and at least ten independent inter-species instances of HGT involving this gene have been identified (Coelho et al. 2013).

The HGT between fungi has been used to study how frequent the gene transference is. Thus, *Magnaporthales* is a monophyletic in the Ascomycota that includes infecting pathogens of roots and above ground tissues of monocot plants [e.g. rice blast fungus *Pyricularia oryzae* (=*Magnaporthe oryzae*)]. This order presents massive gene transfers to the genus *Colletotrichum*, which includes anthracnose pathogens of various plants. Thus, a conservative estimation presented 93 putative gene transfers between *Magnaporthiopsis incrustans* and *Colletotrichum*. The anastomosis (fusing of the same or different hyphae) leading physical connections between cells from different species could explain this HGT between fungal species (Qiu et al. 2016).

1.5 HGT from Fungi to Arthropods: Carotenoid Genes

Carotenoids are a very diverse group of secondary metabolites, which define a subfamily of isoprenoids (also called “terpenoids”). The economical relevance of these natural pigments is mainly due to their uses as colorants, feed supplements, nutraceuticals, as well as medical, cosmetic and biotechnological purposes (Barreiro and Barredo 2018). These lipophilic secondary metabolites, which include carotenes and xanthophylls, range from the yellowish- to the reddish-colour spectrum and play different roles. Thus, in photosynthesizing species, the carotenoids are associated

with the light harvesting complexes, where act as accessory pigments extending the range of absorbed light. Besides, they act as photoprotectors by scavenging singlet oxygen and other toxic oxygen species. In all non-photosynthetic organisms, the carotenoids function seems to be (photo)protection playing a role as antioxidants (Barredo et al. 2017). Natural carotenoids are synthetized by photosynthetic species (including plants and algae) and by some particular classes of fungi and non-photosynthetic bacteria (Sandmann and Misawa 2002; Sieiro et al. 2003; Fraser and Bramley 2004). However, most animals obtain carotenoids from their diet and they are able to modify them producing their own brightly coloured pigments (e.g. feather in birds and cocoon colours in insects) (Bryon et al. 2017). Animals were thought to lack the ability to synthesize carotenoids *de novo*, but this mind was changed when carotenoid biosynthetic genes were detected in the genome sequence of the pea aphid (*Acyrthosiphon pisum*) acquired by horizontal gene transfer from fungi (Moran and Jarvik 2010; Bryon et al. 2017) (Fig. 1).

Aphids are specialized insects in feed on the plant phloem, which, in the function of their host ranges, also transmit economically important viruses to worldwide crops that cause more damage than the aphids themselves (Stern 2008; Rodriguez and Bos 2013). Presence of carotenoids was reported from several species of aphids, which content varies among polymorphic species and conditioned the predators that target them. However, the 464-Mb genome sequence of the pea aphid (*Acyrthosiphon pisum*) allowed detecting, for the first time, carotenoids biosynthetic genes (desaturase homologs and fused carotenoid cyclase–carotenoid synthase enzymes) similar to those of fungi. Intriguingly, while plants and bacteria present homologs of the detected genes, just the fungal ones show a fusion of carotenoid cyclase and carotenoid synthase as the aphids detected genes (Moran and Jarvik 2010). The analyses of the *A. pisum* genome revealed duplication of the carotenoids biosynthetic genes. Thus, four copies of the carotene desaturase genes and three of the fused phytoene synthase/carotene cyclase genes were described, which is rarely detected in bacteria, plants or fungi (Nováková and Moran 2012). Besides, the aphids also bear obligate mutualists, such as the Gammaproteobacteria *Buchnera aphidicola*, which present highly reduced genomes. This fact supports the gene transfer from an ancestor *Buchnera* to the aphid genomes, which presents them as a sort of gene recruiter species (Nikoh et al. 2010). Two functional carotenoid biosynthesis genes (carotenoid cyclase-carotenoid synthase and carotenoid desaturase) derived from fungi were also described in the two-spotted spider mite (*Tetranychus urticae*). Hence, at that time, several explanations for these transfers from fungi to animals have been proposed, such as (i) a single arthropod ancestor of both spider mites and aphids, (ii) direct horizontal transfer between aphids and spider mite, (iii) transference through some microbial intermediary (e.g. *Wolbachia* bacteria that infect both groups); (iv) fungal symbionts (either beneficial or pathogenic) and (v) viruses, which act as horizontal gene transfer vectors (Altincicek et al. 2012). This gene transference is an opened question, which continues unsolved today.

Immediately the HGT from fungi to aphids or spider were observed, a third case of fungal carotenoid biosynthesis gene mobilization was observed in arthropods. Members of the family Cecidomyiidae, which includes the flies commonly known as

gall midges, also carried out those synthesis genes. Cobbs and co-workers in 2013 provided the phylogenetic analyses of these carotenoid biosynthesis genes that established the gene transference moment in a time point after the divergence of the major arthropod lineages (Cobbs et al. 2013). Besides, other aphids close-related arthropods, such as adelgids, which are one of the most destructive introduced pests in the North American coniferous forests, also presented carotene desaturases. In addition, the plant pathogens included in the Phylloxeridae family, which also are other aphids close-related species, carried out carotenoid cyclase/synthase and desaturase genes (Nováková and Moran 2012; Zhao and Nabity 2017). The gene analysis suggested the same carotenoid gene origin in these three taxonomical groups of aphid relatives (Aphididae, Phylloxeridae and Adelgidae). Interestingly, the genes diversification among these arthropods happened through lineage-specific gene duplication, deletion, recombination and occasional positive selection. Thus, the carotenogenic fungal-derived genes are taxonomically closer connected among aphids close-related species than to the microbial homologs, which suggested a single HGT event between a common ancestor and a fungus, probably an endosymbiont (Zhao and Nabity 2017).

As a parallel way of carotenoids self-sufficiency, the presence of carotenoids-synthesizing endosymbiont instead of HGT has been described in some arthropods such as the whiteflies (e.g. *Bemisia tabaci*) (Sloan and Moran 2012). This agricultural pest, which affects a wide range of crop species, has antique relationships with intracellular bacteria located in a brightly pigmented abdominal organ (bacteriome) (De Barro et al. 2011). These endosymbiotic bacteria contain non-fungus-derived genes coding for carotenoid biosynthesis enzymes. This fact suggested distinct evolutionary mechanisms (bacterial endosymbiosis or fungal lateral gene transfer) to acquire the same functional trait (Sloan and Moran 2012).

Carotenoid biosynthetic genes gathering allows to abolish the dietary requirement of carotenoids in spider mites (*T. urticae*), even for diapause, which is essential for survival in temperate regions (Bryon et al. 2017). Besides, the aphids colour conditioned the predators that target the aphids, which highlight the relevance of the HGT beyond the well-known antioxidant effect of these compounds (Moran and Jarvik 2010).

1.6 Endosymbionts: A HGT from/to Inside

The endosymbiotic gene transfer from organelles (e.g. mitochondria or chloroplasts) to the eukaryotic cellular nucleus is well described, but the knowledge about HGT among different individuals within the same species or unrelated species is more restricted and even less about real endosymbiotic species. Sometimes the gene acquisition happened long time ago, which heavily erase their phylogenetic signal limiting their detection and the definition of the different endosymbionts contribution to the host genome (Kleine et al. 2009; Mehrabi et al. 2011; Moreira and Deschamps 2014). Recently, Pawlowska and co-workers reported that “heritable

symbioses, in which endosymbiotic bacteria (EB) are transmitted vertically between host generations, are an important source of evolutionary novelties". These heritable symbioses range from antagonisms to mutualisms, which include vertically transmitted symbionts, as well as horizontal transmission between hosts (Pawlowska et al. 2018).

Fungal and bacterial interactions range from complex soil microbial communities to the mammalian microbiome of the gastrointestinal tract (Valdivia and Heitman 2007). Sometimes, these interactions have moved from the symbiosis to the partial control over specific functions of the fungus. Thus, the ability to form sporangia and spores of the rice-pathogen fungus *Rhizopus microsporus* is controlled by the bacterial endosymbiont *Burkholderia* sp., which questions the fine line between mutualism and parasitism (Valdivia and Heitman 2007; Partida-Martinez et al. 2007). Besides, the endosymbiont has gained the control over an indispensable component of the reproductive development of the fungus, the *ras2–1* gene that encodes the G-protein (GTPase central to fungal reproductive development) (Mondo et al. 2017). Those are not real HGT examples, but indicate a close interaction between endosymbionts and host.

On step further in the HGT is the transference of genes to fungal obligate intracellular parasites. Thus, the fungal phyla *Microsporidia*, obligate intracellular parasites of animals and opportunistic pathogens of immunocompromised individuals, and *Cryptomycota*, obligate intracellular parasites (algae, amoeboids, other fungi), have obtained multiple genes through HGT involved in nucleic acid synthesis and salvage (Alexander et al. 2016). This fact contradicts the idea of few HGT-derived genes present in intracellular parasites (Nakjang et al. 2013). Regarding the mechanisms of gene transference, these are still unclear, but virus-mediated transference seems unlikely due to their intracellular environment. In contrast, the mixture of phagocyted receptive spores of the fungus and the nucleic acids from lysed environmental bacteria inside the same intracellular vesicles of the host could justify the HGT. Besides, the reverse-transcription of host mRNA molecules and their subsequent incorporation in the fungal genomes could be a second explanation (Sibley 2011; Alexander et al. 2016) (Fig. 1).

1.7 HGT Between Plants and Fungi

Despite the rarity of horizontal transfers between plants and fungi and vice versa, the enabling of soil environments exploitation has been described and suggested (Tunjic and Korac 2013) (Fig. 1). Richards and co-workers (2009) compared the genomes of six plant species with those of 159 prokaryotic and eukaryotic species. As a result, they identified 1689 genes showing the highest similarity to corresponding genes from fungi. It presented five fungi-to-plant transfers (none involved angiosperm recipients) and four plant-to-fungi HGTs. Besides, the genes functional suggested that two fungi-to-plant transfers added relevant phenotypes for life in a soil environment. The transference from plants to *Batrachochytrium dendrobatidis* and

Laccaria bicolor involved a potential prokaryote intermediate, which justifies these exceedingly rare events (Richards et al. 2009; Tunjic and Korac 2013).

More recently, it was shown the massive lateral gene transfer of genes encoding for plant cell wall-degrading carbohydrate enzymes and auxiliary proteins from plants to associated filamentous fungi, as for example *Trichoderma* spp. This phenomenon has been related to the ability of some *Trichoderma* species to parasitize a broad range of Ascomycota. Thus, the currently known *Trichoderma* parasitic species should have evolved from an ancestor with a limited cellulolytic capacity, which acquired many of these new hydrolytic activities by LGT from the host plants (Druzhinina et al. 2018).

Arbuscular mycorrhizal fungi act as mutualistic symbionts of many land plants, which establish a robust tripartite with associated bacteria. This lifestyle where plant, bacteria and fungi coexist in a concrete environment increases the chance of HGT. However, very few HGT events have been reported from bacteria or plants to these fungi. Recently, Li and co-workers (2018) have described the 19 fungal genes of the mycorrhizal fungi *Rhizophagus irregularis* transferred between fungi and bacteria/plants. Besides, seven of them were obtained from bacteria. The gene duplication of some of these genes has contributed to the expansion of three of the transferred genes. In addition, the transcriptomic experiments showed the functional role of these genes in *R. irregularis* involved in fundamental biological processes such as gene expression regulation, mitosis and signal transduction (Li et al. 2018).

The analysis of the moss genome of *Physcomitrella patens* identified 57 families of nuclear genes acquired from prokaryotes, fungi or viruses. The fungus genes correspond to a NRPS-like enzyme involved in oxidative stress resistance and a heterokaryon incompatibility superfamily gene related to the heterokaryon formation (Yue et al. 2012).

1.8 Fungal Terpene Biosynthetic Genes: A Case of Study

Fungal terpenes include hundreds of different compounds with a great diversity of physiological and metabolic roles. In fact this is one of the largest groups of natural products (Sivasithamparam and Ghisalberti 1998). It includes mainly secondary but also some important primary metabolites. Among the latter are the sterols (e.g. ergosterol), which are essential for cell membrane structure, and whose biosynthetic pathway is one of the main targets used in the antifungal treatments. In addition, farnesyl diphosphate (FPP) and geranylgeranyl diphosphate (GGPP) are essential not only as precursors or as intermediates in some terpenic secondary and primary biosynthetic pathways but are also essential in the processes of protein prenylation, including farnesylation and geranylgeranylation, respectively. The prenylation processes are needed for the activity of some proteins of Ras and Rho families, which play critical roles, among others, for cell ultrastructure organization, secretion and protein location (Haney et al. 2017). Regarding the secondary metabolites of terpenic nature, many of them have important pharmacological activities,

e.g. antiviral, antibacterial, antimalarial, anti-inflammatory, and anticancer. These compounds have been also involved in some other activities, as would be the case of the biocontrol processes and antifungal activities. In the opposite, some others can have strong toxic activities, as it is the case of the sesquiterpenoids trichothecenes and botrianes, produced by a great diversity of filamentous fungi (Calvo et al. 2002; Collado et al. 2007; Proctor et al. 2018).

The industrial interest of many of these fungal terpenes, together with the exponentially growing knowledge of fungal genome sequences (Cuomo and Birren 2010), has contributed to the characterization of genes, proteins and entire pathways involved in their biosynthesis. This knowledge has allowed the description of some examples involving HGT as a mechanism explaining the inter-kingdom and inter-phylum transfer of terpene biosynthetic genes, which has been widely recognized as a mechanism contributing to the fungal evolution and also to the fungal metabolic diversification (Wisecaver et al. 2014).

In one of the examples, HGT would be more likely involved in the transfer of a sesquiterpene synthase, and some putative tailoring cytochrome P450(s) monooxygenase encoding genes, involved in the biosynthesis of the sesquiterpene Δ6-protoilludene from Basidiomycota to Ascomycota (de Sena Filho et al. 2016). The analysis of the genome sequence of the endophyte Ascomycete *Diaporthe* (*Phomopsis*) sp. BR109 led to the identification of 17 genes encoding for putative sesquiterpene synthases. From a phylogenetic point of view, most of these sesquiterpene synthases clustered in clades with Ascomycota terpene synthases. However, one of these putative sesquiterpene synthases, Dia1, exhibited a closer phylogenetic relationship with previously described Basidiomycota Δ6-protoilludene synthases (Quin et al. 2013). The Dia1 encoding gene (*dia1*) also share a most common intron splicing pattern with orthologous Basidiomycota genes. Thus, all these data supported the hypothesis of very infrequent cross-phyla HGT between Basidiomycota to Ascomycota for Dia1 encoding gene. However, this gene transfer mechanism was not only restricted to the sesquiterpene synthase gene, but also for other tailoring enzymes, i.e. at least a cytochrome P450 monooxygenase encoding gene has been also acquired by *Diaporthe* sp. from Basidiomycota. This fact indicates a common phenomenon by which gene clusters, mainly involved in secondary metabolite biosynthesis, can be partially or completely transferred to the host, given to it a fitness advantage (Slot and Rokas 2011).

Another example, still obscure, refers to genes involved in the biosynthesis of paclitaxel (Taxol™), which is a diterpene metabolite with antitumor properties, whose production was initially described in the yew *Taxus brevifolia* (Wani et al. 1971). Several years later it was reported the production of this compound also by *Taxomyces andreanae*, an endophytic fungus associated with *T. brevifolia* (Stierle et al. 1993), and in several other endophytic *Taxus*- and non-*Taxus*-related fungi. Initial studies indicated that sequences of genes from plants and endophytic fungi were highly conserved, raising the possibility of a plant-fungus inter-kingdom lateral gene transfer (LGT). However, more recent studies on a wider sample of taxol-producing fungi did not find a general high homology between plant and fungal taxol biosynthetic genes (Xiong et al. 2013; Yang et al. 2014), found unlikely the

hypothesis of a lateral gene transfer between plants and fungi for these genes. Nevertheless, the hypothesis of a totally independent origin for taxol biosynthesis genes in plants and fungi seems to be highly improbable. Therefore, the possibility of a lateral trans-kingdom gene transfer between plants and fungi has to be still considered. Furthermore, a plausible hypothesis would be that an ancestral plant gene encoding the terpene cyclase (taxadiene synthase) (Koepp et al. 1995; Jennewein et al. 2004) should be moved to an ancestral fungus. And subsequently, this would have given rise to the whole pathway by recruiting genes in the genome (Heinig and Jennewein 2009), followed by processes of gene paralogy, evolution and/or further gene neofunctionalization, as has been described for other fungal secondary metabolite biosynthetic gene clusters (Proctor et al. 2018).

In a wider study regarding the origin of fungal diterpene synthases (di-Tps), the distribution and origin of di-Tps encoding genes in plants and fungi were analysed. A huge variation in the number of this kind of enzymes was found in plants, with only one in *Phycomitrella patens* (Hayashi et al. 2006) to 66 in *Selaginella moellondorffii* (Li et al. 2012). Furthermore, fungal *di-tps* are only present in Ascomycota and Basidiomycota members. Initial studies indicated that di-Tps involved in the first steps of gibberellin (GA), a plant hormone of diterpenic nature, biosynthesis are highly similar between plants and fungi, which would suggest a common evolutionary origin. However, strong divergence was found in the enzymes/genes involved in the tailoring late steps of GA biosynthesis between plants and fungi. Thus, being unlikely their acquisition by HGT by fungi from plants or even from bacteria. Phylogenetic analysis of fungal di-Tps suggested that they have diverged from a common origin, and that their evolutionary relationships are not in agreement with the species phylogenetic relationship. Production of GAs has been observed in all higher plants, but only in relatively few fungal species, which would indicate that genes involved in their biosynthesis were not present in the ancestor of eukaryotes. Thus, the presence of *di-tps* only in few fungal species, and the lack of correspondence between the phylogenetic relationships of di-Tps with the species phylogeny, would indicate that ancestral *di-tps* gene/s have been acquired by fungi from plants by HGT/LGT, and later they would have evolved independently in fungi. Furthermore, the GA biosynthetic genes are organized in fungal gene clusters, while they are spread in the genome in plants, which further indicates that genes involved in the GA biosynthetic pathway have evolved independently in these two kingdoms and that *di-tps* were not acquired by fungi as a whole gene cluster (Fischer et al. 2015).

2 Conclusions and Future Perspectives

Probably, because of the higher number of bacterial genomes and genes present in databases the HGT has been better described in prokaryotes. However, in eukaryotes just the transference of genes from endosymbionts to the host nuclear genome was properly defined. Nowadays, the increase in the sequenced fungal genomes, better algorithms and new phylogenetic analyses are bringing to light unsuspected events

of HGT in eukaryotes such as fungi. Thus, gene exchange between bacteria and fungi, which are the most commonly detected; fungi and plants; fungi and fungi (e.g. endosymbionts); or even fungi and arthropods have been published in last decades. This fact opens the skyline to the discovery of new genes, which confer ecological advantages to the fungal receptors. Even though the evolution of the transferred genes is currently more easily defined by phylogenetic methodologies, their specific function based on omics analyses as a result of their integration in the fungal host gene pool could be next step of the HGT studies in fungi.

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Transfer of Secondary Metabolite Gene Clusters: Assembly and Reorganization of the β-Lactam Gene Cluster from Bacteria to Fungi and Arthropods

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Abstract Filamentous fungi are able to produce a wide array of secondary metabolites with important biological and medical activities. The biosynthesis of secondary metabolites is encoded by gene clusters that include structural, regulatory and transport genes. Complete sets of genes are transferred by HGT from bacteria to fungi and to higher eukaryotes. In addition, genes have been laterally transferred by LGT between phylogenetically distant fungi.

Many of these HGTs involve genes derived from bacteria that have been modified in fungi by incorporating new genes of eukaryote origin. Important examples of HGT from bacteria to fungi include the transfer of the 6-methylsalicylic acid gene cluster and the three-gene cluster for the reduction of nitrate to ammonia. Examples of lateral transfer of NRPS and PKS gene clusters are discussed. The evolution of β-lactam gene clusters constitutes a paradigmatic example of the assembly, transfer, modification and conservation of gene clusters. In the *Eurotiales* (*Penicillium*/*Aspergillus*), *the pcbAB* and *pcbC* genes were integrated next to a three intron-containing gene, *penDE*, encoding an acyltransferase. In the *Hypocreales* (*Acremonium*/*Kallichroma*/*Pochonia*), a larger gene cluster was assembled that contains also the bifunctional isopenicillin N expandase-hydroxylase gene, which derives from homologous genes in bacteria. Since the bidirectional divergent arrangement of *pcbAB-pcbC* genes is common to all fungi, in contrast to the head to tail organization of these genes in all bacteria, we conclude that a major reorganization of these two genes took place during the initial transfer from bacteria to a β-lactam ancestor in fungi. The phylogenetic studies indicate that a single ancient HGT event took place from Gram-negative bacteria followed by a lateral transfer from a receptor fungus from the *Hypocreales* order to other fungi. Interestingly, the first two genes of the penicillin-cephalosporin gene cluster have been found in the arthropod *Folsomia candida*. The phylogenetic tree supports the conclusion that these β-lactam genes were transferred to *F. candida* in an early separated event.

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1 Introduction: Horizontal Gene Transfer and Lateral Gene Transfer in Evolution of Bacteria and Fungi

Horizontal gene transfer (HGT) is defined as the transfer of genes between organisms of different kingdoms, e.g. from bacteria to fungi or plants and animals. The gene transfer between distant species of the same kingdom, e.g. between families of filamentous fungi, is named Lateral gene transfer (LGT) (Andersson 2005; Druzhinina et al. 2016).

Both HGT and LGT imply a stable integration of foreign DNA in the genome of the receptor strain and they differ from vertical inheritance which is the normal way of gene transfer from parents to offspring. HGT and LGT are powerful evolutionary tools to increase the genomic diversity of living beings (Dunning Hotopp et al. 2007; Dunning Hotopp 2011).

There are numerous examples of horizontal gene transfer between bacteria and yeast or filamentous fungi, but HGT from bacteria to animals is rare. In simple cases, HGT might involve the transfer of a single gene encoding an important enzyme for the metabolism of the receptor strain (Wenzl et al. 2005; Coelho et al. 2013). However, more important is the transfer of gene clusters or chromosome fragments that encode complete biosynthetic pathways (Wisecaver and Rokas 2015). In this article, we focus on the transfer, either by HGT or LGT, of gene clusters that confers novel biosynthetic capabilities to the receptor organisms (Walton 2000; Osbourn 2010).

2 Criteria for Identification of HGT Phenomena

Although there are hundreds of examples of putative of gene transfer phenomena in all living beings, solid evidence of the involvement of explicit gene transfer events is scarce (Ragan et al. 2006; Marcet-Houben and Gabaldón 2010). It is difficult to obtain evidence for unequivocal proof of the HGTs. There are a number of criteria that allows to support that a putative transfer event is authentic. Several observations were made by Fitzpatrick (2012) to identify putative gene transfers and he proposed a set of bioinformatic and genetic studies to confirm these events. Criteria to be considered are:

1. The absence of a particular gene (or gene cluster) in close related strains, while orthologues of the gene/cluster are found only in distantly related species.

2. Variations in GC content of a gene or gene cluster, particularly in the third codon position, with respect to the DNA of the receptor strain.
3. Codon use variation of a gene/gene cluster compared to the whole genome.
4. Location in eukaryotes of intron-less genes or, vice versa, the presence of introns in genes derived from bacteria.
5. Presence of genes of interest located in conserved synteny and absent from closely related species.
6. Phylogenetic inference of linking of a gene to a cluster of genes in organisms that map distantly in the phylogenetic tree.
7. Another feature is the location of the putative transferred genes in genome regions with the tendency to recombine, such as subtelomeric regions (Palmer and Keller 2010) or mobile genetic elements (Shaaban et al. 2010).

The transferred gene clusters are identified as genome islands (Fedorova et al. 2008; Cheeseman et al. 2014) with atypical codon usage and/or differences in GC content.

3 Biosynthetic Gene Clusters

Clusters of biosynthetically related genes are frequent in bacteria, both encoding enzymes for primary metabolism and also for secondary metabolite biosynthesis, also named specialized metabolites (Martín and Liras 1989; Osbourn 2010). In filamentous fungi and plants, there are numerous biosynthetic gene clusters, although they are not as common as in bacteria.

Primary metabolism gene clusters in eukaryotes are rare. Most genes encoding enzymes for primary metabolism are scattered throughout the genome; there are, however, a few exceptional examples such as the genes related to quinic acid utilization (Hawkins et al. 1988) or to proline catabolism in fungi (Cubero et al. 2000).

By contrast, gene clusters for secondary metabolite biosynthesis are common in filamentous fungi and plants (Hurst et al. 2004). Filamentous fungi are able to produce an impressive array of secondary metabolites (Zeilinger et al. 2015; Martín et al. 2014; Nielsen et al. 2017). Genes for secondary metabolites are characteristically dispensable for growth, although they may play important roles in specific habitats (Martín et al. 2000). Many Ascomycetes contain about 20–50 secondary metabolites gene clusters (Osbourn 2010). Up to 67 gene clusters have been identified bioinformatically in the genome of *F. graminearum*, a major pathogen of cultivated cereals, although many of them could not be correlated with production of specific secondary metabolites (Sieber et al. 2014). A well-known example is that of *Penicillium chrysogenum* that contain gene clusters to produce penicillins, roquefortines C and D, andrastin A, meleagrin, sorbicillins and chrysogine (also named chrysogenin) (Houbraken et al. 2011). The secondary metabolites synthesized may influence growth and survival of microorganisms and plants in their

natural habitat; for example, *Penicillium expansum* produces several toxin compounds including ochratoxin A, patulin, chaetoglobosins and comunensins (Andersen et al. 2004; Frisvad et al. 2004) and consequently cause diseases in fruit trees.

The organization of secondary metabolites in gene clusters has the advantage of coordinated expression of the entire set of genes. Co-regulation of expression either by reprogramming the heterochromatin or by using regulatory transcriptional factors for expression of the entire gene cluster (Hurst et al. 2002; Singer et al. 2005) may be a factor facilitating the conservation of SM biosynthesis gene clusters (Keller and Hohn 1997; Keller et al. 2005). For example, in *Aspergillus nidulans* the sterigmatocystin gene cluster contains 23 genes that extend for 54 kb (Yu et al. 2004). Sterigmatocystin is a precursor of the highly toxic aflatoxin, which in *Aspergillus flavus* is formed from a complete set of 26 *afl* genes. The enzymes encoded by each gene in these clusters are conserved in *A. nidulans* and *A. flavus*, although the order of the genes in the clusters is different. In both fungi the sterigmatocystin and aflatoxin gene clusters are co-regulated by a transcriptional factor AflR (Fernandes et al. 1998).

An interesting point regarding the LGT within filamentous fungi is whether entire gene clusters can be transferred from a fungus to a distant unrelated species what favours the formation of secondary metabolites in new producer strains. This is the case of the β-lactam gene cluster transfer in between the *Hypocreaceae* and *Trichocomaceae* families. This raises the question of how these gene clusters were formed, whether they were transferred as entire clusters and modified later and how they are conserved through generations (Walton 2000; Fitzpatrick 2012; Martín and Liras 2016a).

4 Assembly and Conservation of Clusters of Genes for Secondary Metabolites Biosynthesis

The initial formation of secondary metabolite gene clusters and their possible modification during the evolution are still obscure. The gain or loss of single genes from a previously existing gene cluster generates neofunctionalization of the final product (Coelho et al. 2013). A limitation to fully understand the evolution of secondary metabolite gene clusters is the lack of molecular information on the physiological role of many secondary metabolites. Indeed, in a few cases it is clear that some secondary metabolites, like the β-lactam antibiotics, work as arms to combat other bacteria or competing microorganisms, but in other cases the specialized metabolites appear to be communication signals between different species or between cells of the same species (Yim et al. 2007).

Several scenarios have been proposed to explain the initial formation of secondary metabolite gene clusters. One scenario is the assembling of a gene cluster from scattered genes. The *novo* assembly of large gene clusters by joint separated genes in a genome is probably a rare event but, in any case, the original formation of a simple

gene cluster is likely to have occurred previously. A classic example is the birth in *S. cerevisiae* of a novel gene cluster for the catabolism of allantoin by recruitment of scattered genes (Wong and Wolfe 2005).

A second hypothesis is the assembly of more complex gene clusters by gaining new genes which are linked to previous simple clusters (Hall and Dietrich 2007).

A third scenario is gene duplication of a progenitor simple gene cluster and further divergent assembly of different, but still somehow related, gene clusters. This third hypothesis has been proposed by Khaldi and Wolfe (2011) to explain the origin of the fumonisin gene cluster in *Aspergillus niger* and *Fusarium verticillioides*. Another example of this hypothesis is the assembly of the present cephalosporin genes in several filamentous fungi by incorporating eukaryotic epimerase genes (see below).

A fourth hypothesis is that large gene clusters have been transferred from a donor strain to a receptor strain and modified in the receptor strain during the evolution. This is the case of the roquefortine-meleagrin gene cluster. The loss or gain of the genes *nox* and *sro* that convert roquefortine C to intermediates of meleagrine biosynthesis provides an interesting example of the transformation of a long into a short biosynthetic pathway or vice versa (Kosalková et al. 2015; Martín and Liras 2016a; Proctor et al. 2009).

Other interesting examples of modification of gene clusters that results in a different set of final products is the loss or gain of P450 monooxygenases, which is documented in a number of cases, e.g. a monooxygenase involved in the conversion of a clavine intermediate into lysergic acid, a precursor of ergot alkaloids (Haarmann et al. 2006), or the cycloclavine synthase, a monooxygenase that forms the rare alkaloid cycloclavine from chanoclavine I aldehyde (Jakubczyk et al. 2016). In these cases, the P450 monooxygenase encoding gene has been recruited and added to the corresponding alkaloid biosynthetic genes. Also, enzymes such as carbamoylases, methoxylases or dehydrogenases, present in the β-lactam clusters of *Actinobacteria* or Gram-negative bacteria, affect the formation of the final products that have been lost during the transfer of the β-lactam gene cluster from bacteria to fungi (Martín 1998; Liras et al. 1998).

Gene clusters have been conserved throughout evolution, independently of whether they are functional or not. As indicated above some gene clusters suffer deletion or rearrangement that results in the lack of functional biosynthetic products. It is unclear whether the conservation of these clusters confers advantages to the producing cells. A hypothesis is that conservation of the clusters confers an advantage per se (Walton 2000; Lawrence and Roth 1996). These authors propose the selfish cluster or selfish operon hypothesis meaning that the conservation of gene clusters is based on advantages conferred by the gene cluster itself, that favours its propagation as a complete cluster and not only in the advantages conferred by the cluster product.

5 Gene Transfer from Bacteria to Fungi

In the last decades, a significant amount of information has been accumulated in gene transfer between prokaryotes and eukaryotes, particularly between bacteria and fungi. Some representative examples of these HGT events are listed in Table 1. One of the reasons for the large number of well-reported HGT cases is the good knowledge of bacterial metabolism as compared to that of higher eukaryotes.

Table 1 Representative examples of HGTs from bacteria to fungi and LTG between phylogenetically distant fungi

	Donor	Receptor	
(I) HGTs from bacteria to fungi			
Aryl, alkyl sulphatases	*Lactobacillaceae*	*S. cerevisiae*	Hall et al. (2005)
Biotin biosynthesis	Unidentified prokaryote	*S. cerevisiae*	Hall and Dietrich (2007)
β-Lactam biosynthesis genes	*Actinobacteria*	*Hypocreales* (*Acremonium*, *Pochonia*)	Cohen et al. (1990), Buades and Moya (1996), this work
NRPS-PKS genes	*Burkholderia*	Several *Ascomycota*	Lawrence et al. (2011)
6-MSA-type PKS gene	Putative *Actinobacteria*	Several *Ascomycetes*	Schmitt and Torsten-Lumbsch (2009)
(II) LGTs between distantly related fungi			
Sterigmatocystin/ Aflatoxin cluster	*Euromycetes* (*A. flavus*)	*Sordariomycetes* (*Podospora*)	Slot and Rokas (2011)
Penicillin gene cluster	*Hypocreales* (*Acremonium*, *Pochonia*)	*Euromycetes* (*Penicillium*, *Aspergillus*)	This work
Fumonisin gene cluster	*Sordariomycetes* ancestor (*Fusarium verticillioides*)	*Euromycetes* (*A. niger*)	Khaldi and Wolfe (2011)
ACE gene cluster	*Magnaporthe grisea* ancestor	*A. clavatus* ancestor	Khaldi et al. (2008)
Nitrate reductase cluster	Basidiomycetes ancestor	*T. reesei* ancestor	Slot and Hibbett (2007)
Ergot alkaloids and lolins cluster	*Eurotiales* fungi	*Clavicipitaceae* fungi	Marcet-Houben and Gabaldón (2016)
Sorbicillin gene cluster	*Hypocreales* ancestor	*Trichoderma*, *Acremonium*, *Penicillium* sp.	Druzhinina et al. (2016)
Several PKSs and NRPSs	Several fungi	*F. graminearum*	Sieber et al. (2014)
(III) LGTs of large fragments of DNA			
565 kb *wallaby* island	Dairy products associated fungi	*P. chrysogenum*, *P. roqueforti*, *P. camemberti*	Cheeseman et al. (2014)

Early studies on HGT between bacteria and fungi correspond to *S. cerevisiae*. Some wild-type strains of this yeast lost the ability to synthesize biotin during evolution. The reacquisition of the biotin pathway occurred by HGT, gene duplication and neofunctionalization (Hall and Dietrich 2007). In another example, the transference of aryl- and alkyl-sulphatases from *Lactobacillaceae* to *S. cerevisiae* allows this yeast to utilize sulphate, at the difference of what occurs in the closely related yeast *Ashbya gossypii* (Hall et al. 2005).

Transfer of NRPS-PKS Genes A hybrid non-ribosomal peptide synthetase-polyketide synthase (NRPS-PKS) gene was reported to be transferred from a bacterium from the *Burkholderia* class to fungi of the class *Ascomycota*, such as *Cochliobolus heterotrophus*, *Microsporum canis* and species of *Aspergillus*, *Trichophyton* and *Metarhizium* (Lawrence et al. 2011). These authors concluded that the NRPS-PKS encoding gene was transferred horizontally from bacteria very early in the evolution and has been kept by vertical inheritance and further modification in several fungi of the *Pezizomycotina* subdivision.

Transfer of the Methylsalicylic Acid PKS Six-methylsalicylic acid (6-MSA)-type PKS are present in different classes of high Ascomycota fungi, including *Cochliobolus*, *Aspergillus*, *Penicillium* or *Xylaria* and many lichenized fungi. Phylogenetic analysis of 165 keto synthase domain sequences of 6-methylsalicylic acid PKSs, and comparison with bacterial PKSs, suggested that the origin of the 6-MSA-PKS in fungi is likely to derive from *Actinobacteria*. The absence of introns in fungal 6-MSA-type PKSs and their presence in other types of PKS in the same fungi support the hypothesis of this transfer (Schmitt and Torsten-Lumbsch 2009).

Transfer of the β-Lactam Biosynthetic Genes One of the best-known examples of HGT between bacteria and filamentous fungi is the transfer of the β-lactam biosynthetic genes between bacteria to *Acremonium chrysogenum* and *Penicillium* species. This transfer (Aharonowitz et al. 1992) involves the two penicillin biosynthetic genes *pcbAB* and *pcbC*, encoding ACV synthetase and isopenicillin N synthase. In addition, genes encoding deacetoxycephalosporin C synthase and deacetoxy hydroxylase (*cefEF*) were also transferred (see below, Sect. 8).

6 Lateral Transfer of Genes Between Phylogenetically Distant Filamentous Fungi

In the last two decades, several examples of putative LGT between phylogenetically distant fungal classes have been reported (Richards et al. 2006), suggesting that these are frequent events. LGT between fungi may explain the genome plasticity observed in these microorganisms. Here, we describe some of the more relevant examples of these LGTs (Table 1).

The Sterigmatocystin Gene Cluster Transfer The transfer of the sterigmatocystin (ST) cluster from *A. flavus* or *A. nidulans* to *Podospora anserina* has been described by Slot and Rokas (2011). In fact, sterigmatocystin has been found to be produced by some *Podospora* strains (Matasyoh et al. 2011). The ST gene cluster is largely conserved in these two fungi. Interestingly, binding sites for the regulatory protein AflR of *A. flavus* are conserved upstream of several genes in the *Podospora* cluster. The average amino acid identity in proteins encoded by ST genes in *A. nidulans* and *Podospora* is 63%, and both ST clusters are highly conserved in sequence and synteny. In contrast similarity of amino acid of primary metabolism proteins between these genera is only 53%. The putative LGT was confirmed by phylogenetic analysis of the *Podospora* ST cluster which fits into the clade of *Aspergillus* ST genes. This is a clear example of LGT, because *Podospora* belongs to the class *Sordariomycetes*, while *Aspergillus* is a member of *Eurotiomycetes* (Slot and Rokas 2011).

The Fumonisin Gene Cluster The PKS-containing fumonisin gene cluster is formed by 17 genes and has been studied in detail in *F. verticillioides* and *Fusarium proliferatum*. This cluster is not present in all *Fusarium* species, i.e. *Fusarium graminearum* is not able to produce fumonisin (Proctor et al. 2003; Brown et al. 2007). Production of fumonisin has been also reported in *A. niger* (Frisvad et al. 2007). The overall fumonisin gene cluster is similar in both *F. verticillioides* and *A. niger*, despite the large phylogenetic distance between these two genera. Therefore, in a study of the fumonisin gene cluster in both organisms, Khaldi and Wolfe (2011) described that most likely the fumonisin gene cluster originates from a precursor cluster in an ancestor of *Sordariomycetes*. After duplication of the cluster in the ancestor strain, one copy was transferred to *A. niger* and *F. verticillioides*. This proposal is based on the fact that several of the fumonisin genes fit in the branch of *Sordariomycetes* genes in the phylogenetic tree. However, the exact nature of the progenitor *Sordariomycetes* strain is not known (Khaldi and Wolfe 2011).

The ACE Gene Cluster The rice blast fungi *Magnaporthe grisea* contains a fifteen gene cluster encoding a metabolite of the hybrid PKS-NRPS type that is an avirulence factor recognized by some rice plant cultivars resistant to infections by this fungus. The ACE gene cluster is present in a limited number of fungi; in *Aspergillus clavatus*, only six out of the fifteen ACE genes are present. Phylogenetic analysis of the ACE genes in different filamentous fungi led to the conclusion that an original simple gene cluster experienced duplication events, gene transfer, gene rearrangements or gene losses. However, an alternative hypothesis suggests that a simplified ACE cluster was transferred from a progenitor of *M. grisea* to an ancestor of *A. clavatus*. In the alternative hypothesis, the *M. grisea* cluster was duplicated giving rise to the present cluster (Khaldi et al. 2008).

LGT of the Sorbicillin Cluster Sorbicillins are cyclic polyketide produced by a restricted number of filamentous fungi including mainly *Trichoderma*, *Acremonium* and *Penicillium* species. These compounds are synthesized by a gene cluster of eight genes including two PKS genes. A common stretch of six genes was found in

A. chrysogenum, some *Trichoderma* sp., *Verticillium* sp. and *Penicillium rubens* (syn. *P. chrysogenum*) (Druzhinina et al. 2016). Moreover, *Colletotrichum graminicola* contains the two *pks* genes but not the other genes of the sorbicillin cluster. In summary, these authors conclude that the core sorbicillin cluster was assembled in a progenitor of the *Hypocreales* order and was complemented with genes from other fungi by LGT.

Transfer of Alkaloid Gene Clusters A possible lateral gene transfer of ergot alkaloids and lolins occurred between *Eurotiales*, particularly *Aspergillus* species, and plant endophytic fungi (Marcet-Houben and Gabaldón 2016). The family *Clavicipitaceae* include fungi that interact closely with plants and synthesize ergot alkaloids that contribute to the defence of plants against herbivores. They are also toxic to humans and have historically caused human diseases such as ergotism and migraines. The gene cluster for the biosynthesis of ergot alkaloids and lolins is present in *Clavicipitaceae* fungi, and also the related clavine alkaloids cluster occurs in *Aspergillus* species and *Penicillium roqueforti* (Martín et al. 2017). Marcet-Houben and Gabaldón (2016) propose that these gene clusters were laterally transferred from the order *Eurotiales* to the family *Clavicipitaceae*, although the *Eurotiales* cluster synthesizes only clavine alkaloids but not ergopeptines. The origin of these gene clusters in fungi and how they were initially formed is still unclear.

Phylogenetic Analysis of the Nitrate Reductase Cluster Gene Transfer Three genes form a cluster in filamentous fungi that are involved in nitrate assimilation and its reduction to ammonia. They include *nrt2*, for a high-affinity nitrate transporter; *euknr*, for a nitrate reductase; and *nir*, encoding a nitrite reductase. The orthologous genes are not clustered in bacteria or higher eukaryotes. The three-gene cluster is only present in *Ascomycetes*, *Basidiomycetes* and the oomycete *Phytophthora* (Slot and Hibbett 2007). The clustering of these genes appears to favour the utilization of nitrate in filamentous fungi. Phylogenetic analysis revealed that an ancestor of the cluster was originated in a basidiomycete and transferred by LGT to an ancestor of the ascomycete *Trichoderma reesei*. The conservation of this nitrate gene cluster is in agreement with the selfish cluster hypothesis (Price et al. 2005; Lawrence 1997, 1999) which is based in the advantages of co-regulation that increases the fitness of the strains. This arrangement favours the adaptation of fungi to new niches using nitrate as the only nitrogen source.

The β-Lactam Gene Cluster The β-lactam gene cluster is present in a few species of *Penicillium* and *Aspergillus* and in some phylogenetically distant fungi as *A. chrysogenum* and *Kallichroma tethys* that belong to the order *Hypocreales*. The transfer of the β-lactam gene cluster between *Hypocreales* (e.g. *A. chrysogenum*) and *Eurotiales* (e.g. *A. nidulans* or *P. chrysogenum*) will be discussed in Sect. 8.

7 Transfer of Large Genomic Island Between Species of *Penicillium*

A very large DNA region of 565 kb, named *wallaby*, was found to be present in *Penicillium roqueforti*, *Penicillium camemberti* and *P. chrysogenum* NRRL 1951, species of the triverticilliate clade isolated from dairy factories' environments, particularly from cheese (Cheeseman et al. 2014). This island is extensively conserved in these three species but is absent in *Penicillium* species of the same genus isolated from different soil habitats or from meat products, such as *Penicillium carneum*, a strain very closely related to *P. roqueforti*. The *wallaby* island is also absent from *P. chrysogenum* strains other than *P. chrysogenum* NRRL 1951. The integration site of the *wallaby* region in these *Penicillium* species is not conserved. This large DNA fragment derived from an unknown fungal genome and, interestingly, it contains open reading frames encoding mostly unknown proteins. The *wallaby* island may provide some benefits for the cheese fungi that contain this region since it carries the *Paf* gene that encodes the antifungal PAF protein (Binder et al. 2010). Surprisingly, this DNA island does not contain clusters for secondary metabolites biosynthesis, despite the fact that *P. roqueforti* and *P. chrysogenum* habour at least ten well-characterized secondary metabolites gene clusters (Martín and Liras 2016b; Martín and Coton 2016; García-Estrada and Martín 2016). Cheeseman et al. (2016) proposed that this LGT has taken place recently, since the *wallaby* region is not present in most of the *Penicillium* species, i.e. it is not an LGT to an ancestral *Penicillium* strain. However, although the large genome island is 90–95% conserved in those species carrying the *wallaby* region, there are small differences between *P. roqueforti*, *P. camemberti* and *P. chrysogenum wallaby* islands indicating that further rearrangements, gains or deletions have occurred very recently after the LGT event that introduced this region.

8 β-Lactam Antibiotic Gene Clusters

The transfer of β-lactam gene clusters from bacteria to fungi and also to some arthropods is a typical case of HGT, but in addition, there are interesting LTG events, involving some of the genes of the cluster between distant filamentous fungi. There is an excellent knowledge, since three decades ago, of the molecular genetics of the β-lactam gene clusters both in cephamycins/cephabacins producing bacteria and in penicillin/cephalosporin producing fungi (Aharonowitz et al. 1992). In addition, more than one hundred articles on the enzyme activities encoded by the β-lactam genes and the regulation of gene expression in the β-lactam gene cluster (Martín 2000a; Brakhage et al. 2009) provide a solid base to understand how this paradigmatic HGT/LGT works to transmit the genetic information that is found at present in some of these organisms.

β-Lactam antibiotics (penicillins and cephalosporins) are active on enzymes involved in peptidoglycan biosynthesis and therefore affect bacterial cell-wall integrity. Production of this type of antibiotics by fungi constitutes a sophisticated survival mechanism to antagonize competing bacteria in the soil.

The number of fungal species producing penicillin or cephalosporin is restricted to a few fungal families. Penicillin is produced by members of the *Trichocomaceae* family (*Penicillium* and *Aspergillus* species) (Laich et al. 1999, 2002) and the *Arthrodermataceae* family (*Trichophyton* and *Arthroderma* species) (Elander 1983; Aharonowitz et al. 1992). Cephalosporin C is produced by *Cephalosporium acremonium* (now named *Acremonium chrysogenum*), *Pochonia chlamydosporia*, *Kallichroma tethys* and *Paecilomyces persicinus* (Elander 1983; Kim et al. 2003), the first three of them belonging to the *Hypocreaceae* family.

Knowledge of the enzymes encoded by these genes allows a better understanding of the final product formed by each producer organism. A brief scheme of the penicillin and cephalosporin C biosynthesis is shown in Fig. 1. The amino acids α-aminoadipic acid (A), cysteine (C) and valine (V) are condensed by the non-ribosomal peptide synthetase ACVS, encoded by *pcbAB*, forming the tripeptide δ-(α-aminoadipyl-L-cysteinyl-D-valine, ACV) (Díez et al. 1990; Smith et al. 1990; Martín 2000b; Gutiérrez et al. 1991a). ACV is then cyclized by the isopenicillin N synthase, encoded by *pcbC*, to form isopenicillin N (IPN). The structure of this compound (see Fig. 1) consists of a four-membered β-lactam ring and a five-membered thiazolidine ring and possesses weak antibiotic activity. In penicillin G-producing fungi, a third gene, *penDE*, of eukaryotic origin carrying three introns encodes the IPN acyltransferase. This enzyme exchanges the lateral chain of α-aminoadipic with phenylacetic acid to form the aromatic penicillin G, a potent antibiotic (Fig. 1). The cephalosporin C (CPC) structure contains, in addition to the β-lactam ring, a six-membered dehydrothiazine acid ring (Fig. 1) and a lateral chain of D-α-aminoadipic acid. Consequently, the CPC pathway has a higher number of steps than that of penicillins. In *A. chrysogenum* the two initial CPC pathway steps are identical to those of penicillin-producing fungi. The following step, specific of *A. chrysogenum*, is the epimerization of isopenicillin N to penicillin N, performed by two enzymes, CefD1 and CefD2. These are encoded by typical, intron-containing, eukaryotic genes with similarity to long-chain acyl-CoA synthetases and acyl-CoA racemases, respectively (Ullán et al. 2002; Martín et al. 2004). The expansion of the five-membered thiazolidine ring to the six-membered dehydrothiazine ring is carried out in *A. chrysogenum* by the penicillin N expandase-deacetoxycephalosporin C hydroxylase. This enzyme, encoded by the *cefEF* gene, has fully bifunctional activity since it expands the five-membered ring and is able to hydroxylate the six-membered dehydrothiazine ring at the C-3′ position (Dotzlaf and Yeh 1989).

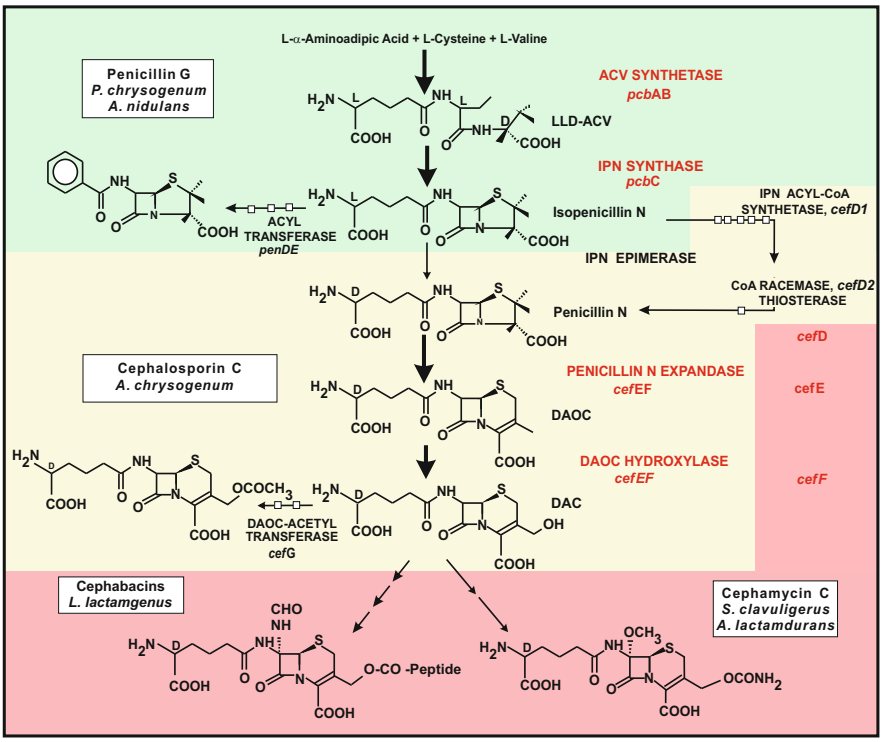

Fig. 1 Biosynthesis pathway for penicillins in *Penicillium* and *Aspergillus* strains (shaded in green), for cephalosporin C in *Acremonium chrysogenum* (shaded in yellow) and for cephamycins and cephamycin by *Streptomyces clavuligerus* and *Lysobacter lactamgenus*, respectively (shaded in pink). The essential steps, products, enzymes and genes are detailed. The introns, in those gene carrying introns, are indicated with small open squares. Thick arrows correspond to the steps detailed in the text

8.1 β-Lactam Gene Clusters in Actinobacteria *and Gram-Negative Bacteria*

Surprisingly, in 1976, it was found that several Gram-positive *Actinobacteria*, mostly *Streptomyces* and *Amycolatopsis* species, produced the antibacterial cephamycins and later that some Gram-negative bacteria, e.g. *Lysobacter lactamgenus* or *Flavobacterium* sp., were able to synthesize cephabacins. Cephamycins and cephabacins have structures similar to cephalosporin C (see Fig. 1) and are active on peptidoglycan biosynthesis. In *Actinobacteria*, the β-lactam clusters carry β-lactamase genes and they are frequently located side by side to β-lactamase inhibitors' gene clusters, forming a supercluster, e.g. cephamycin-clavulanic supercluster (Ward and Hodgson 1993). It is likely that the antibacterial activity produced by these organisms is modulated by the β-lactamase and β-lactamase inhibitors in a delicate balance to avoid self-suicide.

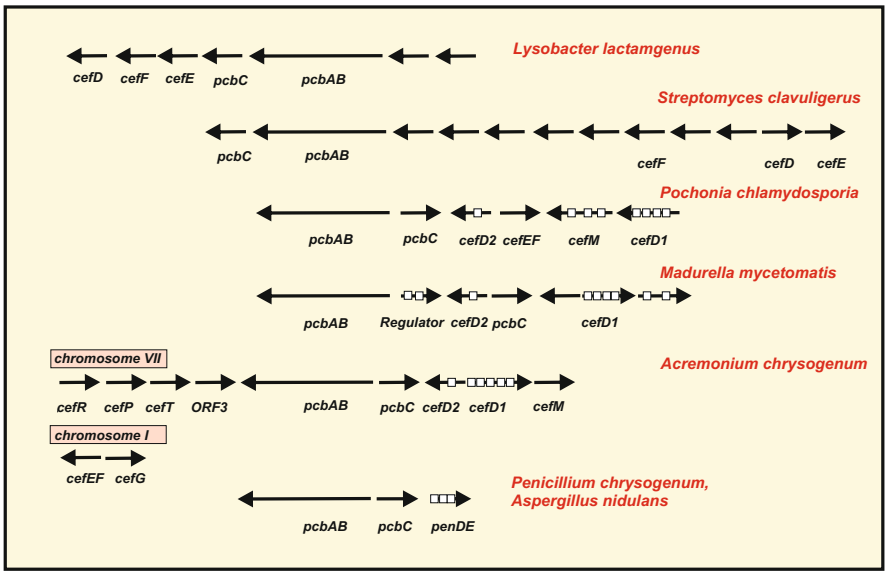

Fig. 2 Organization of the β-lactam cluster of genes in the Gram-negative bacteria *Lysobacter lactamgenus*, the *Actinobacteria Streptomyces clavuligerus* and the fungi *Pochonia chlamydosporia, Madurella mycetomatis, Acremonium chrysogenum, Penicillium chrysogenum* and *Aspergillus nidulans.* The introns in the genes of eukaryotic origin are labelled with open squares

The cephamycins/cephabacins pathways include the two initial steps of penicillins and cephalosporins biosynthesis, but the epimerization of isopenicillin N to penicillin N is carried out by a typical prokaryotic pyridoxal-phosphate-dependent IPN epimerase, encoded by *cefD*, similar to bacterial amino acid racemases. In Gram-positive *Actinobacteria* and in Gram-negative *Lysobacter*, two different enzymes deacetoxycephalosporin C (DAOC) synthase and DAOC hydroxylase carry out the expansion of the thiazolidine ring and the C-3′ hydroxylation of the resulting dehydrothiazine ring, respectively. These enzymes are encoded by two genes, *cefE* and *cefF* (Fig. 2), that were probably formed by gene duplication of an ancestral gene and further specialization (Baker et al. 1991). Each of these enzymes has a residual activity of the other type. The biosynthesis pathway for cephamycins or cephabacins contains more steps than those for cephalosporins and carries additional modification specific steps (late steps, not detailed in Fig. 1).

8.2 *Horizontal Transfer of β-Lactam Genes from Bacteria to Fungi*

The transfer of the *pcbC* gene, encoding the IPN synthase, was one of the first examples of HGT, from bacteria to fungi, described (Weigel et al. 1988; Landan et al. 1990; Cohen et al. 1990). Using the maximum-likelihood phylogenetic analysis of the *pcbC* gene, Buades and Moya (1996) concluded that *pcbC* was transferred from prokaryotes to fungi about 852 +/− 106 million years ago. Comparison of the IPN synthases and the ACV synthetases between *Actinobacteria* and fungi shows amino acid identities in the order of 56–58%. A comparison of enzymes for primary metabolism between *Actinobacteria* and fungi, i.e. the arginine biosynthesis enzymes (ArgBCDJ) of *Streptomyces clavuligerus* and those of *P. chrysogenum*, shows amino acid identities of 29–35%. This analysis reveals large differences in amino acid identity between the enzymes for primary metabolism and enzymes of secondary metabolism (IPN synthases and ACV synthetases). In addition, the GC content of *pcbAB* (58.1% and 54.3% in *Penicillium* and *Acremonium*, respectively) and *pcbC* gene is higher in relation to the CG content in fungal genomes (average 48.9% in *P. chrysogenum* exons) (van den Berg et al. 2008) and closer to what is expected in high GC content organism such as *Actinobacteria*. However, the use of codons in the fungal β-lactam genes has changed with respect to the GC-rich *Actinobacteria* (close to 70% CG) or *Lysobacter* (about 65%), suggesting that it has been adapted progressively for millions of years to the consensus fungal use (Weigel et al. 1988; Buades and Moya 1996). Phylogenetic analyses of the IPNS proteins reveal that the enzymes of Gram-negative bacteria are closer to the IPNSs of *Hypocreales* and *Eurotiales* fungi than those of Gram-positive bacteria (Fig. 3). In summary, all evidence supports the conclusion that the *pcbAB* and *pcbC* genes were transferred from bacteria to fungi as an early ancestor of the β-lactam cluster that has been later modified both in bacteria and in fungi.

8.3 *A Major Rearrangement of the* pcbAB-pcbC *Head to Tail Cluster Occurred During Transfer to Fungi*

The organization of the cephamycin and cephabacins gene clusters in *S. clavuligerus* and *L. lactamgenus* is shown in Fig. 2. These clusters contain the genes for the early steps of the pathway, *pcbAB* and *pcbC*, in a head to tail organization, expressed from a common upstream promoter. Also present are genes for the formation of the dehydrothiazine ring of the cephalosporins (*cefD*, *cefE*, *cefF*), genes for the cephamycin and cephabacins specific late steps of the pathways, and regulatory genes (not detailed in Figs. 1 and 2).

The organization of the penicillin biosynthesis genes follows the classical pattern *pcbAB-pcbC-penDE*, being *pcbAB-pcbC* organized in a bidirectional divergent arrangement. This organization is conserved in all the *Penicillium* species

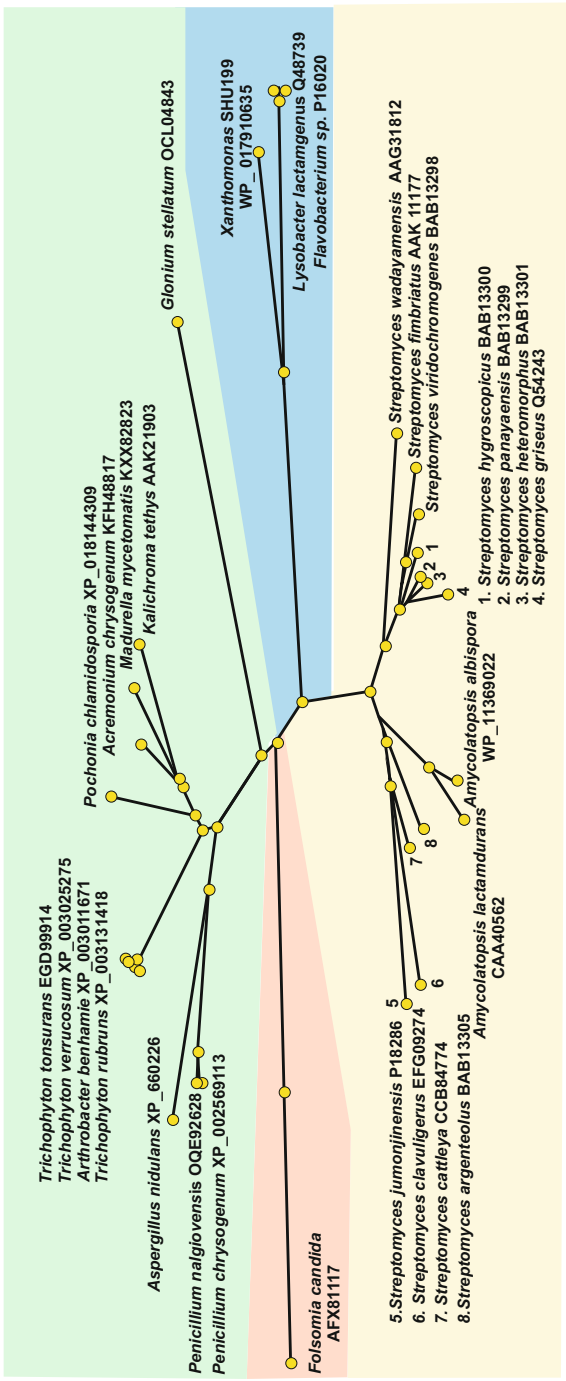

Fig. 3 Phylogenetic tree of the 29 IPN synthases of bacteria, fungi and the arthropod *Folsomia candida*. The tree has been obtained with the COBALT Program, NCBI. The IPNSs of fungi (shaded in green), *Actinobacteria* (in yellow), Gram-negative bacteria (in purple) and the arthropod *F. candida* (in blue) are shown. The numbers following the species indicate accession number of the proteins

(*P. notatum*, *P. chrysogenum*, *P. griseofulvum*, *P. nalgiovense*, *P. verrucosum*), in *Aspergillus* species (*A. nidulans*, *A. sydowii*, *A. bombycis*, *A. nomius*, *A. oryzae* and *A. flavus*) and in *Trichophyton* species (*T. rubrum*, *T. benhamiae*, *T. verrucosum* and *T. tonsurans*). It is important to note that the two genes *pcbAB* and *pcbC* are sufficient to produce the antibiotic isopenicillin N and the third gene, *penDE*, is not required to produce active antibiotic that may explain the presence of only two genes in the clusters of some fungi (Gutiérrez et al. 1991b). The genes *pcbAB* (11 kb) and *pcbC* (1 kb) lack introns (Díez et al. 1990; Gutiérrez et al. 1991a). In contrast, *penDE* is a classical eukaryotic gene containing three introns, suggesting that the penicillin gene cluster has been assembled by recruitment of an acyltransferase gene from an uncharacterized fungal genome. Indeed, a paralogous gene that may have evolved to form *penDE* is present in most Ascomycete genomes, although its function does not appear to be directly related to penicillin biosynthesis (García-Estrada et al. 2009; Spröte et al. 2008).

The different organization of the *pcbAB* and *pcbC* genes in bacteria and fungi suggests that a major rearrangement of these two genes occurred during the HGT event from bacteria to an early β-lactam cluster progenitor in fungi.

A remaining question is the directionality of the LGT between members of the *Hypocreales* and the *Eurotiales*. As indicated above the β-lactam gene cluster is present in two phylogenetically distant orders of fungi, the *Hypocreales* and the *Eurotiales*. In the *Hypocreales* order, the cephalosporin gene cluster was reported in *A. chrysogenum* and *K. tethys* and recently has been found also in *Pochonia chlamydosporia*, another member of the *Hypocreales*, and *Madurella mycetomatis* (Liras and Martín, unpublished results), included now in the order *Sordariales*, phylogenetically close to *Hypocreales*. In members of the order *Eurotiales* the *pcbAB-pcbC* cluster (but not the late genes specific for cephalosporin biosynthesis), is present in several species of *Penicillium* and *Aspergillus*.

The important question is whether there was a single HGT event from bacteria to fungi, or if were two or more HGT transfers from bacteria to phylogenetically distant fungi. Analyses of the phylogenetic tree (Fig. 3) reveal that *Hypocreales* and *Eurotiales* diverge from a common branch in the tree, indicating that all of them probably derived from a single transfer event from bacteria to fungi.

The species *A. chrysogenum*, *P. chlamydosporia*, *K. tethys* and *M. mycetomatis* contain cephalosporin biosynthetic genes although there are important differences between them. In *A. chrysogenum* the intron-less *pcbC* and *pcbAB* genes, located in chromosome VII, were integrated next to two typical eukaryotic genes (*cefD1*, *cefD2*) containing introns, that were functionalized to epimerize isopenicillin N to penicillin N (Ullán et al. 2002; Martín et al. 2004). The same organization *pcbAB-pcbC-cefD2* has been found in *Kallichroma tethys* (Kim et al. 2003; Martín et al. 2004).

Interestingly, *P. chlamydosporia* contains in a single chromosome locus a complete cephalosporin gene cluster that is closely related to those of bacteria, including the *pcbAB, pcbC* and *cefEF* genes, genes *cefD1* and *cefD2* for epimerization of penicillin N and *cefM*, a gene encoding a transporter protein, essential for cephalosporin formation. These genes are separated into two chromosomes in

A. chrysogenum (Fig. 2). *P. chlamydosporia* and *M. mycetomatis* lack the *cefG* gene, encoding the last enzyme of cephalosporin C biosynthesis in *A. chrysogenum.* These findings allow us to propose that the first HGT bacterial β-lactam genes were incorporated in an ancestor of *P. chlamydosporia.* Since *P. chlamydosporia* contains all relevant genes of the cephalosporin gene cluster in one single location, it seems that the initial transfer was from bacteria to an ancestor of the *Hypocreales* order, that has been maintained in recent isolates of *P. chlamydosporia* (Fig. 2). The division in *A. chrysogenum* of cephalosporin biosynthesis genes in two separated subclusters (chromosomes I and VII) may have resulted from a rearrangement and translocation of the previous unsplitted cluster. Indeed, chromosome plasticity is well documented in *A. chrysogenum* (Smith et al. 1991; Gutiérrez et al. 1999). On the other hand, members of the *Eurotiales* and *Mytilinidiales* orders (*Glonium stellatum*) contain only the first two genes of the penicillin-cephalosporin pathway *pcbAB-pcbC* that are likely to have been transferred by an LGT event from an ancestor of the cephalosporin cluster in the *Hypocreales* order. This hypothesis integrates all available evidence.

9 Genes *pcbAB* and *pcbC* in the Soil Arthropod *Folsomia candida*

A provocative example of rare HGT from bacteria to animal cells involving β-lactam biosynthesis genes has been reported (Roelof et al. 2012). A gene encoding an IPN synthase was found in the detritivorous and fungivorous soil arthropod *Folsomia candida*, of the order *Collembola*. The encoded IPNS conserves all the consensus amino acids in the active centre of this enzyme in bacteria and filamentous fungi. The *Folsomia pcbC* gene contained three introns and typical eukaryotic splicing signals. This result is very interesting since all *pcbC* genes described so far in bacteria and filamentous fungi lack introns. The presence of introns in the *pcbC* gene of *Folsomia* supports the conclusion that this enzyme is produced by the arthropod cells and not by bacteria inhabiting its gut or the skin. Indeed, no IPN-forming activity was observed in the microbial gut population of *Folsomia.* Recombinant IPN synthase was obtained by the expression of *Folsomia pcbC* cDNA gene in *E. coli*. The IPN synthase was functional and able to produce isopenicillin N from the substrate ACV (Roelof et al. 2012).

The *pcbC* phylogenetic analysis suggested a much ancient transfer from bacteria to *Folsomia* than to fungi (Fig. 3). This analysis showed that the *Folsomia pcbC* gene diverged from bacterial and fungal and followed its own evolutionary pathway. The most likely *pcbC* donors are soil bacteria or filamentous fungi associated with the arthropod diet.

At this time, it is unclear whether *Folsomia* has a complete *pcbAB-pcbC* cluster. This possibility is supported by evidence that suggests that some expressed sequence tags (EST) of the *Folsomia* genome correspond to the *pcbAB* gene. Transcription of

these *pcbAB* ESTs in the *Folsomia* epithelial cells is triggered by phenanthrene or by toxic metals (Nota et al. 2008). An important question is whether the arthropod benefits from penicillin production by its own cells. Even if the entire *pcbAB* gene is present, it is still unclear whether the amount of isopenicillin N produced is sufficient to exert a significant biological action in combating infection of the arthropod by undesirable bacteria that would explain the conservation of these genes in the *Folsomia* genome. It is possible that other arthropods, particularly of the *Folsomia* genus, contain the *pcbAB* and *pcbC* genes and are able to produce isopenicillin N (Roelof et al. 2012), but, so far, this has not been unequivocally established.

10 Future Outlook

Filamentous fungi have an impressive capability to produce an enormous range of secondary metabolites. In the last decades, it has become clear that genes for the production of secondary metabolites are arranged in clusters that contain not only the biosynthetic genes but also regulatory genes and transporter genes involved in the secretion of the secondary metabolite. Many of the gene clusters are still unexplored and therefore our knowledge of the ability to produce secondary metabolites is still limited. The secondary metabolite gene clusters may be transferred between different organisms thereby acting as caravans of genes travelling across bacterial and fungal genomes, and therefore providing abilities that allow adaptation of the host fungi to new habitats (Wisecaver and Rokas 2015). Interestingly, although interkingdom HGT from bacteria to eukaryotes provides the basis for the acquisition of secondary metabolites gene cluster, there are also numerous LGT events that have contributed to the dissemination of these clusters between phylogenetically distant fungi. Additional genes are frequently recruited or loss from the clusters but the origin and integration mechanisms of these extra genes is very poorly known. The β-lactam gene cluster is an interesting model to understand the formation and distribution of secondary metabolites gene clusters. However, the phylogenetic relationship of the β-lactam gene cluster between bacteria and filamentous fungi is still not completely elucidated. It is possible that this cluster was transferred from bacteria in a single HGT event although still there is the possibility of two or more parallel HGT events. The presence of two genes of the β-lactam cluster, in the arthropod *Folsomia candida* cells, provides a very interesting example of gene transfer from bacteria to animals. It is however unclear whether the presence of these two genes in *Folsomia candida* confers production of an active β-lactam in this arthropod. Further research, including the analyses of rare fungal genomes, may provide novel evidence on how these gene clusters have evolved in filamentous fungi and also in animals.

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Horizontal Gene Transfer Among *Neisseria* Species and Humans

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Abstract The genus *Neisseria* is included among those organisms containing a large number of foreign or exogenous genes in its genome. In most cases, the incorporation of these foreign genes is the consequence of recent events of horizontal gene transfer (HGT) occurring between different species of *Neisseria* but also with other bacterial genera such as *Haemophilus influenzae* and even with its exclusive host, the human. In this chapter, we review the transformation process as the main mechanism of HGT in *Neisseria* and its role in the acquisition of virulence factors and antibiotic resistance markers, mainly from commensal to pathogen *Neisseria* spp. In addition, we review evidence of gene exchange between both pathogenic *Neisseria* spp. and human, showing that HGT can occur between mammal's host and their associated bacteria.

Keywords *Neisseria* spp. · Horizontal gene transfer · Transformation · *Neisseria meningitidis* · *Neisseria gonorrhoeae* · Antibiotic resistance

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1 Introduction

The genus *Neisseria* includes capsulated (Clemence et al. 2018), Gram-negative oxidase-positive β proteobacteria that are part of the commensal flora of mucosal membranes of humans and some animals. This genus is generally defined to be formed by diplococci with flattened opposing sides but also includes rod-shaped species as *N. elongata* (Bovre and Holten 1970), *N. weaveri* (Holmes et al. 1993; Andersen et al. 1993), *N. bacilliformis* (Han et al. 2006) and *N. shayeganii* (Wolfgang et al. 2011). Generally considered non-pathogenic except for *N. meningitidis* and *N. gonorrhoeae*, *Neisseria* species colonize the human upper respiratory tract and the human urogenital tract (Wolfgang et al. 2011). Current knowledge of the members of the *Neisseria* genus was recently reviewed by Liu et al. (2015) showing that the *Neisseria* genus includes a large number of species successful in finding niches in a broad range of hosts including humans, other mammals, birds or even insects [suggesting insects might act as a vector (Weinstein 1991)]. Although the main non-human habitat for *Neisseria* is primarily in the upper airways, it is also found in the lower intestinal tract of avian hosts. In their review Liu et al. also refer to *Neisseria* species in the environment with no obvious association with a host (free-living *Neisseria*) and with the ability to degrade organic pollutants.

Many of these *Neisseria* species share a niche along with the rest of the microbiota. The members of the genus *Neisseria* are closely related, and it is likely that all or most species cause asymptomatic colonization. Carriage colonization with multiple strains provides an opportunity for horizontal genetic exchange. The most studied species are the two that are considered human pathogens, and the study of mechanisms of horizontal gene transfer concerning the genus has primarily focused on them. *Neisseria meningitidis* is carried asymptomatically in the nasopharynx. In Europe and the United States, it was estimated that carriage rates are between 10 and 35% in young adults (Caugant and Maiden 2009; Cartwright et al. 1987; Caugant et al. 1994; Stephens 1999; Claus et al. 2005). It is an opportunistic pathogen which occasionally penetrates the mucosal membrane and gains access to the bloodstream causing invasive meningococcal disease. The most common clinical presentations of the disease are meningitis and septicaemia, and the WHO estimates that 8–15% of patients die within 24–48 h of developing symptoms. Twelve serogroups of *N. meningitidis* have been identified based on the chemical composition of their polysaccharides, six of which (A, B, C, W, X and Y) can cause disease. *Neisseria gonorrhoeae* is the etiologic agent of disease gonorrhoea, a sexually transmitted infection commonly manifested by urethritis, cervicitis, proctitis, salpingitis or pharyngitis. Infection may be asymptomatic, so lack of treatment can lead in women to develop pelvic inflammatory disease (PID), chronic pelvic pain, ectopic pregnancy and infertility. Each year, there are an estimated 78 million new infections with *N. gonorrhoeae* (WHO Guidelines for the Treatment of *Neisseria gonorrhoeae*, World Health Organization, Geneva, 2016). Due to multiple resistance to available antibiotics, infection by *N. gonorrhoeae* has become a serious health problem worldwide (Lewis 2010).

Neisseria are naturally competent for the uptake of exogenous DNA during all phases of growth (Biswas et al. 1989), and DNA transformation is the most effective mechanism for horizontal genetic exchange in *Neisseria* spp. Processes of recombination and genetic exchange contribute to variability in major surface antigens as Opa (Hobbs et al. 1994) or the PorA (Feavers et al. 1992) which has implications for design of vaccines. HGT also has led to clinical isolates becoming less susceptible to antibiotics, especially in *N. gonorrhoeae* (Spratt et al. 1992; Unemo and Shafer 2011), and to the transfer of essential virulence determinants (Frosch and Meyer 1992). Furthermore, HGT in *Neisseria* seems to be a mechanism used by bacteria to maintain the integrity of the genome by replacing damaged or lost regions of the chromosome with new copies from exogenous DNA templates (Mongold 1992; Davidsen et al. 2004; Aas et al. 2002b).

2 Mechanisms for Horizontal Gene Transfer in *Neisseria*

2.1 Transformation

Transformation is the most effective mechanism for horizontal genetic exchange in *Neisseria* spp. and has been widely described (Sparling 1966; Lorenz and Wackernagel 1994; Hamilton and Dillard 2006; Obergfell and Seifert 2015; Chen and Dubnau 2004). Also in most of the Gram-negative bacteria, transformation in *Neisseria* spp. is generally accepted to be a process tightly associated with the expression of type IV pili (Tfp) because there is a correlation between the presence of pili and competence (Biswas et al. 1977; Chen and Dubnau 2004). However, more recent studies suggest that simply a pseudopilus apparatus, consisting of the type IV pilus complex proteins, is sufficient for transformation (Obergfell and Seifert 2015). Pilus type IV is a fibre implicated in twitching motility, cellular adherence and microcolony formation (Merz et al. 2000; Swanson 1973; Dietrich et al. 2011). It is composed mainly of major pilin (PilE) that is processed by the prepilin peptidase PilD. The NTPase PilF provides energy to assemble the pilin subunits into a filament (Lauer et al. 1993). This filament is anchored in the inner membrane by PilG and extends to the bacterial surface of the cell through a pore formed by a homododecameric complex of the outer membrane PilQ secretin (Collins et al. 2003). The PilT NTPase mediates retraction of the pilus through depolymerization of the pilin subunits, and it is required for twitching motility (Whitchurch et al. 1991). Other proteins and lipoproteins have also been implicated in the transformation process as ComE, ComL, Tpc and ComA. This HGT mechanism takes place in sequential steps: binding of exogenous DNA, uptake into a DNase-resistant state and translocation across the outer membrane, the murein layer and the inner membrane to gain access to the cytoplasm and finally RecA-dependent homologous recombination (Fig. 1).

Like *Haemophilus influenzae*, *Neisseria* preferentially take up exogenous DNA containing a non-palindromic and specific DNA uptake sequence (DUS) (Goodman

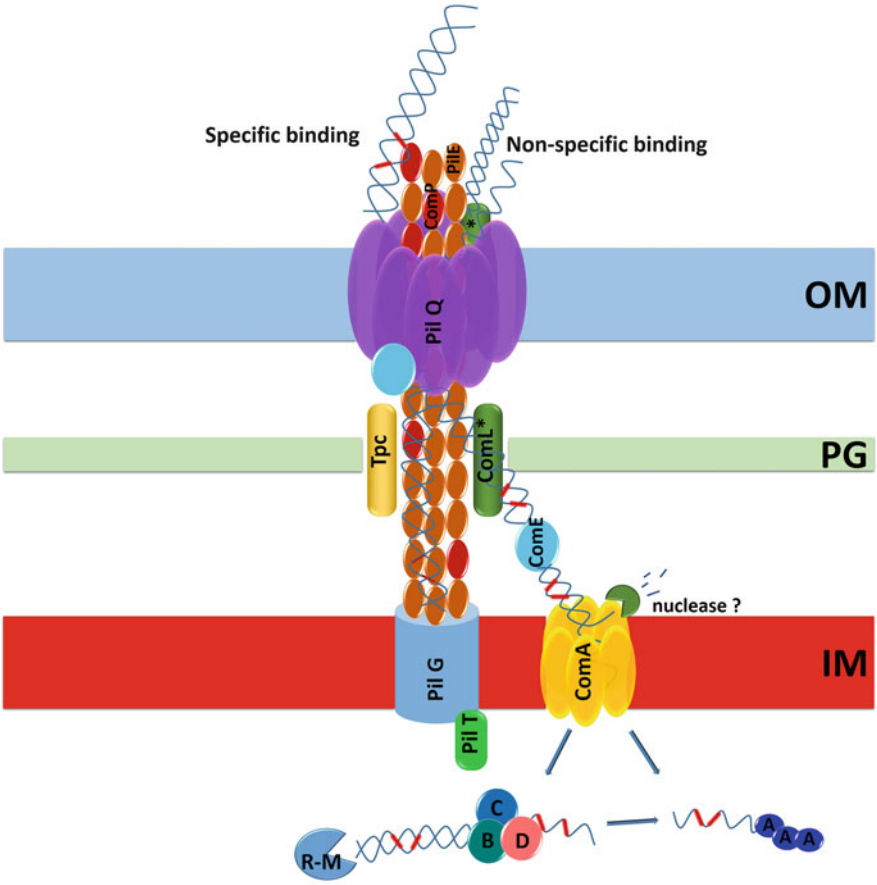

Fig. 1 Type IV pilus-like *Neisseria* DNA uptake. The pilin ComP binds specifically dsDNA DUS-containing sequences (indicated as red boxes in DNA). PilQ ring might bind no DUS-containing DNA with the binding sites located in the central portions of the ring. Also, the protein ComL (if outer membrane protein) might uptake no DUS-containing DNA. Alternative to the model of pseudopilus retraction due to PilT, a ratchet model with ComE acting as a ratcheting chaperone is proposed. The protein ComE is the secondary receptor in the periplasm. Proteins Tpc and ComL (if periplasmic protein) are involved in the transport of the DNA through the peptidoglycan layer, and transport of DNA to the cytoplasm takes places through protein ComA. The protein PilG might cooperate with ComA in the incoming of DNA. In the cytoplasm transforming DNA is incorporated to the bacterial chromosome by RecA-dependent homologous recombination. It is thought that unknown nuclesase processes dsDNA in the periplasm, and the majority of the incoming DNA is ssDNA. When the incoming DNA is dsDNA, it is thought to be processed by restriction-modification (R-M) systems and RecBCD

and Scoccat 1988; Elkins et al. 1991). The DUS motif appears approximately once every kilobase within the genomes of all *Neisseria* spp., occurs on both strands in equal number (Smith et al. 1999) and concentrates with core sequences in inverted pairs forming transcription terminators (Ambur et al. 2007). DUS was initially

defined as a 10-base sequence (5′GCCGTCTGAA3′), but in 2007 Ambur et al. proposed to extend the 10-mer identity of DUS to a 12-mer DUS by the inclusion of two semi-conserved bases into the DUS sequences (5′atGCCGTCTGAA3′) (Ambur et al. 2007). It has been shown that 12-mer DUS enhances transformation rates compared to 10-mer DUS and that transformation with homologous DNA is strand specific, the single-strand Watson 12-mer DUS being more efficient than the single-strand Crick 12-mer DUS (Duffin and Seifert 2012). Later, Frye et al. (2013) established the term DUS dialects, which define the eight variants of DUS revealed by bioinformatic analyses of different *Neisseriaceae* genomes. This study also allowed the identification of a DUS core 5′CTG3′ conserved in all eight DUS dialects and strictly required for specific transformation.

One of the biggest questions about the transformation process in *Neisseria* has been the identity of the receptor of DUS-containing DNA. Because overexpression of ComP was associated to an increase in frequency transformation, this pilin was initially pointed as a possible specific DNA receptor (Aas et al. 2002b). The role of ComP in specific binding of the DUS was confirmed by Cehovin et al. showing that ComP is highly conserved among *Neisseria* spp. and binds dsDNA via an electropositive stripe with higher affinity for DUS-containing DNA (Cehovin et al. 2013). Some studies indicate that the expression of a functional pilus or a pseudopilus is determined by the minor pilin expressed, so the transformation would be inhibited by the expression of PilV acting as antagonist, whereas transformation is enhanced by the expression of ComP (Aas et al. 2002a). Further studies of ComP showed that several single-base substitutions in DUS inner bases resulted in the loss of interaction with ComP and loss of efficiency in transformation. Different DUS variants are also tailored to their cognate ComP receptor, promoting intraspecies gene transfer (Berry et al. 2013). Although ComP can bind both DUS-containing DNA and no DUS-containing DNA, the higher affinity for the former makes the presence of the specific uptake sequence results in more efficient transformation. It was considered that Tfp/pseudo-Tfp retraction, mediated by PilT NTpase, dragged the bound dsDNA through the PilQ secretin pore from the bacterial surface to the periplasm crossing the outer membrane in this way. An alternative to the retraction model was described by Hepp et al. who proposed a translocation ratchet model with ComE acting as a ratcheting chaperone (Hepp and Maier 2016). This study showed the DNA uptake velocity depends on the concentration of the periplasmic protein ComE but the velocity–force relations of Tfp retraction and DNA uptake were not comparable. Tfp/pseudo-Tfp would be the trigger for opening the secretin PilQ pore once ComP bound exogenous DNA. Both models probably coexist as it has been shown for *N. meningitidis* that the PilQ ring binds non-DUS-containing DNA with the binding sites located in the central portions of the ring to be later taken up by pilus retraction (Assalkhou et al. 2007; Frye et al. 2015).

ComE is a protein with reversible DNA-binding activity and predicted HhH motifs, which interact with DNA in a non-sequence-specific manner (Doherty et al. 1996). The *comE* gene is present in four copies in the *N. gonorrhoeae* and *N. meningitidis* genomes, the expression of one copy being necessary for transformation to take place (Chen and Gotschlich 2001). It is postulated that ComE acts as

the secondary receptor in the periplasm of the already selected DUS-containing DNA and as the ratcheting chaperone that allows entry of that DNA to the periplasm. Once within the periplasm, another two proteins are considered to be involved in transport of the DNA through the peptidoglycan layer, ComL and Tpc. Mutations in *comL* and *Tpc* genes affect natural transformation but not DNA uptake. ComL is a lipoprotein exclusively associated with the murein, and Tpc is a protein considered to have murein-hydrolase activity that is implicated in cell division. Therefore, ComL and Tpc could serve in the transformation process as murein hydrolases by making openings that allow donor DNA to cross the peptidoglycan layer (Fussenegger et al. 1997). However, Benam et al. demonstrated that ComL is co-purified with the outer membrane after sucrose density gradient centrifugation and also it binds DNA (Benam et al. 2011). Therefore, this lipoprotein could be implicated in the binding of DNA rather than transport through the peptidoglycan layer. A study of the spatiotemporal dynamics of DNA uptake revealed that binding and transport through the outer membrane of DNA take place all over the bacterial surface but the secondary receptor ComE concentrates most of the imported DNA locally to the septum at the centre of diplococci, so exogenous DNA is accumulated in a short period of time in the periplasm, independent of the transport through the inner membrane to the cytoplasm (Gangel et al. 2014). Finally, the inner membrane protein ComA is responsible for transporting the DNA to the cytosol where it will be recombined with the human chromosome. Mutants in *comA* gene were DNA uptake proficient but blocked for translocation of DNA into the cytoplasm (Facius and Meyer 1993). It was found that the inner membrane protein PilG interacts with DNA and PilQ, so it might be that PilG cooperates with the ComA pore to facilitate the DNA uptake (Frye et al. 2015). In the cytoplasm, transforming DNA is incorporated to the bacterial chromosome by RecA-dependent homologous recombination (Mehr and Seifert 1998; Hamilton and Dillard 2006). It was confirmed that protein DprA is directly involved in competence for natural transformation via RecA loading and protection of the incoming ssDNA from degradation by restriction endonucleases (Beyene et al. 2017). It was also demonstrated that DrpA from *N. meningitidis* binds ssDNA, with lower affinity dsDNA, and has no specificity for DUS-containing DNA (Hovland et al. 2017). It is thought that the majority of donor DNA in *N. gonorrhoeae* entering the cell is double stranded (Biswas et al. 1989), although Assalkhou et al. found that *N. meningitidis* binds ssDNA non-specifically better than dsDNA (Assalkhou et al. 2007). As the recombinase RecA binds ssDNA, at some point ssDNA must be formed during the transformation process. A study of *N. gonorrhoeae comA* mutants suggests that it is initially formed within the periplasm (Chaussee and Hill 1998), but the responsible nuclease was not identified. When the incoming DNA is double stranded, it is thought to be processed by restriction-modification (R-M) systems and RecBCD (Mehr and Seifert 1998). R-M systems are constituted by a methyltransferase (Mod) that recognizes self-DNA and a restriction endonuclease (Res) and are considered to stabilize clonal linages by inhibiting DNA transfer from unrelated clones (Claus et al. 2000; Budroni et al. 2011). Clustered, regularly interspaced, short palindromic repeat (CRISPR)

was also demonstrated to be a mechanism to limit the integration of foreign nucleic acids in *N. meningitidis* (Zhang et al. 2013).

How is DNA donated? DNA can be acquired from autolysis or cell death. However, *N. gonorrhoeae* like other Gram-negative bacteria can also donate DNA using the type IV secretion system (TSS4) which secretes ssDNA into the extracellular milieu (Ramsey et al. 2011; Callaghan et al. 2017).

Secreted membrane vesicles (MVs) may provide an alternative source of donated DNA. Many bacteria produce MVs, and most Gram-negative bacteria secrete outer membrane vesicles (OMVs); however, OMVs contain a range of other components such as inner membrane and periplasmic and cytoplasmic components including genetic material. Dorward et al. have demonstrated MV-mediated transfer of plasmid DNA among *N. gonorrhoeae* suggesting that OMVs might be a previously unexplored genetic exchange mechanism (Dorward et al. 1989). Genetic material may be bound to the MV surface or in the lumen, protected against nucleases, thermo-degradation and other harmful agents. Different possibilities have been suggested for transferring of genetic material to recipient bacteria: MV may present the DNA by attaching to the OM surface of the recipient cell, DNA may be released to the medium after MV lysis or the MV may attach to the OM and be internalized by the recipient cell (Domingues and Nielsen 2017), constituting a possible new genetic exchange mechanism.

2.2 Other Horizontal Gene Transfer Mechanisms

Although DNA transformation is the most effective mechanism for horizontal genetic exchange in *Neisseria* spp., it can also take place through bacterial conjugation, and this is the most common mechanism for mobilization of antibiotic resistance plasmids (Sparling 1986; Biswas et al. 1989; Roberts 1989). Although many phage-related genes have been identified in most species of *Neisseria* (Goodman et al. 2006; Piekarowicz et al. 2014; Kłyż and Piekarowicz 2018; Steinberg et al. 1976), horizontal genetic exchange via transduction seems to be less frequent and not as effective as DNA transformation.

3 Horizontal Gene Transfer Among *Neisseria* spp. and Other Bacteria

The development of classical and high-throughput sequencing technologies in recent decades has allowed access to complete genomes, providing powerful tools for the study of horizontal intra- and interspecies genetic transfer events by comparing a wide range of nucleotide sequences. Many studies have compared genomes of *Neisseria* spp. using different techniques such as microarray comparative genome

hybridization (mCGH) (Joseph et al. 2011; Hotopp et al. 2006), multilocus sequence typing (MLST) (Maiden et al. 1998; Mulhall et al. 2016; Xu et al. 2015; Yero et al. 2010) or pulsed-field gel electrophoresis (PFGE) (Ohnishi et al. 2010), together with bioinformatic tools and available databases.

Comparison of nucleotide sequences allows detection of mosaic gene structures that are interpreted as having arisen from the horizontal transfer of blocks of DNA by transformation (Spratt et al. 1992). As *Neisseria* spp. are naturally competent for transformation, mosaic genes occur frequently, and several genes are mosaics, including major outer membrane proteins PorA (Feavers et al. 1992; Bart and van der Ende 1999) and Por B (Dyet and Martin 2005) and the fHbp (Beernink and Granoff 2009), which are components of meningococcal vaccines.

Horizontal genetic exchange promotes the generation of antigenic diversity and the transmission of virulence factors, so coexistence with asymptomatic or commensal strains of the oropharynx microbiota might lead to the emergence of new virulent strains not covered by vaccines. An example might be the consecutive outbreaks of meningitidis in Nigeria, since 2013, caused by a novel strain of serogroup C *N. meningitidis* (NmC) (Brynildsrud et al. 2018) that also spread to the neighbouring country Niger in 2015. NmC cases have been rare in the meningitis belt for many decades. The last NmC reported outbreak in Nigeria was in 1975. Because meningococcal disease outbreaks in the African meningitis belt were mainly caused by serogroup A *N. meningitidis* (NmA), a monovalent meningococcal A conjugate vaccine (MenAfriVac) was introduced in 2010. Brynildsrud et al. characterized the evolution and spread of this new outbreak clone in Nigeria and Niger and concluded that the emergence of this virulent strain was due to horizontal acquisition of a serogroup C capsule and the filamentous bacteriophage MDAϕ (a virulence factor that allows increased colonization on epithelial cells in the nasopharynx) by a noncapsulated carrier strain circulating in the region before the introduction of MenAfriVac. There have been nearly 16,000 suspected cases in 2017 in Nigeria requiring a new massive vaccination campaign with a multivalent conjugate vaccine. Changes in the distribution of molecular types among different serogroups before and after vaccine introduction due to horizontal gene transfer events were also suggested by Wang et al. (2015).

The implication of HGT in capsule acquisition is discussed by Clemence et al. (2018) in a study that identified genes for capsular transport and translocation in *N. subflava* and *N. elongata* homologous to those of *N. meningitidis.* This study also revealed novel putative capsule synthesis genes in these commensal species. In contrast with the previous hypothesis that *N. meningitidis* has emerged as an unencapsulated human commensal from a common ancestor with *N.gonorrhoeae* and *N. lactamica* and subsequently acquired the genes responsible for capsule synthesis via HGT (Schoen et al. 2008), Clemence et al. suggest that some meningococci reacquired capsule transport genes that were initially lost in the common ancestor, from a close relative that has either not been previously isolated or is extinct.

Other virulence factors also appear to be acquired by HGT, and some of them are examples of interspecific genetic exchange. This is the case of *tbpB* gene whose

variants were found in various *Neisseria* species including pathogenic and commensal species (Linz et al. 2000) and have been repeatedly exchanged among these species over very short periods (Harrison et al. 2008). Lipooligosaccharide (LOS) is another major virulence factor, and *lgt* genes encode the glycosyltransferase responsible for its biosynthesis. Nine *lgt* genes at three chromosomal loci were examined in 95 strains of *N. meningitidis*, *N. gonorrhoeae* and commensal *Neisseria* by Zhu et al. (2002). This study demonstrated that pathogenic and commensal *Neisseria* share a common *lgt* gene pool, and it is suggested that HGT contributes to the genetic diversity of *lgt* loci. Also, in the FadL-like protein, a cell-surface protein with vaccine potential, similarities between regions of amino acid sequence in *N. meningitidis*, *N. lactamica*, *N. polysaccharea* and *N. sicca* provide evidence of interspecies recombination (Yero et al. 2010). A different G + C content compared to the rest of the genome indicates HGT. Phylogenetic and G + C content analyses of pilin sequences also indicate fully virulent strains of meningococci may possess pilin sequences derived from commensal *Neisseria* species (Aho et al. 2005).

Apart from the implications for the design of vaccines, HGT is implicated in antibiotic resistance, a problem of particular concern for *N. gonorrhoeae*, one of the pathogens included in the global priority list of antibiotic-resistant bacteria published by the WHO (priority 2: high). Alterations in chromosomal genes encoding the high molecular weight penicillin-binding proteins (PBP) are responsible for the acquisition of penicillin resistance in both *N. gonorrhoeae* and *N. meningitidis*, and the mosaic structure of the gene in resistant strains is interpreted to be the result of horizontal genetic exchange events (Spratt et al. 1992). Different studies have compared and analyzed the penicillin-binding protein 2 (*penA*) gene among *Neisseria* species from both susceptible and resistant strains. Pathogen species appear to have increased resistance to penicillin by totally or partially replacing their genes with the *penA* genes of related species that decrease the affinity for the antibiotic. The commensal species identified as donor in these interspecies recombinational events are in most cases *N. cinerea* or *N. flavescens* (Spratt et al. 1992). Bowler et al. used chromosomal DNA from an *N. flavescens* isolate from the pre-antibiotic era to transform a penicillin-susceptible meningococcus strain. About 50% of the resulting transformants had replaced their *penA* genes with that from *N. flavescens* (Bowler et al. 1994). However, transformants using chromosomal DNA from *N. cinerea* failed to acquire the commensal *penA* gene. Later, these alterations in *penA* that lead to penicillin resistance were associated with five specific mutations in the transpeptidase region of PBP2 (Thulin et al. 2006). Kart et al. detected three of the five mutations in a study analyzing 123 *N. lactamica* strains and 129 *N. meningitidis* strains. Data showed that 64 *N. lactamica* strains exhibited all the 5 mutations and 56 showed 3 of the 5 mutations. Just three mutations were exclusive to *N. lactamica* and exhibited mostly lower penicillin MICs than those exhibited by strains with all of the mutations. Genetic transformation of *N. lactamica penA* alleles in meningococci was only possible for alleles encoding five mutations. In contrast, alleles with all five mutations were detected just in seven *N. meningitidis* strains suggesting that horizontal gene transfer is uniformly directed from *N. lactamica* to *N. meningitidis* (Karch et al. 2015). In *N. gonorrhoeae*, the mosaic

type of penA was also associated with reduced susceptibility to oral cephalosporins, and the horizontal transfer of the penA-X allele can explain the clonality of Cef^{Rs}-associated PBP2 even in isolates of different STs (Ohnishi et al. 2010).

Sulfonamide resistance in *N. meningitidis* is mediated by altered chromosomal *folP* genes, and various studies again showed that resistance might be acquired by HGT of new alleles from commensal *Neisseria* species as *N. lactamica*, *N. sicca*, *N. subflava* and *N. mucosa*. This commensal flora might act as a reservoir of sulfonamide resistance genes (Qvarnstrom and Swedberg 2006; Fermer et al. 1995; Rådström et al. 1992).

The same role for a "reservoir" of virulence factors has been postulated for Opa outer membrane proteins, Tfp systems, iron acquisition proteins and other mentioned above (Marri et al. 2010).

Apart from the genetic exchange among *Neisseria* species, there are examples of intergeneric exchange implicating other nasopharyngeal colonizers such as *Haemophilus influenzae*. Serino et al. correlated the acquisition of four genes putatively involved in phosphorylcholine biogenesis (licA, licB, licC and licD) by *N. lactamica* with horizontal exchange from *H. influenzae* (Serino and Virji 2002). Kroll et al. reported natural transfer of *Haemophilus* sequences to *N. meningitidis* by detecting the presence of *Haemophilus* uptake sequences (HmUS) in *N. meningitidis* and *Neisseria* uptake sequences (nUS) in *Haemophilus*. Another example of HGT among *Haemophilus* and *N. meningitidis* is the horizontal transfer of the *lav* gene that encodes a virulence-associated autotransporter transferred from *H. influenzae* to *Neisseria* (Davis et al. 2001). A recent publication describes the microevolution of *N. lactamica* during nasal colonization of up to 6 months following intranasal inoculation of human volunteers (Pandey et al. 2018). Phase variation was the principal mechanism by which diversity arose. However, a large hypothetical protein with a G + C content that suggested possible acquisition from *H. influenzae* (L-hp) was a region where mutations persisted. It may be that the deletion of this protein was beneficial, perhaps metabolically or as a site for host recognition.

The Brazilian purpuric fever (BPF) is a fulminant paediatric disease caused by *H. influenzae* biogroup aegyptius (Hae) first described in Brazil during the 1980s. The clinical picture is similar to the meningococcal septicaemia. Before BPF, Hae was only associated with conjunctivitis. Meningococcal conserved sequences were identified in the Hae strains associated with BPF, so the emergence of invasive Hae strains seems to be the dramatic consequence of horizontal genetic exchange between both bacteria (Li et al. 2003; Cury et al. 2014).

4 Horizontal Transfer of Genetic Information Between Humans and *Neisseria*

Most *Neisseria* species colonize either the human upper respiratory tract or the human urogenital tract, and, for both pathogenic species, *N. meningitidis* and *N. gonorrhoeae*, humans are the only natural host. We have reviewed the evidence that horizontal gene transfer exists in *Neisseria* and that genetic exchange can be intraspecies or interspecies as well as intergeneric. But does horizontal transfer of genetic information occur between *Neisseria* spp. and their host? To date, only the possible acquisition of a small fragment of human DNA sequence into *N. gonorrhoeae* (nL1) has been reported (Anderson and Seifert 2011a, b). This fragment is 685-bp long, and it is 98–100% identical to the human long interspersed nuclear element (LINE) L1, and apart from this human DNA does not appear to have been acquired by other *Neisseria* species. This, together with the low frequency of the nL1 allele among the 62 *N. gonorrhoeae* included in the study (11%), suggests a recent HGT event which occurred subsequent to the divergence of *N. gonorrhoeae*, *N. meningitidis* and another *Neisseria* spp. Possible sequence contaminations were discarded using a combination of PCR amplifications specific for the nL1 sequence and flanking gonococcal genetic region, DNA hybridization and independent sequencing. In all nL1-positive isolates, the insert sequences and locations of insertion are identical. The insertion site was adjacent to the irg4 gene, which encodes a phage transposase. This conservation suggests that rather than different HGT events, a single HGT event occurred and was propagated within the gonococcal population. There is no homology between the gonococcal insertion site and the L1 sequences flanking the nL1 fragment, so the mechanism for integration of nL1 cannot be homologous recombination. Transcripts containing the nL1 sequence were detected in *N. gonorrhoeae* RNA preparations, but the product and the hypothetical function are unknown and further studies are needed. How did *N. gonorrhoeae* gain access to this human DNA? Because *N. gonorrhoeae* can be found intracellularly and extracellularly associated with neutrophils and epithelial cells, two possibilities were considered: (1) from DNA within chromatin-rich neutrophil extracellular traps (NETs) and (2) from DNA released by host cells undergoing apoptosis or a necrotic event.

Many questions remain unclear, but the insertion of human L1 in the gonococcal chromosome indicates that genetic exchange between *Neisseri*a and humans is possible in spite of all the barriers that this genus has to avoid acquisition of foreign genes.

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Implications of Lateral or Horizontal Gene Transfer from Bacteria to the Human Gastrointestinal System for Cancer Development and Treatment

A. G. Abril, P. G. Lanzi, and V. Notario

Abstract The ultimate consequence of horizontal, or lateral, gene transfer, as it is usually understood, is the transient or permanent acquisition by the recipient cell(s) of new, positive or negative, functional characteristics by virtue of the incorporation of the DNA from the donor into their own genome. In reality, it is simply the manifestation of a novel gene expression repertoire(s). It has been recognized for some time that gene expression changes in human cells were primarily the result of structural alterations of their own DNA (mutations, deletions, amplification, or major rearrangements). At present, despite some degree of controversy, it is becoming an increasingly accepted notion that gene expression changes in human cells may be brought about also by their acquisition of exogenous DNA from microorganisms, particularly bacteria, present in the human microbiome. Recent published analyses of information deposited in publicly available data bases of human normal and tumor genome sequences reported a high frequency of detection of bacterial DNA integrated in the human DNA, thus providing solid evidence in support for bacteria-to-human lateral gene transfer. In addition, and most importantly, these studies also showed a much more frequent presence of bacterial DNA in human cancer samples (e.g., acute myeloid leukemia, gastric cancers) than in the DNA samples from healthy individuals, raising the possibility that the bacterial sequences might be directly or indirectly involved in the development of cancer, either by encoding protein/enzyme products with pro-carcinogenic activity or by causing epigenetic alterations that ultimately could lead to genomic instability in the host cells and, later, to carcinogenic progression. This chapter will examine the current status and landmark developments in this still growing and highly innovative research field, focusing on the role of resident bacteria in the onset and/or progression of human gastrointestinal

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malignancies. We will also discuss the exciting possibility of exploiting bacteria-to-human lateral gene transfer to deliver anticancer therapeutic tools to human tumors.

Keywords Gene transfer · Human microbiota · Gene expression · Gastrointestinal malignancies · Anti-cancer therapeutics

1 Cancer Etiology

The vast majority of human cancers are caused by exposure to environmental agents, while only about 10% of cancers are driven by heritable genetic alterations (Lichtenstein et al. 2000; Hemminki and Boffetta 2004; Perduca et al. 2018). Cancer risk increases by exposure to known extrinsic chemical, physical, or biological cancer-causing agents such as pollutants, radiation, and oncogenic viruses, but the ultimate health outcome will be influenced by intrinsic factors (e.g., detoxifying capacity, immune competence, DNA-repair efficiency) that vary between populations and individuals (Wu et al. 2018a). The gastrointestinal (GI) mucosa is one of the body surfaces nearly constantly being exposed to potentially carcinogenic substances that either directly associate with food intake and other lifestyle factors (Kim et al. 2016) or indirectly result from abnormalities of various GI-specific intrinsic determinants (Dziki et al. 2017). Consequently, GI tumors constitute one of the most prominent cancer types in humans.

1.1 Gastrointestinal Cancer

According to statistics released in 2018 by the American Cancer Society (Cancer Facts and Figures 2018, ACS), taken together, cancers of the GI tract (esophageal, gastric, and colorectal tumors) account for nearly two thirds (63.6%) of cancers of the entire digestive system. In the USA, colorectal cancer (CRC) remains in third position, for both males and females, with regard to the total number of new cases (9% of all cases) and to the total number of deaths (8.3% of all deaths). Significantly lower numbers of new cases and deaths are reported for esophageal (1% of new cases, 2.6% of deaths), gastric (1.5% of new cases, 1.8% of deaths), and small intestine (0.6% of new cases, 0.2% of deaths) cancers. With regard to the overall trends, the incidence and deaths of CRC have continued to decline among adults 55 years of age and older, but they have continued to increase among individuals younger than age 55, for not fully understood reasons (Siegel et al. 2017, 2018). The origin of CRC has been related to alterations in three types of mechanistic factors: genetic, epigenetic, and immune signaling (Fearon 2011; Goel and Boland 2012; Arends 2013). However, each and every one of them can be triggered by imbalances, the so-called dysbiosis, in the GI intestinal microbiota (Couturier-Maillard et al. 2013; Yang et al. 2013).

1.2 Gastrointestinal Microbiota

It is now experimentally demonstrated and widely accepted that the gut microbiota plays a pivotal role in intestinal homeostasis (Cario 2013). The intestinal microbiota is necessary for a healthy immune system and, therefore, for the prevention of disease states (Sekirov et al. 2010; Kelly and Mulder 2012). In reality, the intestinal microbiota plays a dual role with regard to human health and disease. On the one hand, gut microorganisms are involved in complex mutualistic interactions with the host mucosal epithelial and immune cells, in a way that under normal physiological conditions, intestinal homeostasis is achieved by striking a balance between the stimulation of the immune system to fight against potential pathogens and the immunosuppressive signaling necessary to allow the survival of the gut commensal microbes. Both the number of microbes and the identity of the species living in the gut ecosystem are contributing factors to maintaining intestinal homeostasis.

Most organs in the human body contain microorganisms (bacteria, fungi, archaea, and viruses) that live in a mutualistic interaction with the host (Blaser and Kirshner 2007; Turnbaugh et al. 2007; Sender et al. 2016b). Recent developments in more advanced sampling and microbial metagenomics (Narihiro and Kanagata 2017; Cani 2018) and other technologies (Foster et al. 2012; Biteen et al. 2016; Xu et al. 2017; Burns and Blekhman 2018; Song et al. 2018) have been highly instrumental in providing continuously improved information on the composition and community structures of the human microbiome (Gilbert et al. 2018). The human body undergoes colonization by the mother's microbiota at birth, with *Bifidobacteria* being among the first microorganisms to colonize newborn children's guts, and acting to promote colonic epithelial cell proliferation and overall gut maturity by forming an extracellular scaffold structure built with a pilus-associated protein (O'Connell Motherway et al. 2019). The human microbiota stabilizes during childhood (Faith et al. 2013; Gilbert et al. 2018) and later evolves through old age (Dominguez-Bello et al. 2010; Claesson et al. 2011). The diversity and abundance of microorganisms vary at different sites and within regions of each site (Weinstock 2012; Goodrich et al. 2014). The most prominent microbiota is that of the GI tract, particularly in the gut, which contains up to thousands of species of microorganisms (Turnbaugh et al. 2007; Dominguez Bello et al. 2018). Although the number of bacterial cells relative to host cells in the gut has been frequently reported as a 10:1 ratio on the basis of initial overestimations (Luckey 1972), most recent calculations have brought that ratio down to a 1.3–1.94 range (Rosner 2014; Sender et al. 2016a, b). Nevertheless, more recent reports describe numerous and divergent uncharacterized components of the human microbiota (Kowarsky et al. 2017).

The overall composition and community structure of the human microbiota vary from individuals to individual (Jones et al. 2018), as it can be modified by personal factors such as diet, medication intake, intestinal transit time, or exercise as well as by extrinsic influences such as increasing industrialization (Paul et al. 2015; Falony et al. 2016; Tuan and Chen 2016; Flint et al. 2017; Allen et al. 2018; Dominguez Bello et al. 2018; Maier et al. 2018; Niederreiter et al. 2018). In the gut, the

microbiota is the most influential environmental factor, as the epithelial cells are constantly exposed to high numbers of microorganisms (Bhatt et al. 2017), and it plays important physiological functions that have both localized and systemic effects. The main function of the normal colorectal microbiota is the maintenance of the host health (Wang et al. 2017; Reticker-Flynn and Engleman 2019), and, to that end, its most important role is aiding the development of the immune system (Chung et al. 2012). Studies with animal models and a limited number of bacterial strains showed that bacteria boosted the immune response to pathogenic microorganisms and cancer (Tanoue et al. 2019). Similar studies suggested that pairwise interactions between bacterial strains may be a major driver in multi-species dynamics and that interactions between species influence the microbiome functions (Venturelli et al. 2018). Importantly, the gut microbiota varies in health and disease states (Cho and Blaser 2012; Bhatt et al. 2017). Changes in the microbiome, microbial metabolome, and its interactions with the immune, endocrine, and nervous systems are correlated with disease conditions such as obesity (Paul et al. 2015; Maruvada et al. 2017; Schmidt et al. 2018), chronic inflammation (Frank et al. 2007; Gevers et al. 2014; Ni et al. 2017; Dejea et al. 2018; Yilmaz et al. 2019), cancer (Kostic et al. 2013; Yu et al. 2017a, b; Goodman and Gardner 2018; Kaakush 2018; Maisonneuve et al. 2018), depression (Jiang et al. 2015; Zheng et al. 2016; Cheung et al. 2019; Strandwitz et al. 2019), lupus (Azzouz et al. 2019), and other health disorders (Gilbert et al. 2018).

1.3 Microbiome Association with Gastrointestinal Cancer Progression

As indicated above, recent improvements in microbiota analysis technologies (Foster et al. 2012; Biteen et al. 2016; Xu et al. 2017; Burns and Blekhman 2018; Song et al. 2018) have allowed a more detailed examination of the nature of organization of the human microbiota in different tissues but also of the changes that are associated with the onset and progression of human cancer. Although associations between the resident microbiota and cancer development have been established in other organs such as the lungs (Greathouse et al. 2018; Jin et al. 2019), the majority of information on microbiota and human cancer derives from studies in the GI, particularly the gut (Bultman 2014; Nistal et al. 2015; Bhatt et al. 2017; Raza et al. 2019), not only about GI cancers themselves but also about the influence of the gut microbiota on the development of tumors in distant organs, such as the liver (Ma et al. 2018), breast (Fernández et al. 2018), prostate (Golombos et al. 2018), and others.

The influence of the microbiota on cancer development has been established for all main segments of the human GI tract. Barrett's esophagus and esophageal cancer have been associated not only with the esophageal resident microbiome (Di Pilato et al. 2016; Corning et al. 2018; Desphande et al. 2018) but also with

the microbiome in gastric reflux and the oral microbiome (Yang et al. 2014; Peters et al. 2017; Snider et al. 2018), in particular with specific bacterial species such as *Tannerella forsythia* and *Porphyromonas gingivalis*, two anaerobic, Gram-negative bacteria known to be implicated in periodontal diseases (Malinowski et al. 2019). With regard to gastric cancer, the microbiome involvement in cancer development has been documented also (Brawner et al. 2014; Nardone and Compare 2015; Yu et al. 2017a), not only about the well-established participation of *Helicobacter pylori* in the process but also in relation to the contribution of mucosal microbiota dysbiosis to gastric carcinogenesis (Di Pilato et al. 2016; Baba et al. 2017; Corning et al. 2018). The involvement of the gut microbiota in CRC development has been solidly established (Dahmus et al. 2018; Maisonneuve et al. 2018; Tilg et al. 2018), even to the point of identifying the association of CRC carcinogenesis with particular bacterial communities (Flemer et al. 2017; Burns et al. 2018).

2 Microbiome-Mediated Mechanisms of Gastrointestinal Cancer Progression

Under physiological conditions, commensal members of the normal GI microbiota perform various beneficial roles for the host, including the synthesis of certain vitamins, the utilization of dietary fiber to produce short-chain fatty acids (SCFAs), the help of metabolizing certain compound, and the modulation of the immune system to protect against pathogenic microorganisms (Bhatt et al. 2017; Gilbert et al. 2018, Raza et al. 2019). In fact, the fecal metabolome has been described to represent a functional readout of the gut microbiome (Zierer et al. 2018). However, disruption of the GI microbiota (dysbiosis) may result in CRC initiation and progression (Couturier-Maillard et al. 2013; Yang et al. 2013) as well as the development of cancer at distant sites as a consequence of the release of bacterial pro-carcinogenic metabolites and toxins into the blood and/or lymphatic circulation (Louis et al. 2014; Niederreiter et al. 2018; Wu et al. 2018b) or the transfer of immune cells (Ma et al. 2018; Jin et al. 2019). As indicated above, CRC development has been related to three main types of mechanisms: genetic, epigenetic, and immune alterations (Fearon 2011; Goel and Boland 2012; Arends 2013; Zitvogel 2016; Cervantes-Barragán et al. 2017; Routy et al. 2018a). However, all of them can be triggered by alterations in the composition, population size, relative numbers, niche distribution, and metabolic activities of the colonic microbiota (Abreu and Peek 2014; Carding et al. 2015; Nistal et al. 2015; Sun and Kato 2016).

2.1 Carcinogenesis by Infectious Microbes in Humans

Most of our current knowledge on mechanisms of cancer development induced by microbes derives from studies of infectious agents, which are estimated to cause nearly 20% of all sporadic human cancers (Bhatt et al. 2017; van Elsland and Neefjes 2018; Raza et al. 2019). The role of certain viruses as etiological agents for several types of human cancer has been conclusively demonstrated. Human papillomaviruses (HPV) are associated with cervical tumors and head and neck cancer, hepatitis viruses (HBV and HCV) are associated with hepatocellular carcinoma, Epstein-Barr viruses (EBV) and human T-cell lymphotropic type 1 viruses (HTLV-1) are associated with some hematologic malignancies, human herpesvirus 8 (HHV8, also known as KSHV) is associated with Kaposi's sarcoma, and Merkel cell polyomavirus (MCPyV) is associated with Merkel cell carcinoma (Schwarzenbach et al. 2011; White et al. 2014; Liu et al. 2016; Thierry et al. 2016; Bhatt et al. 2017). The role of infectious bacteria as drivers of human cancer development has remained less prominent; until recently, there is strong epidemiological and experimental evidence supporting the association between several infectious bacteria and various types of cancer (Bierne et al. 2012; Bhatt et al. 2017; van Elsland and Neefjes 2018; Raza et al. 2019). The best known case relates to the causative role for human gastric cancer played by *Helicobacter pylori*, an infectious agent initially detected in patients with gastritis and peptic ulcers (Marshall and Warren 1984). Since then, the strong lines of evidence provided by epidemiological analyses (Kikuchi 2002), experiments with several animal models (Koga et al. 2002; Werawatganon 2014), and continuing eradication studies (Vakil and Megraud 2007; Choi et al. 2018) have led to the definition of *H. pylori* as a class 1 human carcinogen (IARC 2012; Moss 2017). In addition to *H. pylori*, other infectious bacteria have been associated with cancer development also. In this regard, *Salmonella* infections have been reported to increase CRC risk (Lu et al. 2017; Mughini-Gras et al. 2018)

The use of metagenomics analyses for case control studies during the last 5–10 years has established an association between some commensal bacteria and the incidence of cancer, in general, and of CRC in particular. In this cases, cancer occurrence correlates with changes in bacterial diversity (some genera or species increase or decrease in the microbiota) as well as in alterations of community stability (changes in binary interactions between bacterial species). *Fusobacterium nucleatum* and other *Fusobacterium* spp. are frequent participants in dysbiotic processes leading to colorectal polyps, adenomas, adenocarcinomas, and metastatic CRC (Kostic et al. 2012; Castellarin et al. 2012; McCoy et al. 2013; Zackular et al. 2014; Mira-Pascual et al. 2015; Flemer et al. 2017; Bullman et al. 2017). In fact, it has been reported that *F. nucleatum* travels with the CRC cells to the metastatic sites, and it is maintained within the metastatic niches (Bullman et al. 2017). Animal studies have conclusively demonstrated a pro-tumorigenic role for *Fusobacterium* spp. in human carcinogenesis (Kostic et al. 2013; Rubinstein et al. 2013; Yu et al. 2015; Chen et al. 2017; Yang et al. 2017; Yu et al. 2017b).

2.2 *Pathogenic and Commensal Microbes Utilize Similar Carcinogenesis Mechanisms*

Dysbiosis increases the relative proportion of gut bacteria with pathogenic potential (Lee et al. 2017) and also increases intestinal barrier permeability, which results in increased secretion of bacterial enzymes (Lerner et al. 2017). The GI mucosa defines the separation between the internal milieu and the external non-sterile environment. The intestinal barrier plays a physical and immune protective role against infections (Doran et al. 2013; Citi 2018; Mohanan et al. 2018). Invasion of epithelial cells by bacterial cells (Kim et al. 2018) may allow the persistence of infection, their IgA-mediated colonization (Donaldson et al. 2018), and the ultimate membrane translocation by pathobionts (Vieira et al. 2018). Regardless of whether microorganisms remain outside or invade the host cells, the primary mechanisms by which cancer development is stimulated can be divided into two main, broad classes: genetic and epigenetic.

Genetic mechanisms of microbiota-driven carcinogenesis are primarily related to the induction of DNA damage and the interference with the DNA-damage response, thereby favoring the maintenance and transmission of host DNA mutations. Microbiota dysbiosis frequently causes chronic inflammation that, subsequently, increases the levels of reactive oxygen species (ROS), ultimately promoting genotoxicity effects such as DNA mutations and other DNA alterations leading to genomic instability (van Elsland and Neefjes 2018). Bacterial toxins such as the cytolethal distending toxin (CDT), secreted by several species of *Proteobacteria*, such as *Escherichia coli*, *S. typhi*, *Shigella dysenteriae*, and *Campylobacter jejuni* (Bezine et al. 2014; Graillot et al. 2016), and the *E. coli*-secreted colibactin (Wilson et al. 2019), are known to cause double-stranded DNA breaks while simultaneously impairing the cellular DNA damage response, stimulating cell proliferation and survival to apoptotic signals, thereby resulting in the accumulation of DNA mutations and other DNA repair errors, leading ultimately to cancer development (van Elsland and Neefjes 2018). In addition, microbiota alterations have been shown to have substantial epigenetic effects (Yang et al. 2013; Lee et al. 2017), including changes in DNA methylation (Park et al. 2009) as well as whole genome methylation (Kumar et al. 2014), histone acetylation (Ding et al. 2010), chromatin remodeling (Bierne and Cossart 2012), expression of specific miRNAs that modulate intestinal epithelial permeability (Nakata et al. 2017), global epigenetic reprogramming at multiple host tissues (Krautkramer et al. 2016), and other epigenetic abnormalities (Fearon 2011).

Some of these genetic and epigenetic pro-carcinogenic effects can be triggered by enzymes (Raza et al. 2019), toxins (Lemichez and Barbieri 2013), metabolites such as short-chain fatty acids (SCFAs), or other products either secreted by gut microbes (Louis et al. 2014) or resulting from their metabolic conversion of dietary components (Hagland and Søreide 2015; Johnson et al. 2015) and other ingested xenobiotics (Das et al. 2016; Koppel et al. 2017). Many of these effectors drive GI carcinogenesis indirectly through various mechanisms, including modulating cellular

stemness (Sun 2010; Munro et al. 2018), stimulating cell proliferation (Wroblewski et al. 2016; Naito et al. 2017), and enhancing cell death resistance (Rosadi et al. 2016; van Elsland and Neefjes 2018). Effectors from different bacteria may act at the same time and in some cases elicit synergistic actions on the host epithelial and/or immune cells (Fulbright et al. 2017; Dejea et al. 2018), thereby modifying the homeostatic balance both locally in the GI and systemically, including the nervous system (Savidge 2016; Strandwitz et al. 2019). Microbiota-derived effectors frequently carry out their carcinogenic-stimulating role by directly interacting with surface or nuclear receptors (Abreu 2010; Cohen et al. 2017; Sivaprakasam et al. 2017; Raza et al. 2019), and turning on in an unscheduled way, or enhancing, diverse signaling pathways via MAPK (Baek 2010), NF-κB (González-Sarrías 2010), Wnt/β-catenin (Moosavi 2014), JAK/STAT (Kesselring et al. 2016), or others.

It is through these types of interactions with cellular receptors and cell signaling cascades that the microbiota plays a role, positive or negative, in modulating the response of GI cancer patients to the various treatment modalities (Goubet et al. 2018; Kroemer and Zitvogel 2018), including radiotherapy (Touchefeu et al. 2014; Cui et al. 2016, 2017; Xiao et al. 2018), chemotherapy (Alexander et al. 2017; Roy and Trinchieri 2017), and immunotherapy, particularly with regard to the efficacy of immune checkpoint blockers (Gopalakrishnan et al. 2018; Matson et al. 2018; Routy et al. 2018b).

2.3 Insertional Mutagenesis in Microbiota-Driven Gastrointestinal Carcinogenesis

As indicated above, the role of diverse types of viruses in human cancer has been fully demonstrated, while the involvement of bacteria in human carcinogenesis has been mainly associated with infectious microorganisms. An important difference between the processes of virally and bacterially driven mechanisms of human carcinogenesis is the fact that vital genetic materials integrate into the host genome thereby resulting in insertional mutagenesis and ultimately leading to carcinogenesis depending on the integration sites (Chen et al. 2014). While bacteria are known to contribute to the development of cancer by the diverse mechanisms discussed earlier, the possibility that commensal bacteria may transfer DNA to human cells and promote cancer either by insertional mutagenesis or by the functional expression of pro-oncogenic sequences has not been as extensively documented as viral carcinogenesis to date.

2.3.1 Contribution of Lateral Gene Transfer

The notion of lateral gene transfer (LGT), also called horizontal gene transfer (HGT), refers to the transmission of genetic material between organisms that do not belong

to the same species, in contrast with vertical gene transfer, which is the acquisition of genetic material by the offspring from its ancestors. LGT is a widespread process among bacteria (Ochman et al. 2000; Moreira and Lopez-Garcia 2017), but it seemed to play a much less prominent role in the diversification of eukaryotes. Nevertheless, the existence of LGT from bacteria to eukaryotes has been frequently documented, including LGT instances to unicellular and multicellular eukaryotes, and all domains of life (Duning Hotopp et al. 2007; Dunning Hotopp 2011; Boto 2014; Metcalf et al. 2014; Chou et al. 2015; Lacroix and Citovsky 2016; Sieber et al. 2017; Kominek et al. 2019). Although LGT was described for a number of metazoans (Boto 2014), its existence in vertebrates still remained questionable due to the shortage of such studies and to the generally perceived limitation to HGT in higher organisms, including humans, by the presence of specialized and protected germ cells, which would effectively prevent the transmission of any possibly acquired new traits

Taking into consideration that (1) LGT takes place between organisms which are in close proximity (Beiko et al. 2005); (2) there are more bacterial cells than host cells in the human GI tract, as described above; (3) some bacteria can transform human cells in vitro (Llosa et al. 2012); and (4) under appropriate environmental conditions, pathogenic bacteria may transfer oncogenic sequences to commensal bacteria in the human gut (Stecher et al. 2012), it seems perfectly reasonable to suggest (Rubinstein et al. 2013) that "non-inherited bacterial DNA integration into chromosomes in somatic human cells could induce mutations leading to cancer." In fact, testing this hypothesis, by analyzing publically available databases the possible presence of bacterial DNA integration in a wide variety of cancers, Riley et al. (2013) reported the existence of integrations of bacterial sequences, all derived from the rRNA operon from in human cases of acute myeloid leukemia (AML) and from *Pseudomonas* in cases of stomach adenocarcinoma (Fig. 1). With regard to its implications for GI cancer, these findings are very significant for three main reasons: (1) rRNA would not be recognized by the human innate immune system (Wu and Chen 2014), (2) *Pseudomonas aeruginosa* had been described previously as a promoter of gastric carcinogenesis in a rat model and associated to human gastric cancer (Morishita and Shimizu 1983; Noto and Peek 2017), and all integrations occurred into genes known to be overexpressed in gastric cancers and having oncogenic potential (Sieber et al. 2016). Further characterization of features of the integration sites of the *Pseudomonas*-like sequences in stomach cancer samples (Robinson and Dunning Hotopp 2014) established a clear similarity between the carcinogenic effect of this LGT from bacteria to humans with the integration of mobile elements and viral sequences in CRC (Hancks and Kazazian 2012; Solyom et al. 2012; Benard et al. 2013; Chen et al. 2014). More recently, integrations of 30–32 bp fragments of the *H. pylori* genome into the *PREX2* gene on chromosome 18, in human gastric cancer, have been reported (Cui et al. 2015). The findings about this pro-carcinogenic LGT are quite significant, as PREX2 is known to interact with PTEN in breast cancer (Fine et al. 2009) and hepatocellular carcinoma (He et al. 2016), as well as being frequently mutated in melanoma (Chroncinski et al. 2014; Lissanu Deribe 2016) and pancreatic carcinoma (Yang et al. 2016).

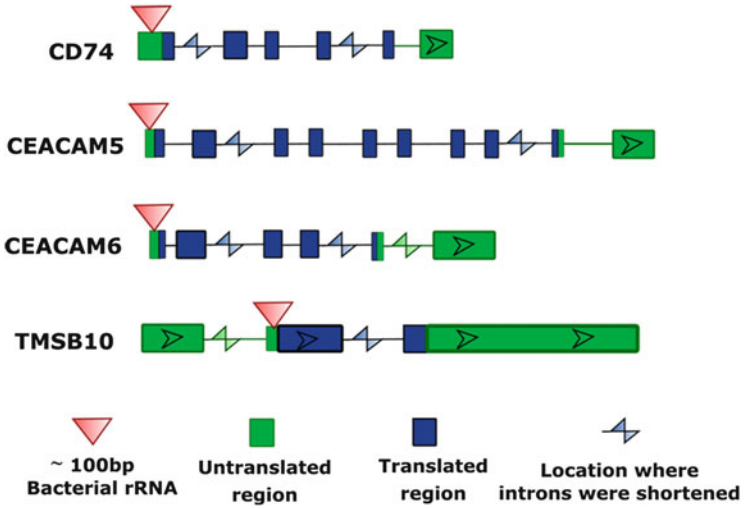

Fig. 1 *Pseudomonas*-like DNA integrations by LGT into stomach adenocarcinoma genes. The four genes shown (*CD74*, *CEACAM5*, *CEACAM6*, and *TMSB10*) are known to be overexpressed in stomach cancer. This, along with their established relevant functions, qualifies them as proto-oncogenes. Modified from Robinson and Dunning Hotopp (2014)

It was the completion of the Human Genome Project that initially stimulated research on LGT from bacteria to humans, as it uncovered the presence of a number of presumably bacterially derived sequences incorporated into the human genome (Lander et al. 2001). Although initial validation studies refuted most of these sequences as possibly not being of bacterial origin or not resulting from an LGT process (Salzberg et al. 2001; Huerta-Cepas et al. 2007), some of them could not be eliminated at that time. Further studies designed to reanalyze previous data claim to had identified up to hundreds of active foreign genes in animals, including humans (Crisp et al. 2015), which created a scientific controversy, as evaluations by other researchers suggested that the sequences presumably originated by HGT into human cells may rather be the result of gene loss rather than LGT (Salzberg 2017). Nevertheless, the data reported recently described evidence for widespread HGT in the human genome (Huang et al. 2017), and most recent reevaluation studies of previous data confirmed that ACY3/ASA, one of the original disputed results (Crisp et al. 2015; Salzberg 2017), is a bacterial-animal LGT that occurred millions of years ago (Dunning Hotopp 2018).

As all general conditions in the human GI tract provide a favorable environment for the efficient LGT from bacteria to the host cells, adaptive LGT is expected to happen at a greater rate than what it has been reported to date. It seems highly likely that this situation may be due to the fact that only a relatively small proportion of epithelial cells are undergoing cell division, which would facilitate the stable integration of bacterially derived DNA into the host genome. As a consequence, very few host cells that might actually be subject to bacterial LGT may be

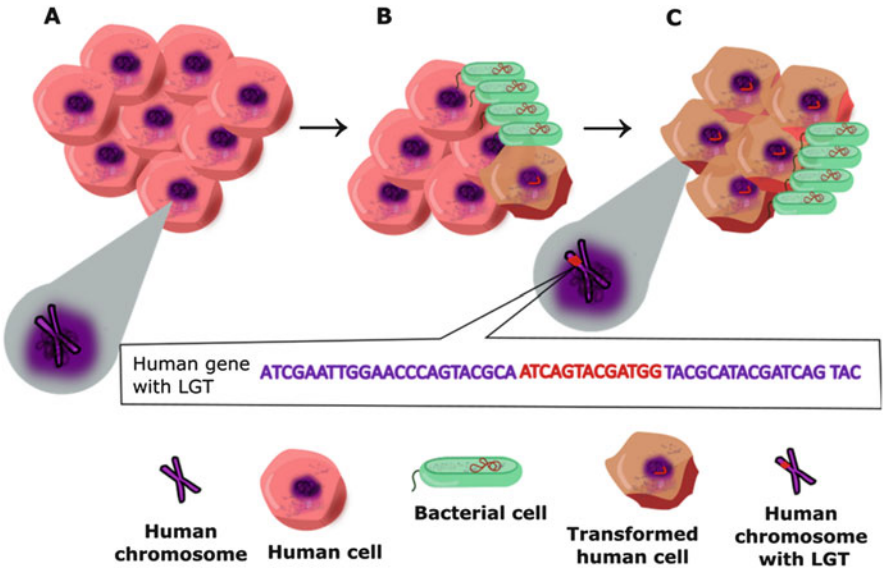

Fig. 2 Bacteria to human LGT and carcinogenesis. (**a**) Normal human somatic cells. (**b**) Human cells coexist with bacterial cells and occasionally, bacterial DNA is transferred by LTG (lateral gene transfer) from bacteria to human cells. The integration of this DNA may transform normal cells which then expressing cancerous traits. (**c**) These cancerous cells undergo clonal proliferation, thereby amplifying the bacterial sequences in the tumor cells and facilitating their detection. Modified from Robinson, Sieber, Dunning Hotopp (2013)

represented in the samples collected for genomic analysis. Such dilution factor may hinder the detection of LGT insertions. New approaches to analyze the extent of LGT (vs. gene loss or other processes) in eukaryotes will require the use of more vigorous methodologies (Dunning Hotopp 2018), with highly effective methods for targeted enrichment and sequencing of possible LGT events (Dunning Hotopp et al. 2017), and fully reliable and unbiased procedures to distinguish true associations from noise generated by data analysis from different taxa.

The higher frequency of detection of LGT in human cancers in general, and GI tumors in particular, may be related to the increased proliferation rate characteristic of malignant cells, which will greatly facilitate the integration of the bacterial sequences and, as reported by Robinson et al. (2013) to the clonal expansion of the cancer cells (Fig. 2), which will amplify the levels of the target sequences to be identified. It seems clear that further experimentation in this field has the potential of establishing any possible causal role played by LGT in GI cancer progression.

2.3.2 Virome and Carcinogenic Progression

An aspect of LGT research that remains underinvestigated relates to the possible transfer of genetic materials from the viral communities abundant in humans (the

virome) into the host cells, both in what relates to the possible transfer of viral sequences by bacteria (Aggarwala et al. 2017; Lerner et al. 2017) and to the possible transduction into human cells by bacteriophages of either their own DNA or of DNA from the bacterial species they infect (Lacroix and Citovsky 2016). The ability of bacteriophage DNA to integrate into the genome of cultured human cells and the role of phage-specific integrases in the process were previously described (Wenger et al. 1981; Groth et al. 2000), and the ability of bacteriophages to bind to components of the human extracellular matrix (ECM) has been characterized more recently (Porayath et al. 2018). Bacteriophages are known to play highly influential roles as regulators of the composition, structure, and dynamics of bacterial populations in experimental settings (Mitarai et al. 2016) as well as in natural ecosystems, including the human body (Navarro and Muniesa 2017). In fact, it has been reported that bacteriophages are more virulent to bacteria in the presence of human cells (Shan et al. 2018). The fact that bacteriophages are the most numerous viruses in the human virome (Navarro and Muniesa 2017) along with their ability to get into epithelial cells and cross epithelial cell layers (Nguyen et al. 2017) has led to the current notion that bacteriophages are the new human pathogens (Tetz and Tetz 2018), possibly acting directly via LGT as mobile elements driving insertional mutagenesis events and, also indirectly, affecting various aspects of human health through their ability to modify the composition, structure, and dynamics of the human microbiota.

3 Conclusions and Future Directions

Although the number of cases reported is still small, the preponderant evidence suggests that LGT events involving bacterial DNA are associated with gastric cancer and CRC. At this time, it is not possible to determine whether the transfer of genetic material took place on untransformed GI cells, or whether LGT happened to already pre-transformed cells, thus pushing their carcinogenic progression. As the field expands, it will be important to move from associations to causation and mechanisms of action (Fischbach 2018). Nevertheless, the expectation is that additional instances of LGT from bacteria to GI epithelial cells will be identified, and their mechanism of induction of carcinogenesis will be elucidated. There should be also an increase in studies focusing on the potential direct and indirect carcinogenic roles of bacteriophages. In addition, emphasis should be placed on the development of strategies to modulate the activities of the microbiota (Schmidt et al. 2018). In this regard, the notion that microbiota alterations may have significant health effects has become part of the public domain (Hodgetts et al. 2018), and now it is possible to have access to prebiotics and probiotics (Behsen et al. 2013; Zitvogel et al. 2017; Ding et al. 2018) in regular grocery stores, and their use and advantages are frequent topic of conversation among lay individuals. Our current knowledge of the role of the GI microbiota in cancer should be utilized to modify nursing practices (Kelly et al. 2016) and to foster precision medicine approaches (Jobin 2018). Ultimately, the idea is to exploit the known anticancer actions of the gut microbiota (Zitvogel

et al. 2017) while simultaneously preventing its negative effects. A variety of methodologies have been devised to facilitate microbiota modifications, including the use recombinant vectors (Suzuki et al. 2015; Celec and Gardlik 2017), genetically modified bacteria (Baban et al. 2010; Wegmann et al. 2017), and bacteria-derived vesicles (Bitto et al. 2017; Toyofuku et al. 2019) as well as fecal transplantation techniques (Cohen and Maharshak 2017). Results from the use of these approaches should hopefully result in human health improvements in the not too distant future.

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Role of Horizontal Gene Transfer in Cancer Progression

A. G. Abril and V. Notario

Abstract Different forms of programmed cell death are known to happen at different stages in the course of normal development as well as during the life of healthy adult organisms to maintain physiologic homeostasis. Cell death causes the release into the surrounding environment of nucleic acids, proteins, and other macromolecules that can be discharged as free entities or incorporated into vesicles derived from intracellular membranes, including nuclear fragments, or from the cell membrane. Nucleic acids (DNA, RNA, non-coding RNAs) can be taken up by neighboring or distant cells and may promote in them significant changes in fundamental biological processes such as stress-response, gene expression, proliferation, differentiation, secretory patterns, substrate attachment, and many other functions. In the context of cancer progression, once tumors reach a certain size, prior to the establishment of a tumor-associated vasculature system, cancer cells in the inner portion of the tumor mass are subjected to stress conditions (e.g., hypoxia, acidic pH, limited nutrient availability), which in many cases result in cell death, with the consequent release of cellular malignancy-promoting materials (e.g., oncogenes, oncogenic miRNAs, and others) that, when taken up by normal cells, can promote their malignant transformation. In addition to such form of cell death-associated horizontal gene transfer, cancer cells have been shown to be particularly proficient at releasing cell membrane-derived vesicles, most frequently of the exosome type, carrying a variety of cancer-promoting cellular constituents. Exosomes are used by the tumor cells to shuttle reciprocal signals to stromal cells proximal to the tumors to favor the creation of a more favorable environment for cancer growth or to transport oncogenic molecules to remote destinations where they contribute to create a pro-metastatic niche in healthy tissues. In addition, exosome-mediated horizontal gene transfer has been shown to contribute to provide and spread resistance in

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response to diverse anticancer therapies. This review will summarize our current knowledge on the contribution of horizontal gene transfer from circulating cell-free nucleic acids (CNAs) or from exosome-encapsulated materials to the various stages of human tumor progression.

Keywords Carcinogenesis · Cell death · Circulating nucleic acids · Exosomes · Extracellular vesicles · Malignant transformation

1 Multistage Carcinogenesis

It has been clearly established that, from a mechanistic perspective, cancer onset and development constitute a multistep process. This notion has been demonstrated not only in experimental settings exposing animals to chemical, physical, or biological carcinogens, which allowed the distinction between tumor initiation and promotion, but also in studies of human tumorigenesis (Weston and Harris 2003; Weiss 2004) that showed the sequential accumulation of molecular alterations in oncogenes and tumor suppressor genes in most human cancer types, leading to the acquisition of increasingly malignant properties (Yuspa 2000; Vogelstein et al. 1988). Our current understanding of the carcinogenic process distinguishes at least four distinct stages, initiation, promotion, malignant conversion, and progression, and has expanded to include alternative cellular and molecular mechanisms involving not only genetic alterations but also the contribution of epigenetic shifts as possible contributors to the dedifferentiation of cells, thereby allowing them to behave as cancer stem cells and ultimately drive the oncogenic process (Chaffer and Weinberg 2015; Baylin and Ohm 2006; Calin and Croce 2006). From a clinical standpoint, the acquisition by cancer cells of therapeutic resistance leading to local tumor recurrence and their ability to migrate away from the primary tumor site and home and proliferate in distant organs, the process known as metastasis, are widely considered as the main landmarks of tumor progression (Steeg 2016; Califano and Alvarez 2017) as well as the two major reasons for tumor relapse and therapeutic failure that result in cancer deaths. In fact, metastasis is the final stage in the progression of solid tumors, and is responsible for the majority (around 90%) of cancer deaths associated with solid tumors (American Cancer Society 2018; Siegel et al. 2018; Bray et al. 2018).

2 Tumor-Derived Carcinogenesis Effectors

As the size of primary tumors increases, cancer cells located in the innermost areas of the tumor mass are subjected to stress conditions due to limited oxygen and nutrients, pH acidification, and biophysical constrains, which may result in cell death, particularly prior to the establishment of a tumor-associated vasculature. The death of tumor cells causes the release into the tumor microenvironment

(TME) of biological materials that can be either taken up by other tumor or normal cells, or reach bodily fluids that will facilitate their distribution to other anatomic locations. Although the existence of circulating cell-free DNA (cfDNA) was initially discovered in the blood of presumably healthy individuals (Mandel and Metais 1948; Volik et al. 2016), it became clear later that the levels of cfDNA were much higher in the blood of cancer patients, being substantially higher in patients with metastatic disease (Volik et al. 2016; Bettegowda et al. 2014; Newman et al. 2014; Blood 2016). Recently, the presence of various RNA types (mRNA and miRNA) has been also demonstrated in the bloodstream (Gonzalez-Masia et al. 2013; Gupta 2017). Tumor-derived CNAs, particularly cfDNA, have been shown to enter into healthy cells, integrate into their genomes, and, by promoting genomic instability and DNA damage and modifying their apoptotic response, result in the transmission of neoplastic, even malignant, properties to the recipient cells (Gahan 2013; Trejo-Becerril et al. 2012; García-Olmo 2010; Mittra et al. 2015; Gravina et al. 2016). In this regard, it has become quite clear that carcinogenesis can be stimulated, perhaps even at the level of initiation, by the lateral transmission of nucleic acids from tumor to normal cells or to other tumor-associated cells, in a way similar to the process of horizontal gene transfer (HGT), which has been well established in a vast majority of cellular organisms (Soucy et al. 2015), although it is not a hallmark of the variability of the normal human genome (Salzberg 2017).

Under stressful conditions in the TME, tumor cells may detach from the primary tumor mass and, once tumor vasculature develops, may be disseminated to other parts of the body after entering the lymphatic and blood circulation systems (Steeg 2016; Le et al. 2004). The fact that particular types of cancer would preferentially metastasize to certain organs led to the proposal of the so-called "seed" and "soil" theory (Langley and Fidler 2011) to explain the lack of randomness in the final outcome of the dissemination process. According to such theory, the microenvironment of some organs (the "soil") would provide a more favorable milieu for the growth of cells (the "seed") derived from certain primary tumors. Over time, as additional mechanistic knowledge became available, this theory had to be modified to integrate the established contributions from tumor-stroma interactions (Paget 1989), from the biophysical features of the circulatory vessels (Ewing 1928; Goetz 2018), and from cell-free, molecular factors reaching a given homing location to the formation of specific premetastatic niches (PMNs) in distant organs (Kaplan et al. 2005; Peinado et al. 2017) prior to the arrival of the circulating tumor cells (CTCs), so they may survive and proliferate (Peinado et al. 2017; Psaila and Lyden 2009; Sleeman 2015; Chin and Wang 2016). Collectively, these factors may be envisioned as providing the optimum "fertilizer" to increase the known typically low efficiency of the metastatic process (Weiss 1986). Vascular leakiness plays an important role early in the generation of PMNs, as it allows the recruitment of nonresident cells (e.g., bone marrow-derived cells), thereby contributing to modify resident fibroblasts and promote local immune deregulation and the remodeling of the extracellular matrix (ECM) to make a receptive homing place for the CTCs (Peinado et al. 2017; Ordonez-Moran and Huelsken 2014; Joyce and Pollard 2009; Shibue and Weinberg 2011; Sleeman 2012). In addition to shedding the CTCs themselves, tumors are also known to secrete soluble factors such as TGF-β, tumor necrosis factor (TNF),

various cytokines, proangiogenic factors (e.g., VEGF), granulocyte colony-stimulating factor (G-CSF), and other factors (Kaplan et al. 2005; Peinado et al. 2017; Hiratsuka et al. 2002, 2008; Shojaei et al. 2009) that enhance their metastatic ability. Other systemic factors related to PMN formation and specificity are released by tumor cells or by tumor-associated stromal cells, in a programmed manner, as cargo encapsulated within different types of membrane-surrounded extracellular vesicles (EVs). These EVs carry malignancy-regulating, cell-derived materials that can be exported into the intercellular space adjacent to the tumor or distributed through the lymphatic and, primarily, the blood circulation to other parts of the body, where they contribute to cell-cell communication (Van Niel et al. 2018; Xu et al. 2018).

2.1 Significance of Cell-Free CNAs in Cancer

Although cfDNA was identified in the blood of healthy individuals in 1948 for the first time (Mandel and Metais 1948), it was not generally recognized as a potential marker for cancer detection until about 50 years later (Blood 2016; Thierry et al. 2016; Heitzer et al. 2015; Schwarzenbach et al. 2011). CfDNA is released into the blood by necrotic and apoptotic cells. These cells are usually phagocytosed by macrophages that engulf necrotic cells and release partly digested DNA. Yet, it is a well-established fact that all living cells release low levels of DNA into the circulation; increased cfDNA levels may be a consequence of pathological processes such as benign lesions, inflammatory diseases, tissue trauma, metabolic alterations, or cancer (Heitzer et al. 2015). Cancer patients, particularly those with metastatic disease, have been reported to have markedly high blood levels of cfDNA, and CNAs in general (Blood 2016; Thierry et al. 2016; Heitzer et al. 2015; Khier and Lohan 2018; Abels and Breakefield 2016).

2.1.1 Integrity of CNAs

Liver and kidney are the organs responsible for the clearance of CNAs from blood. CNAs have been shown to have variable half-lives, as their nature (DNA vs. RNA) and certain structural features allow some forms to survive longer than others (Thierry et al. 2016; Khier and Lohan 2018). Circulating cell-free RNAs (cfRNAs) are more labile than cfDNAs due to the widespread presence of RNAses in most biological compartments. From a structural point of view, double-stranded DNAs are known to remain longer in circulation than single-stranded DNAs. In addition, it has been shown that nuclease activity plays a role in DNA integrity, as it was reported that DNA in plasma shows a predominant fragmentation pattern reminiscent of nuclease-cleaved nucleosomes. The sizes of cfDNA are known to vary between 70 and 200 base-pairs, for small fragments, while large fragments can reach up to 21 kilobase-pairs (Blood 2016; Thierry et al. 2016; Khier and Lohan 2018). Obviously, the larger the cfDNA fragments may be, the greater is the

probability that they may encompass an entire gene and, consequently, there is a better opportunity for true HGT when taken up by a recipient cell. It has been reported that there is a correlation between the size of cfDNAs and the nature of their generation process. For instance, the fact that more than 80% of cfDNA fragments detected in plasma samples from colorectal cancer (CRC) patients were smaller than 145 bp seemed to correlate with the high level of DNA fragmentation characteristic of apoptotic mechanisms in cancer (Heitzer et al. 2015; Mouliere et al. 2014). On the other hand, studies of cfDNA levels and integrity in plasma samples from 383 primary and metastatic breast cancer patients showed that metastatic breast cancer patients could be divided into two groups, depending on whether their plasma contained or not detectable tumor-derived cfDNA (Madhavan et al. 2014). Moreover, it was reported that cfDNA concentration and integrity differed between normal control individuals and patients with primary tumors, and that cfDNA integrity correlates with progression-free and overall survival (Madhavan et al. 2014; O'Leary and Turner 2016; Jiang and Lo 2016).

2.1.2 CNAs as Biomarkers in Cancer

It was reported that the features of CNAs varied during the process of CRC development in mice, with a progressive increase of the proportion of circulating tumor-derived DNA (ctDNA) in the population of CNAs, and that the ctDNA exhibited a significant increase in its extent of fragmentation (Heitzer et al. 2015; Mouliere et al. 2011). These findings suggested that ctDNA may participate in tumor development and metastasis, in agreement with the fact that ctDNA levels are up to nearly 20% higher in patients with advanced disease (Heitzer et al. 2015) compared to patients at earlier disease stages (<1%), and with data from another study showing the existence of a correlation between patient relapse within 1 year after a surgery and the detectability of ctDNA in their plasma (Heitzer et al. 2015; Schwarzenbach et al. 2011). It was estimated that a patient with a 100-g tumor (about 3 x 10^{10} tumor cells) could release up to 3.3% of tumor DNA into the circulation every day (Schwarzenbach et al. 2011; Diehl et al. 2005). CTCs and micrometastatic deposits also contribute to the release of ctDNA into the blood (Blood 2016; Thierry et al. 2016; Khier and Lohan 2018). The released ctDNA is a heterogeneous mixture of genomic, mitochondrial, and epigenetic materials, some forming part of protein complexes (Bronkhorst et al. 2016), derived from different cancer cell clones and from tumor-associated cells (Heitzer et al. 2015; Schwarzenbach et al. 2011), and may have features with potential to lead to a number of molecular alterations including loss of heterozygosity (LOH) and different mutations in tumor suppressor genes and oncogenes (Thierry et al. 2016; Heitzer et al. 2015; Schwarzenbach et al. 2011). Thus, analyses of CNAs facilitate the detection of genetic and epigenetic alterations that could be relevant in the development of cancer. Even though serum has anywhere between 2- and 24-fold higher cfDNA concentration, plasma is a better source for cfDNA/ctDNA analysis than serum, due to the absence of interference in plasma of contaminants derived from changes that take place in association

with clotting processes in serum (Blood 2016; Thierry et al. 2016; Jiang and Lo 2016; Bronkhorst et al. 2016).

A liquid biopsy in cancer patients using CNAs as biomarkers can demonstrate not only the presence of cancer at different progression stages, but may be used also to monitor the effectiveness of any given therapeutic treatment plan. In addition, the majority of research data reported to date suggests that analysis of CNAs provides better overall representation of malignant disease and could be an important diagnostic and prognostic tool (Kuo et al. 2014). However, it is important to understand that the simple quantification of the levels of CNAs may not be enough for tumor diagnosis and prognosis. However, determination of levels of CNAs could be a better prognostic marker than the CTC count, as sequencing of CNAs-derived PCR products may yield information on possible tumor-specific mutations. Highly informative results were reported on patients with advanced non-small cell lung cancer (NSCLC) using the combination of CNA analyses and CTC counts (Heitzer et al. 2015). One of the most important applications of liquid biopsies is the monitoring of the patients response to therapy, in particular when focusing on therapies for which known resistance mechanisms have been identified. Specific gene mutations with high mutation frequencies in a variety of tumors, such as KRAS, TP53, epidermal growth factor (EGFR), adenomatous polyposis coli (APC), and BRAF, have been studied using CNA analysis techniques. A study of KRAS hotspot mutations and cyclin-dependent kinase inhibitor 2A (CDKN2A) hypermethylation status in CRC patients showed that 40% of CRC patients carried KRAS mutations and 20–50% of them had CDKN2A gene promoter hypermethylation. Results demonstrated that 100% of patients for whom there was no evidence of ctDNA carrying any of those alterations survived for at least 2 years (Lecomte et al. 2002). The presence of EGFR mutations in lung cancer sensitizes this tumor type to EGRF-targeted therapies (Diehl et al. 2005). Similarly, KRAS wild-type CRC tumors are initially sensitive to EGFR blockade, but over time, they typically develop resistance to relevant therapeutic agents. It was reported that KRAS mutations were detectable in about 38% of CRC patients after their initial 5–6-month treatment with panitumumab (Diaz et al. 2012). Studies of metastatic CRC patients before treatment demonstrated 100% diagnostic specificity and sensitivity for the BRAF V600E mutation and over 90% specificity and sensitivity for all tested KRAS mutations, thus demonstrating the high prognostic value of CNAs analyses (Thierry et al. 2014). KRAS mutations carrying CNAs, which are typically detectable in circulation before tumor progression, have been found to be responsible for the development of resistance to anti-EGFR therapies, although mutations of other genes such as the *MET* proto-oncogene and *ERBB2* have been reported to be involved also in the development and clinical manifestation of anti-EGFR therapeutic resistance (Heitzer et al. 2015).

2.1.3 Noncoding CNAs as Methylation and DNA Integrity Markers

The mammalian genome contains a variety of noncoding DNA sequences which are very frequently repeated, sometimes at highly regular intervals. Some of the best

characterized human repetitive sequences, such as the ALU and LINE1 elements, are highly distributed throughout the human genome and play important roles in physiological processes such as DNA repair, transcriptional regulation, epigenetic control, and transposon-based genome dynamics. Because these repetitive sequences can be detected as part of the CNAs, having different sizes and being methylated or unmethylated, they can be used also as possible blood biomarkers for various cancer types. In fact, it has been reported that the extent of methylation of these non-coding DNA repetitive sequences is lower in cancer cells than in normal cells (Tangkijvanich et al. 2007). LINE 1 elements are CpG-rich sequences and their 5′ regions can act as an internal transcriptional promoters. In normal cells, heavy methylation of LINE 1 sequences restricts their capacity to function as retrotransposable elements, thus contributing to the maintenance of genomic stability (Schulz et al. 2006). Changes in the methylation status of LINE1 sequences circulating in the blood of cancer patients yield relevant information on possible increases in genomic instability, a typically characteristic of advancing stages of tumor progression. The CpG methylation status of any given DNA, coding or noncoding, sequence can be detected by methylation-specific PCR and DNA sequencing after sodium bisulfite treatment, which converts unmethylated cytosines to uracil in the target DNA molecules. In addition, the use of PCR-based assays to determine the integrity of ALU sequences circulating in blood has been reported as a useful approach to assess the various progression stages of breast, testicular, ovarian, prostate, and nasopharyngeal cancer as well as the presence of micrometastases (Umetani et al. 2006a, b).

2.1.4 Circulating Nucleosomes

Tumor-derived cfDNA can be found in blood either as free monomeric or oligomeric nucleosomes, either retaining their binding to chromatin proteins (Laktionov et al. 2004), or bound to specific nucleic acid-binding proteins located on the surface of blood cells. Circulating nucleosomes can be detected using ELISA-based methodologies. Under normal physiological conditions, circulating nucleosomes are arranged in apoptotic particles which are phagocytosed by macrophages (Stollar and Stephenson 2002). However, under conditions resulting in increased levels of apoptosis, such as those taking place during the growth of large or fast proliferating tumors or after therapeutic treatments, increased nucleosome levels can become detectable in the blood (Ward et al. 2008), as macrophage-mediated phagocytosis may not be sufficiently effective to carry out their complete clearing from blood circulation. Because of this, quantification of circulating nucleosomes seems in fact particularly useful for monitoring the efficacy of apoptosis-inducing anticancer therapies (Holdenrieder et al. 2008). Nevertheless, it is important to point out that, although increased levels of circulating nucleosome have been associated with the progression of certain tumor types, they are not specific to cancer and may be linked to other nonneoplastic disease processes (Schwarzenbach et al. 2011).

2.1.5 Other Forms of cfDNAs: Mitochondrial and Viral DNAs

Mitochondrial and viral DNAs are also detectable in the population of human cfDNAs. Mitochondrial DNA (mtDNA), a circular molecule present in each cell in hundreds of copies, can be detected in the blood of normal individuals as well as in the serum of patients with different diseases, including cancer (Thierry et al. 2016; Chiu et al. 2003). General apoptosis mechanisms as well as mitophagy cause the release of mtDNA into the circulation (Ding and Yin 2012). Detection of mtDNA levels has become a biomarker for the exposure to carcinogenic agents (Budnik et al. 2013), for the progression of certain types of tumors (Kohler et al. 2009; Uzawa et al. 2012), and for monitoring the response to anticancer therapies (Huang et al. 2014). On the other hand, it has been conclusively established that viruses are etiological agents for certain types of human cancer. Human papillomaviruses (HPV) are associated with cervical tumors and head-and-neck cancer; hepatitis viruses (HBV and HCV) are associated with hepatocellular carcinoma; and Epstein-Barr viruses (EBV) are associated with some hematologic malignancies (Thierry et al. 2016; Schwarzenbach et al. 2011). Levels of circulating viral DNA can be used as biomarkers for those tumor types. However, it needs to be taken into consideration that, particularly for diagnostic purposes, the interpretation of data from assays for circulating viral DNA may be confounded by the simultaneous presence in patients or normal individuals of infections caused by the same viruses. This has become the main limitation for the diagnostic application of the detection of circulating viral DNAs to clinical oncological settings, although detecting the presence or absence of viral proteins in solid biopsies has become part of routine currently performed prognostic tests.

2.1.6 Circulating RNAs: mRNA and microRNA

The presence of both mRNA transcripts (cfmRNAs) and noncoding microRNAs (miRNAs) among the CNAs has been established for some time (Schwarzenbach et al. 2011; Lawrie et al. 2008). Interestingly, despite the abundant presence of RNAses in blood, cfRNAs were found to be particularly resistant to degradation. This unexpected stability seems mainly due to the fact that cells release cfRNAs after packaging them into extracellular vesicles (EV) of various types, including the so-called exosomes. Although, due to this release mechanism, the role of the lateral transfer of tumor-derived cfRNAs in cancer progression will be examined later in this chapter, it is important to state at this point that circulating miRNA profiling has become a much more reliable tool than the quantification of cfmRNAs to identify potentially useful diagnostic, prognostic, and predictive biomarkers for cancer progression and therapeutic response. This is particularly significant because the preponderant evidence derived from the extensive body of miRNA literature published in recent years demonstrates that miRNAs play critical roles as positive or negative effectors in the modulation of cancer onset, development, and

progression (Adams et al. 2017; Falcone et al. 2015; Treiber et al. 2019; Gebert and MacRae 2019).

3 Role of Horizontal Transfer of Cell-Free CNAs in Cancer Progression

It has been widely accepted that the main function of CNAs is to behave as agents of intercellular communication (Thierry et al. 2016; Peters and Pretorius 2012), because they can be readily transferred from cell to cell. In this context, the notion that the transfer of tumor-derived CNAs to normal cells may result in their acquisition of some malignant properties, or even of a fully transformed cancer phenotype, became an obvious possibility. This concept was initially applied to suggest that cfDNAs could play an important role in the development of metastasis (García-Olmo 2010), and the horizontal transfer of cfDNAs, including oncogenes, was indeed demonstrated to occur both in vitro (García-Olmo 2010; Bergsmedh et al. 2001) and in vivo (Trejo-Becerril et al. 2012). However, the so-called "genometastasis" theory had to be expanded later as it was also demonstrated that the lateral transfer of tumor-derived cfDNAs was able to induce in normal cell levels of genomic instability permissive for the development of malignancy, as described above (e.g., highly repetitive DNA sequences). Moreover, it has been also demonstrated that the lateral transfer of cfDNAs from normal cells to tumor cells resulted in the blockade of tumor growth (Gahan 2013; Garcia-Arranz et al. 2017). The preponderant evidence from published literature is that the lateral transfer from cfDNAs plays important modulatory roles, positive or negative, in tumor development, with tumor-derived cfDNAs being able to stimulate cancer development at each and every canonical stage of the carcinogenic process (Volik et al. 2016; Gupta 2017; Gravina et al. 2016; Heitzer et al. 2015; Schwarzenbach et al. 2011; Bronkhorst et al. 2016). In addition, it has been reported recently that cfDNA transfer from tumor to other cells can activate some of their prosurvival stress signaling pathways and thus modify their response to anticancer therapy (Kostyuk et al. 2012; Glebova et al. 2015). Similar roles have been described for cfRNAs, particularly for miRNAs. However, as their cell-to-cell transfer is mostly mediated by various EV types, their involvement in cancer progression will be examined below.

4 Cell Membrane-Derived Extracellular Vesicles, Exosomes

It has been known for quite some time that membranous vesicles of different sizes could be detected in the culture media of normal and cancer cells as well as in mammalian intercellular spaces in tissues and bodily fluids (Mathieu et al. 2019). Although the presence of such vesicles was interpreted at first as the consequence of a controlled mechanism used by cells to get rid of unnecessary or defective (e.g., misfolded) proteins (Harding et al. 1983; Pan et al. 1985), it has become abundantly clear in recent times that the release of EVs is a highly regulated process that, through different mechanisms, yields several EV types loaded with a variety of cargo components, with the primary function of facilitating diverse forms of intercellular communication (Van Niel et al. 2018; Mathieu et al. 2019). Different types of EVs have been distinguished on the basis of size, including exosomes (about 50–100 nm), microsomes, ectosomes, and microparticles (~100–1000 nm, in some cases grouped as microvesicles), and apoptotic EVs or apoptotic bodies (up to 5000 nm). As shown schematically in Fig. 1, exosomes are formed by means of an intracellular endocytic mechanism that results in the fusion of late multivesicular bodies (MVBs) with the cellular membrane, followed by the release of their intraluminal vesicles (ILVs) to the extracellular milieu (Van Niel et al. 2018; Abels and Breakefield 2016; Mathieu et al. 2019). The lipid content has been reported to play an important role in exosome packaging and delivery (Abels and Breakefield 2016). Larger EVs are generally released from cells by the formation of outward budding structures from the plasma membrane (Colombo et al. 2014; Raposo and Stoorvogel 2013). The predominant EV release mechanism seems to vary with the cell type of origin and environmental conditions (Van Niel et al. 2018; Mathieu et al. 2019; Colombo et al. 2013; Atkin-Smith et al. 2015; Menck et al. 2017). Regardless of their size, EVs have been demonstrated to contain a variety of cargo, including DNA, various types of RNAs, and proteins (Van Niel et al. 2018; Mathieu et al. 2019; French et al. 2017; Thèry et al. 2002), the abundance of which is also cell-type specific and influenced by the molecular mechanisms of their biogenesis (Van Niel et al. 2018; Kalra et al. 2016; Minciacchi et al. 2015). Nevertheless, typically, EV populations isolated from different sources are heterogeneous with regard to both their size and their content, and this circumstance still represents an important technical challenge, as the ability to isolate pure populations of any given EV type is an obvious prerequisite for their full structural and functional characterization (Schorey et al. 2015; Konoshenko et al. 2018; Momen-Heravi 2017; Heath et al. 2018). In this regard, it is important to realize that preparations of exosomes, the most widely studied EV type, which in theory could be more easily isolated to purity on the basis of their smaller size, likely incorporate both exosomal and non-exosomal EVs (Thèry et al. 2018). This chapter section focuses on the role of exosomes (or "small EVs") as vehicles for the horizontal transfer of procarcinogenic molecular effectors and their role in cancer progression.

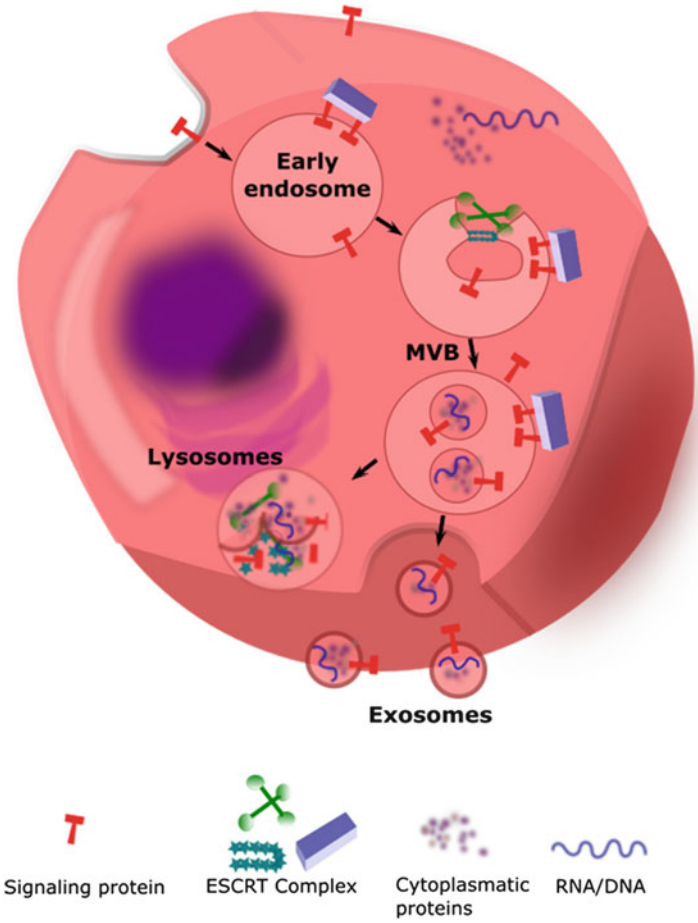

Fig. 1 Exosome origin, biogenesis, and secretion. An early endosome is formed by endocytosis of the plasma membrane. Exosomes are formed within multivesicular bodies (MVBs) generated by invaginations of late endosomes. The Endosomal Sorting Complex Required for Transport (ESCRT complex) recognizes ubiquitinated membrane proteins and receptors leading to the internalization of these tagged components into the MVBs. Consequently, exosomes are enriched in cytosolic material and endosome-associated signaling proteins. Finally, exosomes are released when MVBs fuse with the plasma membrane, and travel to recipient cells delivering cytosol components and genetic information from the cell of origin

Exosome membranes are enriched in cholesterol, sphingolipids, ceramide, and glycerophospholipids. Exosomes have been shown to carry a large variety of cargo, including growth factor receptors, tetraspanins (CD63, CD81), integrins, immunoglobulins, and cytoskeletal, ESCRT-related, and heat-shock proteins as well as proteins involved in vesicle biogenesis and trafficking and signaling molecules (Van Niel et al. 2018; French et al. 2017). Most importantly, exosomes are also enriched in single- and double-stranded genomic DNA (Xu et al. 2018; Thèry et al.

2002; Thakur et al. 2014; Waldenström et al. 2012; Ronquist et al. 2009; Lázaro-Ibáñez et al. 2014; Boyiadzis and Whiteside 2017) and mtDNA (Xu et al. 2018; Sansone et al. 2017), coding mRNAs (Xu et al. 2018; Lasser et al. 2011; Batagov and Kurochkin 2013) and noncoding RNAs (Xu et al. 2018; Falcone et al. 2015; Silva and Melo 2015) such as miRNAs and long noncoding RNAs (lncRNAs), and ribonucleoprotein particles (Valadi et al. 2007) such as vault RNA. Proteins and RNA species identified in exosomes are deposited in the ExoCarta database (http://www.exocarta.org) accessible online (Simpson et al. 2012; Mathivanan et al. 2012). In the context of cell-to-cell communication, it has been shown that exosomes display target selectivity in vitro and in vivo for particular types of cells and organs (French et al. 2017), and that such preference may be dependent on specific properties of the recipient cells (Lee et al. 2016; Prada et al. 2016; Wiklander et al. 2015). Most interactions between exosomes and recipient cells involve proteins in the exosomal surface (e.g., integrins, proteoglycans, lectins, tetraspanins) and the appropriate ligands or receptors (e.g., ICAMS, integrins, ECM adhesion receptors) present in recipient cells that facilitate exosome docking and provide cell/organ selectivity to the process (Mulcahy et al. 2014). This contact activates intracellular signaling pathways and exosome entry into their recipient cells by different ways. The binding of exosomes and the recipient cell can be also mediated by adhesion molecules, such as integrins and ICAMs and adhesion receptors (ECM). Although in some cases it was shown that cargo delivery was not necessary for the exosomes to transmit information to the recipient cells, the majority of studies reported full delivery of cargo and exosome internalization following docking on the target cells. Exosomes can be internalized through various mechanisms including endocytosis by clathrin-dependent and clathrin-independent pathways (Costa Verdera et al. 2017; Svensson et al. 2013; Horibe et al. 2018). Actin-mediated pinocytosis and lamellipodia-facilitated phagocytosis are documented alternative ways for exosomes to enter into the recipient cells, fusing their membranes and directly releasing their content. In most cases, after exosomes enter the recipient cell, they are directed to the lysosomes to be degraded (Chen et al. 2016; Tian et al. 2010), although in some instances, exosomes fuse with the endosomal membranes and deliver their contents to the recipient cell (Van Niel et al. 2018; French et al. 2017; Bissig and Gruenberg 2014; Kanada et al. 2015).

4.1 Exosome Roles in Malignant Transformation and Cancer Progression

Tumor cell-derived exosomes (TEX) carry messages from tumor cells either to other malignant cells or to normal cells. Tumor cells are known to be more proficient than normal cells in secreting TEX and other EVs than normal cells (Thèry et al. 2002; Xu et al. 2018; Peinado et al. 2012; Luga et al. 2012; Wendler et al. 2017). The ratio between TEX and normal cell-derived exosomes in plasma samples of cancer

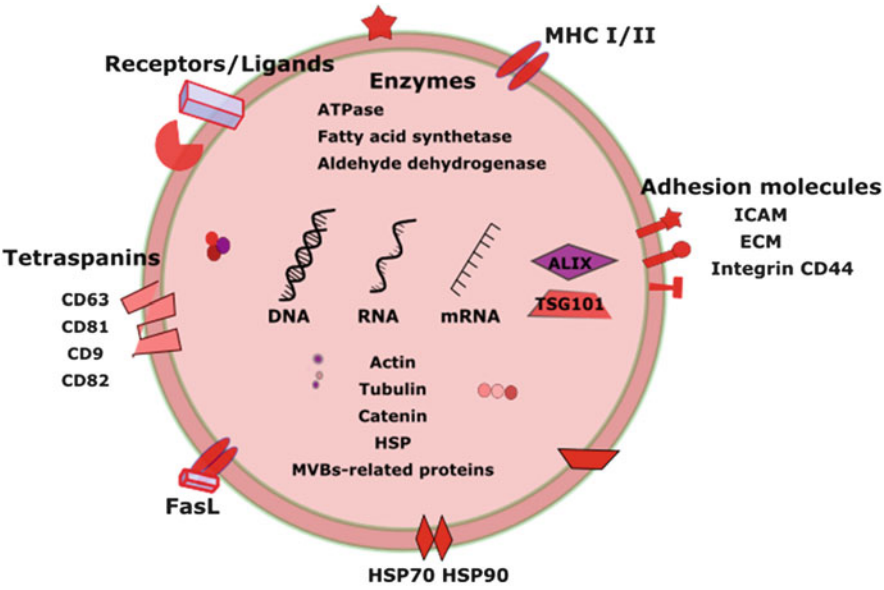

Fig. 2 Typical TEX (Tumor cell-derived exosomes) content. TEX cargo varies among different tumor types. However, it consistently shows the presence of cancer-relevant molecules such as FasL (Fas ligand), ALIX, tetraspanins, TSG101 (tumor susceptibility gene 101), and HSPs (heat shock proteins). In addition, TEX carry a high content of cytoskeletal proteins, enzymes, adhesion molecules, and nucleic acids. Modified from Boyiadzis & Whiteside (108)

patients varies for different tumor types and for individual patients of each case, but the invariable fact is that the majority of detectable exosomes proceed from tumor cells (Boyiadzis and Whiteside 2017; Whiteside 2016). When examined by electron microscopy (EM), TEX do not differ from other exosomes, but their molecular content comes from the parent tumor cells and includes a variety of proteins that differ from those in normal exosomes (Fig. 2). Immuno-EM showed the presence of cancer-relevant molecules such as FasL, glypican, MVB-related proteins, ALIX, TSG101, tetraspanins, heat-shock proteins, CD9, CD63, CD81, and CD82, which are typically used as exosome markers. They have also a high content of actin, tubulin, and components of cellular signaling pathway such as β-catenin, WNT, and/or Notch (Boyiadzis and Whiteside 2017). The nature of the TEX content varies among different tumor cell lines and tumor types. Comparative analyses suggested that TEX cargo incorporation is not a random process, but rather involves selective packaging (Abels and Breakefield 2016; Boyiadzis and Whiteside 2017).

It has been reported that exosomes have pleiotropic functions in the TME and participate in pathological events such as malignant transformation, tumor growth, immune escape, angiogenesis, invasion, and drug resistance (Whiteside 2016; Challagundla et al. 2015; Chen et al. 2014a, b; Giallombardo et al. 2016). The presence of DNA, mRNA, and miRNA as TEX cargo has been associated also with the inhibition of the antitumor immune cell response. In addition, at least three

different mechanisms of induction of therapeutic drug resistance in tumor cells by TEX have been described, including drug export via the exosome pathway, neutralization of antibody-based drugs, and exosome-mediated transfer of miRNAs (Giallombardo et al. 2016). It was reported also that intratumoral hypoxic conditions increase TEX release by some tumor types, such as breast cancer, and that changes in the pH of the TME modulate the lipid composition of TEX, thereby modulating their docking selectivity and efficiency of cargo delivery (Wendler et al. 2017). Comparatively speaking, our current knowledge on the role of TEX-encapsulated DNA in cancer is limited relative to the abundant information on the involvement of TEX-encapsulated RNAs. This is due to the fact that not only TEX have been reported to contain thousands of mRNA species but also to the frequent detection of noncoding RNAs (miRNAs and lncRNAs) as TEX cargo, coupled with the enormous amount of information concerning their cancer-promoting or cancer-suppressing effects reported in recent years. The overall picture shows that the transfer of exosomal NAs from tumor cells to normal cells or to other tumor cells has the potential to modify the phenotype and/or behavior of the recipient cells by genetic (true HGT) or epigenetic (gene expression-dependent) mechanisms, thereby priming them to move along the various stages of the carcinogenic process, from initiation (malignant transformation) to progression, by enhancing migration, invasiveness, PMN formation, angiogenesis, immune escape, or therapy resistance.

4.1.1 Horizontal Transfer of TEX DNAs

As indicated above, TEX may carry genomic DNA and mtDNA (Xu et al. 2018; Boyiadzis and Whiteside 2017; Sansone et al. 2017; Chennakrishnaiah et al. 2018). Genomic double-stranded DNA fragments of 10 Kbp or longer have been described as TEX cargo (Thakur et al. 2014; Boyiadzis and Whiteside 2017). It has been shown in vitro that TEX-linked DNA did not derive from apoptotic or necrotic cells and that it was mainly associated with the outer vesicular membrane, although some could be detected also inside the TEX (Fischer et al. 2016). The use of plant-derived DNA with TEX-like vesicles and human recipient cells provided proof-of-principle support for the HGT of donor TEX-derived DNA to the target cells (Fischer et al. 2016). The presence of chromosomal DNA containing enriched oncogene or tumor suppressor sequences has been described in TEX secreted by different tumor types (Ronquist et al. 2009; Lázaro-Ibáñez et al. 2014; Kahlert et al. 2014; Choi et al. 2017; Balaj et al. 2011) as well as in EVs derived from normal cells (Yang et al. 2017). In fact, it has been reported that presumably, normal leukocytes constitute an important circulating reservoir of oncogenic DNAs (Chennakrishnaiah et al. 2018). The intercellular transfer of oncogenic sequences has been demonstrated also (Balaj et al. 2011; Al-Nedawi et al. 2008; Lee et al. 2016). Similarly, the horizontal transmission (HGT) of mtDNA has been documented also, as having either promalignancy (Sansone et al. 2017; Esquilin et al. 2012; Szczesny et al. 2018) or antitumor (Berridge et al. 2018) consequences. The involvement of mutated oncogenes or tumor suppressor genes has been obviously related to the direct malignant

transformation of the recipient cells, by becoming able to express dominant-transforming proteins (e.g., mutant H- or K-Ras) or by the loss of tumor-suppressive potential. However, an indirect effect on cancer progression seems also very likely, as the transfer of TEX-associated single-stranded DNA (Thèry et al. 2002) may act as a signal for DNA damage in the recipient cell, which may deregulate stress-response pathways, and lead to genomic instability, in a similar fashion to the way that the transfer of cfDNA-containing repetitive sequences was reported to act (Schulz et al. 2006; Umetani et al. 2006a, b) but in a methylation-independent context.

4.1.2 Horizontal Transfer of Epigenetic Effectors: Effects on Cancer Progression

The functional transfer of TEX-derived coding or noncoding RNAs and proteins may result in the alteration of the gene-expression repertoires of the recipient cells, in the absence of any genetic alteration. The epigenetic consequences for the recipient cells of the transfer of TEX-associated proteins seem to be more direct (Peinado et al. 2012; Luga et al. 2012; Hosseini-Beheshti et al. 2012; Nilsson et al. 2009), but they are obviously limited by the intrinsic half-life of the proteins themselves, although transient bursts of cancer-relevant enzymatic activities (e.g., DNA methylases, histone deacetylases, protein kinases) may have lasting effects on the recipient cells, which may be passed along to the next generations of cells (Dielmann-Gessner et al. 2014).

Horizontal Transfer of Tumor-Derived Exosomal RNAs

The presence of mRNAs as TEX cargo and their transfer to recipient cells have been frequently reported (Xu et al. 2018; Lasser et al. 2011; Valadi et al. 2007; Huber et al. 2005; Ratajczak et al. 2006). It has been established that TEX may transfer functional mRNAs that can be translated into their encoded proteins by the recipient cells (Valadi et al. 2007; Ridder 2015) which will then acquire their corresponding functions. It has been reported that TEX-transferred mRNA populations are enriched in 3′-untranslated (3′UT) sequences (Lasser et al. 2011; Batagov and Kurochkin 2013) and their presence, along with the concomitant presence of zipcode mRNA localization sequences (Bolukbasi et al. 2012), has been interpreted as a reflection of their possible role in the selective packaging of certain mRNA species during TEX biogenesis. However, the abundance of 3′UT sequences may have also important gene-expression regulatory consequences. It is a well-known fact that the vast majority of cellular mRNA sequences targeted by miRNAs and other noncoding RNAs are located within their 3′UT region (Catalanotto et al. 2016). Therefore, the cell-to-cell exchange of abundant 3′UT sequences mediated by TEX transfer might create an imbalance in the ratio of endogenous/exogenous 3′UT sequences available for miRNA targeting in the recipient cells, and that could have immediate effects on

their gene-expression repertoire and, consequently, on their malignancy-related properties. However, whether that plays a real regulatory role in TEX-mediated cancer progression remains to be determined. Regardless, there is abundant documented evidence supporting a variety of phenotypic effects of TEX-transferred mRNAs on the recipient cells. The transfer by TEX of functional mRNAs has been reported to enhance tumor growth by different mechanisms, including the enhancement of cell cycle activities by M-phase mRNAs (Huber et al. 2005; Skog et al. 2008). The transfer of tumor-associated fusion-gene transcripts, such as the prostate cancer-derived *TMPRSS2:ERG* or the lung cancer-related *EML4-ALK*, has been shown to enhance tumor growth and modify the response to chemotherapeutic agents (Hosseini-Beheshti et al. 2012; Nilsson et al. 2009, 2016). Other cancer progression-related functions affected by TEX-mediated mRNA transfer include tumor dissemination (Yokoi et al. 2017), angiogenesis (Skog et al. 2008; Vader et al. 2014; Grange et al. 2011), cell reprogramming (Ratajczak et al. 2006), modulation of the immune response (Ridder 2015), and drug resistance (Ma et al. 2014).

Horizontal Transfer of Tumor-Derived Exosomal miRNAs and Other Noncoding RNAs

The miRNA class of noncoding RNAs includes a number of small RNA molecules (19–25 nucleotides) that have been shown to exert positive or negative modulatory effects on the expression and translation of target mRNAs by binding their 3'UT sequences (Catalanotto et al. 2016). Diverse miRNAs have been described to play important roles in fundamental physiological processes such as cell differentiation, metabolism, proliferation, and apoptosis. In addition, miRNAs and other noncoding mRNAs have been reported to play key roles in the development of various diseases (Adams et al. 2017), including cancer (Falcone et al. 2015). The expression of miRNAs is deregulated in diverse tumor types, and, depending on the cellular context, miRNAs have been reported to either have tumor-suppressive activity or act as oncogenes ("oncomiRs") and also to play critical roles in cancer inflammation and drug resistance as well as in the regulation of cancer stem cells (Falcone et al. 2015). Diverse miRNAs can be found inside cells; secreted as free molecular entities in serum saliva, plasma, or urine; and packaged into exosomes both in normal individuals and in cancer patients (Lawrie et al. 2008). Their membrane encapsulation provides exosomal miRNAs with greater stability than cellular miRNAs or cfmiRNAs, and that environmental protection makes them nearly ideal vehicles for the transfer of gene-expression regulatory capabilities to the exosome-recipient cells (Lawrie et al. 2008; Falcone et al. 2015).

Exosomes of different origins carry unique miRNA profiles. For instance, human colon carcinoma cells purified by immunocapture with different antibodies were shown to contain two distinct exosome populations that differ in the proteins and miRNA cargo (Villarroya-Beltri et al. 2013). The fact that one of the types contained mainly miRNAs suggested that miRNA packaging during exosome biosynthesis is

not a random process. Both pre-miRNAs and mature miRNAs have been found in exosomes, and it has been suggested that the exosome sorting of pre-miRNAs is likely to be controlled in a sequence-dependent manner. Breast cancer-derived exosomes were shown to carry pre-miRNAs along with the core RNA-induced silencing complex (RISC) proteins Dicer, Ago2, and TRBP and displayed a cell-independent capacity to process pre-miRNAs into mature miRNAs (Melo et al. 2014). It has been reported also that the availability of miRNAs in exosomes and their secretion depend on the cellular levels of their endogenous target transcripts, suggesting that exosomal miRNA secretion is a mechanism to maintain miRNA/mRNA homeostasis in the cells and tissues or tumors (Squadrito et al. 2014; Ohshima et al. 2010). In fact, it has been reported that sorting of mature miRNAs into exosomes is controlled by miRNA sequence motifs, which are recognized and bound during the sorting process by the ribonucleoprotein A2B1 (hnRNPA2B1) (Villarroya-Beltri et al. 2013).

Despite their recognized dual, positive or negative, role in tumor progression, most of the literature reports focus on the role of TEX miRNAs in enhancing tumorigenesis and tumor progression (Falcone et al. 2015; Melo et al. 2014), although there are also some reports indicating that TEX miRNAs can also work in tumor suppression (Falcone et al. 2015). Even an individual miRNA can work on tumor promotion and on tumor suppression depending on the tumor type of origin. That is the case of miR-23b and others that have tumor-suppressive functions on bladder carcinoma by inhibiting angiogenesis and decrease lymph node metastasis (Ostenfeld et al. 2014), whereas in gastric cancer, they maintain or stimulate their metastatic potential (Ohshima et al. 2010). TEX-miRNAs have been reported to enhance tumor progression by having suppressive effects on the antitumor immune response system. For instance, overexpression of miR-9 in multiple cancer types inhibits the transcription of *MHC* class I genes, thus preventing tumor cell recognition. Similarly, miR-212 targets the regulatory factor X-associated protein (RFXAP), which is a transcription factor of *MHC* class II genes. Also, miR-222 downregulates the expression of intracellular cell adhesion molecule 1 (ICAM-1), the binding of which to lymphocyte function-associated antigen (LFA-1) is essential for activation of cytotoxic T cells and necessary for tumor cell lysis (Falcone et al. 2015), and TEX miR-23 from lung carcinoma cells inhibits NK cell functions mediated by TGF-β (Yang et al. 2018). Moreover, some TEX miRNAs have the ability of epigenetically affect target genes in dendritic cells, and T-cell-derived exosomal miRNAs can suppress T-H1-mediated immune responses (Falcone et al. 2015) and ultimately favor immune escape of the tumor cells (Yang et al. 2018). TEX individual miRNAs can work sometimes in tumor suppression and others in enhancing tumor progression. For instance, high levels of TEX miR-23b secreted by bladder carcinoma have tumor-suppressive effects by inhibiting angiogenesis and lymph node metastases (Ohshima et al. 2010), whereas TEX miR-23b derived from metastatic gastric cancer has tumor-promoting activities, by stimulating the acquisition of metastatic properties (Ostenfeld et al. 2014). In other cases, TEX miRNAs have been reported to stimulate metastasis by enhancing angiogenesis (Kosaka et al. 2013) or by causing the destruction of the vascular endothelial barrier, thereby

facilitating the invasiveness of tumor cells (Zhou et al. 2014) in different cancer types (Le et al. 2014).

It was reported that, in the absence of tumor cells, some TEX miRNAs are involved in the modulation of PMN formation by acting on target organ stromal cells and facilitating tumor cell homing, their active growth, and the onset of metastasis. For instance, high expression of miR-105, a potent regulator of the ZO-1 tight junction protein, in breast cancer cells led to the destruction of the vascular endothelial cell layer, the induction of vascular permeability, and the extravasation of otherwise nonmetastatic cancer cells (Zhou et al. 2014). Similarly, miR-200 acts by regulating the mesenchymal-to-epithelial transition (MET) process, and when transferred TEX confers the capacity of tumor growth at metastatic lesions (Le et al. 2014). In CRC, TEX miR-9 enhances tumor angiogenesis and endothelial cell migration by inhibiting the expression of suppressor of cytokine signaling 5 (SOCS 5) and by promoting the activation of the Janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling (JAK/STAT), a known driver of endothelial cell migration (Zhuang et al. 2012). Furthermore, it was reported that miR-210 can suppress the expression of genes such as *EFNA3* (coding for Ephrin-A3) in endothelial cells, thereby supporting neoangiogenesis. Moreover, sphingomyelinase-2 (nSMase2) was demonstrated to be required to regulate TEX miRNAs secretion and enhance angiogenesis (Kosaka et al. 2013). It is also known that tumor oxygen levels may modify the components of TEX cargo. Certain proteins and miRNAs exert proangiogenic functions in response to hypoxia (Umezu et al. 2014), as in the case of TEX miRNA-135b from hypoxic multiple myeloma, which may decrease the expression of hypoxia-inducible factor-1 (HIF-1) and reduce its activity (Fan 2014).

Finally, TEX miRNAs have the ability to horizontally transfer drug resistance to the recipient cells. Many tumors with various drug resistances show up- or downregulation in expression of miRNAs. It was reported that many breast cancer cells resistant to several drugs (docetaxel, adriamycin, tamoxifen) transferred such resistances to recipient cells by TEX miRNAs exchange. TEX from drug-resistant breast cancer cells released a pool of miR-100, miR-222, and miR-30a into the target cells, resulting in the modulation of target genes critical to determine their drug response. Particularly, TEX miR-222 is known to downregulate PTEN mRNA and thereby decrease the apoptotic response of ovarian cancer cells to doxorubicin, while miR-21-3p concomitantly increases cisplatin resistance by targeting the *NAV3* gene (Pink et al. 2015). A number of other TEX miRNAs derived from different types of tumor cells (Challagundla et al. 2015; Chen et al. 2014a, b) have been associated with the transmission of drug resistance to the recipient cells by various mechanisms (Giallombardo et al. 2016).

5 Clinical Applications of Cell-Free and Exosomal CNAs

The use of CNAs and TEX cargo components as tumor biomarkers (Melo et al. 2015; Lai et al. 2017) with utility in the diagnosis, prognosis, and staging of cancer has become more accessible to the oncology clinics in recent times (Volik et al. 2016; Blood 2016; O'Leary and Turner 2016), and information for biomarker analyses has been used to make treatment decisions and to predict and monitor therapeutic response outcomes (Volik et al. 2016; Van Niel et al. 2018). Although these applications provided great noninvasive advances for the clinical management of oncology patients, their universal use still must overcome certain challenges, including some related to achieving reliable levels of sensitivity and specificity, to establishing the relative contributions of tumor vs. normal tissues in cancer patients, and to understanding the influence of tumor heterogeneity (Mao et al. 2018). While progress is being made to address such challenges, the most attractive goal in this research area is to become able to use TEX or other EVs as tumor-targeting vehicles capable of delivering effective antitumor agents in a controlled fashion. In fact, the use of TEX and other EVs as delivery vehicles has several advantages when compared with other delivery system (e.g., liposomes or polymeric nanoparticles) used in the past (Van Niel et al. 2018; Xu et al. 2018; Mao et al. 2018). TEX and other EVs have demonstrated ability to target their cargo recipient cells, low intrinsic toxicity, biocompatibility, and longer circulating half-lives (Haney et al. 2015; Gilligan and Dwyer 2017; Tian et al. 2014; El Andaloussi et al. 2013). However, there are also some drawbacks, perhaps the most important being the need for a complete standardization of relevant methodologies for their preparation, analysis, controlled targeting, and regulated cargo delivery (Van Niel et al. 2018; Xu et al. 2018; Mao et al. 2018). Also, as indicated above, it is essential for these purposes to improve preparation methods to the point that they may yield pure populations of TEX or other EVs (Heath et al. 2018; Thèry et al. 2018). Progress during the last 15–20 years has been remarkable. Not only very exciting preclinical results have been reported on the use antitumor activity of TEX loaded with RNAi-mediators, apoptosis-inducing suicide drugs, miRNAs, and toxic drugs (Ohno et al. 2013; Mizrak et al. 2013; Pan et al. 2014) or used for trapping CTCs (Wendler et al. 2017), but also some of these and other approaches, such as the generation and delivery of antitumor vaccines (Pitt et al. 2014; Besse et al. 2016), have moved to clinical trials (Colombo et al. 2014; Lo Cicero et al. 2015; Fais et al. 2016) designed to test their efficacy against a variety of tumor types (Van Niel et al. 2018; O'Leary and Turner 2016; Xu et al. 2018; Milane et al. 2015). On the basis of the wealth of knowledge gained to date on their role as possible positive and negative effectors of tumor progression, substantial progress has been made on the targeting of noncoding RNAs in cancer, an approach that has moved recently also to clinical trials (Adams et al. 2017). It seems obvious that some of the recent reports describing the role of EVs in acting as migratory role-models for cancer cells (Steenbeek et al. 2018) or the process of tumor dissemination through tumor spheres of cells with reverse polarity (Zajac et al. 2018) will be utilized in the near future to design anticancer strategies.

In summary, HGT has been conclusively demonstrated to play an important role in cancer onset and progression. However, substantial progress has been made beyond establishing such key finding itself. Information obtained from studies designed to understand the underlying HGT-mediated cancer-enhancing mechanisms has led the scientific community in the direction of using HGT to our advantage, to develop improved approaches for the clinical management of cancer.

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