Handbook of Experimental Pharmacology 211

Anthony R.M. Coates *Editor*

Antibiotic Resistance

Springer

Handbook of Experimental Pharmacology

Volume 211

Editor-in-Chief

F.B. Hofmann, München

Editorial Board

J.A. Beavo, Seattle
J.E. Barrett, Philadelphia
D. Ganten, Berlin
P. Geppetti, Florence
M.C. Michel, Ingelheim
C.P. Page, London
W. Rosenthal, Berlin

For further volumes:
http://www.springer.com/series/164

Anthony R.M. Coates
Editor

Antibiotic Resistance

Editor
Anthony R.M Coates
Division of Clinical Sciences
St. George's, University of London
Cranmer Terrace
London SW17 ORE
United Kingdom

ISSN 0171-2004 ISSN 1865-0325 (electronic)
ISBN 978-3-642-28950-7 ISBN 978-3-642-28951-4 (eBook)
DOI 10.1007/978-3-642-28951-4
Springer Heidelberg New York Dordrecht London

Library of Congress Control Number: 2012950770

© Springer-Verlag Berlin Heidelberg 2012
This work is subject to copyright. All rights are reserved by the Publisher, whether the whole or part of the material is concerned, specifically the rights of translation, reprinting, reuse of illustrations, recitation, broadcasting, reproduction on microfilms or in any other physical way, and transmission or information storage and retrieval, electronic adaptation, computer software, or by similar or dissimilar methodology now known or hereafter developed. Exempted from this legal reservation are brief excerpts in connection with reviews or scholarly analysis or material supplied specifically for the purpose of being entered and executed on a computer system, for exclusive use by the purchaser of the work. Duplication of this publication or parts thereof is permitted only under the provisions of the Copyright Law of the Publisher's location, in its current version, and permission for use must always be obtained from Springer. Permissions for use may be obtained through RightsLink at the Copyright Clearance Center. Violations are liable to prosecution under the respective Copyright Law.
The use of general descriptive names, registered names, trademarks, service marks, etc. in this publication does not imply, even in the absence of a specific statement, that such names are exempt from the relevant protective laws and regulations and therefore free for general use.
While the advice and information in this book are believed to be true and accurate at the date of publication, neither the authors nor the editors nor the publisher can accept any legal responsibility for any errors or omissions that may be made. The publisher makes no warranty, express or implied, with respect to the material contained herein.

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

Preface

Antibiotics have changed the face of medicine since the early part of the twentieth century. The Achilles' heel of antibiotics is resistance which has arisen to all marketed compounds. This sets antibiotics apart from other groups of medicines, because the former become obsolete over time as resistance to them increases. So, in order to maintain an effective arsenal of anti-infectives, it is necessary, at regular intervals, to invent new ones, develop them and then market them. Unfortunately, the emergence of resistance has outpaced the rate of replacement of obsolete antibiotics with new effective compounds. This situation threatens the practice of modern medicine.

Bacteria have existed on earth for over 3 billion years. During that time they have produced numerous antibiotics, and in order to survive, they have developed resistance to these compounds. Whilst we tend to think of individual species which are multi-drug resistant, bacteria are thought to operate together as a "Resistome" which contains pathogenic and non-pathogenic bacteria that cooperate in the exchange of resistance genes.

What hope is there for the human race? The first rule in any war is that intelligence is paramount. Surveillance of the enemy, in this case bacterial resistance, is the bedrock of the fight. The medical microbiologists and infectious disease clinics use this information to choose the right antibiotics to treat infected patients. Companies, which make new antibiotics, are advised which beta-lactamase to block and what species of bacteria are becoming most resistant. It is a slowly moving front, taking decades to shift; the Gram-positive antibiotics are currently pushing back their bacterial foes, whilst the Gram-negatives seem to be in the ascendant. The scientists are trying to devise new ways to win the war but have suffered a series of setbacks including the failure of the genomics programme to lead to any novel marketed antibiotics. However, it is possible that we can learn from the tuberculosis treatment arena, in which combinations have proven a success, although resistance is also now becoming a major problem in this disease. A new dimension in the battle may come from targeting non-multiplying bacteria, which are also called persisters. This is because virtually all clinical infections contain non-multiplying bacteria which exist alongside multiplying ones. The problem with non-multiplying bacteria

is that they cannot be readily killed by most marketed antibiotics, which means that the therapy is prolonged, and this can lead to more genetic-resistant mutants. Alternatively, perhaps we can reduce the overall emergence of resistance by simply reducing our usage of antibiotics in medicine, textiles and animals or maybe we can harness the power of, for example, efflux pumps to make new effective antibiotics.

I hope that this volume will give the reader a new angle on the subject of antibiotic resistance, which will be valuable to both those who have many years of experience and those who are new to the field. It describes the problem of growing resistance, our failure to market enough antibiotics to cope with this resistance, particularly for Gram-negatives, and its origins. The volume also covers ways that we can deal with antibiotic resistance, such as surveillance, infection control, combinations of antibiotics, targeting non-multiplying persisters or focussing on efflux pumps. Finally it describes how we can perhaps reduce usage of antibiotics and so decrease the rate of emergence of resistance.

I also hope that this book will highlight the excitement in the antibiotic resistance field and the successes of what has been and will continue to be one of the pillars of modern medicine. The future will bring unimaginable ingenuity of new bacterial resistance mechanisms and, hopefully, equally brilliant scientific advances in antibiotic research.

London, UK Anthony Coates

Contents

Introduction to Antibiotic Resistance 1
Richard Bax and David Griffin

The Origins of Antibiotic Resistance 13
Gerard D. Wright

Surveillance Programmes and Antibiotic Resistance: Worldwide and Regional Monitoring of Antibiotic Resistance Trends 31
Stephen Hawser

Current and Future Challenges in the Development of Antimicrobial Agents 45
Robert P. Rennie

The Role of the Outer Membrane of Gram-negative Bacteria in Antibiotic Resistance: Ajax' Shield or Achilles' Heel? 67
Malcolm G.P. Page

Prevention of Drug Resistance by Combined Drug Treatment of Tuberculosis 87
Denis A. Mitchison

Nonmultiplying Bacteria are Profoundly Tolerant to Antibiotics 99
Yanmin Hu and Anthony Coates

Persister Cells: Molecular Mechanisms Related to Antibiotic Tolerance . . . 121
Kim Lewis

Antimicrobial Textiles 135
J. Vaun McArthur, R.C. Tuckfield, and C. Baker-Austin

Efflux: How Bacteria Use Pumps to Control Their Microenvironment . . . 153
E. David G. McIntosh

Antibiotics in Phase II and III Clinical Trials 167
Anthony R.M. Coates and Gerry Halls

Index 185

Introduction to Antibiotic Resistance

Richard Bax and David Griffin

Contents

1 What Is Happening to Antibiotic Resistance? ... 3
2 Epidemiology of Resistance ... 4
3 Concerns and Activities Occurring to Reduce This Threat ... 5
4 US Congress ... 6
5 European Medicines Agency (EMA) Activities ... 6
6 React ... 7
7 Present State of Antibiotic Research Constraints ... 8
8 Proposals on Co-development of Antibiotics ... 10
9 Abstracts ... 10
10 Conclusion ... 11
References ... 11

Abstract The inexorable rise in multidrug-resistant Gram-negative bacteria has been widely reported. Multiple modes of resistance often present in a single strain of bacteria, and this may also be combined with an increase in virulence, both of which are leading to a significant increase in morbidity and mortality in patients. Against this background, the absolute number of new antibiotics licensed has declined especially for Gram-negative multidrug-resistant pathogens. The reasons for this failure are presented here: market issues, big pharma changes, regulatory constraints, difficulties in finding drugable targets and, lastly, suitable compounds worthy of full development.

Keywords Antibiotic resistance • Gram-positive bacteria • Gram-negative bacteria • Regulatory

R. Bax (✉) • D. Griffin
TranScrip Partners LLP, 400 Thames Valley, Park Drive, Reading RG6 1PT, UK
e-mail: richard.bax@transcrip-partners.com

A.R.M. Coates (ed.), *Antibiotic Resistance*, Handbook of Experimental Pharmacology 211, DOI 10.1007/978-3-642-28951-4_1,
© Springer-Verlag Berlin Heidelberg 2012

Antibiotics have saved millions of lives and eased the suffering of patients of all ages for more than 60 years. These “wonder drugs” deserve much of the credit for the dramatic increase in life expectancy in the United States and around the world in the twentieth century. They prevent amputations and blindness, advance our ability to perform surgery, enable new cancer treatments to be used and protect the lives of our military men and women. A famous infectious disease expert once noted that the discovery of penicillin in the early 1940s gave more curative power to a lone provider than the collective talent of all the physicians in New York City at that time. Unfortunately, it is inevitable that, over time, bacteria develop resistance to existing antibiotics, making infections more difficult to treat.

Antibiotic resistance is not a new phenomenon. National surveillance data and independent studies show that drug-resistant, disease-causing bacteria have multiplied and spread at alarming rates in recent decades. A diverse range of patients is affected. The Institute of Medicine (IOM), Centers for Disease Control and Prevention (CDC), National Institutes of Health (NIH) and Food and Drug Administration (FDA) warn that drug-resistant bacteria are a serious public health threat, especially considering that there are a few novel drugs in the pipeline to combat them.

Infections that were once easily curable with antibiotics are becoming difficult, even impossible, to treat, and an increasing number of people are suffering severe illness—or dying—as a result. This year, nearly two million people in the United States will acquire bacterial infections whilst in hospital, and about 90,000 of them will die, according to CDC estimates. More than 70% of the bacteria that cause these infections will be resistant to at least one of the drugs commonly used to fight them. In a growing and frightening number of cases, these bacteria are resistant to many approved drugs, and patients have to be treated with new, investigational compounds or older, possibly more toxic alternatives. For many patients, there simply are no drugs that work.

Furthermore, some strains of resistant bacteria are no longer confined to hospitals and are occurring in otherwise healthy individuals in communities across the United States and other countries.

In 1998, Bax and others noted the increase in MDR pathogens leading to antibiotics in general losing their effectiveness. According to IOM and FDA, only two new classes of antibiotics have been developed in the past 30 years, and resistance to one class emerged even before the FDA approved the drug. Nalidixic acid launched in 1962 and then daptomycin launched in the USA in 2003, these are the last two systemic antibiotics with a completely new mode of action introduced to the market (Bax et al. 1998). The recommendations include researching and developing new antibiotics, clarifying the strengths and weakness of clinical trials with the aim of improving their relevance. This included measuring the precise impact of the use of antibiotics on the prevalence of resistance, the effect of resistance on a range of clinical outcomes and the effect of outcomes on costs.

As resistant bacteria multiply, so does the burden they place on our healthcare system. The economic cost has reached billions of dollars annually in the United States, according to estimates from IOM and the former Congressional Office of Technology Assessment. The human cost in terms of pain, grief and suffering, however, is incalculable.

1 What Is Happening to Antibiotic Resistance?

Multidrug resistance (MDR) is increasing (Arias and Murray 2012; So et al. 2012). Amongst Gram-negative bacteria, the increasing incidence of bacteria which carry carbapenemases, such as New Delhi metallo-beta-lactamase (NDM-1) and extended-spectrum beta-lactamases (ESBL) (Pitout and Laupland 2008), is particularly worrying because very few antibiotics remain that are effective against such MDR bacteria. In addition, for some MDR strains there may be an increase in virulence (Walsh 2011; Poirel et al. 2007).

The phenomenon of more than one resistance mechanism occurring in one mutant strain of bacteria is increasing the threat. For example, enzymes which destroy antibiotics, porin defects, alteration in cell wall structure, changes in RNA and efflux pumps can all occur in MDR strains (Taubes 2008). Plasmids which encode MDR genes are an important part of the rapid transfer of resistance between bacteria within a species and even between species. In Gram-negative bacteria, beta-lactamases in the periplasmic space destroy beta-lactams including, in some cases, carbapenems. Overexpression of transmembrane efflux pumps can reduce the efficacy of beta-lactams including meropenem, aminoglycosides, quinolones and tetracyclines. Antibiotic-modifying enzymes can blunt the activity of aminoglycosides and ciprofloxacin, and mutations of the DNA gyrase and topoisomerase IV genes induce resistance to quinolones (Giamarellou 2009).

Other mechanisms of antibiotic activity include ribosomal mutation or modification which reduces the effect of tetracyclines and aminoglycosides, mutations in lipopolysaccharide structure which affects the efficacy of polymyxin and loss of porins which reduces the efficacy of carbapenems. Bypassing of dihydrofolate reductase leads to resistance to trimethoprim, and bypassing dihydropteroate synthase results in sulphonamide resistance (Livermore 2009).

The traditional way of developing a broad-spectrum, blockbuster, antibacterial drug is probably a thing of the past, and targeted treatments are now required. This requires different regulatory routes and these are currently being considered but need significant development (Livermore 2004). The current considerations include a greater use of PK/PD to support clinical trials and conditional approval for compounds with a high medical need to risk ratio. Pathogen-specific indications require significant discussion and development with regulatory authorities and experts in the field. Pathogen-specific clinical studies may be more scientifically valid than indication studies because an anti-infective targets infecting organisms and the clinical expression of disease varies by pathogen, site of infection, virulence and host response. Perhaps the orphan drug route could be used for rare life-threatening infections such as those caused by NDM-1? Should surrogate markers be developed? What is the place of rapid diagnostics? Another question is whether approval, which is based on extrapolations, could be arranged for compounds with similar microbiological, pharmacological and disease characteristics, for example, intra-abdominal sepsis and gynaecological infections.

Drug-resistant pathogens are responsible for more and more infections. Indeed, it is impossible to study even a single MDR species in all indications. An indication-only

approach may frustrate wider application, and the corollary will follow: that a pathogen-only licence may actually extend use. Importantly, the ratio of benefit-risk is the basis of medical need and of regulatory support. Both individual benefit and public health need to be considered. The licensed indications of recently marketed antibacterials (since 2000) are helpful, but many are not indicated for infections caused by Gram-negative bacteria. The exceptions are gemifloxacin and tigecycline which are also active against certain MDR pathogens. All remaining antibiotics, except ertapenem, were licensed for MRSA (Livermore et al. 2003).

The licensed indications of antibiotics since 2000 are useful for indications such as acute bacterial skin and skin structure infections (ABSSSI) and Gram-positive community-acquired pneumonia (CAP) but not indications in which MDR Gram-negative bacteria cause the infection. And this is where medical need is highest.

2 Epidemiology of Resistance

MDR organisms are now present in every continent and in virtually every hospital in the world. There is a marked regional variation in the incidence of MDR strains, and indeed, the number of clinically significant infections caused by them fluctuates between each hospital. In other words, the intra-hospital infection rate due to MDR Gram-negatives varies (Gopalakrishnan and Sureshkumar 2010). Some countries have higher levels of resistance than others. For example, the incidence of MRSA in Greece is higher than that in Sweden (Sader et al. 2006).

The species of MDR bacteria which are most prevalent and are responsible for two-thirds of all healthcare-associated infections (HIAs) are conveniently described by the acronym, ESKAPE (Bouchett et al. 2009). The six pathogens that make up this acronym are *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter spp*.

Intensive care units (ICUs) are an important focus of antimicrobial resistance because patients there are particularly vulnerable to infection. Recent data from ICUs in Europe (Vincent et al. 2009) show that the incidence of Gram-positive infections, such as MRSA, has either decreased or remained stable and the prevalence of bacteria resistant to vancomycin, daptomycin or linezolid has remained at a low level. Unfortunately, the prevalence of MDR Gram-negative bacteria is increasing, and this is creating a particularly serious situation in ICUs. MDR *Escherichia coli* and *Klebsiella pneumoniae* are a special problem; these organisms have transferable resistance genes residing on plasmids and produce enzymes such as extended-spectrum β-lactamases and carbapenemases. Carbapenemase-expressing bacteria, such as *Klebsiella pneumoniae* (EMA/ECDC Joint Technical Report, the bacterial challenge: time to react http://ecdc.europa.eu/), are a particular problem because carbapenems are the antibiotics of last resort for some MDR Gram-negative infections. The alternatives, such as polymyxins, are quite toxic and are associated with a less favourable outcome for the patient (Falagas et al. 2005).

This problem is currently such a major public health threat that the British Health Protection Agency (HPA) and the Advisory Committee on Antimicrobial Resistance

and Healthcare Associated Infection (ARHAI) have issued an advisory note on the detection of carbapenemases, infection control and treatment which is available at. http://www.hpa.org.uk/Topics/InfectiousDiseases/InfectionsAZ/CarbapenemResistance/GuidanceOnCarbapenamProducers/.

NDM-1, the New Delhi metallo-carbapenemase, has been observed in Enterobacteriaceae since 2008 (Yong et al. 2010). In 2010, Kumarasamy 2010 described 29 cases in the UK, 17 of which were associated with visits to India and Pakistan. This was supported by data showing that NDM-1-carrying Enterobacteriaceae are widespread across the Indian subcontinent (http://www.ncbi.nlm.nih.gov/pubmed/20705517). Street water in New Delhi is contaminated with NDM bacteria, which suggests that travellers become colonised and then carry these MDR organisms to other countries (http://www.channel4.com/news/drug-resistant-superbug-threatens-ukhospitals). Also, it is reported that in a hospital in Mumbai, 5–7% of Enterobacteriaceae carry NDM-1 (http://www.ncbi.nlm.nih.gov/pubmed/20964525).

Not only does NDM-1 destroy the main group of last-resort antibiotics but it can also be transferred between different species of bacteria (Potron et al. 2011).

The emergence of NDM-1 marks a serious deterioration in the range of effective antibiotics which are currently available to treat MDR-resistant Gram-negative bacteria.

Recent data suggest that dissemination of antibiotic resistance amongst MDR Gram-negative bacteria is associated with high-risk clones (Woodford 2008). In some cases, it is thought that single clones spread; for example, multilocus sequence typing has shown that KPC carbapenemase-positive *Klebsiella pneumoniae* ST258 spread from Greece to northwest Europe. In other cases, clones may repeatedly and independently acquire resistance. Woodford and colleagues looked at the interplay between clone and resistance for *E. coli, K. pneumoniae, Acinetobacter baumannii* and *Pseudomonas aeruginosa* and observed that high-risk clones play a major role in the spread of resistance. These clones seem to accumulate and switch resistance.

3 Concerns and Activities Occurring to Reduce This Threat

In September 2001, WHO launched the first global strategy for combating the serious problems caused by the emergence and spread of antimicrobial resistance. Known as the WHO Global Strategy for Containment of Antimicrobial Resistance, it recognised that antimicrobial resistance is a global problem that must be addressed in all countries. No single nation, however effective it is at containing resistance within its borders, can protect itself from the importation of resistant pathogens through travel and trade. Poor prescribing practised in any country now threatens to undermine the potency of vital antimicrobials everywhere.

The WHO strategy recommends interventions that can be used to both slow the emergence and reduce the spread of resistance in a diverse range of settings. These interventions are organised according to groups of people whose practices and behaviours contribute to resistance and where changes are judged likely to have a

significant impact at both national and international levels. These include consumers, prescribers and dispensers, veterinarians and managers of hospitals and diagnostic laboratories, as well as national governments, pharmaceutical industry, professional societies and international agencies (http://www.who.int/mediacentre/factsheets/fs194/en sourced 15th Jan 2011).

4 US Congress

Until recently, research and development (R&D) efforts have provided new drugs in time to treat bacteria that became resistant to older antibiotics. That is no longer the case. Unfortunately, both the public and private sectors appear to have been lulled into a false sense of security based on past successes. The potential crisis at hand is the result of a marked decrease in industry R&D, government inaction and the increasing prevalence of resistant bacteria. Infectious disease physicians are alarmed by the prospect that effective antibiotics may not be available to treat seriously ill patients in the near future.

The Infectious Disease Society of America report in July 2004 published the report entitled “Bad Bugs, No Drugs” with a subtitle: “As Antibiotic Discovery Stagnates. . . A Public Health Crisis Brews” (Infectious Disease Society of America 2004). This report discussed possible incentives most likely to spur R&D within major pharmaceutical companies. The committee also considered the FDA as pivotal partners along with their “critical path” initiatives. Other groups who needed to invest new resources include the congress, the administration, CDC, NIAID and public-private research efforts.

5 European Medicines Agency (EMA) Activities

MDR bacteria remain a major issue in hospital-associated infections, and new reservoirs for such organisms have arisen both in the community and in animals (Dufour et al. 2002; Cuny et al. 2010).

Alarmingly, the number of infections that now require treatment with either carbapenems or polymyxin is increasing (Meyer et al. 2010). In contrast, the number of new antibiotic applications has decreased, and the level of R&D within Europe has declined over the last few years (ECDC EMA Joint Report 2009).

Several requests have been made to the EMA to provide more detailed guidance on the requirements to support issues such as patient selection and primary endpoints. For some indications, there is no established position on data required because either they are rarely studied or because existing guidance does not cover such issues.

In February 2010, the EMA released, for comment, revised draft guidelines for the evaluation of medicinal products indicated for the treatment of bacterial infections (CPMP/EWP/558/95 rev2). In preparation for the next revision of these guidelines (rev3), the EMA Efficacy Working Party (EWP) organised a meeting on

7th–8th February 2011 with key European opinion leaders, industry representatives and the FDA. The objectives of this meeting were to receive comment on the Rev2 guidelines, to debate ways to improve the regulatory process so that the decreasing rate of new antibiotic applications and declining R&D within Europe might be reversed and to discuss important differences between the EMA and FDA opinions on clinical trial design with a view to achieving harmonisation.

Meeting participants included members of the EMA, European regulatory authority experts, the Anti-Infective Scientific Advisory Group (SAG), selected experts from the European Federation of Pharmaceutical Industries and Associations (EFPIA) and other experts including RB.

The report of the workshop was published on the EMA website on the 17th March 2011 (EMA/257650/2011) as a concept paper, and an addition to the note for guidance on evaluation of medicinal products published on 22nd September 2011 (EMA/CHMP/EWP/736904/2011) proposed the development of the addendum.

Many issues were discussed including non-inferiority/superiority studies, bacteraemia, febrile neutropenia and MDR bacteria. These activities and related documents are a genuine attempt by the EMA to address some major issues that antibiotic developers have experienced including changes in goalposts, unrealistic expectations, increased bureaucracy and increases in complexity and size of clinical trials. Regulators are oblivious to the increasingly limited resources and financial burden of most of the antibiotic developers. Added to this is that many of the primary endpoints required by the EMA for indications such as pneumonia and skin and soft tissue bacterial infections are different to those required by the FDA. This state of affairs is bad for the regulators, bad for the antibiotic developers and bad for patients!

Williams and Bax (2009) state that regulatory activities have had a major negative impact on the R&D and availability of new antibiotics. Pharmaceutical companies have abandoned investment in this area. Mass redundancies have resulted with no large pharmaceutical companies now working in antibacterial research. This has now resulted in significant loss of expertise in antibiotic R&D.

6 React

React (reactgroup.org) is an independent global network for concerted action on antibiotic resistance which is funded by the Swedish government and Uppsala University. React acts as a forum for ideas, debate and collaboration between diverse stakeholders in order to manage concerted global action on antibiotic resistance.

There are numerous other groups involved.

TATFAR comprise of European and US members of the Transatlantic Task Force for Antimicrobial resistance (ecdc.europa.en/activities/diseaseprogrammes/tatfar). TATFAR are looking at challenges and solutions in the development of ways to manage the increasingly difficult issue of antimicrobial resistance. These activities are funded via the European Centre for Disease Prevention and Control (ECDC) located in Stockholm, Sweden. Their mission includes the strengthening of

Europe's defences against infectious diseases. Specific activities include the European Antimicrobial Resistance network (EARS-NET).

The Infectious Disease Society of America (IDSA) (http://www.idsociety.org) represents 9,000 physicians and healthcare professionals who specialise in infectious diseases. Initiatives to address the growing antimicrobial resistance problem include proposals to build sustainable antibiotic R&D included in the short term to produce the 10 × 20 initiative, whereby leading to the delivery of ten new systemic antibiotics by 2020. This has led to detailing the US Congress and Food and Drug Agency (FDA). In fact, this has resulted in over 60 communications. Included in these are efforts to provide the input into the FDA in order to bring regulatory clarity to the antibiotic approval pathway. The IDSA also lobbied US Congress to advance the adaption of statutorily defined incentives with sufficient power to encourage manufacturers to engage in antibiotic and related diagnostic R&D.

The British Society for Antimicrobial Chemotherapy (BSAC) with 700 members exists to facilitate the acquisition and dissemination of knowledge in the field of antimicrobial therapy. The BSAC (http://www.bsac.org.uk) in Urgent Need initiative identified the barriers discouraging participation in antibacterial R&D and considered what opportunities exist to stimulate interest in the field looking at three major components required to bring antibacterial agents to the market.

7 Present State of Antibiotic Research Constraints

Generally, infections caused by Gram-positives such as methicillin-resistant Staphylococci (MRSA) are still susceptible to a range of old and new antibiotics (Boucher et al. 2009).

Two new novel antibiotic classes active against multi-resistant Gram-positive infections were commercialised in the decade starting in the year 2000. Unfortunately, this is not the same situation for multi-resistant Gram-negative bacillary bacteria. The last truly novel active antibiotic against Gram-negative bacteria was nalidixic acid, the forerunner of the plethora of quinolone antibiotics. The development of resistance of common bacteria including *Staphylococcus aureus* and Gram-negative bacteria such as *Pseudomonas aeruginosa* has been rapid. A large number of analogues have been developed in each class of antibiotics although market delivery of analogues has been more feasible for some classes, such as cephalosporins and quinolones, than others, for example, the macrolides.

New analogue development, however, has not been able to keep up with the rise in MDR Gram-negative organisms for the last decade or so. New antibiotics must attack multiple targets within each bacterial species which also are increasing over time. These bacteria are now expressing multiple-resistant determinants against a large number of established antibiotic classes such as beta-lactams and beta-lactam inhibitors, aminoglycosides, quinolones and tetracyclines. In addition, the acquisition of multidrug resistance determinants by bacteria appears to increase the pathogenicity of the bacteria. Part of the problem of the lack of new novel agents is because in the

1990s big pharma companies adopted a strategy of R&D which was beguiling opportunity of the new techniques in molecular biology which included bacterial genomics, combinatorial chemistry and high-throughput screening enabling the identification of lethal targets and virulence factors. Unfortunately, this led to a wasted decade or more with no new useful classes of antibiotics being identified (Silver 2011). Perhaps the antibiotic researchers confused activity with progress!

Economic barriers to the development of new novel antibiotics have increased. A large part of this problem is that, unlike any other classes of medicines, significant use of antibiotics leads to development of resistance to those agents and, therefore, increasing clinical failure. Antibiotics that are highly effective against more resistant bacteria will, by definition, be used only when the causative bacteria have been identified, thus limiting its use very significantly as most antibiotics are prescribed empirically. Because of the lack of novel new agents, the great majority of available antibiotics are now generic, resulting in low prices. Launching a new, expensive antibiotic, including one with high activity versus MDR Gram-negative bacteria, is not to be recommended!

Prescribing doctors are constantly urged to use antibiotics appropriately and prudently so as to optimise patient outcomes and minimise the acquisition and spread of antibiotic resistance (Bax et al. 1998). But the evidence base expected to be derived from clinical trials usually fails to give them the guidance required to achieve this end. Unfortunately, many calls for appropriate use merely mean a non-specific reduction in use (Bax et al. 1999).

Since the amalgamation of large pharmaceutical companies such as GlaxoSmithKline, Sanofi-Aventis and Pfizer Wyeth, the research groups of large pharma have generally left Europe to set up anew in the USA. Indeed, only a few large pharmas have now significant research in the antibacterial area, most are concentrated in the USA, and Pfizer has considered moving to China; unfortunately, this decision was reversed. In the meantime, most of the US-based research has left Pfizer.

Progress in R&D for new novel agents active against Gram-negative pathogens has been dire. Currently, there are no new agents active against these key pathogens in phase 3 or phase 4 clinical studies (Williams and Bax 2009).

Regulatory guidelines for clinical development have a major impact on decisions regarding research and development. Approval of new antibacterials in Europe and the USA has decreased significantly in recent years along with a large increase in development costs largely through the requirement for increasingly larger clinical trials and often complicated guidelines. Antibiotic clinical development is extremely complex and onerous but with the prospect of a small market return! Criticisms of regulatory agencies, largely by the pharma industry, include the ever increasing stringency in the licensing requirements: a licensing process that is bureaucratic, costly and also inconsistent in its requirement, consequent upon the lack of international harmonisation, notably between the Food and Drug Agency and the European Medicine Agency (Finch 2011).

It is possible that no new, novel, broad-spectrum antibiotics active against a broad range of MDR Gram-negative bacteria will be available in the next 10 years. Even after discovery, it takes 8–10 years before an antibiotic becomes generally available for use!

Antibiotics active against selected resistant Gram-negative bacteria include beta-lactamase inhibitors such as Novexel 104 and Bas 30072 which are active against KPC and NDM-1 beta-lactamase, respectively (Livermore et al. 2011; Page et al. 2012).

Unfortunately, they will have specific activity against bacteria which produce those specific beta-lactamases but not against other carbapenemases. They will, therefore, become narrow-spectrum antibiotics even though Novexel 104 is to be combined with ceftazidime and BAL30072 is to be combined with meropenem.

8 Proposals on Co-development of Antibiotics

In many conditions, developing narrow-spectrum antibiotics creates difficulties if the causative organism might not be covered with the novel compound (e.g. a Gram-positive antibiotic monotherapy for CAP treatment).

Recent FDA draft guidelines outline methods for the co-development of novel antibiotic agents and help address certain scientific and regulatory issues that will arise during co-development.

It is intended where there is:

- The need to treat a serious condition
- A rationale for combination of agents to be used
- Preclinical data suggesting synergistic effects in either efficacy or preventing resistance development
- Compelling reason why the agents cannot be developed individually, for example, increased risk or resistance or limited activity as monotherapy

In many cases, co-development would facilitate trial design where monotherapy would prove difficult. Targeted treatment will require methods for quick, cheap and accurate methods of diagnosis, which currently are not available. Currently, blood cultures are initially taken but can take up to five days to be returned, and so blind treatment is required in most cases for all likely organisms.

Due to advances in "lab on a chip", starting targeted treatment within the first hour of presentation will increasingly be possible. Rates of detection in a sample of blood of biotin (vitamin B7) at a concentration of about 1 part per 40 billion were achieved in 10 minutes (http://www.sciencedaily.com/releases/2011/03/110318102243.htm) (Peleg and Hooper 2010; van Duijn et al. 2011; Woodford et al. 2011).

9 Abstracts

"High-risk clones" play a major role in the spread of resistance, with the risk lying in their tenacity—deriving from poorly understood survival traits—and a flexible ability to accumulate and switch resistance, rather than to constant resistance batteries (Woodford et al. 2011).

As numbers of published results from national/international surveillance studies rise rapidly, the amount of new information may be overwhelming. Therefore, the

authors reviewed recent trends in antibiotic resistance in ICUs across Europe over 18 months (Woodford et al. 2011).

Antibiotic resistance in ICUs is rapidly increasing in both epidemics and endemicity of multi- and panresistant Gram-negative pathogens. Better infection control and improved diagnostics will become even more important than before (Woodford et al. 2011).

10 Conclusion

At least 10 years are required after compound selection to achieve regulatory approval. It follows therefore that the prospects for the delivery to the market in the next 10 years for general use of a new important antibiotic active against even a moderate range of MDR Gram-negative pathogens are not good. Many proposals have been made by governments, academics, non-government organisation, companies and researchers, but in spite of this, little has been achieved so far.

It remains to be seen if the world can combine its vast resources to combat the bacterial challenge which is increasing daily.

References

Arias CA, Murray BE (2012) Antibiotic-resistant bugs in the 21st century – a clinical super challenge. N Engl J Med 360(5):439–43

Bax RP, Anderson R, Crew J et al (1998) Antibiotic resistance—What can we do? Nat Med 4(5):545–6

Bax RP, Gabbay F, Phillips I (1999) The Witley Park Study Group. Clin Microbiol Infect 5(12):774–778

Boucher HW, Talbot GH, Bradey JS, Edwards JE, Gilbert D, Rise LB et al (2009) Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America. Clin Infectious Dis 48:1–12

Bouchett W, Talbot GH, Bradley JS (2009) Bad bugs, no drugs: no ESCAPE. Clin Infect Dis 48:1–12

Cuny C, Friedrich A, Kozytska S, Layer F, Nübel U, Ohlsen K, Strommenger B, Walther B, Wieler L, Witte W (2010) Emergence of methicillin-resistant Staphylococcus aureus (MRSA) in different animal species. Int J Med Microbiol 300(2–3):109–17

Dufour P, Gillet Y, Bes M, Lina G, Vandenesch F, Floret D, Etienne J, Richet H (2002) Community-acquired methicillin-resistant Staphylococcus aureus infections in France: emergence of a single clone that produces Panton-Valentine leukocidin. Clin Infect Dis 35 (7):819–24

Falagas ME, Rizos M, Bliziotis A (2005) Toxicity after prolonged administration of intravenous colistin. BML Infect Dis 5(1):1–8

Finch R (2011) J Antimicrob Chemother 66:1945–1947

Giamarellou H (2009) Multi-drug resistant Gram-negative infections. Drugs 69(14):1879–1901

Gopalakrishnan R, Sureshkumar D (2010) Changing trends in antimicrobial susceptibility and hospital acquired infections over an 8 year period in a tertiary care hospital in relation to introduction of an infection control programme. J Assoc Physicians India 58(Suppl):25–31

Infectious Disease Society of America (2004) Bad bugs, no drugs. As antibiotic discovery stagnates… A Public Health Crisis Brews

Kumarasamy KK, Toleman MA, Walsh TR, Bagaria J, Butt F, Balakrishnan R, Chaudhary U, Doumith M, Giske CG, Irfan S, Krishnan P, Kumar AV, Maharjan S, Mushtaq S, Noorie T, Paterson DL, Pearson A, Perry C, Pike R, Rao B, Ray U, Sarma JB, Sharma M, Sheridan E, Thirunarayan MA, Turton J, Upadhyay S, Warner M, Welfare W, Livermore DM, Woodford N (2010) Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study. The Lancet Infectious Diseases 10(9):597–602

Livermore DM (2004) The need for new antibiotics. Clin Microbiol Infect 10(suppl 4):1–9

Livermore DM (2009) Has the era of unbeatable infections arrived? J Antimicrob Chemother 64 (suppl 1):i29–i36

Livermore DM, Sefton AM, Scott GM (2003) Properties and potential of ertapenem. J Antimicrob Chemother 52(3):331–44

Livermore DM, Mushtag S, Warner M et al (2011) Activities of NXL 104 combination with ceftazidime and azthreonam against carbapenemase-producing Enterobacteriaceae. Antimicrob Agents Chemother 55:390–4

Meyer E, Schwab F, Schroeren-Boersch B, Gastmeier P (2010) Dramatic increase of third-generation cephalosporin-resistant E. coli in German intensive care units: secular trends in antibiotic drug use and bacterial resistance, 2001 to 2008. Crit Care 14(3):113

Page MGP, Clothilde D, Eric D (2012) In vitro properties of BAL30072, a novel siderophore sulfactin with activity against multiresistant Gram-negative Bacilli. Antimicrob Agents Chemother 6:2291–2302

Peleg AY, Hooper DC (2010) Hospital-acquired infections due to Gram-negative bacteria. N Engl J Med 362(19):1804–1813

Pitout JDD, Laupland KB (2008) Extended B-lactamase producing Enterobacteriaceae: an emerging problem. Lancet Infect Dis 8:159–66

Poirel L, Pitout JD, Nordmann P (2007) Carbapenemases: molecular diversity and clinical consequences. Fut Microbiol 2:501–12

Potron A, Poirel L, Nordmann P (2011) Plasmid-mediated transfer of the bla(NDM-1) gene in Gram-negative rods. FEMS Microbiol Lett 324(2):111–6

Sader HS, Streit JM, Fritsche TR, Jones RN (2006) Antimicrobial susceptibility of gram-positive bacteria isolated from European medical centres: results of the Daptomycin Surveillance Programme (2002–2004). Clin Microbiol Infect 12(9):844–52

Silver LL (2011) Challenges of antibacterial discovery. Clin Microbiol Rev 24:71–109

So AD, Gupta N, Cars O (2012) Tackling antibiotic resistance. BMJ 340:1091–96

Taubes G (2008) The bacteria fight back. Science 321(5887):356–61

van Duijn PJ, Dautzenberg MJ, Oostdijk EA (2011) Recent trends in antibiotic resistance in European ICUs. Curr Opin Crit Care 17(6):658–65

Vincent JL, Rello J, Marshall J (2009) International Study of the prevalence and outcomes of infection in intensive care Units 2009. JAMA 302(21):2323–29

Walsh TR, Weeks J, Livermore DM, Toleman MA (2011) Dissemination of NDM-1 positive bacteria in the New Delhi environment and its implications for human health: an environmental point prevalence study. The Lancet Infectious Diseases 11(5):355–362

Williams KJ, Bax RP (2009) Challenges in developing new antibacterial drugs. Curr Opin Invest Drugs 10(2):157–163

Woodford N (2008) Successful, multiresistant clones. J Antimicrob Chemother 61(2):233–4

Woodford N, Turton JF, Livermore DM (2011) Multiresistant Gram-negative bacteria: the role of high-risk clones in the dissemination of antibiotic resistance. FEMS Microbiol Rev 35(5):736–55

Yong D, Toleman MA, Giske CG, Cho HS, Sundman K, Lee K, Walsh TR (December 2009) Characterization of a new metallo-beta-lactamase gene, bla(NDM-1), and a novel erythromycin esterase gene carried on a unique genetic structure in Klebsiella pneumoniae sequence type 14 from India. Antimicrob Agents Chemother 53(12):5046–5054

The Origins of Antibiotic Resistance

Gerard D. Wright

Contents

1 Antibiotics: Essential Medicines in Peril 14
2 The Antibiotic Resistome 16
  2.1 Highly Efficient Resistance Elements in Nonpathogenic Bacteria 17
  2.2 De Novo Evolution of Resistance 21
  2.3 Proto-Resistance 23
3 Summary, Challenges and Opportunities 25
References 26

Abstract Antibiotics remain one of our most important pharmacological tools for the control of infectious disease. However, unlike most other drugs, the use of antibiotics selects for resistant organisms and erodes their clinical utility. Resistance can emerge within populations of bacteria by mutation and be retained by subsequent selection or by the acquisition of resistance elements laterally from other organisms. The source of these resistance genes is only now being understood. The evidence supports a large bacterial resistome—the collection of all resistance genes and their precursors in both pathogenic and nonpathogenic bacteria. These genes have arisen by various means including self-protection in the case of antibiotic producers, transport of small molecules for various reasons including nutrition and detoxification of noxious chemicals, and to accomplish other goals, such as metabolism, and demonstrate serendipitous selectivity for antibiotics. Regardless of their origins, resistance genes can rapidly move through bacterial populations and emerge in pathogenic bacteria. Understanding the processes that contribute to the evolution and selection of resistance is essential to mange current stocks of antibiotics and develop new ones.

G.D. Wright (✉)
Department of Biochemistry and Biomedical Sciences, Michael G. DeGroote Institute for Infectious Disease Research, McMaster University, Hamilton, ON, Canada L8N 3Z5,
e-mail: wrightge@mcmaster.ca

A.R.M. Coates (ed.), *Antibiotic Resistance*, Handbook of Experimental Pharmacology 211, DOI 10.1007/978-3-642-28951-4_2,
© Springer-Verlag Berlin Heidelberg 2012

Keywords Resistome • Efflux • Evolution • Lateral gene transfer

1 Antibiotics: Essential Medicines in Peril

The first compounds purposely discovered to combat infection were the arsenicals such as Salvarsan reported by Paul Ehrlich at the turn of the twentieth century. These were highly toxic molecules that nevertheless were the first successful drugs for the treatment of syphilis, then a worldwide scourge caused by the bacterial pathogen *Treponema pallidum* (and now in the twenty-first century re-emerging in antibiotic-resistant forms). The success of these compounds in infection control inspired the development of the sulpha drugs 15 years later and ushered in the "Age of Antibiotics" that we continue to enjoy. The mid-twentieth century saw the discovery of the vast majority of antibiotic chemical scaffolds now in clinical use. This era of discovery, roughly from 1940 to 1960, was followed by a period of great innovation where medicinal chemists modified these scaffolds to improve the pharmacological properties of antibiotics, making them better medicines. At the same time, chemical modification of antibiotic scaffolds was a means to overcome another problem associated with antibiotics—resistance.

Resistance to all classes of antibiotics occurs after their use in the clinic or on the farm. In the case of penicillin, resistance was actually characterized before clinical deployment (Abraham and Chain 1940)! The emergence of resistance, often quite rapidly after being made available to the clinical community, helps to stimulate the discovery of new antibiotic scaffolds and their subsequent chemical modification (when possible) to structures less susceptible to resistance. This has resulted in many cases in several "generations" of antibiotics (e.g., Fig. 1) each with improved properties including expanded bacterial spectrum, favorable pharmacology, and "resistance to resistance."

Despite these cycles of antibiotic discovery and innovation, resistance remains a significant and growing challenge to the successful treatment of infectious disease. A principal reason for this is that new drug development over the past decade has lagged behind the unavoidable selection for resistance. Challenged with fewer leads for new antibiotic scaffolds and in some cases approaching the limits of productive chemical modification of known scaffolds, the growing costs of drug development, and a challenging regulatory environment, many players in the pharmaceutical industry have abandoned new antibiotic discovery (Spellberg et al. 2004; Williams and Bax 2009; Gwynn et al. 2010). The result is a growing unmet clinical need and an ever-widening gap in early stage antibiotic innovation and delivery of new antibiotics to the clinic (Fischbach and Walsh 2009).

This alarming trend has occurred at the same time that resistance to all antibiotics continues to evolve and spread across the globe (Boucher et al. 2009). Furthermore, pathogens are now very often multidrug resistant, harboring many resistance genes (sometimes dozens) and frequently they are associated with increased virulence. In some cases, highly successful multidrug resistant bacteria

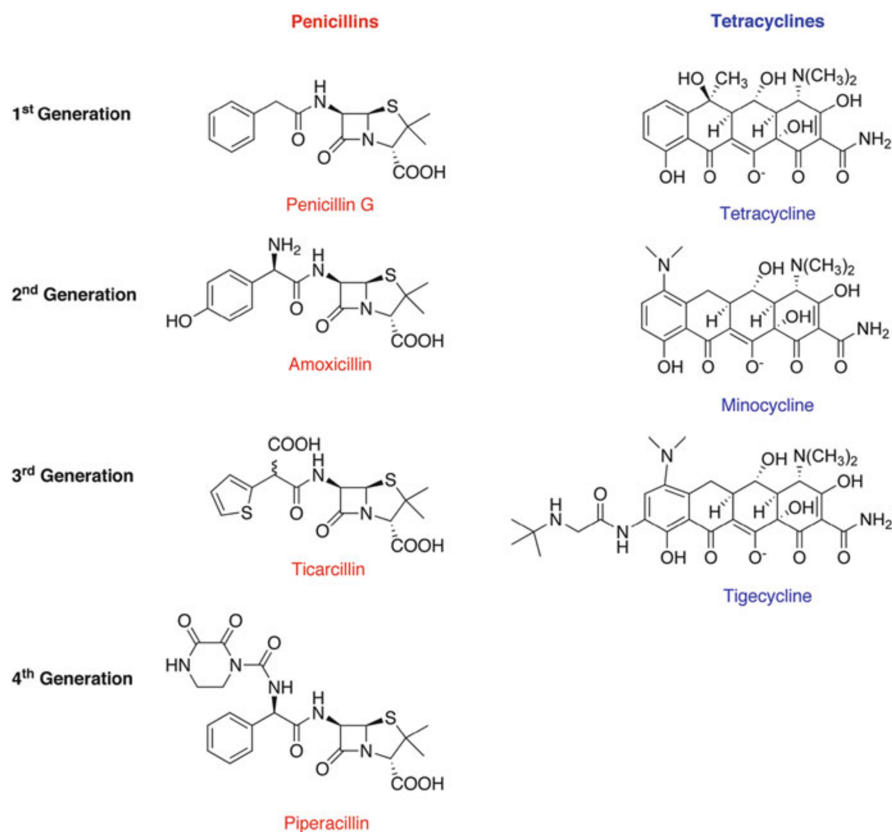


Fig. 1 *Antibiotic improvement through chemical derivatization*. The majority of antibiotics are natural products that were first introduced in the form isolated from producing organisms (First Generation). Subsequent modification of the active core scaffold by medicinal chemistry, as shown for the examples in the penicillin and tetracycline classes improve their drug properties and avoid prominent resistance mechanisms

are emerging as epidemic and pandemic strains (Croucher et al. 2009; Freitas et al. 2010; Peirano and Pitout 2010). The resulting erosion of drug efficacy is now being widely viewed as a global health crisis that needs to be recognized and addressed with new drug discovery (Infectious Diseases Society of America 2010).

The root cause of this crisis is antibiotic resistance. Recent efforts to understand the evolution and origins of resistance are building on decades of research in epidemiology and fundamental biology to formulate a unifying hypothesis that ties drug resistance to bacterial genomics and physiology. This concept, the *antibiotic resistome*, provides a framework to understand how and why resistance emerges and offers the prospect for clinicians and drug discoverers to predict the emergence of clinical resistance and probe the long-term efficacy of new drugs. The origins and evolution of antibiotic resistance elements that comprise the resistome are the focus of this chapter.

2 The Antibiotic Resistome

The predictable and often rapid emergence of antibiotic resistance in previously susceptible pathogenic bacteria was recognized very early in the history of antibiotic drug discovery. Indeed, Fleming, the discoverer of penicillin, warned of the prospect of resistance in his Nobel Prize speech in 1945 noting: “It is not difficult to make microbes resistant to penicillin in the laboratory by exposing them to concentrations not sufficient to kill them, and the same thing has occasionally happened in the body.” (Fleming 1945). Why are all antibiotics susceptible to resistance and why does resistance appear so quickly?

Antibiotics are small organic molecules (MW ~100–2,000 Da) composed of C, H, N, O, S, and other biologically available elements. Microbes live their lives surrounded by these and countless similar molecules. Among them are nutrient sources (carbohydrates, peptides, lipids, vitamins, and cofactors), communication compounds produced by bacteria and other microbes (e.g., quorum-sensing molecules), siderophores made by fungi and bacteria to secure iron that is essential for their growth, assorted compounds produced by microbes (including antibiotics, surfactants, hormones, etc.), and countless molecules including terpenoids, steroids, and alkaloids produced by plants and other eukaryotes such as protists and algae. The world of bacteria is a world of small molecules. They are their language and their life source.

Many of these compounds, however, are toxic to bacteria. As a result, they have developed strategic countermeasures to overcome the noxious properties of chemicals that they may come across during their life cycle. These include nonspecific mechanisms to block the entry of toxic compounds into cells or decrease their intracellular concentrations. Such mechanisms are a first line of defense against toxins that a bacterium may encounter in its environment. Additionally, highly specific mechanisms to evade the activities of certain toxic molecules (among them many naturally produced antibiotics) have also evolved. Examples of such mechanisms include the alteration or mutation of the cellular targets of antibiotics and the development of degrading or modifying enzymes. The evolution of microbial defenses to guard against both general toxins and specific molecules has occurred over the millennia. The fossil record shows that stromatolites, the remnants of cyanobacteria, prodigious producers of toxic small molecules, are greater than 3.4 billion years old (Allwood et al. 2006) and land plants, equally important producers of small molecules, emerged 475 million years ago (Wellman et al. 2003). Consequently, all bacteria have developed some ability to resist toxic small molecules; indeed some microbes are prodigiously equipped to evade them.

The focus of much of the antibiotic resistance literature has (not surprisingly) been on the emergence of resistance in previously susceptible pathogens in the clinic. However, this focus has disproportionately emphasized a small subgroup of microbes. The vast majority of bacteria do not cause human or animal disease; nevertheless, they are continuously exposed to toxic small molecules, including naturally occurring antibiotics, and have consequently developed resistance.

For example, a survey of ~500 soil microbes against a panel of 21 antibiotics including natural products, semisynthetic derivatives of these, and completely synthetic molecules revealed a remarkable ability of these organisms to resist antibiotics (D'Costa et al. 2006). All strains in this study were resistant to multiple drugs and on average they were impervious to 7–8 antibiotics. The source of the antibiotic did not matter; natural products or synthetic molecules were equally susceptible. This observation is consistent with evolution of both general mechanisms of detoxification, e.g., the efflux of molecules from the cell, and highly specific mechanisms such as enzymatic detoxification.

This study led us to propose the concept of an antibiotic *resistome* (D'Costa et al. 2006, 2007; Wright 2007, 2010). The resistome includes all the genes that directly or indirectly act to confer antibiotic resistance in bacteria. It includes the resistance elements in pathogenic bacteria and also the much more diverse and populous nonpathogenic organisms. Furthermore, the resistome includes the microbial genes that contribute to inherent antibiotic resistance in a particular organism, the intrinsic resistome (Fajardo et al. 2008). Finally, the "housekeeping" genes that can give rise to resistance elements either by mutation or overexpression are also constituents of the resistome. We have termed these "proto-resistance" elements in functional analogy to proto-oncogenes in cancer (Morar et al. 2009; Morar and Wright 2010).

The components of the resistome therefore vastly exceed the traditional narrow focus of the clinical and scientific communities on resistance in pathogens. A growing number of studies of microbes that can subsist on antibiotics (Dantas et al. 2008) and on the prevalence of resistance genes in the human microbiome (Sommer et al. 2009) and in the environmental metagenome (Riesenfeld et al. 2004; Allen et al. 2009; Donato et al. 2010; Lang et al. 2010) emphasize the point. Importantly, we now understand that unlike most complex organisms, gene flow in bacteria can readily occur vertically (from mother to daughter cell) and also horizontally (between cells) (Roy 1999; Barlow 2009). Therefore, antibiotic resistance that evolves in one bacterium will be passed on to its progeny as is common to all organisms, but it can also be mobilized (by transduction, transformation, or conjugation) into unrelated bacteria (Fig. 2). In this fashion, resistance in nonpathogenic bacteria can "escape" and be captured by pathogens. Given the vast numbers of prokaryotic cells on the planet [$\sim 5 \times 10^{30}$ (Whitman et al. 1998)], their innate and evolved abilities to evade the lethal effects of antibiotics and other toxic small molecules developed over millions of years, and the relative ease of gene movement in microbes, the rapid and inevitable emergence of resistance to antibiotics in the clinic becomes completely predictable.

2.1 Highly Efficient Resistance Elements in Nonpathogenic Bacteria

In 1973, Benveniste and Davies noted the similarity between the activity of aminoglycoside antibiotic modifying enzymes in the soil microbes that produce these drugs and the enzymes responsible for resistance in pathogens

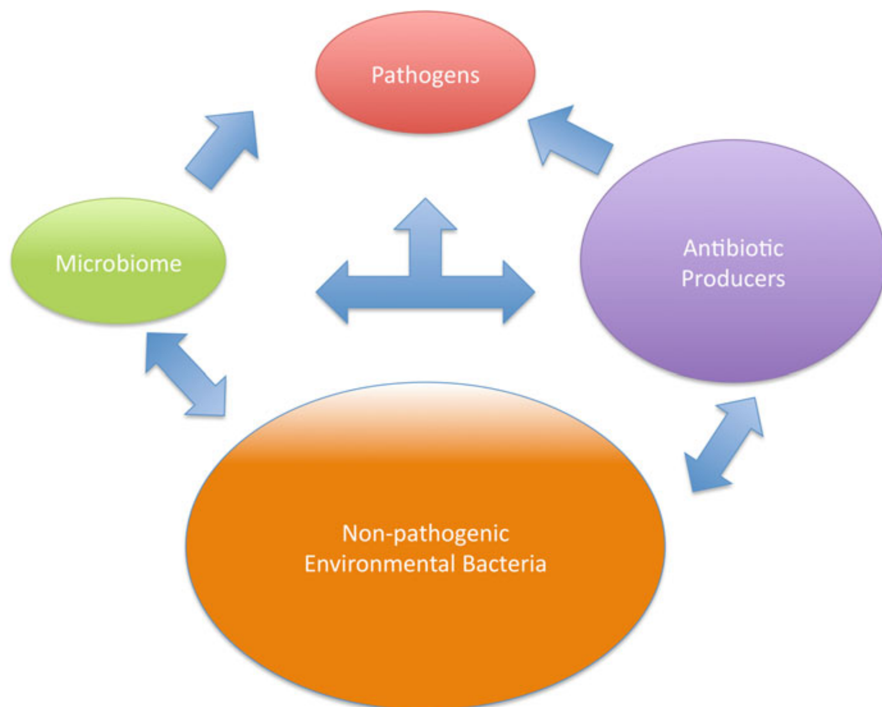


Fig. 2 *Genes move laterally through the resistome*. Antibiotics provide a powerful selection pressure to mobilize and capture resistance elements in the resistome. The ability of genes to cross species and genus barriers by transformation, conjugation, and transduction provides a large gene pool for pathogens to draw on

(Benveniste and Davies 1973). They identified the activities of aminoglycoside acetyltransferases, adenylyltransferases and phosphotransferases in cell free extracts of a number of producing organisms. The specificity of these enzymes matched the biochemical profiles of aminoglycoside resistance enzymes that had begun to emerge as a significant clinical problem since their first detection as horizontally acquired resistance elements in the 1950s (Davies 1995). The subsequent cloning and sequencing of aminoglycoside resistance genes from both pathogens and producers showed that the associated enzymes were closely related and demonstrated for the first time an unexpected link between mechanisms of resistance in antibiotic producers and clinically important pathogens.

In retrospect, it should not have been surprising to discover that antibiotic producers would also require intrinsic resistance elements to avoid committing suicide during drug production (Cundliffe 1989). It is now well established that secondary metabolites of bacterial origin, including antibiotics, are the products of large biosynthetic gene clusters. These clusters include the genes required for the generation of the individual components of the secondary metabolites (sugars, unusual amino acids, etc.), the assembly of these components into the proper

scaffold structure (e.g., by nonribosomal peptide or polyketide synthetases), the modification of the scaffold (e.g., acylation, glycosylation, halogenation), transport of the compound from the cell, and in the case of antibiotics, resistance elements. The number of characterized biosynthetic gene clusters has increased dramatically over the past 15 years and frequently these are associated with genes that encode resistance elements that share function and structure with those found in pathogens.

Sequencing of the complete genome of the erythromycin producer *Saccharopolyspora erythraea* provides an example (Oliynyk et al. 2007). The genome sequence revealed the expected ~55 kb erythromycin biosynthetic gene cluster including *ermE*, an associated resistance gene. Erm genes are common resistance elements in pathogenic bacteria, associated with the MLS_B drug resistance phenotype, so called because they provide overlapping resistance to the structurally unrelated *m*acrolide antibiotics such as erythromycin, the *l*incosamides such as clindamycin, and the type B *s*treptogramins (Maravic 2004). Erm gene products are methyltransferases that specifically modify the exocyclic amine of a key adenosine residue (A2058 *Escherichia coli* numbering) of the 23S rRNA that is an essential component of the large ribosomal subunit (Mukhtar and Wright 2005). The structure of the ribosome in complex with MLS_B antibiotics reveals that these compounds bind to the same region of the ribosome, the peptide exit tunnel, and that methylation of A2058 results in a steric and ionic barrier to drug binding (Tu et al. 2005). The presence of *erm* genes in many other macrolide producers (Cundliffe et al. 2001; Ward et al. 2004; Karray et al. 2007) demonstrates that the 23S rRNA methylation is a common link between pathogens and antibiotic-producing environmental bacteria.

Resistance to glycopeptide antibiotics such as vancomycin provides yet another example of the links between the clinic and antibiotic resistance in producing organisms. Vancomycin binds to the acyl-D-Alanine-D-Alanine terminus of the bacterial peptidoglycan, an essential component of the cell wall that protects bacteria from cell lysis. This noncovalent interaction through five hydrogen bonds prevents the growth of the peptidoglycan that is essential during cell division providing the rationale for the antibacterial activity of glycopeptides. Resistance to vancomycin emerged in the late 1980s in clinical isolates of *Enterococci* (Leclercq et al. 1988; Courvalin 2006). Subsequent determination of the mechanism of resistance on associated genes revealed that resistance was the result of the concerted action of five gene products (Walsh et al. 1996). Two of these, VanR and VanS, make up a two-component regulatory system that senses the presence of the antibiotic (Koteva et al. 2010). This triggers the expression of three genes: *vanH*, *vanA,* and *vanX*. VanH produces D-Lactate from pyruvate and VanA is an ATP-dependent ligase that forms an ester bond between D-Alanine and D-Lactate. The D-Ala-D-Lac ester (depsipeptide) is a substrate for MurF that adds the depsipeptide to the peptidoglycan monomer, which is then exported to the exterior of the cell to be grafted onto the growing peptidoglycan polymer.

Replacing D-Ala-D-Ala-terminating peptidoglycan with a D-Ala-D-Lac terminus has a dramatic effect on antibiotic action. The loss of the amide hydrogen of D-Ala-D-Ala (replaced by an ester oxygen in D-Ala-D-Lac) removes one of the key

hydrogen bond interactions between the drug and the peptidoglycan (Bugg et al. 1991). The result is a 1,000-fold decrease in drug affinity and clinical resistance. Because the cell continues to make D-Ala-D-Ala even in the presence of vancomycin, the cell wall could be a mixture of D-Ala-D-Ala and D-Ala-D-Lac terminating peptidoglycan, thereby diminishing resistance. The expression of VanX ensures that the peptidoglycan is dominated by D-Ala-D-Lac by selectively hydrolyzing any D-Ala-D-Ala in the cell (Wu et al. 1995).

The origin of this highly effective and complex mechanism of resistance was the subject of significant debate when vancomycin resistance first emerged in the clinic. The resistance genes in *Enterococci* were associated with a transposon and mobilized on a plasmid. Subsequent sequencing of glycopeptide antibiotic biosynthetic gene clusters and the associated resistance genes revealed that the likely origins of the *vanHAX* operon are in drug-producing bacteria (Marshall et al. 1998). The operon has now been found in most glycopeptide producers. Furthermore, *vanHAX* was later identified in glycopeptide-resistant environmental strains of *Paenibacillus* and many other soil organisms, which do no impact human health, demonstrating that glycopeptide resistance was not restricted to human pathogens (Hong et al. 2004; Guardabassi et al. 2005; D'Costa et al. 2006; Guardabassi and Agerso 2006). The most parsimonious explanation of the available evidence suggests that the *vanHAX* operon originated in glycopeptide producers as a means of self-resistance and has spread laterally to neighboring nonproducing organisms and, with the selection offered in hospitals due to increased use of vancomycin to treat infections caused by drug-resistant Gram-positive pathogens, eventually to pathogenic *Enterococci*. However, the high GC content of the *vanHAX* genes in producing bacteria is significantly different to that in the operons found in *Enterococci* suggesting that gene escape and transfer was not a recent event. Given the distribution of *vanHAX* in nonproducing bacteria, it is likely that there is a very large pool of these genes in environmental bacteria.

The similarity of resistance elements in antibiotic-producing bacteria and pathogenic bacteria demonstrates a clear link between the environment and the clinic. However, as noted earlier for the vancomycin resistance genes, even nonproducing bacteria have acquired the operon. Similarly, in addition to *ermE*, the sequence of the *S. erythraea* genome revealed many predicted antibiotic resistance genes, including 17 β-lactamases, 2 aminoglycoside phosphotransferases, and an aminoglycoside acetyltransferase, even though this organism does not produce β-lactams or aminoglycosides. This observation is recapitulated in the sequences of many bacterial genomes. Antibiotic resistance elements are not rare.

The question of whether these environmental organisms present a reservoir for contemporary transfer of resistance genes to pathogens has been answered with the case of the emergence of the CTX-M β-lactamases. These enzymes emerged in the 1980s as elements that confer resistance to the oxyimino-cephalosporins such as cefotaxime and have become significant clinical problems (Canton and Coque 2006). The genes were associated with plasmids that were horizontally transferred between *Enterobacteriaceae*. The origins of the *ctx-m* genes were unknown as their products showed only ~40% similarity to known β-lactamases. This changed with the

cloning of the β-lactamases genes from several species of the nonpathogenic bacterium *Kluyvera*, which are often found in the environment. These chromosomal genes showed up to 100% identify to the *ctx-m* genes found in clinical pathogens and often included flanking genes shared between *Kluyvera* chromosomes and circulating plasmids in *Enterobacteriaceae* (Decousser et al. 2001; Humeniuk et al. 2002; Poirel et al. 2002; Olson et al. 2005). These results point to a recent escape of CTX-M from *Kluyvera* with subsequent spread among pathogenic bacteria via lateral gene transfer. The net result has been termed the CTX-M pandemic (Canton and Coque 2006).

Thus nonpathogenic bacteria, including antibiotic producers, are reservoirs of antibiotic resistance genes that can make their way into populations of pathogens by later gene transfer.

2.2 De Novo Evolution of Resistance

Resistance can also emerge spontaneously within bacterial populations. This can be the result of mutagenesis with subsequent selection of favorable resistance traits, for example in a patient undergoing antibiotic therapy. The error rate of DNA polymerase is ~1 base in 10^9. Therefore for *E. coli* with a genome size of ~5×10^6, one random mutation occurs in the genome every 200 cell divisions. In a population of bacteria in an infection, the number of mutants can be considerable. Of course many of these are silent or even fatal, but some may offer some advantage. This is especially true for slow growing organisms such as *Mycobacterium tuberculosis*, where mutations are the principal cause of drug resistance and chemotherapy lasts for months. In a study on the development of resistance to vancomycin and other antibiotics in a patient undergoing chemotherapy, Tomasz's group sequenced the genomes of increasingly resistant *Staphylococcus aureus* isolates recovered from a patient's bloodstream over a period of ~4 months (Mwangi et al. 2007). During this time, the strain accumulated 35 point mutations in 31 loci. The susceptibility to vancomycin decreased from 1 μg/mL at the beginning of therapy to 8 1 μg/mL after 12 weeks when the patient died. Furthermore, these mutations also conferred increased resistance to daptomycin.

The accumulation of point mutations with selection therefore has an important role in the evolution of resistance in pathogens. Another example of the significance of point mutations is in the development of resistance to the quinolone antibiotics. These are synthetic compounds, first identified in the early 1960s and developed in earnest in the 1970s, 1980s, and 1990s when 6-fluoroderivatives (the fluoroquinolones) were found to have excellent broad spectrum activity against a number of important pathogens, including multidrug resistant strains. These antibiotics target bacterial topoisomerases that catalyze double-strand breaks in DNA such as the type IIA enzymes DNA gyrase (induces supercoiling and unwinds strands at the replication fork; it is comprised of dimers of two subunits, GyrA and GyrB) and type IV topoisomerases (also a tetramer, e.g., of ParC/ParE, that decatenates daughter strands following DNA replication). These enzymes form a covalent adduct with the DNA

during cleavage to enable controlled strand passage during catalysis (Laponogov et al. 2010). Quinolones have little affinity for the free enzyme, but rather bind to this cleavage complex, blocking enzyme activity. This potently arrests cell division and results in cell death (Hooper 2001).

Resistance to the quinolones arises most frequently as a result of point mutations in *gyrA* or *parC*. A region of the enzyme termed the Quinolone Resistance Determining Region (QRDR) is most frequently associated with resistance. Mutations in key residues such as Ser83 and Asp87 (*E. coli* GyrA numbering) are among the most common in the clinic. Additional mutations in *parC* and secondary mutations in both genes incrementally increase resistance (Hooper 1999). Mutations in the QRDR region are not confined to resistant pathogenic bacteria, but these have also been found in environmental organisms (Waters and Davies 1997; D'Costa et al. 2006). These presumably arise without the selective pressure of exposure to the synthetic fluoroquinolones and thus reflect either natural variation in these genes, or the selective pressure of an as yet undiscovered class of natural products that impact the same target.

The presumption has been that such mutations have associated with them fitness costs that will be selected against in the absence of antibiotics. Indeed, this is true in many cases [reviewed in (Andersson 2006; Andersson and Hughes 2010)]. This would imply that removal of antibiotics would select for loss of resistance. However in many cases, compensatory secondary mutations in the target gene or others on the chromosome improve the fitness of resistant bacteria [see Table 2 in ref (Andersson and Hughes 2010)]. Thus, resistant mutations can propagate vertically in bacteria populations even in the absence of selection.

Antibiotic exposure is stressful to bacteria, and there have been several studies showing that exposure to an antibiotic or biocide alters the expression of hundreds of genes (Shaw et al. 2003; Cirz et al. 2006; Dwyer et al. 2007; Bailey et al. 2010). This complex response can in some cases converge on important pathways. For example, *E. coli* produced toxic reactive oxygen species including hydroxyl radicals in response to bactericidal antibiotics (Dwyer et al. 2007). Importantly, this was further correlated with increases in the genome mutation rate leading to the hypothesis that antibiotics transiently increase the bacterial mutation rate by generating DNA damage (Kaufmann and Hung 2010; Kohanski et al. 2010). Similar results we noted upon exposure of *Pseudomonas aeruginosa* to ciprofloxacin where the error-prone DNA repair and synthesis proteins were up-regulated (Cirz et al. 2006). This could allow antibiotic-resistant bacteria to be selected at an elevated frequency while under pressure from antibiotics and could account for the emergence of resistance by mutation during chemotherapy.

Point mutations are not the only mechanism of de novo resistance development. Gene duplication or higher order amplification is also common and has been linked to gene evolution by providing "substrate" for mutation and innovation (Sandegren and Andersson 2009). For example, some antibiotics (for example, fluoroquinolones) are often associated with the selection in vitro and in vivo of multidrug resistant bacteria due to over-production of RND (*R*esistance-*N*odulation-cell *D*ivision) type efflux pumps in Gram-negative bacteria (e.g., in

Enterobacteriaceae AcrAB–TolC, MexAB–OprM in *P. aeruginosa* and MtrCDE in *Neisseria* spp.) (Piddock 2006). These pumps confer innate resistance to many of the agents commonly used to treat infections by Gram-positive bacteria, and when deleted not only does the Gram-negative bacterium become hyper-drug susceptible but virulence can also be attenuated (Piddock 2006). Control and regulation of expression of the components of MDR efflux pumps is still not fully understood but can occur at either a local gene level (e.g., for *acrAB* by AcrR and *mexAB-oprM* by MexR) or at a global level such as in *E. coli* via MarA (White et al. 1997; Pomposiello and Demple 2000), RamA in *Klebsiella pneumoniae* and *Salmonella enterica* (Ricci and Piddock 2009) and MtrR in *Neisseria gonorrheae* (Folster et al. 2009). Mutations in these control elements can result in the amplification of RND pump expression and associated resistance.

A direct screen of the potential role of gene amplification in antibiotic resistance was recently reported (Soo et al. 2011). The authors screened an *E. coli* library of ~4,000 overexpressing clones for increased resistance to 237 antibiotics and other toxic compounds. Resistance to 86 of these was observed including many antibiotics (β-lactams, aminoglycosides, macrolides, and antifolates) upon overexpression of 61 genes. Eighteen of these genes included global mechanisms of resistance (efflux, regulators, stress proteins, capsule synthesis). The remaining 41 genes were specific to individual antibiotics including 12 that encode metabolic enzymes that could contribute to compound modification. These results in a "wild type" drug susceptible organism point to the relative ease of selecting resistance by gene amplification mechanisms and points to the importance of such events as a first step in the evolution of highly specific resistance elements that can be transferred vertically to progeny and can escape to move laterally in bacterial populations.

2.3 Proto-Resistance

The case described earlier where 12 metabolic genes were identified that can confer antibiotic resistance upon gene amplification in *E. coli* (Soo et al. 2011) demonstrates the capacity of microorganisms to capitalize on weak, cryptic, or promiscuous activities to evolve new metabolism. This has been termed the innovation–amplification–divergence model of gene evolution (Bergthorsson et al. 2007). The challenge in the evolution of genes with new functions after a gene duplication event is the maintenance of both the original gene and the duplicate while enabling divergence in the duplicate. Antibiotics provide the strong selective pressure to ensure that the duplicate gene is not lost in subsequent generations. Furthermore, it provides the requisite selection for innovation and evolution in the new gene. The determination of the gene (and protein) sequences of highly efficient resistance elements along with their biochemical and structural properties provides an opportunity to hypothesize on the origins of many resistance proteins. We have termed these proto-resistance genes and proteins (Morar and Wright 2010).

Among the first links between resistance biochemistry and metabolic processes was noted between peptidoglycan biosynthesis proteins and β-lactamases (Kelly et al. 1986). The β-lactamases utilize one of two general mechanisms to hydrolytically cleave the β-lactam ring of the antibiotics. Metallo-β-lactamases use an active site Zn^{2+} ion (more often two) to active a water molecule for attack on the β-lactam carbonyl carbon resulting in a ring-opening drug inactivation. These enzymes were previously rare in the clinic but are growing in importance as they confer resistance to virtually all β-lactams including the "last resort" carbapenems (De Pascale and Wright 2010). The more prevalent mechanism in pathogens operates through an active site Asp-His-Ser catalytic triad. The Ser hydroxyl group attacks the carbonyl of the β-lactam ring forming a covalent-enzyme intermediate and subsequent cleavage of this complex by water generates the inactive antibiotic. The first step of the reaction, formation of the acyl-enzyme intermediate, is highly reminiscent of the activity of β-lactams on their cellular targets. β-Lactam antibiotics covalently inactivate enzymes involved in peptidoglycan biosynthesis (Massova and Mobashery 1998). These so-called *p*enicillin-*b*inding *p*roteins (PBPs) also operate by an active site Asp-His-Ser catalytic triad. Analogously, the Ser attacks the β-lactam ring resulting in a covalent intermediate. However unlike the β-lactamases, subsequent hydrolysis does not occur and the enzyme is permanently in the inactive form. This results in an inability to generate new peptidoglycan and subsequent inhibition of cell division.

This convergence in biochemical function was further strengthened with the determination of the structures of β-lactamases and PBPs. The first was the comparison of the 3D structure of the D-alanyl-D-alanine-peptidase from *Streptomyces* R61 with the β-lactamase from *Bacillus licheniformis* (Kelly et al. 1986). Despite a lack of significant amino acid sequence similarity, these enzymes showed remarkable conservation of structure and mechanism. This 25-year-old observation has been supported by the determination of the structure and function of many PBPs and β-lactamases (Maveyraud et al. 1998). PBPs are therefore the most likely β-lactamase proto-resistance elements.

Similarly, aminoglycoside phosphotransferases have the same protein fold as Ser/Thr/Tyr protein kinases despite a lack of sequence similarity (Hon et al. 1997; Nurizzo et al. 2003; Young et al. 2009; Fong et al. 2010; Toth et al. 2010; Stogios et al. 2011). Furthermore, these enzymes have the capacity to phosphorylate peptides and proteins (Daigle et al. 1998) and their activities blocked by protein kinase inhibitors (Daigle et al. 1997). These enzyme families likely share a common ancestor.

There are many other examples emerging of the ties between resistance and metabolic processes [reviewed in (Morar and Wright 2010)]. A contemporary example is the emergence of a variant of an aminoglycoside antibiotic inactivating acetyltransferase that has the ability to inactivate a subset of fluoroquinolone antibiotics (Robicsek et al. 2006; Vetting et al. 2008). This determinant has since spread worldwide by lateral gene transfer (Strahilevitz et al. 2009). The strong selective pressure provided by antibiotics along with the number of both pathogens and nonpathogens exposed to antibiotics across the globe predicts that the innovation–amplification–divergence model of evolution

will continue to offer new examples of the co-opting of metabolic genes for drug resistance.

3 Summary, Challenges and Opportunities

Antibiotic resistance has proven to be inevitable. The reasons are now clear. The movements of genes horizontally through bacterial populations do not respect the artificial pathogen/nonpathogen boundary. Bacteria are ancient organisms that have adapted to virtually all environmental challenges on the planet. They live in environments dominated by small molecules and have evolved both specific and nonspecific mechanisms to evade or detoxify noxious compounds including antibiotics. In addition to these ancient resistance elements, contemporary resistance to any new drug can arise by gene mutation and amplification. From these processes new alleles can emerge that confer drug resistance. There are no irresistible antibiotics.

This fact explains our experience with 6 decades of antibiotic discovery and use. Nevertheless, despite the prevalence of resistance and the mechanisms that can rise to it, antibiotics remain foundational medicines for modern health care. A world without antibiotics would mean a world without many surgeries, transplants, cancer treatments, longevity of patients with cystic fibrosis, etc.; it would mean increased deaths of infants and the elderly by diseases now commonly tractable by antibiotics. Our clinical experience with antibiotics over the past decades and our growing understanding of the molecular basis of resistance must inform the discovery and use of future antibiotics.

The concept of a global antibiotic resistome offers an ecological view of resistance that can be applied in new drug development. The screening of candidate molecules based not only on their activity against traditional panels of pathogens but also nonpathogenic environmental bacteria will serve to identify "weak spots" in antibiotic structures and targets. The early identification of resistance elements can propel the development of diagnostics that will ensure epidemiological monitoring of resistance and the proper use of drugs at the bedside.

The discovery of an *intrinsic* resistome also offers a number of important opportunities for drug development. The systematic inactivation of nonessential genes, either through precise gene deletions (Baba et al. 2006) or by more traditional transposon mutant libraries, has offered an opportunity to examine the antibiotic susceptibility of bacteria upon inactivation of selected genes—so-called chemical synthetic interactions. By screens such libraries in *E. coli* (Liu et al. 2010) or *P. aeruginosa* (Breidenstein et al. 2008; Alvarez-Ortega et al. 2010), a large number of genes whose inactivation sensitizes bacteria to antibiotics have been identified. These offer a unique set of potential targets for the development of combination drugs to extend the efficacy of our current arsenal of antibiotics well into the future. Similarly, compounds that block resistance elements, already proven as successful clinical agents with the inhibitors of β-lactamases, can also be explored.

Whether such approaches will be adopted by physicians and the drug development industry remains to be seen. What is certain is that innovation and understanding of the molecule basis of the problem is vital. As noted by Joshua Lederberg: "Pitted against microbial genes, we have mainly our wits."

Acknowledgements Research in the author's lab on antibiotic resistance is supported by a Canada Research Chair, the Canadian Institutes of Health Research and the Natural Sciences and Engineering Research Council of Canada.

References

Abraham EP, Chain E (1940) An enzyme from bacteria able to destroy penicillin. Nature 146:837

Allen HK, Moe LA et al (2009) Functional metagenomics reveals diverse beta-lactamases in a remote Alaskan soil. ISME J 3(2):243–51

Allwood AC, Walter MR et al (2006) Stromatolite reef from the Early Archaean era of Australia. Nature 441(7094):714–8

Alvarez-Ortega C, Wiegand I et al (2010) Genetic determinants involved in the susceptibility of *Pseudomonas aeruginosa* to beta-lactam antibiotics. Antimicrob Agents Chemother 54(10):4159–67

Andersson DI (2006) The biological cost of mutational antibiotic resistance: any practical conclusions? Curr Opin Microbiol 9(5):461–5

Andersson DI, Hughes D (2010) Antibiotic resistance and its cost: is it possible to reverse resistance? Nat Rev Microbiol 8(4):260–71

Baba T, Ara T et al (2006) Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol Syst Biol 2:0008

Bailey AM, Ivens A et al (2010) RamA, a member of the AraC/XylS family, influences both virulence and efflux in *Salmonella enterica* serovar Typhimurium. J Bacteriol 192(6):1607–16

Barlow M (2009) What antimicrobial resistance has taught us about horizontal gene transfer. Methods Mol Biol 532:397–411

Benveniste R, Davies J (1973) Aminoglycoside antibiotic-inactivating enzymes in actinomycetes similar to those present in clinical isolates of antibiotic-resistant bacteria. Proc Natl Acad Sci USA 70:2276–2280

Bergthorsson U, Andersson DI et al (2007) Ohno's dilemma: evolution of new genes under continuous selection. Proc Natl Acad Sci USA 104(43):17004–9

Boucher HW, Talbot GH et al (2009) Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America. Clin Infect Dis 48(1):1–12

Breidenstein EB, Khaira BK et al (2008) Complex ciprofloxacin resistome revealed by screening a *Pseudomonas aeruginosa* mutant library for altered susceptibility. Antimicrob Agents Chemother 52(12):4486–91

Bugg TDH, Wright GD et al (1991) Molecular basis for vancomycin resistance in *Enterococcus faecium* BM4147: biosynthesis of a depsipeptide peptidoglycan precursor by vancomycin resistance proteins VanH and VanA. Biochemistry 30:10408–10415

Canton R, Coque TM (2006) The CTX-M beta-lactamase pandemic. Curr Opin Microbiol 9(5):466–75

Cirz RT, O'Neill BM et al (2006) Defining the *Pseudomonas aeruginosa* SOS Response and Its Role in the Global Response to the Antibiotic Ciprofloxacin. J Bacteriol 188(20):7101–10

Courvalin P (2006) Vancomycin resistance in gram-positive cocci. Clin Infect Dis 42(Suppl 1): S25–34

Croucher NJ, Walker D et al (2009) Role of conjugative elements in the evolution of the multidrug-resistant pandemic clone *Streptococcus pneumoniae*Spain23F ST81. J Bacteriol 191(5):1480–9
Cundliffe E (1989) How antibiotic-producing organisms avoid suicide. Annu Rev Microbiol 43:207–33
Cundliffe E, Bate N et al (2001) The tylosin-biosynthetic genes of *Streptomyces fradiae*. Antonie Van Leeuwenhoek 79(3–4):229–34
Daigle DM, McKay GA et al (1997) Inhibition of aminoglycoside antibiotic resistance enzymes by protein kinase inhibitors. J Biol Chem 272:24755–24758
Daigle DM, McKay GA et al (1998) Aminoglycoside phosphotransferases required for antibiotic resistance are also Serine protein kinases. Chem Biol 6:11–18
Dantas G, Sommer MO et al (2008) Bacteria subsisting on antibiotics. Science 320(5872):100–3
Davies J (1995) Vicious circles: looking back on resistance plasmids. Genetics 139(4):1465–8
D'Costa VM, McGrann KM et al (2006) Sampling the antibiotic resistome. Science 311 (5759):374–7
D'Costa VM, Griffiths E et al (2007) Expanding the soil antibiotic resistome: exploring environmental diversity. Curr Opin Microbiol 10(5):481–9
De Pascale G, Wright GD (2010) Antibiotic resistance by enzyme inactivation: from mechanisms to solutions. Chembiochem 11(10):1325–34
Decousser JW, Poirel L et al (2001) Characterization of a chromosomally encoded extended-spectrum class A beta-lactamase from *Kluyvera cryocrescens*. Antimicrob Agents Chemother 45(12):3595–8
Donato JJ, Moe LA et al (2010) Metagenomics reveals antibiotic resistance genes encoding predicted bifunctional proteins in apple orchard soil. Appl Environ Microbiol 76:4396–4401
Dwyer DJ, Kohanski MA et al (2007) Gyrase inhibitors induce an oxidative damage cellular death pathway in *Escherichia coli*. Mol Syst Biol 3:91
Fajardo A, Martinez-Martin N et al (2008) The neglected intrinsic resistome of bacterial pathogens. PLoS One 3(2):e1619
Fischbach MA, Walsh CT (2009) Antibiotics for emerging pathogens. Science 325 (5944):1089–93
Fleming A (1945) Penicillin. Nobel Prize Lecture http://nobelprize.org/nobel_prizes/medicine/laureates/1945/fleming-lecture.pdf
Folster JP, Johnson PJ et al (2009) MtrR modulates *rpoH* expression and levels of antimicrobial resistance in *Neisseria gonorrhoeae*. J Bacteriol 191(1):287–97
Fong DH, Lemke CT et al (2010) Structure of the antibiotic resistance factor spectinomycin phosphotransferase from *Legionella pheumophila*. J Biol Chem 285(13):9545–55
Freitas AR, Tedim AP et al (2010) Global spread of the hyl(Efm) colonization-virulence gene in megaplasmids of the *Enterococcus faecium* CC17 polyclonal subcluster. Antimicrob Agents Chemother 54(6):2660–5
Guardabassi L, Agerso Y (2006) Genes homologous to glycopeptide resistance *vanA* are widespread in soil microbial communities. FEMS Microbiol Lett 259(2):221–5
Guardabassi L, Perichon B et al (2005) Glycopeptide resistance *vanA* operons in *Paenibacillus* strains isolated from soil. Antimicrob Agents Chemother 49(10):4227–33
Gwynn MN, Portnoy A et al (2010) Challenges of antibacterial discovery revisited. Ann N Y Acad Sci 1213:5–19
Hon WC, McKay GA et al (1997) Structure of an enzyme required for aminoglycoside antibiotic resistance reveals homology to eukaryotic protein kinases. Cell 89(6):887–95
Hong HJ, Hutchings MI et al (2004) Characterization of an inducible vancomycin resistance system in *Streptomyces coelicolor* reveals a novel gene (*vanK*) required for drug resistance. Mol Microbiol 52(4):1107–21
Hooper DC (1999) Mechanisms of fluoroquinolone resistance. Drug Resist Updat 2(1):38–55
Hooper DC (2001) Mechanisms of action of antimicrobials: focus on fluoroquinolones. Clin Infect Dis 32(Suppl 1):S9–S15

Humeniuk C, Arlet G et al (2002) Beta-lactamases of *Kluyvera ascorbata*, probable progenitors of some plasmid-encoded CTX-M types. Antimicrob Agents Chemother 46(9):3045–9

Infectious Diseases Society of America (2010) The 10 × '20 Initiative: pursuing a global commitment to develop 10 new antibacterial drugs by 2020. Clin Infect Dis 50(8):1081–3

Karray F, Darbon E et al (2007) Organization of the biosynthetic gene cluster for the macrolide antibiotic spiramycin in *Streptomyces ambofaciens*. Microbiology 153(Pt 12):4111–22

Kaufmann BB, Hung DT (2010) The fast track to multidrug resistance. Mol Cell 37(3):297–8

Kelly JA, Dideberg O et al (1986) On the origin of bacterial resistance to penicillin: comparison of a beta-lactamase and a penicillin target. Science 231(4744):1429–31

Kohanski MA, DePristo MA et al (2010) Sublethal antibiotic treatment leads to multidrug resistance via radical-induced mutagenesis. Mol Cell 37(3):311–20

Koteva K, Hong HJ et al (2010) A vancomycin photoprobe identifies the histidine kinase VanSsc as a vancomycin receptor. Nat Chem Biol 6(5):327–9

Lang KS, Anderson JM et al (2010) Novel florfenicol and chloramphenicol resistance gene discovered in Alaskan soil using functional metagenomics. Appl Environ Microbiol 76:5321–5326

Laponogov I, Pan XS et al (2010) Structural basis of gate-DNA breakage and resealing by type II topoisomerases. PLoS One 5(6):e11338

Leclercq R, Derlot E et al (1988) Plasmid-mediated resistance to vancomycin and teicoplanin in *Enterococcus faecium*. N Engl J Med 319(3):157–61

Liu A, Tran L et al (2010) Antibiotic sensitivity profiles determined with an *Escherichia coli* gene knockout collection: generating an antibiotic bar code. Antimicrob Agents Chemother 54 (4):1393–403

Maravic G (2004) Macrolide resistance based on the Erm-mediated rRNA methylation. Curr Drug Targets Infect Disord 4(3):193–202

Marshall CG, Lessard IA et al (1998) Glycopeptide antibiotic resistance genes in glycopeptide-producing organisms. Antimicrob Agents Chemother 42(9):2215–20

Massova I, Mobashery S (1998) Kinship and diversification of bacterial penicillin-binding proteins and beta-lactamases. Antimicrob Agents Chemother 42(1):1–17

Maveyraud L, Mourey L et al (1998) Structural basis for the clinical longevity of carbapenem antibiotics in the face of challenge by the common A beta-lactamasees from the antibiotic-resistnat bacteria. J Am Chem Soc 120:9748–9752

Morar M, Wright GD (2010) The genomic enzymology of antibiotic resistance. Annu Rev Genet 44:25–51

Morar M, Bhullar K et al (2009) Structure and mechanism of the lincosamide antibiotic adenylyltransferase LinB. Structure 17(12):1649–59

Mukhtar TA, Wright GD (2005) Streptogramins, oxazolidinones, and other inhibitors of bacterial protein synthesis. Chem Rev 105(2):529–42

Mwangi MM, Wu SW et al (2007) Tracking the in vivo evolution of multidrug resistance in *Staphylococcus aureus* by whole-genome sequencing. Proc Natl Acad Sci USA 104 (22):9451–6

Nurizzo D, Shewry SC et al (2003) The crystal structure of aminoglycoside-3'-phosphotransferase-IIa, an enzyme responsible for antibiotic resistance. J Mol Biol 327(2):491–506

Oliynyk M, Samborskyy M et al (2007) Complete genome sequence of the erythromycin-producing bacterium *Saccharopolyspora erythraea* NRRL23338. Nat Biotechnol 25(4):447–453

Olson AB, Silverman M et al (2005) Identification of a progenitor of the CTX-M-9 group of extended-spectrum beta-lactamases from *Kluyvera georgiana* isolated in Guyana. Antimicrob Agents Chemother 49(5):2112–5

Peirano G, Pitout JD (2010) Molecular epidemiology of *Escherichia coli* producing CTX-M beta-lactamases: the worldwide emergence of clone ST131 O25:H4. Int J Antimicrob Agents 35(4):316–21

Piddock LJ (2006) Multidrug-resistance efflux pumps – not just for resistance. Nat Rev Microbiol 4(8):629–36

Poirel L, Kampfer P et al (2002) Chromosome-encoded Ambler class A beta-lactamase of *Kluyvera georgiana*, a probable progenitor of a subgroup of CTX-M extended-spectrum beta-lactamases. Antimicrob Agents Chemother 46(12):4038–40
Pomposiello PJ, Demple B (2000) Identification of SoxS-regulated genes in *Salmonella enterica* serovar typhimurium. J Bacteriol 182(1):23–9
Ricci V, Piddock LJ (2009) Ciprofloxacin selects for multidrug resistance in *Salmonella enterica* serovar Typhimurium mediated by at least two different pathways. J Antimicrob Chemother 63 (5):909–16
Riesenfeld CS, Goodman RM et al (2004) Uncultured soil bacteria are a reservoir of new antibiotic resistance genes. Environ Microbiol 6(9):981–9
Robicsek A, Strahilevitz J et al (2006) Fluoroquinolone-modifying enzyme: a new adaptation of a common aminoglycoside acetyltransferase. Nat Med 12(1):83–8
Roy PH (1999) Horizontal transfer of genes in bacteria. Microbiol Today 26:168–170
Sandegren L, Andersson DI (2009) Bacterial gene amplification: implications for the evolution of antibiotic resistance. Nat Rev Microbiol 7(8):578–88
Shaw KJ, Miller N et al (2003) Comparison of the changes in global gene expression of *Escherichia coli* induced by four bactericidal agents. J Mol Microbiol Biotechnol 5(2):105–22
Sommer MO, Dantas G et al (2009) Functional characterization of the antibiotic resistance reservoir in the human microflora. Science 325(5944):1128–31
Soo VW, Hanson-Manful P et al (2011) From the cover: artificial gene amplification reveals an abundance of promiscuous resistance determinants in *Escherichia coli*. Proc Natl Acad Sci USA 108(4):1484–9
Spellberg B, Powers JH et al (2004) Trends in antimicrobial drug development: implications for the future. Clin Infect Dis 38(9):1279–86
Stogios PJ, Shakya T et al (2011) Structure and function of APH(4)-Ia, a hygromycin B resistance enzyme. J Biol Chem 286(3):1966–75
Strahilevitz J, Jacoby GA et al (2009) Plasmid-mediated quinolone resistance: a multifaceted threat. Clin Microbiol Rev 22(4):664–89
Toth M, Frase H et al (2010) Crystal structure and kinetic mechanism of aminoglycoside phosphotransferase-2″-IVa. Protein Sci 19(8):1565–76
Tu D, Blaha G et al (2005) Structures of MLSBK antibiotics bound to mutated large ribosomal subunits provide a structural explanation for resistance. Cell 121(2):257–70
Vetting MW, Park CH et al (2008) Mechanistic and structural analysis of aminoglycoside N-acetyltransferase AAC(6')-Ib and its bifunctional, fluoroquinolone-active AAC(6′)-Ib-cr variant. Biochemistry 47(37):9825–35
Walsh CT, Fisher SL et al (1996) Bacterial resistance to vancomycin: five genes and one missing hydrogen bond tell the story. Chem Biol 3:21–28
Ward SL, Hu Z et al (2004) Chalcomycin biosynthesis gene cluster from *Streptomyces bikiniensis*: novel features of an unusual ketolide produced through expression of the chm polyketide synthase in *Streptomyces fradiae*. Antimicrob Agents Chemother 48(12):4703–12
Waters B, Davies J (1997) Amino acid variation in the GyrA subunit of bacteria potentially associated with natural resistance to fluoroquinolone antibiotics. Antimicrob Agents Chemother 41(12):2766–9
Wellman CH, Osterloff PL et al (2003) Fragments of the earliest land plants. Nature 425 (6955):282–5
White DG, Goldman JD et al (1997) Role of the *acrAB* locus in organic solvent tolerance mediated by expression of *marA*, *soxS*, or *robA* in *Escherichia coli*. J Bacteriol 179(19):6122–6
Whitman WB, Coleman DC et al (1998) Prokaryotes: the unseen majority. Proc Natl Acad Sci USA 95(12):6578–83
Williams KJ, Bax RP (2009) Challenges in developing new antibacterial drugs. Curr Opin Investig Drugs 10(2):157–63
Wright GD (2007) The antibiotic resistome: the nexus of chemical and genetic diversity. Nat Rev Microbiol 5(3):175–86

Wright GD (2010) The antibiotic resistome. Expert Opin Drug Disc 5:779–788

Wu Z, Wright GD et al (1995) Overexpression, purification, and characterization of VanX, a D-D-dipeptidase which is essential for vancomycin resistance in *Enterococcus faecium* BM4147. Biochemistry 34(8):2455–63

Young PG, Walanj R et al (2009) The crystal structures of substrate and nucleotide complexes of *Enterococcus faecium* aminoglycoside-2″-phosphotransferase-IIa [APH(2″)-IIa] provide insights into substrate selectivity in the APH(2″) subfamily. J Bacteriol 191(13):4133–43

Surveillance Programmes and Antibiotic Resistance: Worldwide and Regional Monitoring of Antibiotic Resistance Trends

Stephen Hawser

Contents

1 Introduction 32
2 Impact of Antibiotic Consumption and bacterial Resistance 32
3 Surveillance of Bacterial Resistance 34
4 Governmental and Institutional Surveillance Programmes 34
5 Industry/Pharmaceutical Surveillance Programmes 38
6 Conclusions 40
References 41

Abstract Since the introduction of the penicillins many decades ago, multiple species of bacteria have responded to the use of antimicrobial agents in their ability to develop and transmit antimicrobial resistance. Increased consumption of antimicrobial agents, their misappropriate use among other factors have further catalysed this resistance phenomenon. As bacterial resistance is a global healthcare issue, appropriate monitoring through governmental, institutional and industry or pharmaceutical led surveillance programmes is essential. This chapter describes the resistance issue, factors affecting this issue and examples of such ongoing resistance surveillance programmes.

Keywords Surveillance • Resistance • Global health • Monitoring • Antibiotic consumption • Government • Institutions • Industry

S. Hawser (✉)
IHMA Europe Sàrl, 9A Route de la Corniche, 1066 Epalinges, Switzerland
e-mail: shawser@ihmainc.com

A.R.M. Coates (ed.), *Antibiotic Resistance*, Handbook of Experimental Pharmacology 211, DOI 10.1007/978-3-642-28951-4_3,
© Springer-Verlag Berlin Heidelberg 2012

1 Introduction

The ongoing issue of antimicrobial resistance has become a significant public health concern throughout the world (Zhang et al. 2006). Importantly, resistance to antimicrobial agents occurs in all types of pathogens, ranging from viruses, mycobacteria, parasites, fungi to bacteria. Concerning the latter, various studies have described the impact of such antimicrobial resistance in bacteria regarding therapeutic success and failure, mortality, morbidity and the pharmaco-economic issues related to extensive hospital length of stay and multiple treatments (Ang et al. 2004; Myrianthefs et al. 2004). While antimicrobial resistance in many types of pathogens is important, for simplicity, this chapter concentrates only on resistance in bacteria.

Many types of bacteria have demonstrated their efficiency in developing resistance to one or more antimicrobial agent. Indeed, following the introduction of penicillin into human therapeutics in the 1940s and throughout the past 7 decades, antibiotic resistance has appeared and has continuously evolved. The emergence of such resistance has revealed multiple and complex mechanisms by which resistance genes spread across the bacterial kingdom, with apparent disregard for species barriers. Interestingly, many resistance determinants were actually present before the introduction of antibiotics for human use. These were mostly isolated from organisms that naturally produce their "own" antibiotics (Levy 1992). Neighbouring species had already likely acquired those genes or developed new mechanisms to protect themselves from the action of antibiotics to which they had been exposed. Bacterial resistance has since evolved with the increasing number, volume and diversity of antimicrobial applications. As new drugs were introduced, clinically resistant strains were identified relatively rapidly soon after. Furthermore, many of these pathogens are not obligate pathogens and play significant roles in the human microflora.

2 Impact of Antibiotic Consumption and bacterial Resistance

A direct relationship between the quantity of antibiotic used and the development of resistance has not been easy to determine. Data on the total antibiotic utilization in particular areas are very limited, often unreliable, or in many instances are non-existent. Both the amount of antibiotics used and precisely how they are used contribute to the development of resistance. For example, the use of broad-spectrum agents as opposed to narrow-spectrum agents is known to favour the emergence of resistance by broadly eliminating competing susceptible flora. An example of such a relationship was shown in neonatal intensive care units (ICUs) whereby the empiric use of amoxicillin/cefotaxime combinations for suspected neonatal sepsis was associated with the emergence of resistant Gram-negative bacilli (De Man et al. 2000). In that study, overall, the risk of colonization with resistant bacteria to the empiric treatment was 18-fold higher for the

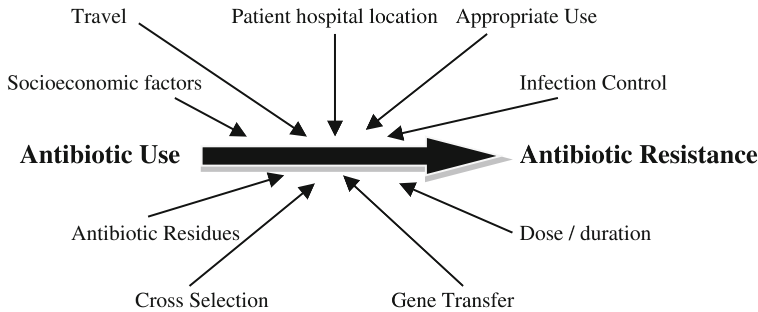


Fig. 1 Relationships between antibiotic use and the development of bacterial resistance

broad-spectrum therapy than for an alternative regimen of narrow-spectrum antibiotics (De Man et al. 2000).

Antibiotics are frequently, and erroneously, prescribed in the treatment of viral infections or at incorrect doses for inappropriate durations. The impact of prescribing practices has been shown to be more closely related to the emergence of resistant bacteria than the actual volume used (Guillemot et al. 1998). Other factors, although difficult to precisely quantify, also impact the relationship between the use of antibiotics and resistance emergence, and these, as depicted in Fig. 1, include socio-economics, travel, education, communal facilities (e.g., child care centres, nursing homes, rehabilitation), duration of hospital stay and intra-hospital stay and so on.

Monitoring antibiotic use or consumption has become increasingly important in recent years. Barbosa and Levy (2000) described the intense use and misuse of antibiotics as being the major forces that drive bacterial resistance. Interestingly, they also described a reverse in this trend whereby decreases in antibiotic resistance frequency following implementation of infection control measures were observed. A national study from Denmark noted that while antibacterial resistance is high, and considering the lack of novel therapies to combat resistance, the major determinant considered useful in salvaging today's therapies is in the control of antibiotic use (Frimodt-Moller et al. 2007). Traditionally, bacterial resistance in Nordic countries like Denmark has been lower than those observed elsewhere in Europe often attributed to infection control measures having been implemented very early and to the low use of antibiotics. However, antibiotic consumption in Denmark even increased by 20% from 1997 to 2005, and this increase was attributed to complexity of new surgical procedures, increased need for antibiotic prophylaxis and higher rates of nosocomial infections (Frimodt-Moller et al. 2007). In a similar way, the European Surveillance of Antimicrobial Consumption (ESAC) has monitored antibiotic consumption throughout Europe with respect to resistance in bacteria (Goossens et al. 2005). It was noted that prescription of antibiotics in primary care in Europe varied greatly from one country to the next. However, a shift in usage from the old narrow-spectrum antibiotics to the more recent broad-spectrum antibiotics was noteworthy. Overall, data from ESAC has shown higher

rates of antibiotic resistance in "high consuming" countries, seen notably in southern and eastern European countries (Goossens et al. 2005). There are many other reports that describe antibiotic consumption and the link with bacterial resistance. To highlight a few, reports concerning the link between use and resistance in *Pseudomonas aeruginosa* (Tresoldi das Neves et al. 2010), the selective pressure of macrolide (Skalet et al. 2010) and quinolone (Garcia-Rey et al. 2006) use and resistance in *Streptococcus pneumoniae* and the use of antibiotics in the community setting and resistance in community settings (Cizman 2003) all share the same phenomenon whereby antibiotic use is strongly linked with bacterial resistance.

3 Surveillance of Bacterial Resistance

The effective surveillance of antimicrobial susceptibility and bacterial resistance is important for developing rational empirical therapy guidelines and for guiding efforts to control and prevent the dissemination of resistant organisms. While the need to monitor such trends was already acknowledged upon the first introduction of antimicrobial agents for human therapy, only relatively recently has the immense issue of antimicrobial resistance catalysed significant interest and efforts at international, national, institutional and professional levels (Felmingham et al. 2000; Vandepitte et al. 1996; Jones 1996, 1999; Standing Medical Advisory Committee Sub-Group on Antimicrobial Resistance 1998; Sahm et al. 1999). Core to this interest is the focus on surveillance as the centralized tool in understanding the nature and scope of bacterial resistance while assisting in controlling the spread of resistance and, through better information and education, leading to improved antimicrobial prescribing. In this context, surveillance programmes consist of compiling, analysing and disseminating data to a variety of antimicrobial agents for a number of target organisms and in a series of clinical settings. Currently, there are various types of ongoing surveillance programmes, several of which are highlighted in the following sections of this chapter.

4 Governmental and Institutional Surveillance Programmes

A number of governmental/institutional surveillance programmes have been performed, of which many of these are ongoing programmes. Examples of several of these are described below and are highlighted in Table 1.

For example, the European Centre for Disease Prevention and Control (ECDC) publishes an annual surveillance report describing antibiotic resistance in Europe. The ECDC annual report of the European Antimicrobial Resistance Surveillance Network (EARS-Net) most recently described surveillance data from 28 European countries which had actively participated (European Centre for Disease Prevention and Control 2010). In 2009, the most concerning resistance data came from the

Table 1 Examples of ongoing governmental/institutional surveillance programmes

Programme	Geography	References
EARS-Net (European Antimicrobial Resistance Surveillance Network)	Europe	European Centre for Disease Prevention and Control (2010)
STRAMA (Swedish Strategic Programme against Antibiotic Resistance)	Sweden	SWEDRES (2009)
CIPARS (Canadian Integrated Program for Antimicrobial Resistance Surveillance)	Canada	Government of Canada (2010)
NARMS (National Antimicrobial Resistance Monitoring System)	USA	Centers for Disease Control and Prevention (2010)
DANMAP (Danish Integrated Antimicrobial Resistance Monitoring and Research Programme)	Denmark	DANMAP (2009)
ABC (Active Bacterial Core Surveillance)	USA	Centers for Disease Control and Prevention (2009a, b, c, d)

rapidly decreasing susceptibility of invasive *Escherichia coli* to all antimicrobial agents under the scope of EARS-Net with the exception of the carbapenem antibiotic class. Moreover, the report described the dramatic increase in multi-drug resistance species, including *Klebsiella pneumoniae* shown to have gained resistance to third-generation cephalosporins, fluoroquinolones and aminoglycoside antibiotics. In addition, while the carbapenems have “traditionally” maintained high activity, resistance to these agents has started appearing in Europe with reports of carbapenem-resistant *K. pneumoniae*, of which resistance has been linked to carbapenemase (VIM, KPC, OXA-48 and most recently NDM-1 carbapenemases) production. Overall, EARS-Net 2009 provides a knowledge baseline on the occurrence of bacterial resistance in multiple pathogens throughout the 28 participating European countries and consequently also documents the diminishing number of effective antibiotic regimens for human use.

A second national programme from Sweden also effectively documents antibiotic use in clinical settings and bacterial resistance. This report, by the Swedish Strategic Programme against Antibiotic Resistance (STRAMA), highlighted the situation with regard to annually increasing antibiotic resistance in Sweden (SWEDRES 2009). STRAM 2009 involved a total of 28 clinical laboratories from 21 counties throughout Sweden. For example, an outbreak of vancomycin-resistant *Enterococcus faecium* which was first detected in 2007 was still ongoing in 2009 in Sweden. Furthermore, community-acquired methicillin-resistant *Staphylococcus aureus* is also increasing in incidence as is the case of resistant isolates of *K. pneumoniae* among others. In 2009 STRAMA consequently set three national targets for antibiotic prescriptions in outpatient care: (1) the total number of prescriptions should not exceed 250 prescriptions per 1,000 inhabitants per year, (2) the proportion of fluoroquinolones prescribed for female urinary tract infections in ages 18–79 years should not exceed 10% and (3) penicillin V should constitute 80% of all prescriptions of antibiotics primarily used for respiratory tract infections in children up to the age of 6 years. Overall, STRAMA has effectively identified

resistance issues and consequently initiated measures that are designed, and expected, to contain and reduce the resistance issue.

The Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) tracks temporal and regional trends in antimicrobial use, and antimicrobial resistance in selected species of enteric bacteria obtained at different points along the food chain and from human cases (Government of Canada 2010). Such information allows CIPARS to create and evaluate policies to contain antimicrobial resistance and to better manage antimicrobial use in human medicine, veterinary medicine and in agricultural sectors. The latest CIPARS report showed that compared with data from 2002 the overall human consumption for 2007 decreased as measured by prescribing rates and defined daily doses per 1,000 inhabitant-days. Moreover, CIPARS reported that from 2002 to 2007 resistance to ampicillin–amoxicillin/clavulanic acid–ceftiofur–cefoxitin (A2C-AMP resistance pattern) was detected in many *Salmonella* isolates recovered from human cases. However, the percentage of human clinical isolates of *S. typhimurium* with the ACSSuT resistance pattern had significantly decreased from 21% in 2003 to 10% in 2007. Notably, the percentage of human *S. typhimurium* isolates exhibiting resistance to nalidixic acid was significantly higher in 2007 than in 2003. The nalidixic acid data is of particular interest because nalidixic acid resistance is often associated with reduced susceptibility to the fluoroquinolone ciprofloxacin and can increase the risk of treatment failures with fluoroquinolones.

A similar report based on Enteric bacteria is published by the national Antimicrobial Resistance Monitoring System (NARMS) which represents a collaboration among the Centers for Disease Control and Prevention (CDC), US Food and Drug Administration's Center for Veterinary Medicine (FDA-CVM) and the US Department of Agriculture (USDA) (Centers for Disease Control and Prevention 2010). In 2008, all 50 states participated in NARMS. Specifically, NARMS 2008 showed a substantial proportion of Enterobacteriaceae demonstrated resistance to clinically important antimicrobial agents included, 2.0% of non-typhoidal *Salmonella* isolates being resistant to nalidixic acid, 2.9% of non-typhoidal *Salmonella* isolates were resistant to ceftriaxone, 59% of *Salmonella* ser. Typhi isolates were resistant to nalidixic acid, 2.2% of *Shigella* isolates were resistant to nalidixic acid and 0.9% were resistant to ciprofloxacin and 1.9% of *Escherichia coli* O157 isolates were resistant to nalidixic acid. The report also highlighted similar resistance in other species, including *Campylobacter* spp., and also the phenomenon of increased incidence in multi-drug-resistant phenotypes (Centers for Disease Control and Prevention 2010).

The Danish Integrated Antimicrobial Resistance Monitoring and Research Programme (DANMAP 2009) was established in 1995 on the initiative of the Danish Ministry of Health and the Danish Ministry of Food, Agriculture and fisheries, as a coordinated national surveillance and research programme for antimicrobial consumption and antimicrobial resistance in bacteria from animals, food and humans. DANMAP 2009 describes various resistance patterns in different bacteria but mainly focussed on extended-spectrum beta-lactamase (ESBL) producing bacteria in animals and in humans. Bacteria that produce such ESBLs are resistant to a

wide-spectrum of antibiotics including penicillins and third-generation cephalosporins and their occurrence, even at low levels, is considered to be a matter of concern. The latest report has shown that the rise in incidence of ESBLs in animals was also reflected in a similar rise in ESBL occurrence in humans, especially so for *K. pneumoniae* for which prevalence of *K. pneumoniae* increased significantly from 2007 to 2009, reaching 14.6% in 2009 (DANMAP 2009).

The Active Bacterial Core surveillance (ABCs) is a core component of CDC's Emerging Infections Programs network (EIP), a collaboration between CDC, state health departments and universities in the USA. ABCs is an active laboratory- and population-based surveillance system for invasive bacterial pathogens of public health importance. For each case of invasive disease in the surveillance population, a case report with basic demographic information is completed and bacterial isolates are sent to CDC and other reference laboratories for additional laboratory evaluation. ABCs also provides an infrastructure for further public health research, including special studies aiming at identifying risk factors for disease, post-licensure evaluation of vaccine efficacy and monitoring effectiveness of prevention policies.

ABCs was initially established in four states in 1995. It currently operates among ten EIP sites across the United States, representing a population of approximately 41 million persons. At this time, ABCs conducts surveillance for six pathogens: group A and group B *Streptococcus* (GAS, GBS), *Haemophilus influenzae*, *Neisseria meningitidis*, *Streptococcus pneumoniae* and methicillin-resistant *Staphylococcus aureus* (MRSA) (Centers for Disease Control and Prevention 2009a, b, c, d). ABCs data have been used to track disease trends, including the decline in pneumococcal disease following the introduction of the paediatric pneumococcal conjugate vaccine and the emergence of serogroup Y meningococcal disease. ABCs has also contributed to public health policy by providing information which formed the basis of revised CDC guidelines recommending the use of universal screening of pregnant women to prevent early onset GBS infections and the prevention of GAS infections among household contacts of persons with invasive disease and among postpartum and post-surgical patients. A programme to assist state and local health departments with surveillance for MRSA and drug-resistant *Streptococcus pneumoniae* has been developed, based primarily on lessons learned from ABCs.

A final example of governmental surveillance of bacterial resistance comes from Australia whereby the Australian government regularly publishes the Communicable Diseases Intelligence reports (Australian Government, Department of Health and Ageing 2009). The Communicable Disease Intelligence Reports range from Annual Reports describing the Australian Meningococcal Surveillance Program and reports specific to methicillin-resistant *S. aureus*. The reports also document useful surveillance summaries as well as short reports that largely describe outbreaks of dengue fever, pertussis, viral infections, Q fever and *Salmonella* outbreaks throughout the Australian continent. Concerning the meningococcal surveillance the most recent report from 2007 described of a total of 281 laboratory-confirmed cases of invasive meningococcal disease analysed almost 75% of isolates exhibited decreased susceptibility to the penicillin group of

antibiotics. Secondly, data on methicillin-resistant *S. aureus* have shown that the incidence of this phenotype significantly increased from 10.3% in 2000 to 16% in 2006. The increase was also detected throughout Australia and attributed largely to the emergence and dissemination of community-associated isolates. These data have shown that the epidemiology of methicillin-resistant *S. aureus* infections throughout Australia is evolving and rapidly changing. The emergence of the community-associated phenotype is now widely recognised in Australia and elsewhere to be of paramount importance in healthcare settings.

5 Industry/Pharmaceutical Surveillance Programmes

In addition to governmental and institutional led surveillance programmes, there are many examples of industry or pharmaceutical led surveillance programmes. While most of these are global surveillance programmes, such programmes not only report global data but also report regional (e.g., Africa, Asia, Europe, Latin America, Middle East, North America and Pacific regions) and also report on surveillance based on individual country data. In this section, a number of these programmes are highlighted in Table 2.

The Study for Monitoring Antimicrobial Resistance Trends (SMART) is an ongoing surveillance study conducted by Merck & Co. to monitor worldwide resistance trends among aerobic and facultative anaerobic Gram-negative bacilli from intra-abdominal infections (IAIs) and urinary tract infections (UTIs). SMART has been monitoring such trends in IAIs since 2002 and more recently in UTIs since 2009. SMART is a global surveillance programme designed to monitor, globally and longitudinally, the in vitro susceptibility of intra-abdominal bacterial clinical isolates collected from all units of an institution. The centres include both teaching hospitals and community hospitals and, as of 2009, comprised more than 170 hospital centres worldwide. Data from SMART has been regularly published and typically has highlighted the occurrence of antibiotic resistance in multiple species from IAIs and UTIs. Importantly, SMART reports the occurrence of ESBL-producing bacteria both globally and regionally, such as in Africa, Asia/Pacific, Europe, Latin America, Middle East and North America (Hsueh et al. 2010; Hawser et al. 2010a, b).

Furthermore, the SMART study involves the testing of multiple antibiotics including the carbapenems, cephalosporins, aminoglycosides, quinolones among others. Recent data from SMART has highlighted the steady increase in ESBL-occurrence both globally and regionally and importantly describes steadily increasing resistance rates to all study antibiotics, with the exception of the carbapenems ertapenem and imipenem whose susceptibility rates have remained high (Hoban et al. 2010; Hawser et al. 2009). In addition, molecular microbiological data have also been described from SMART, with particular emphasis in describing the molecular mechanisms associated with carbapenem resistance. SMART data can be accessed both in the public domain through publications in the peer-reviewed literature, scientific posters presented at international conferences and also through

Table 2 Examples of ongoing industry/pharmaceutical led surveillance programmes

Programme	Geography	References
SMART (The Study for Monitoring Antimicrobial Resistance Trends)	Global	Hsueh et al. (2010), Hawser et al. (2010a, b), Hoban et al. (2010), Hawser et al. (2009)
SENTRY	Global	Gales et al. (2009), Sader et al. (2004), Biedenbach et al. (2004), Andrade et al. (2008), Castanheira et al. (2010)
BSAC (British Society for Antimicrobial Chemotherapy)	United Kingdom	Morrissey et al. (2008), Farrell et al. (2008), Hope et al. (2008), Livermore et al. (2008a, b)
T.E.S.T. (Tigecycline Evaluation Surveillance Trial)	Global	Wang and Dowzicky (2010), Darabi et al. (2010), Nagy and Dowzicky (2010), Hawser (2010), Hawser et al. (2010c, d)

a licence-based internet webpage, The Surveillance Data Link Network (SDLN) (http://www.sdln.com/smart/index.aspx).

The SENTRY Antimicrobial Surveillance programme was designed to monitor antimicrobial resistance among various types of infection (Gales et al. 2009; Sader et al. 2004; Biedenbach et al. 2004; Andrade et al. 2008). Similar to SMART, SENTRY differs in that its scope covers Gram-negative and also Gram-positive bacteria. The programme initially sponsored by BMS was initiated in 1997 and today it includes more than 120 medical centres in North America, South America, Europe, Asia and Western Pacific regions. SENTRY data has been regularly reported at international scientific congresses and in the peer-reviewed literature and has described patterns of resistance in multiple Gram-positive and Gram-negative bacterial species, evolving occurrence of ESBL-producing bacteria, evolving occurrence of non-ESBL phenotypes and also reports novel molecular resistance patterns in a wide variety of human pathogens (Castanheira et al. 2010).

The British Society for Antimicrobial Chemotherapy (BSAC) reports two largely industry-funded surveillance programmes, based on pathogens from respiratory infections and on pathogens associated with bacteraemia. The programmes were initiated in response to widespread concerns about rising antimicrobial resistance in the United Kingdom in the late 1990s, and the lack of consistent long-term surveillance of resistance. The BSAC programmes differ from SMART and SENTRY in that (1) geographically SMART and SENTRY are global programmes, while BSAC is a country-wide programme, (2) the number of participating hospitals in BSAC is 25 whereas SMART and SENTRY have at least 170 and 120 centres, respectively, (3) although BSAC covers some Gram-negative and Gram-positive species, these are confined to respiratory and bacteraemia programmes only. Like SMART and SENTRY, the BSAC programmes are longitudinal programmes thus facilitating analyses of data over time and the generation of trending data over time. The respiratory programme is designed to determine the antimicrobial susceptibility of

currently circulating lower respiratory tract isolates of community-acquired *Streptococcus pneumoniae, Haemophilus influenzae* and *Moraxella catarrhalis* and hospital-acquired *S. aureus*, *Acinetobacter* spp., *E. coli, Klebsiella* spp. and *Enterobacter* spp. data of which have been reported in several peer-reviewed journals (Morrissey et al. 2008; Farrell et al. 2008). The bacteraemia programme currently determines the antimicrobial susceptibility of currently circulating bacterial isolates from clinically significant bacteraemia, typically due to *Pseudomonas aeruginosa*, staphylococci and various Enterobacteriaceae (Hope et al. 2008; Livermore et al. 2008a, b). In addition to accessing BSAC data from peer-reviewed publication, international congress presentations and reports, BSAC respiratory and bacteraemia data can be accessed by linking to http://www.bsacsurv.org/.

The Tigecycline Evaluation and Surveillance Trial (T.E.S.T.) run by Pfizer, Inc., operates globally and like SMART and SENTRY is active in all of the major regions including Africa, Asia/Pacific, Europe, Latin America, Middle East and North America. The study has been ongoing since 2004 and data from the study can be freely accessed by linking to http://www.testsurveillance.com/. T.E.S.T is designed to assess the in vitro activity of tigecycline and comparators against a range of important pathogens, from both the community and the hospital comprising isolates from multiple infection sources including various body fluids, central nervous system, cardiovascular, intestinal, genito-urinary, reproductive, respiratory and skeletal among others. As in the case of SMART and SENTRY, TEST comprises a large number of participating hospital centres, currently running at approximately 130. Similar to SENTRY, T.E.S.T covers both Gram-positive and Gram-negative pathogens. There are many peer-reviewed publications that describe T.E.S.T data. Some recent articles have described activity of tigecycline and comparators against bacteraemic Acinetobacter isolates (Wang and Dowzicky 2010), global trends in susceptibility of *H. influenzae* and *S. pneumoniae* from 2004 to 2008 (Darabi et al. 2010) and the in vitro activity of tigecycline against various anaerobes from Europe (Nagy and Dowzicky 2010; Hawser 2010). T.E.S.T also reports data on isolates producing specific mechanisms of resistance, for example the activity of tigecycline against carbapenemase-producing *A. baumannii* isolates (Hawser et al. 2010c) and carbapenem-resistant isolates of *Bacteroides fragilis* (Hawser et al. 2010d).

6 Conclusions

In conclusion, this chapter has highlighted various aspects related to the evolving issue of bacterial resistance, factors influencing the catalysis of resistance and various ongoing resistance surveillance programmes. The combination in monitoring antibiotic consumption, antibiotic-prescribing practices and resistance surveillance already greatly assist our understanding in how to manage the antibiotic resistance crisis. Ongoing surveillance programmes whether national, institutional

or industry led are essential to further monitor resistance trends and to assist in infection control and the optimization of antimicrobial therapy.

References

Andrade S, Sader HS, Barth A (2008) Antimicrobial susceptibility of Gram-negative bacilli isolated in Brazilian hospitals participating in the SENTRY Program (2003–2008). Braz J Infect Dis 12:3–9

Ang JY, Ezike E, Asmar BL (2004) Antibacterial resistance. Indian J Pediatr 71:229–239

Australian Government, Department of Health and Ageing (2009) Quarterly report. Comm Dis Intell 33:1–86

Barbosa TM, Levy SB (2000) The impact of antibiotic use on resistance development and persistence. Drug Resist Updat 3:303–311

Biedenbach DJ, Moet GJ, Jones RN (2004) Occurrence and antimicrobial resistance pattern comparisons among bloodstream infection isolates from the SENTRY Antimicrobial Surveillance Program (1997–2002) Diagn Micro. Infect Dis 50:59–69

Castanheira M, Deshpande LM, Bell JM et al (2010) Early dissemination of NDM-1 and OXA-181-producing Enterobacteriaceae in Indian Hospitals: Report from the SENTRY Antimicrobial Surveillance Program (2006–2007). Antimicrob Agents Chemother. doi:10.1128/AAC.01497-10

Centers for Disease Control and Prevention (2009a) Active Bacterial Core Surveillance Report, Emerging Infections Program Network, Group A *Streptococcus*, 2009

Centers for Disease Control and Prevention (2009b) Active Bacterial Core Surveillance Report, Emerging Infections Program Network, *Neisseria meningiditis*, 2009

Centers for Disease Control and Prevention (2009c) Active Bacterial Core Surveillance Report, Emerging Infections Program Network, *Haemophilus influenzae*, 2009

Centers for Disease Control and Prevention (2009d) Active Bacterial Core Surveillance Report, Emerging Infections Program Network, Methicillin-Resistant *Staphylococcus aureus*, 2008

Centers for Disease Control and Prevention (2010) National Antimicrobial Resistance Monitoring System for Enteric Bacteria (NARMS): Human Isolates Final Report, 2008. US Department of Health and Human Services, CDC, Atlanta, Georgia

Cizman M (2003) The use and resistance to antibiotics in the community. Int J Antimicrob Agents 21:297–307

DANMAP (2009) DANMAP 2009-Use of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, foods and humans in Denmark. ISSN 1600–2032

Darabi A, Hocquet D, Dowzicky MJ (2010) Antimicrobial activity against *Streptococcus pneumoniae* and *Haemophilus influenzae* collected globally between 2004 and 2008 as part of the Tigecycline Evaluation and Surveillance Trial. Diagn Microbiol Infect Dis 67:78–86

De Man P, Verhoeven BA, Verbrugh HA et al (2000) An antibiotic policy to prevent emergence of resistant bacilli. Lancet 355:973–978

European Centre for Disease Prevention and Control (2010) Antimicrobial resistance surveillance in Europe 2009. Annual report of the European Antimicrobial Resistance Surveillance Network (EARS-Net). ECDC, Stockholm

Farrell DJ, Felmingham D, Shackcloth J et al (2008) Non-susceptibility trends and serotype distributions among *Streptococcus pneumoniae* from community-acquired respiratory tract infections and bacteremias in the UK and Ireland, 1999 to 2007. J Antimicrob Chemother 62 (Suppl 2):ii87–ii95

Felmingham D, Grunenburg RN, The Alexander Project Group (2000) The Alexander Project 1996–1997: latest susceptibility data from this international study of bacterial pathogens from community-acquired lower respiratory tract infections. J Antimicrob Chemother 45:191–203

Frimodt-Moller N, Hammerum AM, Hessler JHR et al (2007) Global development of resistance. Dan Med Bull 54:160–162

Gales AC, Sader HS, Ribeiro J et al (2009) Antimicrobial susceptibility of Gram-positive bacteria isolated in Brazilian hospitals participating in the SENTRY program (2005–2008). Braz J Infect Dis 13:90–98

Garcia-Rey C, Martin-Herrero JE, Baquero F (2006) Antibiotic consumption and generation of resistance in *Streptococcus pneumoniae*: the paradoxical impact of quinolones in a complex selective landscape. Clin Micro Infect 12(Suppl 3):55–66

Goossens H, Ferech M, Vander Stichele R et al (2005) Outpatient antibiotic use in Europe and association with resistance: a cross-national database study. Lancet 365:579–587

Government of Canada (2010) Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) 2007. Public Health Agency of Canada

Guillemot D, Carbon C, Balkau B et al (1998) Low dosage and long treatment duration of beta-lactam: risk factors for carriage of penicillin-resistant *Streptococcus pneumonaie*. JAMA 279:365–370

Hawser SP (2010) Activity of tigecycline and comparators against recent clinical isolates of *Finegoldia magna* from Europe. Eur J Clin Microbiol Infect Dis 29:1011–1013

Hawser SP, Bouchillon SK, Hoban DJ et al (2009) Emergence of high levels of extended-spectrum-beta-lactamase-producing gram-negative bacilli in the Asia-Pacific region: data from the Study for Monitoring Antimicrobial Resistance Trends (SMART) program, 2007. Antimicrob Agents Chemother 53:3280–3284

Hawser SP, Bouchillon SK, Hoban DJ et al (2010a) Epidemiologic trends, occurrence of extended-spectrum beta-lactamase production, and performance of ertapenem and comparators in patients with intra-abdominal infections: analysis of global trend data from 2002 to 2007 from the SMART study. Surg Infect 11:371–378

Hawser SP, Bouchillon SK, Hoban DJ et al (2010b) Incidence and antimicrobial susceptibility of Escherichia coli and Klebsiella pneumoniae with extended-spectrum beta-lactamases in community- and hospital-associated intra-abdominal infections in Europe – Results of the 2008 SMART Study. Antimicrob Agents Chemother 54:3043–3046

Hawser SP, Hackel M, Person MB et al (2010c) In vitro activity of tigecycline against carbapenemase-producing *Acinetobacter baumannii*. Int J Antimicrob Agents 36:289–290

Hawser SP, Hackel M, Hoban DJ (2010d) Antibiotic susceptibility profiles of European *Bacteroides fragilis* with reduced carbapenem susceptibility. J Antimicrob Chemother 65:803–804

Hoban DJ, Bouchillon SK, Hawser SP et al (2010) Trends in the frequency of multiple drug-resistant Enterobacteriaceae and their susceptibility to ertapenem, imipenem, and other antimicrobial agents: data from the Study for Monitoring Antimicrobial Resistance Trends 2002 to 2007. Diag Microbiol Infect Dis 66:78–86

Hope R, Livermore DM, Brick G et al (2008) Non-susceptibility trends among staphylococci from bacteraemias in the UK and Ireland, 2001–2006. J Antimicrob Chemother 62(Suppl 2):ii65–ii74

Hsueh PR, Badal RE, Hawser SP et al (2010) Asia–Pacific SMART Group. Epidemiology and antimicrobial susceptibility profiles of aerobic and facultative Gram-negative bacilli isolated from patients with intra-abdominal infections in the Asia-Pacific region: 2008 results from SMART. Int J Antimicrob Agents 36:408–414

Jones RN (1996) The emergent needs for basic research, education and surveillance of antimicrobial resistance. Problems facing the report from the American Society for Microbiology Task Force on Antibiotic Resistance. Diag Micro Infect Dis 25:153–161

Jones RN (1999) Beta-lactam resistance surveillance in the Asia-Western Pacific region. Diag Micro Infect Dis 35:333–338

Levy SB (1992) The antibiotic paradox. Plenum, New York

Livermore DM, Hope R, Brick G et al (2008a) Non-susceptibility trends among *Pseudomonas aeruginosa* and other non-fermentative Gram-negative bacteria from bacteraemias in the UK and Ireland, 2001–2006. J Antimicrob Chemother 62(Suppl 2):ii55–ii63

Livermore DM, Hope R, Brick G et al (2008b) Non-susceptibility trends among Enterobacteriaceae from bacteraemias in the UK and Ireland, 2001–2006. J Antimicrob Chemother 62 (Suppl 2):ii41–ii54

Morrissey I, Maher K, Williams L et al (2008) Non-susceptibility trends amonh *Haemophilus influenzae* and *Moraxella catarrhalis* from community-acquired respiratory tract infections in the UK and Ireland, 1999–2007. J Antimicrob Chemother 62(suppl 2):ii97–ii103

Myrianthefs PM, Kalafati M, Samara I et al (2004) Nosocomial pneumonia. Crit Care Nurs Q 27:241–257

Nagy E, Dowzicky MJ (2010) In vitro activity of tigecycline and comparators against a European compilation of anaerobes collected as part of the Tigecycline Evaluation and Surveillance Trial (TEST). Scand J Infect Dis 42:33–38

Sader HS, Jones RN, Gales AC et al (2004) SENTRY antimicrobial surveillance program report: Latin America and Brazilian results for 1997 through 2001. Braz J Infect Dis 8:25–79

Sahm DF, Marsilio MK, Piazza G (1999) Antimicrobial resistance in key bloodstream bacterial isolates: electronic surveillance with the Surveillance Network Database-USA. Clin Infect Dis 29:259–263

Skalet AH, Cevallos V, Ayele B et al (2010) Antibiotic selection pressure and macrolide resistance in nasopharyngeal *Streptococcus pneumoniae*: A cluster-randomized clinical trial. PLoS Med 7:1–9

Standing Medical Advisory Committee Sub-Group on Antimicrobial Resistance (1998) The path of least resistance. Department of Health, London

SWEDRES (2009) A report on Swedish Antimicrobial Utilisation and Resistance in Human Medicine. Swedish Strategic Programme against Antibiotic Resistance (STRAMA), Stockholm

Tresoldi das Neves M, Pinto de Lorenzo ME, Almeida RAMB et al (2010) Antimicrobial use and incidence of multidrug-resistant *Pseudomonas aeruginosa* in a teaching hospital: an ecological approach. Rev Soc Brasil Med Trop 43:629–632

Vandepitte J, El-Nageh M, Tikhomirov E et al (1996) Guidelines for antimicrobial resistance surveillance; WHO Regional Publications Eastern Mediterranean Series 15. WHO Regional Office for the Eastern Mediterranean, Alexandria

Wang YF, Dowzicky MJ (2010) In vitro activity of tigecycline and comparators on *Acinetobacter* spp. isolates collected from patients with bacteremia and MIC change during the Tigecycline Evaluation and Surveillance Trial, 2004 to 2008. Diagn Microbiol Infect Dis 68:73–79

Zhang R, Eggleston K, Rotimi V et al (2006) Antibiotic resistance as a global threat: evidence from China, Kuwait and the United States. Global Health 2:6

Current and Future Challenges in the Development of Antimicrobial Agents

Robert P. Rennie

Contents

1 Introduction 46
2 Antimicrobial Resistance Overview 47
  2.1 The Problem 47
  2.2 Gram-Positive Bacteria 48
  2.3 Gram-Negative Bacteria 50
  2.4 Resistance that Requires Other Reduction Modalities 54
  2.5 Antifungal Resistance Determinants 54
3 Agents Under Development: What Are We Trying to Fix? 55
4 Models for Determining Efficacy, Safety, Pharmacokinetics and Pharmacodynamics ... 59
  4.1 Animal Models 59
  4.2 Non-mammalian Models 60
5 Antimicrobial Stewardship: Finding the Right Niche for New Antimicrobial Agents 61
6 Summary 61
References 62

Abstract Micro-organisms exist to survive. Even in the absence of antimicrobial agents, many have determinants of resistance that may be expressed phenotypically, should the need arise. With the advent of the antibiotic age, as more and more drugs were developed to treat serious infections, micro-organisms (particularly bacteria) rapidly developed resistance determinants to prevent their own demise.

The most important determinants of resistance have been in the Gram-positive and Gram-negative bacteria. Among Gram-positive bacteria, methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE) and penicillin-resistant *Streptococcus pneumoniae* (PRSP) have taxed researchers and pharmaceutical companies to develop new agents that are effective against these

R.P. Rennie (✉)
Department of Laboratory Medicine and Pathology, University of Alberta, Edmonton, AB, Canada TG6 2B7
e-mail: Robert.rennie@albertahealthservices.ca

A.R.M. Coates (ed.), *Antibiotic Resistance*, Handbook of Experimental Pharmacology 211, DOI 10.1007/978-3-642-28951-4_4,
© Springer-Verlag Berlin Heidelberg 2012

resistant strains. Among the Gram-negative bacteria, extended-spectrum beta-lactamase (ESBL) enzymes, carbapenemases (CREs) and the so-called *amp*-C enzymes that may be readily transferred between species of enterobacteriaceae and other facultative species have created multi-drug resistant organisms that are difficult to treat. Other resistance determinants have been seen in other clinically important bacterial species such as *Neisseria gonorrhoeae, Clostridium difficile, Haemophilus influenzae and Mycobacterium tuberculosis*. These issues have now spread to fungal agents of infection.

A variety of modalities have been used to stem the tide of resistance. These include the development of niche compounds that target specific resistance determinants. Other approaches have been to find new targets for antimicrobial activity, use of combination agents that are effective against more than one target in the cell, or new delivery mechanism to maximize the concentration of antimicrobial agents at the site of infection without causing toxicity to the host. It is important that such new modalities have been proved effective for clinical therapy. Animal models and non-mammalian systems have been developed to determine if new agents will reach sufficient concentrations at infection sites to predict clinical efficacy without toxicity. It will also be key to consider antimicrobial stewardship as an important component of the continuing battle to prevent the development of antimicrobial resistance.

Keywords Antimicrobial resistance • Drug development • Animal models • Stewardship

1 Introduction

In the context of antimicrobial resistance, micro-organisms, particularly bacteria, but also fungi and viruses have been on earth for hundreds of millions of years. Man and higher animals have only been around for a small number of millions of those years.

We tend to believe that because we are a higher species that we are much smarter than those miniscule beasts. In fact, their presence in our environment attests to the fact that despite a genetic code that is tiny compared to ours (4.6 Mbase pairs versus 3.2 Gbase pairs—0.1%) that they are indeed the intelligent ones.

These micro-organisms have only one necessity, and that is "survival". Nothing else matters. So their entire genome is designed to create ways and mechanisms to survive. They do so by one or more of a frightening array of mechanisms. These include, increased or decreased efflux, enzymatic inactivation, target modification, bypass, repair or amplification, sequestration, intracellular localisation or biofilm formation (Davies 2006). Sometimes they do so to their own detriment. They may create a mechanism to develop resistance to antimicrobial agents, but in doing so they become less tolerant of environmental conditions, or less adaptable to a hostile environment. They may make new enzymes to inactivate the latest antimicrobial agent, but at the expense of the ability to colonize or infect a particular body site.

This chapter is an overview of important antimicrobial resistance with a focus on how these determinants affect new antimicrobial agent development. It is not intended to delve deeply into various surveillance systems and methods. Those will vary from place to place and will also vary considerably over time. There is already a plethora of literature available. I am interested in antimicrobial resistance that has the most important effects on management of patient care. I am also interested in how best to understand that resistance and how to manage the development of new agents to prevent or slow the development of resistance.

2 Antimicrobial Resistance Overview

Development of new antimicrobial agents has largely responded to the emergence of resistance in microorganisms, rather than being proactive. Historically, even with the advent of early sulpha drugs and penicillin, the concept of drug resistance was rarely considered. When treatment of some soldiers in World War II failed, it was thought that their wounds were just too severe and unfortunately strains of staphylococci from those early days were rarely saved.

2.1 The Problem

The concept of resistance is a more recent phenomenon. So individual researchers and pharmaceutical companies began to investigate new agents largely after the so-called "horse was out of the barn". Even penicillin derivatives such as ampicillin that were developed for activity against Gram-negative bacteria such as *Escherichia coli* quickly became less effective. From strains that had been isolated from groups who had never been exposed to any antimicrobial agents, we now know that there was already resistance to a number of antimicrobials in some of these isolates (Gardner et al. 1969; D'Costa et al. 2011). What this tells us is that the appearance of naturally occurring agents in the environment, produced usually by fungi (but also by other bacteria or actinomyces) likely forced a response of commonly found bacterial species to develop resistance as a competitive survival mechanism. Clearly there must be genetic determinants already present in most micro-organisms that can be "turned on" when required. We still do not know all the underlying mechanisms, but some have been elucidated.

So let us look at the most clinically important resistance determinants that have been instrumental in the development of new antimicrobial agents. In this overview, we consider bacteria, yeast and fungi. Antiviral resistance is beyond the scope of this chapter. We will also not consider widely the various clinical indications for the various drugs mentioned, either existing or in development, but rather will investigate how resistance may affect the search and development of new, effective agents.

2.2 Gram-Positive Bacteria

2.2.1 Beta-lactam (Including Methicillin) Resistance in Staphylococci

There is ample evidence that strains of *Staphylococcus aureus* and coagulase negative staphylococci can readily develop resistance to beta-lactam antimicrobials. Penicillin resistance was observed not long after the agent was discovered, but penicillinase-producing strains rapidly became the norm. By the early 1970s, that percentage had risen to almost 90% in hospital strains, with only slightly lower rates in community isolates (Chambers 2001). Today that percentage is greater than 95%, and is unlikely to go down.

The significant change in the staphylococci was the advent of methicillin/oxacillin resistance. The first known isolate was discovered in a British hospital in 1961 (Deurenberg et al. 2007). Data gathered from some outbreaks and more sophisticated strain analysis strongly suggest that increased resistance from just a few isolates in the 1960s and 1970s to a situation where all beta-lactams have almost no activity against staphylococci is a result of global clonal spread. This indicates that these strains have a survival fitness that is capable of overcoming strains that are not resistant to the antimicrobials. This has led to the discovery of new beta-lactam agents and combinations that have activity against these strains.

2.2.2 Vancomycin Resistance in Enterococci

The mechanism of resistance to vancomycin found in enterococci involves an alteration of D-alanyl-D-alanine, in the terminal amino-acid sequence of the cell wall to D-alanyl-D-lactate. This change results in a 1,000-fold decrease in affinity for vancomycin. There is also a D-alanyl-D-serine variation that results in six-fold loss of affinity between vancomycin and the peptide. Thus, at least in vitro, differences in MIC are observed between strains that have one of these variations in terminal cell-wall peptides. It is important to understand the mechanisms by which vancomycin resistance developed in enterococcal species. Essentially there are two primary mechanisms: plasmid-mediated transfer from other bacterial species and mutational evolutionary changes in genes affecting their susceptibility.

Vancomycin-resistance was not definitively observed until the late 1980s. It is most likely that plasmids encoding vancomycin resistance were acquired from other species—possibly other streptococci or from Gram-negative bacilli. Although there are six different genes that are involved in the vancomycin resistance cascade, vanA, van B, van C, vanD, VanE and vanF, only vanA, vanB and vanC variants are important clinically. VanA and vanB strains are clearly plasmid-mediated and highly capable of moving those genes to other strains or species. Although not published (for obvious reasons) there have been laboratory attempts to mobilize these plasmids in other organisms such as *Streptococcus pneumoniae*. The genes encoding vanC resistance are intrinsic and a result of mutational events in the more downstream components of the van gene group of genetic elements. So far as we

know, these genes are not transferrable to other strains of enterococci. The vanC genes also confer a lower degree of resistance to the enterococci they inhabit, mainly *Enterococcus gallinarum* and *E. cassiliflavus*. However, it is also clear that these species harbouring vanC genes are capable of acquiring plasmids encoding the vanA gene sequence. The clinical significance of those strains is not clear, although from an epidemiological and infection control perspective, they are treated the same as the plasmid-mediating strains.

From a clinical perspective acquisition of these vancomycin resistance plasmids in the enterococci appears to confer an ability to survive for long periods of time both in the host and on fomites. Patients who acquire VRE may remain colonized for years in the gastrointestinal tract without any untoward effects (Weiss 2006). It is also clear that these strains cause significant morbidity and mortality in patients with underlying disease, and that bacteraemia in such persons often leads to a rapid demise. Like other enterococci, they do not appear to possess additional virulence factors, so the lack of available alternative therapies creates a problematic treatment scenario.

2.2.3 Other Gram-Positive Bacteria

Resistance in *Streptococcus pneumoniae*

Infections caused by *S. pneumoniae* continue to cause high morbidity and mortality. It remains the leading cause of bacterial pneumonia, and is associated with purulent meningitis, not only in young children but also in younger and middle aged adults. Mortality can be swift if diagnosis and appropriate treatment is not instituted quickly. While it is not important here to go into all the reasons for development of resistance in pneumococci, the frequency of strains resistant to penicillin and other agents, such as cephalosporins and macrolides, has been on the increase (Farrell et al. 2004, 2005; Jenkins and Farrell 2009). Even with the advent of vaccines, there are serotypes of *S. pneumoniae* (e.g., serotype 19) where resistance has emerged in spite of the vaccine use. Resistance to penicillin in *S. pneumoniae* varies considerably across the world—from about 5% in some countries in Europe to over 80% in parts of Africa and Asia. As resistance to penicillin increases so does resistance to second- and third-generation cephalosporins. In some parts of the world, strains with penicillin resistance are commonly resistant to macrolides and lincosamides (approximately 25%), to tetracycline (65%) and to co-trimoxazole (90%). Fortunately there is still little resistance to rifampin and ciprofloxacin (<5%) (Bryskier 2005) and vancomycin resistance has not been observed.

Clostridium difficile

Clostridium difficile presents a particular problem for treatment. The clinical symptomatology is associated with an enterotoxin (tcdA) and a cytolytic toxin (tcdB), but the organism must first colonize and bind in the large intestine, so the

toxin may be produced. Development of symptoms almost always occurs (at least in adults) after treatment with antimicrobial agents. Almost every antibiotic has been implicated in *C. difficile* infection. The most interesting aspect is that *C. difficile* infection may occur following treatment with agents to which the bacterium is susceptible at least in vitro. The reasons for this are still speculative, but likely involve inadequate concentration in the intestinal tract, or protection of the micro-organism by the outpouring of fluid and cells into the gut at the site of toxin production, thereby protecting the cells from antimicrobial killing.

Treatment of *C. difficile* infection has classically been either to withdraw the offending antibiotic, or to treat with either metronidazole or oral vancomycin. There are other non-antimicrobial therapies which are considered here. The problem is that up to 30% of patients who are treated with in vitro active agents fail primary therapy, so recurrence is common (author, unpublished data). Further, highly virulent strains have now been reported (the so-called NAP-1 and NAP-2 strains) that cause significant disease including toxic megacolon and death. Increasing resistance to metronidazole and vancomycin is being observed although their prevalence is under-reported because most centres only look for the presence of toxins in faeces (Karlowsky et al. 2012). It is unclear what the mechanisms of resistance to metronidazole are in these strains of *C. difficile*. Presumably vancomycin resistance is due to the same mechanisms as other Gram-positive bacteria. Other new therapies are being investigated including fidaxomicin, a novel macrocyclic antimicrobial (Venugopal and Johnson 2012), and faecal replacement treatment.

2.3 Gram-Negative Bacteria

2.3.1 Enterobacteriaceae

Extended-Spectrum Beta-Lactamases

The extended-spectrum beta-lactamases (ESBLs) are a group (and increasing in numbers of enzymes) of primarily plasmid-mediated enzymes that are capable of inhibiting a broad spectrum of cephalosporin antimicrobials. They were actually described many years ago by Richmond and colleagues (1976), but have more recently been classified according to their major substrates by Bush and colleagues (1995, 2008, 2010). Most of these enzymes have a primary target, often a serine residue in the enzyme that specifies the avidity of the enzyme for its target agent. From a mechanistic perspective, on a molar level, there will be one cephalosporin that is more actively degraded than others. Therefore, the CTX enzymes are most active against cefotaxime (Rodríguez-Baño et al. 2006). However, at achievable concentrations in the host, other cephalosporins are sufficiently inactivated so as to render them not useful clinically. This has prompted new thinking about what the breakpoints for these antimicrobial agents should be. Both international bodies that develop in vitro breakpoints for susceptibility and resistance (Clinical and

Laboratory Standards Institute in the United States [CLSI] and the European Committee on Antimicrobial Susceptibility Testing [EUCAST]) note that strains that harbour ESBLs should be considered resistant to all cephalosporins, but not to the cephamycins (e.g., cefoxitin, cefotetan, cefepime). Clinically these strains appear to be most often recovered from *E. coli* and *Klebsiella*, although other enterobacteriaceae, and even *Salmonellae* can acquire ESBLs. Urinary infections are those most often observed, but patients generally only have low-grade symptoms. Like any other bacteria that colonize the gastrointestinal tract, strains with ESBLs can cause serious infections in the compromised host. However, their virulence does not appear to be enhanced by acquiring an ESBL. Although there is no solid evidence, in most cases, these organisms do not appear to cause re-occurrence of infection following treatment with active agents. Since screening cultures are not routinely performed, unlike VRE where this is common practice in hospitals, we do not know if the ESBL strains remain in the gut for long periods of time. If they do so, purely clinical observations would suggest that they are not a significant threat in compromised patients, any more than in a patient who has undergone a routine cystoscopy.

Carbapenemases

There are a group of enzymes that inactivate carbapenem antimicrobial agents. Such agents include imipenem, meropenem, ertapenem and doripenem. They are now given a designation as CROs (carbapenem-resistant organisms). Enterobacteriaceae that carry these enzymes have recently become a scourge in hospitals. The first descriptions were in *Klebsiellla*, closely followed by *E. coli*. In some hospitals, almost all Klebsiella isolated are CRO strains (Patel et al. 2008). Where these isolates differ from ESBLs is that they cause serious infections, including bacteraemia, pneumonia or pleural infections, wounds and abscesses and urinary infections. So the acquisition of carbapenem inactivating enzyme(s) has not taken a toll on organism fitness.

These bacteria have also been capable of spreading globally, both in their primary species and in other Gram-negative bacteria. Recently, the finding of the so-called NDM-1 (New-Delhi metallo-beta-lactamase) in *E. coli* and *K. pneumoniae* in patients returning to other countries from hospitals in India has shown that these enzymes are capable not only of changing the resistance landscape, but also of causing serious infections in some patients (Yong et al. 2009). A similar observation has been made for strains of *Acinetobacter baumannii* complex isolated primarily from injured military personnel returning their home countries from the Gulf and Afghanistan wars, after initial triage either in the war zones or in triage centres in Germany. These so-called MDR strains are also resistant to carbapenems (and to most other agents, including fluoroquinolones), and also appear to be more virulent than other more susceptible strains. Other enterobacteriaceae have been shown to possess metallo-beta-lactamase enzymes that confer resistance to carbapenems, including etrapenem,

meropenem and imipenem. These are found in *Enterobacter* species (e.g., *E. cloacae*), in *Proteus*, and *Morganella*).

AmpC-Producing Enterobacteriaceae

These beta-lactam inactivating agents are more problematic clinically because they are inserted into the bacterial chromosome. They have arisen either as constitutive enzymes or as integron insertions originally of plasmid origin. The clinical problem is that they may not be expressed phenotypically until the isolate is put under antibiotic pressure, usually from exposure to a cephalosporin. From a clinical perspective, infections with such organisms often may be treated with combinations of a cephalosporin and an agent of another class, but resistance can develop rapidly in individual patients, within days (Rodríguez-Baño et al. 2012). From a practical standpoint most microbiologists and infectious diseases physicians consider these strains to be resistant to all the cephalosporins, and aztreonam. They are not normally resistant to carbapenems, but are capable of harbouring resistance determinants of these agents as well.

2.3.2 Resistance in *Pseudomonas aeruginosa*

This is a most perplexing microorganism. From an antimicrobial treatment perspective, historically it has defied drug development. From a clinical perspective, it is primarily a hospital opportunistic pathogen. Patients at risk are those with underlying compromising conditions such as haematologic cancer, diabetics and persons in burn units or intensive care units. In some conditions such as cystic fibrosis, *P. aeruginosa* is the major microorganism that confounds effective therapy.

In all these situations, treatment is first difficult because of the nature of the patients. Further, there are few antimicrobials for which *P. aeruginosa* is the main target. The classical agents have been ceftazidime, aminoglycosides (mainly tobramycin), fluoroquinolones and ureidopenicillins such as piperacillin. Not much else has shown significant clinical utility. The difficulty is not so much that the organism quickly develops resistance during treatment, but lies in the nature of the patients who are infected. What is observed is either a patient who enters one of these highly specialized units carrying a resistant strain, spreads it rapidly to other patients, or a patient who acquires a susceptible strain (e.g. in a burns unit) and the isolate develops resistance because the patient is treated for a long period of time. In these cases, the antimicrobial agent may not reach adequate bactericidal concentrations at the site of infection.

This phenomenon is illustrated by porin-mediated aminoglycoside resistance in many strains of *P. aeruginosa* (Parr and Bayer 1988). Sub-bactericidal concentrations lead to mutational changes in porin proteins in the membranes of the organism. The result is that the antibiotic cannot be transported through the cell membrane to the site of action, primarily the 30S ribosome complex resulting in

pre-translational termination. Most of these strains have minimal inhibitory concentrations that are greater than achievable peak levels, but are not at levels that would occur in a strain that has acquired a resistance plasmid. These changes to promote resistance in the isolates do not appear to affect their pathogenicity.

An additional issue for antimicrobial therapy is that many microorganisms, bacteria and yeasts in particular, exist as biofilms in infectious processes. It is estimated that in a least 60% of infections the causal agent is in a biofilm or sessile state. The ability of antimicrobials to reach the microbial cells trapped in the biofilm matrix may result in inadequate concentrations at the cell level, with resulting resistance development. Some success clinically, at least with multi-drug resistant pseudomonads, has been observed with detergent-like agents such as colistin and polymyxin so that disrupting the matrix may be important for treatment of these infections. There have also been some interesting but as yet unproven advances made with small molecule peptides that appear to have membrane detergent activity and can penetrate the biofilm matrix. Such biofilms also are important in many other bacterial and fungal infections. The issue of constructing new antimicrobial therapies to effectively treat biofilm-mediated infections must be considered an entire topic on its own.

2.3.3 Neisseria gonorrhoeae

Historically *N. gonorrhoeae* was susceptible to most agents used for treatment of primary gonococcal disease. This included penicillin, tetracyclines, extended activity cephalosporins (cefixime and ceftriaxone) and some fluoroquinolone agents (ciprofloxacin). Like many other bacterial species, either inadequate therapy, acquisition of resistance genes, or clonal spread from isolated pockets of resistant strains has led to more challenging problems in the treatment of this sexually transmitted infection. Data from many sources has clearly documented the development first of penicillin and then tetracycline resistance, to the point that these agents can no longer be considered effective for primary therapy. In recent years strains of *N. gonorrhoeae* resistant to cefixime, ceftriaxone and ciprofloxacin first appeared in Southeast Asia. These have now spread globally. Rates of resistance in some areas now approach 10–20% (Ison and Alexander 2011; Unemo and Shafer 2011; Unemo et al. 2012; Bolan et al. 2012; Katz et al. 2012). Resistance appears to be a result of step-wise mutations. Some strains show only single-step mutations such that they still appear to be susceptible—at least based on current susceptible breakpoints. However, there is increasing evidence that patients infected with such strains may fail standard therapy. In Canada, only cefixime (and ceftriaxone) have maintained their activity. Resistance to ceftriaxone is rare, but the agent is not widely used in clinics because it can only be administered parenterally. Cefixime is a better choice because it is oral and well tolerated, and resistance is still unusual despite continued heavy usage. However, as resistant strains from other parts of the world appear, these agents may no longer be effective.

2.4 Resistance that Requires Other Reduction Modalities

Antimicrobial resistance occurs in a variety of other bacterial species. Microbes associated with respiratory infections and meningitis such as *Haemophilus influenzae* and *Neisseria meningitidis* have both acquired resistance to a number of antimicrobials. Of interest, significant resistance to ampicillin developed in *H. influenzae* with rates in excess of 35–40% in many studies. In our own environment that rate is now down to approximately 10%. Reduced susceptibility to penicillin in *N. meningitidis* is also well described, and there are reports of resistance to rifampin and fluoroquinolones, but the explosion that has been observed with other bacterial species has not occurred to the same degree with this species. Both in *H. influenzae* and in *N. meningitidis*, the advent of large-scale vaccination programmes likely has a more positive effect on preventing resistance than finding new agents to combat antibiotic resistant strains. In developing countries, it will be important to advance vaccine delivery to continue to keep these resistant strains from taking hold.

Obviously most bacteria are capable of acquiring resistance mechanisms. Although it is not of minor consequence, *Mycobacterium tuberculosis* is one organism where early identification of affected patients will go a lot further to reduce the spread of resistant strains. This author expects that new drug development will be taxing. The structure of this microorganism and its snail-like pace of growth make finding suitable targets for new antimicrobial agents extremely difficult. A comprehensive approach to identification and therapy of tuberculosis is required—not just finding new agents for therapy (Zellweger 2011).

2.5 Antifungal Resistance Determinants

The number of antifungal agents is far outweighed by what is available for treatment of bacteria. There are a number of reasons for this difference. For a long time there was a lack of understanding of the role played by yeasts and moulds in infections. As treatment modalities for persons with haematologic and solid organ cancers have advanced, so have the fungi. In many studies fungaemias are now the third or fourth most common blood stream infections. Early treatment options for these infections were amphotericin B or 5-fluorocytosine. The latter had inadequate killing power on its own, and the toxicity of amphotericin B made it a difficult agent to administer without significant renal dysfunction, hypokalaemia and hepatotoxicity.

As eukaryotic microorganisms, the cell structure of the yeasts and moulds are more like mammalian cells so that membrane active agents like amphotericin B might be expected to result in greater toxicity of host cells. These organisms have beta-linked sugars that make up their cell walls and glycoproteins that are intercolated through the walls and membranes. The membrane is composed of significant concentrations of sterols, most prominently ergosterol. They have mitochondria, a

nuclear membrane and a large genome within that membrane. These differences from bacterial cells create challenges for development of new agents for treatment.

Resistance to amphotericin B in the yeasts and moulds has been infrequent, and is not easily detected in the laboratory. Clinical failure may not be due to primary resistance but to patient- or toxicity-related problems. Resistance to 5-fluorocytosine relies on mutational changes in the fungal chromosome or an inability of the agent to penetrate through the fungal cell wall (it is essential that 5-fluorocytosine is administered with another antifungal agent—usually membrane active so it can get into the cell).

The increase in infections, coupled with drug activity and toxicity issues, have prompted the development of new agents, some with different targets (Spanakis et al. 2006). New azole compounds (fluconazole, itraconazole, voriconazole and posaconazole) with sterol membrane activity were developed that had better toxicity profiles, equivalent activity to amphotericin B and in some cases activity against some genera and species that the earlier compounds lacked (Aperis and Alivanis 2011; Bowyer et al. 2011). There has also been development of a number of cell wall active agents—the echinocandins. These include caspofungin, micafungin and anidulafungin (Pitman et al. 2011). The activity of these agents is directed against the enzymes that cause the beta-linkage of cell wall carbohydrates (Pfaller 2012).

Despite these significant changes and advances in antifungal treatment options, resistance has emerged. Resistance in these microorganisms is defined by mutational changes in the chromosome. In such instances, as has occurred with bacteria, the development of resistance has been slow to emerge. Further, these strains do not tend to spread widely throughout hospitals, or the community, so that resistance tends to be focused at the individual patient level in those who are being treated with the particular agent. Ananda-Rajah and colleagues (2012) make a case for antifungal stewardship as has been suggested in many quarters to prevent development of bacterial resistance.

There are a number of mechanisms that lead to antifungal resistance. In *Candida* species, efflux pumps encoded by MDR and CDR genes are common. Point mutations in ERG 11 which encodes for target enzyme(s) for azole drugs is observed. For the echinocandin agents, point mutations in FXS genes have been found. These alterations result in increased minimal inhibitory concentrations without resolution of symptoms and poorer clinical outcomes (Pfaller 2012).

3 Agents Under Development: What Are We Trying to Fix?

The problem with antibiotic resistance is that there are only so many targets. The trick is to develop agents to which the microorganisms either cannot develop resistance or that resistance will develop slowly. When microbial enzymes are involved resistance is likely to develop quite quickly. So it makes sense to find agents that will not have that effect.

The most obvious way to construct this scenario is to avoid agents that primarily interrupt protein synthesis. The best targets should then be to destroy nucleic acid synthesis or to prevent the bacterium or fungus from pumping out the drug by efflux mechanisms. From a physiological basis targeting the microbial membrane would be a likely drug target. The other way is to slow the development of resistance—combination agents that affect more than one target site in the microorganism are more likely to have that effect.

Multi-drug resistant Gram-negative bacilli have caused clinicians and microbiologists to re-think possible agents to target these organisms (Talbot 2008). There has become a renewed interest in peptides, such as colistin and polymyxin, and in some cases these are the only agents to which these strains are at least susceptible in vitro. While colistin resistance is uncommon, investigations are ongoing to construct novel peptides that target the cell membrane in bacteria (Jiang et al. 2008, 2011) or even in yeasts (Maurya et al. 2011). From a physiological perspective it might be expected that it would be more difficult for bacteria or fungi to rapidly develop resistance to a drug that is either merely choking off the cell, or breaking up the cell membrane with detergent activity. Preliminary studies in the author's laboratory have shown that on a weight basis, a number of the candidate peptides produced by Hodges group are up to five times more active than colistin against some species of colistin-resistant Gram-negative bacilli. Further investigations are required to validate these observations, but it may well be possible to prevent resistance development with such compounds.

It might also be expected that preventing the microbial cell from producing proteins in the first place would be a suitable way to present resistance development. This approach may not work as well for fungi for two reasons. As eukaryotic cells, toxicity to the host is more likely to be an issue, and getting the drug to the target requires activity against both nuclear DNA and mitochondrial DNA. For bacteria, new fluoroquinolones are being investigated, and increasing the concentration of drug at the site of infection by inhaled delivery can prevent sub-optimal concentrations with attendant propensity for resistance.

From an activity and pharmacologic perspective, a great deal of investigation continues to develop new agents that will overcome the antimicrobial resistance issues that have been discussed earlier. For the bacteria, new agents are either under development, or in clinical trials or have recently been licenced to provide effective alternatives for treatment of infections. So agents that are pharmacologically active fall into two primary realms, new activity or enhanced delivery. Since the viability of the infecting microorganism still depends on its structure and metabolic activity, such new agents are developed to interrupt cell wall synthesis and development, cell membrane integrity, protein synthesis at various levels, nucleic acid synthesis and dynamics and metabolic activity. For the yeasts and fungi, antimicrobial activity is directed primarily against cell wall synthesis and integrity, the antifungal membrane and nucleic acid synthesis.

For those agents that affect the cell wall of bacteria, most efforts have been focused either on new cephalosporins or carbapenem agents. Additional work has been carried out on glycopeptides and lipo-glycopeptides. For an excellent treatise

on the structure, function and mode of action of many of these classes of agents, the reader is referred to the text by Bryskier (2005).

Penem and carbapenem development has centred on trying to find a niche against those microorganisms that may develop resistance to the major current licenced carbapenems, imipenem and meropenem. Drawbacks of imipenem are its requirement for a carrier (cilastatin) to delay its renal excretion. In addition imipenem and the more recent meropenem both have degrees of neurologic and renal toxicity that can reduce their effectiveness. Further, it has been well documented that isolates that acquire carbapenemases become resistant to both imipenem and meropenem. There are a small number of new penems that have been developed to try to fill this niche. These include doripenem, faropenem and a numbered compound RO-4908463.

We continue to believe that new cephalosporins may be effective to avoid the scourge of resistance. Historically, these agents were created by altering side-chains chemically—mostly to protect the beta-lactam ring from bacterial enzyme degradation. If the antimicrobial agent will not fit into the active site of the enzyme then resistance should be avoidable. So one new agent ceftobiprole, which also has a long half-life and a high $C_{\max}$ to MIC ratio, has showed some considerable promise for treatment even of methicillin-resistant staphylococci (Zhanel et al. 2008). Another cephalosporin, cefdinir, has been licenced by the FDA, and appears to have moderate activity against MRSA (Sader and Jones 2007). Time will tell if these agents maintain that activity. Another cephalosporin-like compound, ceftaroline (formerly PPI-0903), also has significant activity against MRSA and has been licenced by the FDA for a number of indications (Laudano 2011).

Continued development of glycopeptide agents with similar structure and potentially enhanced activity over vancomycin have been developed over the past few years. The most important of these are oritivancin and dalbavancin (a lipo-glycopepetide). Vancomycin has been used for many years, and has been the mainstay of activity against MRSA. Vancomycin, however, suffers from a lower degree of penetration to some sites of infection (e.g., bone, respiratory < etc.), and the result has been an apparent slow change at least in in vitro susceptibility tests (MIC creep) with concomitant clinical failures. There has also been the well-documented resistance in enterococci and some hetero-resistance in staphylococci (GISA and GRSA strains). These new agents have been developed to overcome this issue. While they appear to hold promise, additional studies on complicated skin and soft tissue infections have been requested to ensure that these agents will actually be improvements over existing agents like vancomycin. Such studies require both sufficient numbers of patients infected with MRSA and solid evidence on adverse effects.

Agents affecting protein synthesis at the level of the ribosome and at interruption of initiation or elongation of proteins have not been particularly successful. Two agents, a ketolide, cethromycin, and another topical agent, retapamulin, a member of the pleuromutilin group, which affect binding to the peptidyl transferase component of the 50S ribosomal subunit have received some attention, but only retapamulin has been licenced for topical use Jones et al. 2006). Historically, bacteria have readily developed resistance to most protein synthesis inhibitors either by preventing the agent from entering the cell (porin changes at the level

of the membrane) or by enzymatic inactivation. The aminoglycosides are good examples where both mechanisms have led to impaired activity. Only those agents like amikacin where the primary inactivation occurs by 6′-acetyl transferase are less susceptible to resistance. One new aminoglycoside, plazomicin, has been developed which appears to have increased activity over amikacin and gentamicin against problematic Gram-negative bacilli (Sutcliffe 2011). Further studies are required to determine whether this or other aminoglycosides (e.g., isepamin) will have long-term success against resistant bacterial species (Tsai et al. 2007).

The development over the past decades of new agents affecting bacterial nucleic acid synthesis, particularly the fluoroquinolones, has made an important contribution both to our understanding of the physiology of drug resistance in the bacterial cell, and to the potential for therapeutic response by interrupting bacterial growth at the level of the primary gene. These agents affect DNA gyrase, such that the helix is unable to uncoil during synthesis. The most important aspect of these agents is that resistance results from mutation rather than enzyme inactivation. Thus, resistance may develop more slowly in many bacterial species. Two new agents, garenoxacin and prulifloxacin, both fluoroquinolones, have shown promise for treatment of other quinolone resistant strains. Neither have been licenced in most parts of the world, although use for urinary tract infections and gastroenteritis in Southeast Asia has identified that they may be effective—at least in these infections. Another agent (UB-8902), a piperazine ciprofloxacin derivative, appears to have activity against MDR-*Acinetobacter baumannii* that are resistant to ciprofloxacin (López-Rojas et al. 2011)

The final place to look for new activity is the metabolic activity of the bacterial cell. One new agent, iclaprim, which affects dihydrofolate reductase, in the same pathway as trimethoprim and sulfamethoxazole, appears to have good activity against some co-trimoxazole resistant strains of Gram-negative bacteria. Whether it will survive in the long term is still uncertain.

So the search continues for new agents, for niche compounds that will provide a better mousetrap for specific antimicrobial resistant bacteria. Often in clinical infections single agents to which the organism may be susceptible are not effective. Examples where it is common to use multiple agents are for *P. aeruginosa* infections, in enterococcal sepsis, in tuberculosis and in certain Gram-negative infections (e.g., those caused by SPICEM organisms). The underlying premise is to attack more than one site of activity in or on the bacterial cell. For *P. aeruginosa* infections often an aminoglycoside such as tobramycin is added to a third-generation cephalosporin such as ceftazidime. For bacteria of the SPICEM group (*Serratia*, indole-positive *Proteus, Citrobacter, Enterobacter* and *Morganella*), most recommendations suggest that use only of a third-generation cephalosporin may well result in the development of resistance during treatment even though the isolate may initially test as susceptible in the laboratory.

In this regard one area which is beginning to receive more attention is in the development of combination antibacterials (Talbot et al. 2006). The concept is exciting. If a single administration of a drug which has two sites of activity can effect inhibition or killing of the infecting organism, then resistance should readily be delayed or prevented. One agent combining rifamycin with a fluoroquinolone (CBR-2092) has shown some promise. Another agent (EDP-420) which is a

bicyclolide has been shown to have broad activity in respiratory infections, with potential for reduced resistance development. One agent (CSA-13) which combines a sterol backbone with an antimicrobial active peptide has activity against many strains of MRSA. Other agents consist of heteropolymers. By attaching specific antibodies to a heteropolymer backbone, or by coupling existing agents to other non-drug moieties to target hard to reach infections sites (e.g., bone) that the potential is to clear the pathogen either by antibody binding and clearance or higher effective concentrations. In all these cases there are major pharmacologic issues that determine their effectiveness in clinical infections. First will be that the combined agent is not too complex to get to the site(s) of action in the microbial cell. Second will be that the combination agent is at least no more toxic to the host than the single agents that make up the drug. Third will be that the pharmacokinetics of the combined agent are suitable for human therapy. The promise of little or no resistance development must be tempered by all the issues that may well prevent such combination drugs from clearing all the hurdles to achieve a place in the market.

4 Models for Determining Efficacy, Safety, Pharmacokinetics and Pharmacodynamics

4.1 Animal Models

Unfortunately it is still essential to examine new antimicrobial agents for their efficacy, safety and pharmacokinetic/pharmacodynamic (PK/PD) properties in animal models. Some aspects of pharmacodynamics can be learned from patient simulations (Monte Carlo simulations) once the kinetics of the agent have been worked out, and the MICs are known, but it still requires that probable efficacy and safety are determined in animal models.

There is still no substitute for the animal model. The problem is determining if the animal model actually reflects what is likely to occur in the human host.

There are a few standard models that are used. Some are used because they are easy to quantitate. However, whether they reflect human efficacy may be open to question. An example would be determining the inhibitory effect of agents on organisms such as *S. aureus* or *S. pneumoniae*, and studying the pharmacokinetics of activity using a neutropaenic mouse thigh model (Andes and Craig 2002; Craig 1998, 2001; Andes et al. 2002). It is recognized that non-neutropaenic animals are not easily infected. However, it should also be identified that *S. pneumoniae* does not readily cause skin and soft tissue infections. So while treatment in this model shows that reduction in organism load follows the appropriate pharmacokinetic parameters, that is, time of drug over MIC at appropriate doses, reductions in organism load that support particular dosing regimens may not be sufficient in some instances to clear the organism from sites where the bacterium actually causes infections. Bast et al. (2004) used a respiratory infection model to study the efficacy of antimicrobial activity against *S. pneumoniae*. In this setting, they used surface temperatures to predict outcome and antimicrobial efficacy.

Disease severity was measured prior to and during treatment. Their model correlated with PK/PD and outcomes in moderate and severe pneumonia at least in the animal model. This type of methodology offers the potential for to correlate similar criteria in human infections.

Animal models to study new agents need to reflect the site of infection where the agent will be targeted to fight a particular species or genera of bacteria. The ability of the agent to reach the site of infection may not be identified if the wrong animal model is used. The same applies to how the infection is established in the animal model. So just as a model to test a respiratory infection agent should use a respiratory animal model, so the animal model should reflect how the agent is distributed. There are many examples of how animal models are used that make the agent appear to be effective but may be less effective at the site of infection. One such example is treatment of *Aspergillus* with an echinocandin and amphotericin B. In this experimental model, *Aspergillus* is inoculated intravenously. The highest concentrations of the fungus are found in the kidney (Wasan et al. 2007). As the drugs are excreted renally, efficacy appeared to be excellent. Unfortunately renal aspergillosis is uncommon, and the fungus was not distributed as widely in lung. Models of *Aspergillus* efficacy need to be devised using the lung as a target, and ensuring that sufficient concentrations of conidia are inoculated so that significant decreases in organism load can be observed following therapy.

The other aspect of pharmacology and efficacy that is often overlooked is the animal that is used. Mice for example offer an easy and relatively inexpensive model for identifying antimicrobial PK/PD and efficacy, but their immune system and antigen-processing cells, may not reflect that found in humans or other animal model species (den Braber et al. 2012). While this may not directly affect the short-term pharmacokinetic response to an antimicrobial agent in an artificially induced infection (e.g. the thigh model), it is important to consider carefully how these models are assessed for efficacy, safety and PK/PD parameters as the new agents are moved from the animal models to human testing (Schwartz-Cornil et al. 2011; Fabrino et al. 2011).

4.2 Non-mammalian Models

Some of the issues identified in using mammalian animal models to study antimicrobial efficacy support searches for alternative methods to identify activity, to study effects of antigen-processing cells on pharmacodynamics and to better understand how resistance develops. It is also possible to create perfusion-type cells where nutrients and other cellular components can be introduced to investigate their effects on the activity of the antimicrobial agents. This scientific arena is in its infancy, but there are reports for example of investigating the effects of antimicrobials on bacteria growing in macrophages to study specific disease processes (Subramanian et al. 2008). Others (Mulcahy et al. 2011) have used insects to study infectious processes and the effects of antimicrobial agents.

5 Antimicrobial Stewardship: Finding the Right Niche for New Antimicrobial Agents

There is no doubt that antimicrobial resistance is the major driver for the discovery of new agents. We have identified where the current stress points are. The most important issue is to stay ahead of these organisms.

There are a number of ways to accomplish this. One way is to restrict usage of current antimicrobials. There are a few examples. One might be amikacin. Many pharmacies do not use amikacin. This aminoglycoside is not affected to the same degree by the same aminoglycoside-modifying enzymes as others such as tobramycin and gentamicin. By restricting its usage, resistance rates for amikacin are much lower than the other two agents. It is not well understood what would happen if amikacin became the primary aminoglycoside of choice.

The polymyxins (polymyxin B and colistin) also have reduced levels of resistance. One reason is the potential for increased toxicity. The other is that they have been considered last resort agents. With increased resistance in *Pseudomonas*, and the appearance of other multi-resistant Gram-negatives such as MDR *Acinetobacter* species, colistin and polymyxin B in some cases are the only options. Fortunately resistance is still low, but their increased use may well result in the appearance of pan-resistant strains.

Another way to combat resistance is to cycle current antimicrobials or to use agents in combination instead of single agents. Resistance may also develop readily if single agents are used empirically to treat serious infections. *P. aeruginosa* infections treated only with a cephalosporin may result in resistance. Gram-negatives such as *Enterobacter* species with inducible beta-lactamases can readily develop resistance during therapy if combinations with different modes of action are not used together for treatment. There is some evidence that combination therapy may be changed once antimicrobial susceptibilities are known (Boyd and Nailor 2011) but further studies are needed to determine if this will hold for most bacterial infections.

Enterococci are classic bacteria that require multiple agents for therapy in serious infections. Treatment with an aminoglycoside and a beta-lactam or vancomycin is commonly used to prevent the development of resistance in these microorganisms.

6 Summary

Therefore, finding the right niche for treatment is imperative so that we do not rapidly lose the new agents that are developed. This will involve a whole range of strategies. It is imperative that we are able to identify the agents causing infection quickly, and that local antibiograms can determine the degree of resistance to these agents. There are new technologies such as MALDI-TOF (matrix-assisted desorption/ ionisation—time of flight) which allow identification after primary isolation within

about 2 h. Such rapid identification may be helpful to get patients on the right antimicrobials earlier. Local antibiograms are essential so that treating clinicians have the most up to date information on the patterns of resistance in their institutions. It is also imperative that physicians practice antimicrobial stewardship, treating infections under the correct circumstances, and only when necessary (Diazgranados 2011). Only when these multiple approaches are practiced will we be able to overcome the pervasive increase in resistance that has occurred over the past several decades. Pharmaceutical interests must also review their strategies for new agents so that monies are spent wisely. If it requires a half a billion pounds to bring a new agent to market, then it is imperative that the physiology of the microorganism, the pharmacokinetics/dynamics of the drug–bug combination are considered, and that the appropriate steps are undertaken to ensure that the agents are brought to market and are used wisely.

References

Ananda-Rajah MR, Slavin MA, Thursky KT (2012) The case for antifungal stewardship. Curr Opin Infect Dis 25:107–115

Andes D, Craig WA (2002) Animal model pharmacokinetics and pharmacodynamics: a critical review. Int J Antimicrob Agents 19:261–268

Andes D, van Ogtrop ML, Peng J, Craig WA (2002) In vivo pharmacodynamics of a new oxazolidinone (linezolid). Antimicrob Agents Chemother 46:3484–3489

Aperis G, Alivanis P (2011) Posaconazole: a new antifungal weapon. Rev Recent Clin Trials 6:204–219

Bast D, Yue M, Chen X, Bell D, Dresser L, Saskin R, Mandell LA, Low DE, de Azavedo JCS (2004) Novel murine model of pneumococcal pneumonia: use of temperature as a measure of disease severity to compare the efficacies of moxifloxacin and levofloxacin. Antimicrob Agents Chemother 48:3343–3348

Bolan GA, Sparling PF, Wasserheit JN (2012) The emerging threat of untreatable gonococcal infection. N Engl J Med 366:485–487

Bowyer P, Moore CB, Rautemaa R, Denning DW, Richardson MD (2011) Azole antifungal resistance today: focus on *Aspergillus*. Curr Infect Dis Rep 13:485–491

Boyd N, Nailor MD (2011) Combination antibiotic therapy for empiric and definitive treatment of gram-negative infections: insights from the Society of Infectious Diseases Pharmacists. Pharmacotherapy 31:1073–1084

Bryskier A (2005) Epidemiology of resistance to antimicrobial agents. In: Bryskier A (ed) Antimicrobial agents: antibacterials and antifungals. ASM, Washington, DC, pp 39–92

Bush K, Jacoby GA, Medeiros AA (1995) A functional classification scheme for beta- A functional classification scheme for beta-lactamases and its correlation with molecular structure. Antimicrob Agents Chemother 39:1211–1233

Bush K (2008) Extended-spectrum beta-lactamases in North America, 1987–2006. Clin Microbiol Infect 14(Suppl 1):134–143

Bush K, Jacoby GA (2010) Updated functional classification of beta-lactamases. Antimicrob Agents Chemother 2010(54):969–976

Chambers HF (2001) The changing epidemiology of *Staphylococcus aureus*. Emerg Infect Dis 7:178–182

Craig WA (1998) Pharmacokinetic/pharmacodynamic parameters: rationale for antibacterial dosing of mice and men. Clin Infect Dis 26:1–10

Craig WA (2001) Does the dose matter? Clin Infect Dis 15(Suppl 3):S233–S237

Davies J (2006) Where have all the antibiotics gone? Can J Infect Dis Med Microbiol 17:287–290

D'Costa VM, King CE, Kalan L, Morar M, Sung WW, Schwarz C, Froese D, Zazula G, Calmels F, Debruyne R, Golding GB, Poinar HN, Wright GD (2011) Antibiotic resistance is ancient. Nature 477:457–461

den Braber I, Mugwagwa T, Vrisekoop N, Westera L, Mögling R, Bregje de Boer A, Willems N, Schrijver EH, Spierenburg G, Gaiser K, Mul E, Otto SA, Ruiter AF, Ackermans MT, Miedema F, Borghans JA, de Boer RJ, Tesselaar K (2012) Maintenance of peripheral naive T cells is sustained by thymus output in mice but not humans. Immunity 36:288–297

Deurenberg RH, Vink C, Kalenic S, Friedricjh AW, Bruggeman CA, Stobbeeringh EE (2007) The molecular evolution of methicillin-resistant *Staphylococus aureus*. Clin Microbiol Infect 13:222–235

Diazgranados CA (2011) Prospective audit for antimicrobial stewardship in intensive care: Impact on resistance and clinical outcomes. Am J Infect Control. 2011 Sep 20. Epub ahead of print. In press

Fabrino DL, Leon LL, Genestra M, Parreira GG, Melo RC (2011) Rat models to investigate host macrophage defense against *Trypanosoma cruzi*. J Innate Immun 3:71–82

Farrell DJ, Jenkins SG, Brown SD, Patel M, Lavin BS, Klugman KP (2005) Emergence and spread of *Streptococcus pneumoniae* with erm(B) and mef(A) resistance. Emerg Infect Dis 11:851–858

Farrell DJ, Morrissey I, Bakker S, Morris L, Buckridge S, Felmingham D (2004) Molecular epidemiology of multiresistant *Streptococcus pneumoniae* with both erm(B)- and mef(A)-mediated macrolide resistance. J Clin Microbiol 42:764–768

Gardner P, Smith DH, Beer H, Moellering RC (1969) Recovery of resistance (R) factors from a drug-free community. Lancet 294:774–776

Ison CA, Alexander S (2011) Antimicrobial resistance in *Neisseria gonorrhoeae* in the UK: surveillance and management. Expert Rev Anti Infect Ther 9:867–876

Jenkins SG, Farrell DJ (2009) Increase in pneumococcus macrolide resistance, United States. Emerg Infect Dis 15:1260–1264

Jiang Z, Vasil AI, Hale JD, Hancock RE, Vasil ML, Hodges RS (2008) Effects of net charge and the number of positively charged residues on the biological activity of amphipathic alpha-helical cationic antimicrobial peptides. Biopolymers 90:369–383

Jiang Z, Vasil AI, Gera L, Vasil ML, Hodges RS (2011) Rational design of α-helical antimicrobial peptides to target Gram-negative pathogens, Acinetobacter baumannii and Pseudomonas aeruginosa: utilization of charge, 'specificity determinants', total hydrophobicity, hydrophobe type and location as design parameters to improve the therapeutic ratio. Chem Biol Drug Des 77:225–240

Jones RN, Fritsche TR, Sader HS, Ross JE (2006) Activity of retapamulin (SB-275833), a novel peuromutilin, against selected resistant gram-positive cocci. Antimicrob Agents Chemother 50:2583–22586

Karlowsky J, Zhanel G, Hammond G, Rubinstein E, Wylie J, Du T, Mulvey M, Alfa M (2012) Multidrug-resistant, NAP2 Clostridium difficile was the predominant toxigenic, hospital-acquired strain in the Province of Manitoba, Canada in 2006–2007. J Med Microbiol 61(3): 693–700

Katz AR, Komeya AY, Soge OO, Kiaha MI, Lee MV, Wasserman GM, Maningas EV, Whelen AC, Kirkcaldy RD, Shapiro SJ, Bolan GA, Holmes KK (2012) Neisseria gonorrhoeae with high-level resistance to azithromycin: case report of the first isolate identified in the United States. Clin Infect Dis 54:841–843

Laudano JB (2011) Ceftaroline fosamil: a new broad-spectrum cephalosporin. J Antimicrob Chemother 66(Suppl 3):11–18

López-Rojas R, Sánchez-Céspedes J, Docobo-Pérez F, Domínguez-Herrera J, Vila J, Pachón J (2011) Pre-clinical studies of a new quinolone (UB-8902) against *Acinetobacter baumannii* resistant to ciprofloxacin. Int J Antimicrob Agents 38:355–359

Maurya IK, Pathak S, Sharma M, Sanwal H, Chaudhary P, Tupe S, Deshpande M, Chauhan VS, Prasad R (2011) Antifungal activity of novel synthetic peptides by accumulation of reactive oxygen species (ROS) and disruption of cell wall against *Candida albicans*. Peptides 32 (8):1732–1740

Mulcahy H, Sibley CD, Surette MG, Lewenza S (2011) *Drosophila melanogaster* as an animal model for the study of *Pseudomonas aeruginosa* biofilm infections in vivo. PLoS Pathog 7:e1002299

Parr TR Jr, Bayer AS (1988) Mechanisms of aminoglycoside resistance in variants of *Pseudomonas aeruginosa* isolated during treatment of experimental endocarditis in rabbits. J Infect Dis 158:1003–1010

Patel G, Huprikar S, Factor SH, Jenkins SG, Calfee DP (2008) Outcomes of carbapenem-resistant Klebsiella pneumoniae infection and the impact of antimicrobial and adjunctive therapies. Infect Control Hosp Epidemiol 29:1099–2106

Pfaller MA (2012) Antifungal drug resistance: mechanisms, epidemiology, and consequences for treatment. Am J Med 125(Suppl):S3–13

Pitman SK, Drew RH, Perfect JR (2011) Addressing current medical needs in invasive fungal infection prevention and treatment with new antifungal agents, strategies and formulations. Expert Opin Emerg Drugs. Aug 17 Epub ahead of print

Richmond MH (1976) The presence of a variant of type-IIIa beta-lactamase in a series of strains isolated in a burns unit. J Med Microbiol 9:363–364

Rodríguez-Baño J, Navarro MD, Romero L, Muniain MA, de Cueto M, Ríos MJ, Hernández JR, Pascual A (2006) Bacteremia due to extended-spectrum beta-lactamase-producing *Escherichia coli* in the CTX-M era: a new clinical challenge. Clin Infect Dis 431:407–414

Rodríguez-Baño J, Miró E, Villar M, Coelho A, Gozalo M, Borrell N, Bou G, Conejo MC, Pomar V, Aracil B, Larrosa N, Agüero J, Oliver A, Fernández A, Oteo J, Pascual A, Navarro F (2012) Colonisation and infection due to Enterobacteriaceae producing plasmid-mediated AmpC β-lactamases. J Infect 64:176–183

Sader HS, Jones RN (2007) Cefdinir: an oral cephalosporin for the treatment of respiratory tract infections and skin and skin structure infections. Expert Rev Anti Infect Ther 5:29–43

Schwartz-Cornil I, Bonneau M, Dalod M, Bertho N (2011) Impact of large mammals models in immunology. Reprod Fertil Dev 24:287–288

Spanakis EK, Aperis G, Mylonakis E (2006) New agents for the treatment of fungal infections: clinical efficacy and gaps in coverage. Clin Infect Dis 43:1060–1068

Subramanian S, Roberts CL, Hart CA, Martin HM, Edwards SW, Rhodes JM, Campbell BJ (2008) Replication of colonic Crohn's disease mucosal *Escherichia coli* isolates within macrophages and their susceptibility to antibiotics. Antimicrob Agents Chemother 52:427–434

Sutcliffe JA (2011) Antibiotics in development targeting protein synthesis. Ann N Y Acad Sci 1241:122–152

Talbot GH, Bradley J, Edwards JE Jr, Gilbert D, Scheld M, Bartlett JG (2006) Bad bugs need drugs: an update on the development pipeline from the antimicrobial availability task force of the Infectious Diseases Society of America. Clin Infect Dis 42:657–668

Talbot GH (2008) What is the pipeline for Gram-negative pathogens? Expert Rev Anti Infect Ther 6:39–49

Tsai TY, Chang SC, Hsueh PR, Feng NH, Wang JT (2007) In vitro activity of isepamicin and other aminoglycosides against clinical isolates of Gram-negative bacteria causing nosocomial bloodstream infections. J Microbiol Immunol Infect 40:481–486

Unemo M, Shafer WM (2011) Antibiotic resistance in *Neisseria gonorrhoeae*: origin, evolution, and lessons learned for the future. Ann N Y Acad Sci 1230:E19–28

Unemo M, Golparian D, Nicholas R, Ohnishi M, Gallay A, Sednaoui P (2012) High-level cefixime- and ceftriaxone-resistant *Neisseria gonorrhoeae* in France: Novel penA mosaic allele in a successful international clone causes treatment failure. Antimicrob Agents Chemother 56:1273–1280

Venugopal AA, Johnson S (2012) Fidaxomicin: a novel marcocyclic antibiotic approved for treatment of *Clostridium difficile* infection. Clin Infect Dis 54:568–574

Wasan KM, Sivak O, Rosland M, Risovic V, Bartlett K (2007) Assessing the antifungal activity, pharmacokinetics, and tissue distribution of amphotericin B following the administration of Abelcet and Am Bisome in combination with caspofungin to rats infected with Aspergillus fumigatus. J Pharm Sci 96:1737–1747

Weiss K (2006) Vancomycin-resistant enterococci: the value of infection control and antibiotic control policy. Can J infect Dis Med Microbiol 17(Suppl):9B–12B

Yong D, Toleman MA, Giske CG, Cho HS, Sundman K, Lee K, Walsh TR (2009) Characterization of a new metallo-beta-lactamase gene, bla(NDM-1), and a novel erythromycin esterase gene carried on a unique genetic structure in *Klebsiella pneumoniae* sequence type 14 from India. Antimicrob Agents Chemother 53:5046–5054

Zellweger JP (2011) Multidrug resistant tuberculosis—its extent, hazard and possible solutions. Rev Mal Respir 28:1025–1033

Zhanel GG, Lam A, Schweizer F, Thomson K, Wlakty A, Rubinstein E, Gin AS, Hoban DJ, Noreddin AM, Karlowsky JA (2008) Ceftribiprole: a review of a braod-spectrum and anti-MRSA cephalosporin. Am J Clin Dermatol 9:245–264

The Role of the Outer Membrane of Gram-negative Bacteria in Antibiotic Resistance: Ajax' Shield or Achilles' Heel?

Malcolm G.P. Page

Contents

1 Introduction 68
2 Lipopolysaccharide 68
3 Peptidoglycan 77
4 Porins, Efflux and Other Transport Proteins of the Outer-Membrane 81
References 82

Abstract There has been an enormous increase in our knowledge of the fundamental steps in the biosynthesis and assembly of the outer membrane of Gram-negative bacteria. Lipopolysaccharide is a major component of the outer membrane of Gram-negative bacteria as is peptidoglycan. Porins, efflux pumps and other transport proteins of the outer membrane are also present. It is clear that there are numerous essential proteins that have the potential to be targets for novel antimicrobial agents. Progress, however, has been slow. Much of the emphasis has been on cytoplasmic processes that were better understood earlier on, but have the drawback that two penetration barriers, with different permeability properties, have to be crossed. With the increased understanding of the late-stage events occurring in the periplasm, it may be possible to shift focus to these more accessible targets. Nevertheless, getting drugs across the outer membrane will remain a challenge to the ingenuity of the medicinal chemist.

Keywords Gram-negative bacteria • Outer membrane • Lipopolysaccharide • Lipid A • Peptidoglycan • Transglycosylase • Transpeptidase • Protein secretion

M.G.P. Page (✉)
Basilea Pharmaceutica International Ltd, Grenzacherstrasse 487, CH-4058 Basel, Switzerland
e-mail: malcolm.page@basilea.com

A.R.M. Coates (ed.), *Antibiotic Resistance*, Handbook of Experimental Pharmacology 211, DOI 10.1007/978-3-642-28951-4_5,
© Springer-Verlag Berlin Heidelberg 2012

1 Introduction

The defining characteristic of Gram-negative bacteria is provided by the outer layers of the cell, whose chemical composition underlies the differential response to the Gram staining technique. Gram-negative bacteria have an outer membrane covering a thin peptidoglycan layer: the outer membrane is rich in negative charges that bind the positively charged dye (crystal violet) used in the first step of the Gram procedure (Davies et al. 1983). However, the outer membrane readily dissolves in the ethanolic wash of the second staining step and, thus, the bacteria are more rapidly de-stained than the Gram-positive bacteria, whose accessible, thick layer of peptidoglycan traps precipitated dye. This simple staining technique, originally developed as a way to detect bacteria in clinical samples (Gram 1884), provides insights into the structure and function of the Gram-negative outer membrane, which has an essential role in protecting the bacteria against harmful substances and also contributes greatly to the armamentarium of the organism that can be developed in acquired mechanisms of resistance. Ajax carried a multi-layered shield made from his seven bull's hides capped with bronze, an impenetrable barrier that saw him unscathed through the Trojan War. Achilles, on the other hand, benefitted from his near complete immersion in the River Styx, which was supposed to offer powers of invincibility, until Paris hit his heel, the only unprotected spot on his body, with an arrow. It is in the hope of finding such points of vulnerability in the shield provided by the outer membrane that there has been considerable effort expended in searching for new targets in the biogenesis of the outer layers of Gram-negative bacteria. In this review, the structure and biosynthesis of the components of the outer layers of the Gram-negative bacteria will be discussed in respect of their role in antibiotic susceptibility and resistance as well as the opportunities they provide for discovery of new antimicrobial agents.

2 Lipopolysaccharide

Lipopolysaccharide (LPS) is a major component of the outer membrane of Gram-negative bacteria (Wilkinson 1996). It contributes significantly to the stability of the outer membrane and it functions as a barrier to harmful molecules such as antibiotics. LPS is comprised of three parts: the O-antigen polysaccharide, the core saccharide and lipid A (Fig. 1).

The O-antigen is an important constituent of the LPS molecule: it acts as a binding site for bacteriophages and proteins of host immune response, may play a role in attachment during colonization (Fletcher et al. 1993; Thuruthyil et al. 2001) and the long glycan chains may contribute to the formation of unstirred layers that limit the diffusion of solutes close to the outer membrane. It comprises a number of repeats of an oligosaccharide, the O-antigen unit, that has between two and six sugar residues (Reeves et al. 1996). The O-antigen unit varies extensively between

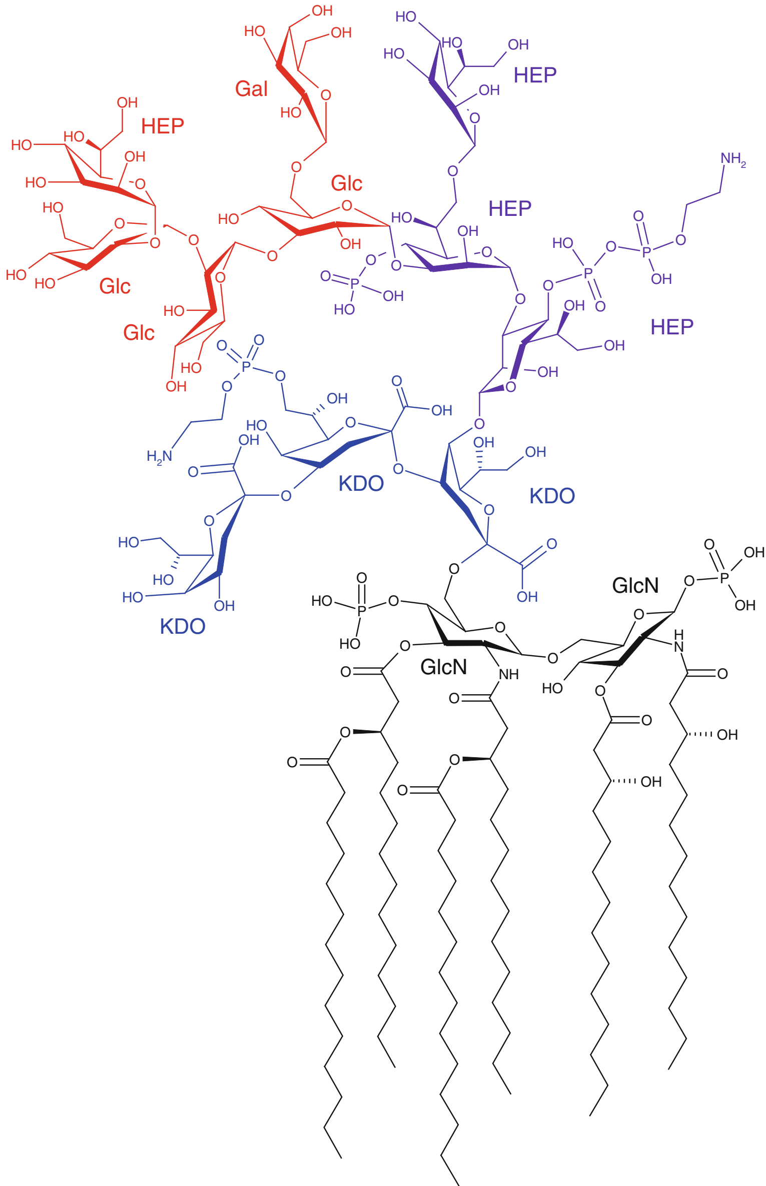


Fig. 1 Structure of the LPS molecule of *E. coli* K-12, which lacks the O-antigen polysaccharide. The lipid A component is shown in *black*, the 3-deoxy-D-manno-oct-2-ulosonic acid (KDO) component of the inner core in blue and the L-glycero-D-manno-heptose (HEP) units in purple; the outer core units are shown in red

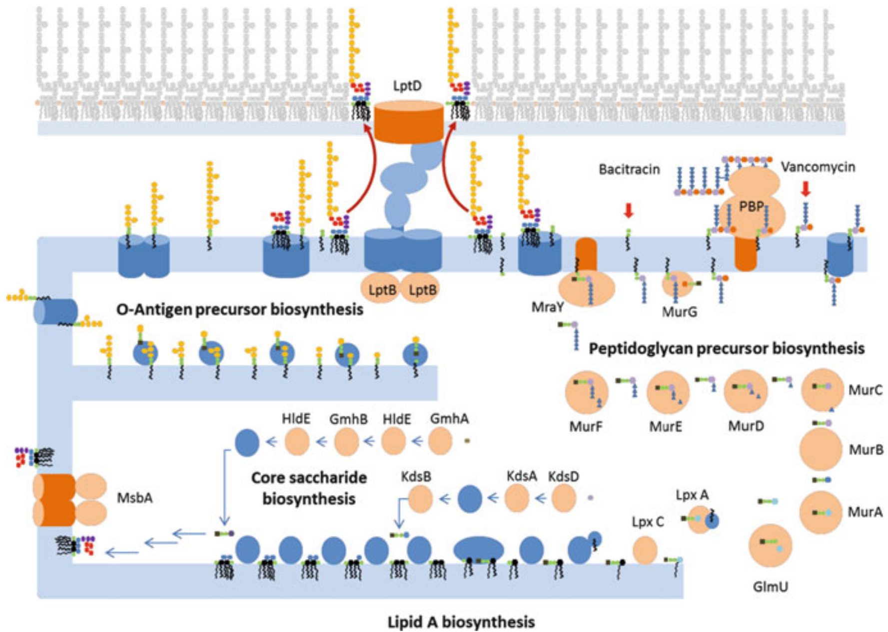


Fig. 2 Schematic representation of the assembly of the outer membrane and peptidoglycan, based on the pathways elucidated for *E. coli*. Proteins that have been studied as potential targets for antimicrobials are named and highlighted in orange

strains in the nature, order and linkage of the different sugars within the oligosaccharide. For example, there are one hundred and eighty-six O-antigens documented in *Escherichia coli* (including *Shigella*) and fifty-four in *Salmonella enterica* (Lior 1994; Popoff and Minor 1997), with only three of these being identical in the two species: *E. coli* O55 and *S. enterica* O50, *E. coli* O111 and *S. enterica* O35, and *E. coli* O157 and *S. enterica* O30 (Kenne et al. 1983; Lindberg et al. 1981; Perry et al. 1986). So far, twenty O-antigen structures have been recognized in *Pseudomonas aeruginosa* (Raymond et al. 2002; Kintz and Goldberg 2008). The majority of the O-antigens found in *E. coli* and all known *P. aeruginosa* O-antigens are heteropolymers, whereas almost all *Klebsiella pneumoniae* O-antigens are homopolymers.

The initial steps in biosynthesis of the O-antigen (Fig. 2) take place in the cytoplasm, where the O-antigen repeat oligosaccharide unit is assembled on a bactoprenol diphosphate carrier in the inner leaflet of the cytoplasmic membrane by a series of glycosyltransferases from the appropriate nucleotide diphosphate-activated sugars (Samuel and Reeves 2003). The completed O-antigen precursor is transferred to the outer leaflet of the cytoplasmic membrane predominantly through the action of the Wzx protein, which is believed to function as a flippase (Liu et al. 1996). Other precursor translocases have been identified and are either Wzx-like flippases or ATP-binding cassette transporters (Hug and Feldman 2011). The latter can consist either of two polypeptides, Wzt and Wzm (Reizer et al. 1992; Kido et al.

1995), or of a single polypeptide homologous to the *Campylobacter jejuni* PglK (Kelly et al. 2006). The O-antigen precursor units are then polymerized through the action of Wzy protein (Yamamoto et al. 1999) and Wzz, the chain length regulator protein (Batchelor et al. 1992; Bastin et al. 1993).

Mutant strains that are defective in the synthesis of the O-antigen have increased susceptibility towards extreme environmental conditions, such as pH (Chen et al. 1993; McGowan et al. 1998; Thomsen et al. 2003; Martinic et al. 2011), and to exposure to antibiotics (Nikaido 1976; Palomar et al. 1995; Banemann et al. 1998; Caroff and Karibian 2003). They may also be considerably less virulent in animal models of infection (Helena Mäkelä et al. 1973). As yet, however, it does not appear that any of the dedicated proteins in the biosynthetic pathway have been targeted as a means for modulating virulence.

The core saccharide is rather more conserved among Gram-negative bacteria than is the O-antigen structure. Two domains are generally recognized (Wilkinson 1996): the inner core, comprising 3-deoxy-D-manno-oct-2-ulosonic acid (KDO) and L-*glycero*-D-*manno*-heptose (HEP) units, and the outer core comprising mostly hexoses (Fig. 1). Only the KDO units of the inner core are essential for bacterial growth, although mutants with this "deep-rough" phenotype have a hyperpermeable outer membrane, exhibit increased susceptibility towards antibiotics such as erythromycin, novobiocin and rifampicin and have decreased virulence in animal models of infection (Nikaido and Vaara 1985; Nikaido 2003).

The biosynthesis of the HEP units takes place in the cytoplasm (Fig. 2) and starts with the synthesis of sedoheptulose 7-phosphate by TktA from fructose 6-phosphate and ribose-5-phosphate (Kneidinger et al. 2001; Valvano et al. 2002). Sedoheptulose 7-phosphate is then isomerized to D-*glycero*-D-*manno*-heptose 7-phosphate by GmhA, which is the first LPS-dedicated enzyme of the pathway. D-*glycero*-D-*manno*-heptose 7-phosphate is phosphorylated by HldE to D-*glycero*-D-*manno*-heptose 1,7-bisphosphate, dephosporylated by GmhB to D-*glycero*-D-*manno*-heptose 1-phosphate, activated by HldE to ADP-D-*glycero*-D-*manno*-heptose and finally converted by HldD to ADP-L-glycero-ß-manno-heptose, which is the precursor used by the glycosyltransferases, WaaC, WaaF and WaaQ, to charge the KDO-lipidA and build the HEP component (Zamyatina et al. 2000; Gronow et al. 2001).

Inhibitors of the biosynthesis of the HEP units have been investigated as potential antivirulence drugs (De Leon et al. 2006; Desroy et al. 2009). De Leon et al. used a pathway screen in which all the individual purified enzymes from TktA to GmhB were recombined in vitro. They identified compound **1** (Fig. 3) from a library of 1,000 small molecules and showed that it was an inhibitor of HldE (IC_{50} 63 μM). Desroy et al. used a specific target assay using synthetic D-*glycero*-D-*manno*-heptose 7-phosphate and ATP as substrates. They screened a library of 40,000 compounds and found compounds **2** and **3** (IC_{50} 51 and 69 μM), from which compound **4** was obtained by chemical modification (IC_{50} 0.27 μM). It was not reported whether any of these compounds had the expected effects on LPS biosynthesis in the intact bacterium and its virulence. Durka et al. (2011) described the synthesis of a series of D-glycero-D-manno-heptopyranose 7-phosphate analogues and found that the epimeric analogues displaying a D-glucopyranose configuration (e.g. compound **5**) were the best inhibitors of GmhA ($IC_{50} = 34$ μM) and HldE ($IC_{50} = 9.4$ μM).

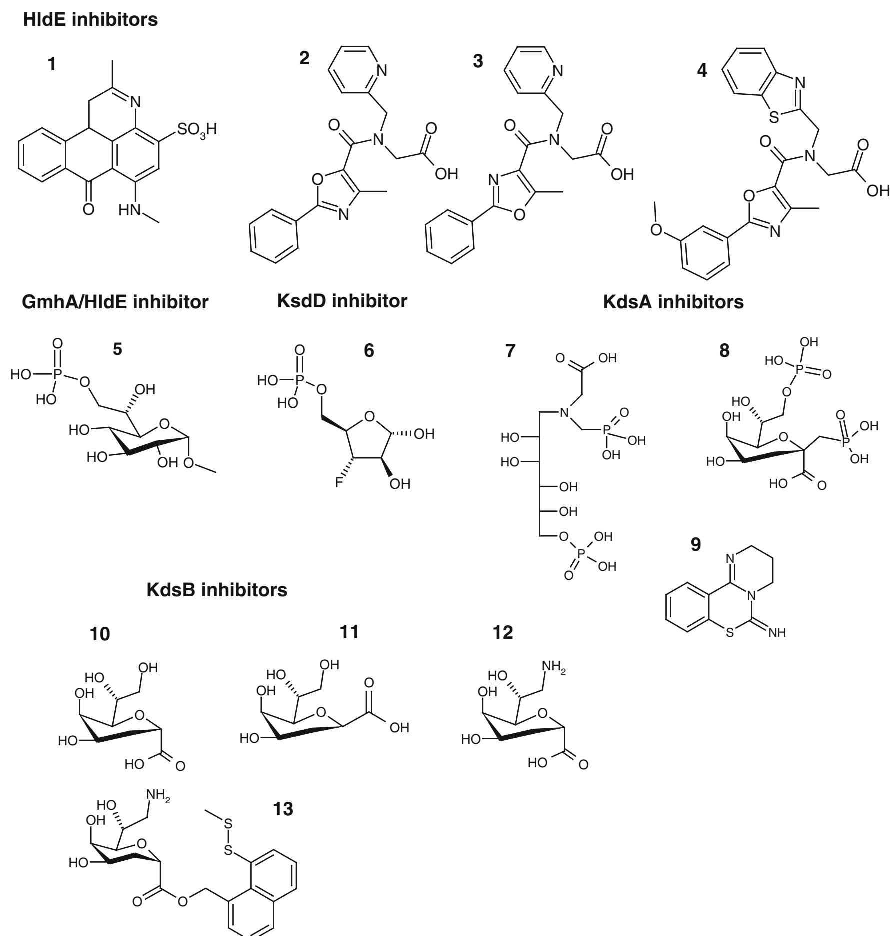


Fig. 3 Inhibitors of biosynthesis of the LPS core saccharide

The biosynthesis of the KDO units takes place in the cytoplasm (Fig. 2) and starts with the conversion of D-ribulose-5-phosphate to D-arabinose-5-phosphate by isomerases, KdsD or GutQ; KdsD is present in the majority of species of Gram-negative bacteria, whereas GutQ is found only in some Enterobacteriaceae. D-Arabinose-5-phosphate is condensed with phosphoenolpyruvate by KdsA to form 3-deoxy-D-manno-oct-2-ulosonic acid 8-phosphate, dephosphorylated by KdsC to give 3-deoxy-D-manno-oct-2-ulosonic acid, which is activated by KdsB to form CMP-3-deoxy-D-manno-oct-2-ulosonic acid, the precursor used by the glycosyltransferase WaaA to charge Lipid A with at least two KDO units (Clementz and Raetz 1991).

The search for inhibitors of the biosynthesis of the KDO units has been pursued in the hope of finding new antimicrobial agents, as the formation of KDO-lipid A is essential for growth of Gram-negative bacteria. Bigham et al. (1984) described a

number of compounds as potential inhibitors of KdsD, including analogues of arabinose, arabinose-5-phosphate, aldonic acid phosphates, alditol phosphates and ribonolactones. None of the compounds was very active; for example, compound **6** was the most active among the arabinose-5-phosphate analogues with an IC_{50} of 61 mM. Baashov and colleagues pursued a series of potential transition-state analogues as inhibitors of KdsA (Sheffer-Dee-Noor et al. 1993; Du et al. 1997; Baasov and Belakov 1999, 2000; Baasov et al. 2001), of which compounds **7** and **8** were among the more active (IC_{50} 3.3 μM and 4.9 μM, respectively). Compound **9** was discovered in a target-based screen of 150,000 compounds (Birck et al. 2000). It was very potent against the purified enzyme (IC_{50} 240 pM) but had low antimicrobial activity (minimum inhibitory concentration (MIC) for *E. coli* 32 mg/L). Sugar analogues (compounds **10–12**) have also been pursued as inhibitors of KdsB (Claesson et al. 1987; Luthman and Claesson 1987; Goldman et al. 1987). Although reasonably potent inhibition (in the low micromolar range) could be achieved against the isolated enzyme, there was little or no activity against the intact bacteria. Compound **12** was investigated in prodrugs either with a short peptide attached to the amino group or esterified with 8-(hydroxymethyl)-1-naphthyl methyl disulphide (Compound **13**). In the former case, delivery of the inhibitor to the intracellular target was achieved by a peptide transport system and antibacterial activity was dependent upon an oligopeptide permease and an intracellular amino-peptidase to release the active inhibitor (Goldman et al. 1987). In the latter case, the compound entered the cell by passive diffusion and the active inhibitor was released after the attack of the disulphide by thiol compounds (e.g. cysteine or glutathione) in the cytoplasm (Norbeck et al. 1989). In addition to the expected effects of suppression of LPS biosynthesis and slowing of bacterial growth, the peptide prodrugs have been shown to be synergistic with kanamycin and fosfomycin in repressing the release of vero toxin from enterohaemorrhagic *E. coli* (Kondo et al. 2004).

Lipid A is essential for bacterial growth and the inhibition of its biosynthesis is lethal to bacteria (Raetz 1993; Wyckoff et al. 1998). Furthermore, mutant strains with a defect in lipid A biosynthesis are hypersensitive to some antibiotics (Nikaido and Vaara 1985; Vaara 1993; Vuorio and Vaara 1992). Lipid A comprises a β-1′,6-linked glucosamine disaccharide acylated on the 2-amino and 3-hydroxyl groups (Fig. 1). Usually, the 4-hydroxyl of the non-reducing sugar and the 1-hydroxyl (reducing end) are phosphorylated.

The biosynthesis of lipid A takes place in the cytoplasm, or at the interface with the inner leaflet of the cytoplasmic membrane (Fig. 2) and starts with the formation of UDP-*N*-acetylglucosamine by GlmU. UDP-*N*-acetylglucosamine is then acylated on the 3-hydroxyl group by LpxA using 3-hydroxymyristoyl-acyl carrier protein as donor. The actetyl group is removed by LpxC and a second 3-hydroxymyristoyl group added to the 2-amino group by LpxD. LpxH removes the UMP from one molecule of UDP-diacyl-glucosamine and this is condensed with a second molecule of the activated intermediate to form a 1-phospho diacyl-disaccharide, which is then phosphorylated on the 4-hydroxyl group of the non-reducing sugar to form lipid A, which serves as the substrate for the WaaA glycosyltransferase for the addition of two KDO units. In *E. coli*, further addition

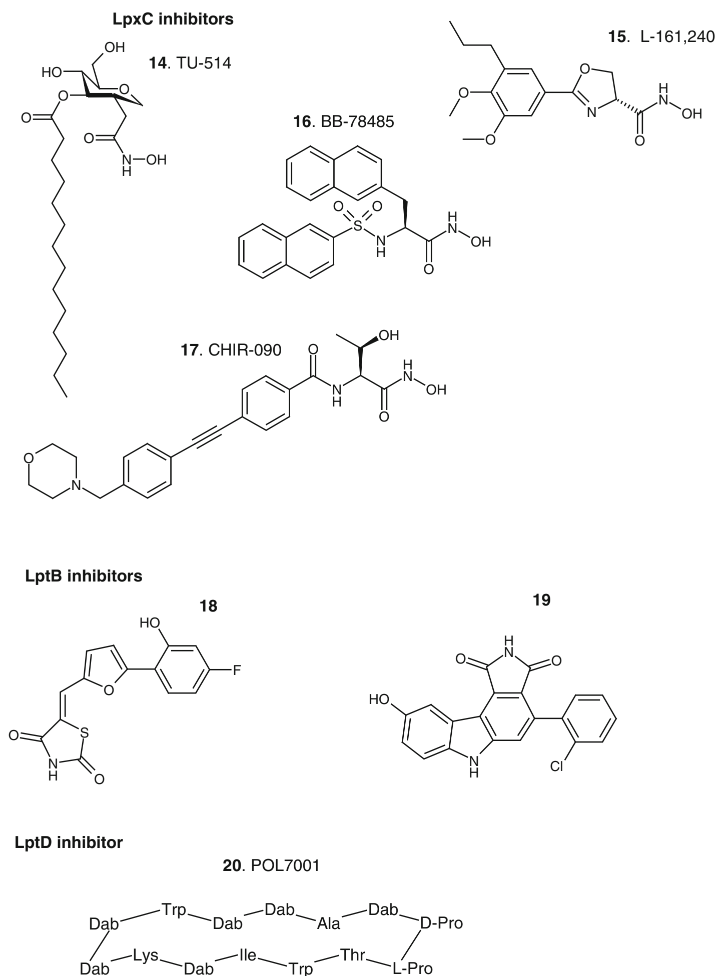


Fig. 4 Inhibitors of biosynthesis of lipid A and translocation of LPS

of lauric acid, by LpxL, and myristoic acid, by LpxM, to the hydroxyl groups of the acyl chains attached to the non-reducing sugar occurs after the addition of the KDO units: this does not occur in *P. aeruginosa*. The lipid A bearing the assembled core saccharide is transferred from the inner leaflet of the cytoplasmic membrane to the outer leaflet through the action of MsbA flippase, an ATP-binding cassette transporter (Doerrler et al. 2001, 2004; Rees et al. 2009; Davidson et al. 2008).

The search for inhibitors of the biosynthesis of lipid A has concentrated on LpxC, although LpxA is certainly under consideration (Benson et al. 2003; Williams et al. 2006; Ulaganathan et al. 2007). LpxC (UDP-3-*O*-(*R*-3-hydroxmyristoyl)-*N*-acetylglucosamine deacetylase) is a zinc-dependent amidase and early work identified inhibitors containing a hydroxamate that could coordinate to the zinc ion (e.g. compounds **14–17**, Fig. 4) (Jackman et al. 2000; Onishi et al. 1996; Clements

et al. 2002; Kline et al. 2002). They are potent inhibitors with K_I values in the nanomolar range for the purified enzyme [650 nM, 50 nM, 20 nM and 2 nM for compounds **14–17**, respectively (Mochalkin et al. 2008)]. However, the very hydrophobic nature of the substrate recognition site and its requirement for a mimic of the myristoyl side chain of the substrate for tight binding (Jackman et al. 1999; Whittington et al. 2003) leaves these molecules with rather a challenge to reach their target in the cytosol. Low MICs were reported for relatively permeable organisms such as *E. coli* or hyperpermeable *P. aeruginosa*, but activity against wild-type *P. aeruginosa* was lacking (Onishi et al. 1996; Clements et al. 2002; Mansoor et al. 2011). The activity of compound **17** against species of the *Burkholderia cepaecia* complex was highly strain dependent, and there was no correlation between the activity of the compound and the LPS profiles of the strains (Bodewits et al. 2010). The MsbA flippase has also been suggested to be a potential target for interfering with LPS biosynthesis (Doerrler and Raetz 2002).

The *mature LPS molecule* is assembled in the periplasmic interface of the cytoplasmic membrane through the action of WaaL ligase (Whitfield et al. 1997) prior to being translocated to the outer membrane by the Lpt ABCDEFG adenosine triphosphate (ATP)-binding cassette transporter (Ruiz et al. 2008). LptF and LptG are inner membrane proteins, LptB is the putative ATP-binding component, LptA is the putative periplasmic binding protein, LptC is an inner membrane protein interacting with LptA, LptE appears to be a kind of chaperone, helping the assembly of LptD by the β-barrel assembly machine in the outer membrane (see below), and Lpt D is the outer membrane components responsible for insertion of the LPS molecule into the outer leaflet of the membrane (Narita and Tokuda 2009; Chimalakonda et al. 2011; Sperandeo et al. 2011). The ATP-binding component, LptB, has been screened against 244 compounds selected from commercial kinase inhibitor libraries using an assay based on its ATPase activity (Gronenberg and Kahne 2010). Two hits with very diverse structures were reported, with K_I values of 7 μM and 5 μM for compounds **18** and **19**, respectively. A remarkable recent discovery was a series of cyclic peptidomimetic antibiotics (e.g. compound **20**, POL7001) that are selective inhibitors of the LptD protein of *P. aeruginosa*. They exhibit extremely potent activity against this organism (MIC $\leq$ 0.008 mg/L) but against no other bacteria (Srinivars et al. 2010).

The outer membrane is maintained as an asymmetric bilayer, with the outer leaflet formed by LPS units bound together largely by Mg^{2+} ions and the inner leaflet formed from the normal phospholipid constituents of the cytoplasmic membrane (reviewed in Nikaido 2003). The LPS units are believed to strongly interact through hydrophobic interactions of the acyl chains of lipid A, hydrogen-bonding between sugars of the core saccharide and ion-bridging between the phosphates of lipid A and the divalent cations. These interactions, and the low fluidity of the hydrocarbon layer, comprised entirely of saturated fatty acids, contribute to making the LPS-leaflet an effective permeability barrier. The asymmetry of the bilayer is actively maintained through import of phospholipids from the outer leaflet of the membrane by the Mla ATP-dependent transport system (Malinverni and Silhavy 2009) and by the action of two outer-membrane enzymes, PldA and PagP. PldA is

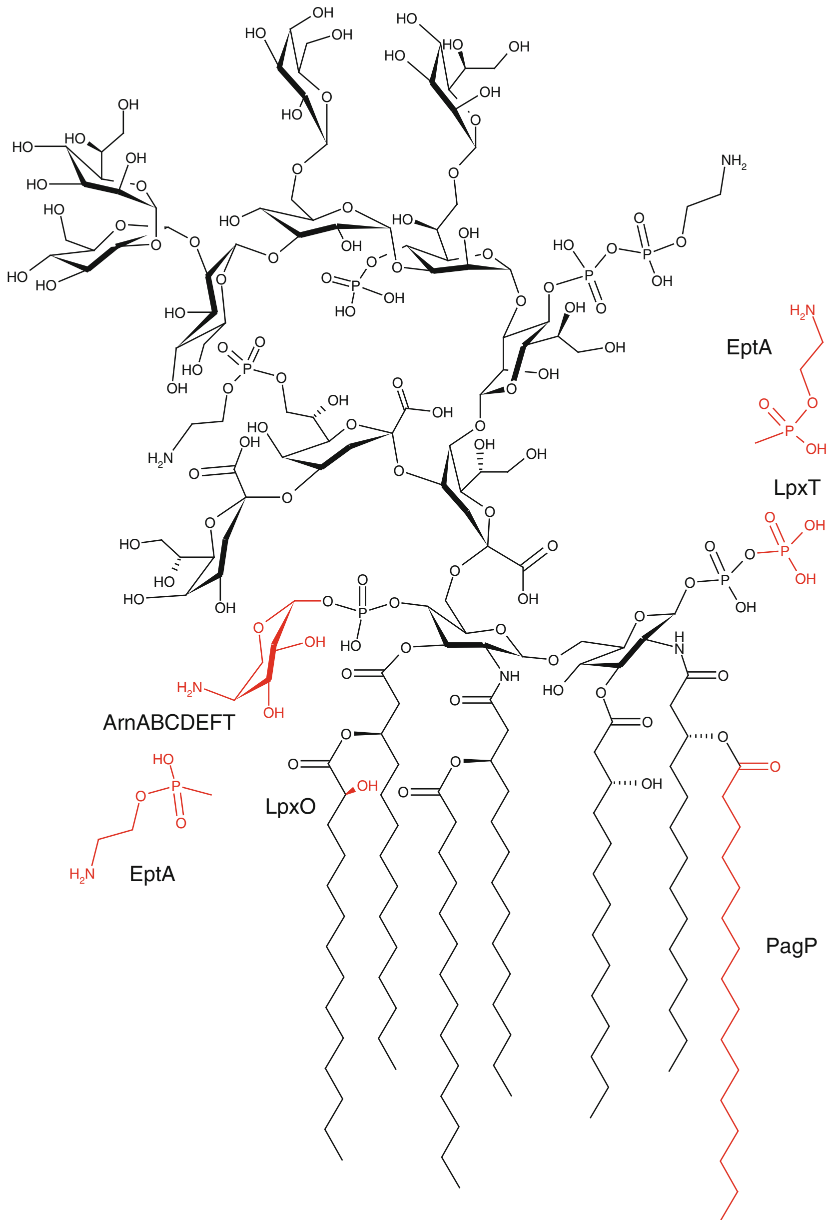


Fig. 5 Structure of the LPS molecule of *E. coli* K-12, in black, with modifications introduced after translaocation into the periplasm shown in red

a phospholipase that hydrolyzes a wide range of phospholipids and lysophospholipids; it is activated by the presence of its substrates (Istivan and Coloe 2006). PagP is induced by the limitation of divalent cations, or exposure to cationic

amphipathic peptides (Bishop 2005); it transfers a palmitate moiety from any available phospholipid to lipid A, forming a hepta-acylated LPS of increased hydrophobicity (Fig. 5) that probably compensates for weakening of the ionic interactions. Other modifications of the LPS may occur during its translocation to the outer membrane. For example, about one-third of the lipid A found in *E. coli* contains a diphosphate at position 1 of the reducing sugar. This is added in the periplasm by LpxT, which uses bactoprenol diphosphate, released during peptidoglycan polymerization, as the substrate donor (Touzé et al. 2008). Further, polymyxin B-resistant strains of Gram-negative bacteria usually become so through acquisition of mutations in the PmrA transcription factor, which controls genes encoding the enzymes responsible for modification of lipid A with phosphatidyl ethanolamine and 4-amino-4-deoxy-L-arabinose (Gibbons et al. 2005). The genes for aminoarabinose modification form an operon of seven genes that encode the enzymes for the conversion of UDP-glucose to bactoprenol phosphate-α-L-4-amino-4-deoxyarabinose (ArnA, ArnB, ArnC, ArnD) in the cytoplasm, its translocation to the periplasmic leaflet of the cytoplasmic membrane (ArnE, ArnF) and transfer of the aminoarabinose moiety to the 4 hydroxyl of the non-reducing glucosamine of lipid A by ArnT (Yan et al. 2007). Phosphatidylethanolamine is added by EPtA (Trent and Raetz 2002; Lee et al. 2004). Both modifications introduce an additional positive charge (at physiological pH) and therefore presumably serve to decrease the avidity of binding of cationic antibiotics.

3 Peptidoglycan

The peptidoglycan cell wall is the other major structural component of the outer layers of the Gram-negative bacterium, providing mechanical strength, for example to resist the internal osmotic pressure. It is a polymer comprising repeating disaccharide units of *N*-acetylmuramic acid β-1′,4-linked to *N*-acetylglucosamine. A pentapeptide chain is attached to the *N*-acetylmuramic acid and can be cross-linked to the peptide chain of another strand forming the 3D mesh-like layer.

The initial steps in the biosynthesis of the peptidoglycan take place in the cytoplasm (Fig. 2), beginning with the formation of UDP-*N*-acetylglucosamine by GlmU, a step shared with lipid A biosynthesis. UDP-*N*-acetylglucosamine is converted to enolpyruvyl-UDP-*N*-acetylglucosamine by MurA with phosphoenolpyruvate as donor substrate. MurB reduces enolpyruvyl-UDP-*N*-acetylglucosamine to UDP-*N*-acetyl muramic acid, which is successively charged with alanine (by MurC), D-glutamate (by MurD), lysine (by MurE) and D-alanyl-D-alanine (by MurF). The UDP-*N*-acetyl muramic acid pentapeptide is transferred to bactoprenol phosphate by MraY to give a membrane-bound intermediate, lipid I, which then condensed with a second molecule of UDP-*N*-acetylglucosamine by MurG, to give the peptidoglycan precursor, lipid II. The completed peptidoglycan precursor has to be transferred from the inner leaflet of the cytoplasmic membrane to its outer leaflet. It has been suggested that the FtsW may be flippase required for this translocation (Mohammadi et al. 2011). The polymerization of peptidoglycan is

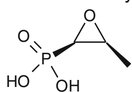
mediated by transglycosylases exposed on the periplasmic face of the cytoplasmic membrane. These may be monofunctional enzymes or a domain of the high-molecular-weight penicillin-binding proteins, PBP 1a and PBP 1b in *E. coli* (Holtje 1998). The formation of the peptidic cross-bridges is accomplished by transpeptidases that are either apparently monofunctional enzymes, for example PBP3 of *E. coli*, or a domain of the high-molecular-weight penicillin-binding proteins, PBP 1a and PBP 1b in *E. coli* (Page 2007).

The biosynthetic pathway for the peptidoglycan precursor, lipid II, is the target of a number of natural antibiotics, many of which were discovered in the early days of antimicrobial development. These include the clinically used antibiotics fosfomycin (compound **21** (Fig. 6), an inhibitor of MurA,) and cycloserine (compound **22** (Fig. 6), an inhibitor of alanine racemase and D-alanyl-D-alanine ligase), as well as a number of antibiotics that have not found clinical use, including amphomycin, tunicamycin, mureidomycin, liposidomycin and muraymycins, all of which inhibit MraY. Fosfomycin and cycloserine appear to cross the outer membrane of Gram-negative bacteria quite readily but are dependent of permeases for entry into the cytoplasm. Mutations that inactivate the non-essential glycerophosphate transporter render *E. coli* resistant to fosfomycin (Kahan et al. 1974; Navas et al. 1990), while mutations in the CycA permease confer resistance to cycloserine (Russell 1972; Cosloy 1973). Fosfomycin contains a reactive epoxide group that is essential for its activity and enzymes that destroy this by catalysing nucleophilic attack by glutathione, cysteine or water render the drug ineffective (Rigsby et al. 2005). In addition, some *Pseudomonas* can phosphorylate fosfomycin, preventing binding to its target (García et al. 1995). Amphomycin and tunicamycin bind to the bactoprenol substrate of MraY, thus inhibiting the reaction by depleting the availability of the acceptor substrate (Banerjee 1989; Brandish et al. 1996). Liposidomycin, mureidomycins and muraymycins are competitive inhibitors of lipid I binding (Brandish et al. 1996; McDonald et al. 2001). However, amphomycin, tunicamycin, liposidomycin and muraymycins apparently do not cross the outer membrane of Gram-negative bacteria and show little or no activity except in strains with LPS defects. The mureidomycins evidently do cross the outer membrane and show quite potent activity against *P. aeruginosa,* with mureidomycin C (compound **23**) being the most active (MICs 0.1–3.1 mg/L for *P. aeruginosa*). They are less active against Enterobacteriaceae, with MICs for *K. pneumoniae* $\geq$12.5 mg/L and *E. coli* $>$200 mg/L (Isono et al. 1989), which has been attributed to the action of the AcrAB-TolC efflux pump (Gotoh et al. 2003).

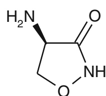
Numerous attempts have been made to find new inhibitors of the soluble enzymes Mura-MurF, but little progress has been made in finding compounds that are active against Gram-negative bacteria, with at best some activity (MIC 8 mg/L) being demonstrable for compound **24** (an inhibitor of MurF) against *E. coli* with an LPS defect (Baum et al. 2007, 2009). There have been also some attempts to discover simplified analogues of liposidomycins (Stachyra et al. 2004) and mureidomycins (Bozzoli et al. 2000) that might have better ability to circumvent the outer-membrane barrier and efflux, but, as yet, these have not delivered new agents.

Inhibitors of Peptidoglycan Precursor Biosynthesis

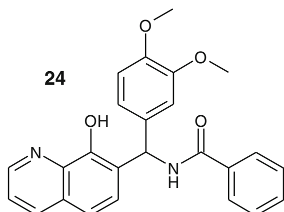
21. Fosfomycin



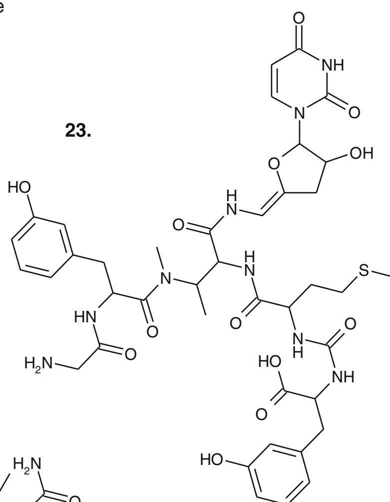
22. Cycloserine



24

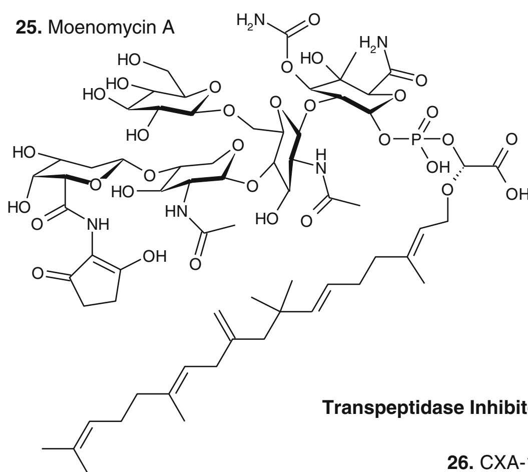


23.



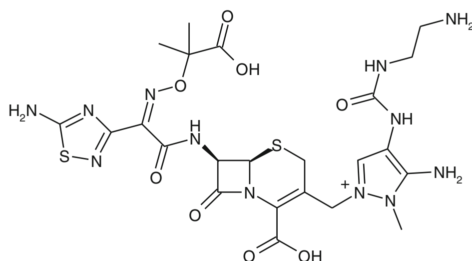
Transglycosylase Inhibitor

25. Moenomycin A



Transpeptidase Inhibitors

26. CXA-101



27. BAL30072

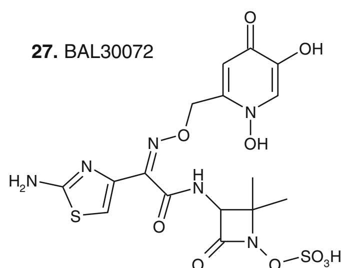


Fig. 6 Inhibitors of peptidoglycan biosynthesis

Lipid II itself is the target for a number of antibiotics that sequester this transglycosylase substrate on the periplasmic surface of the membrane, thus inhibiting peptidoglycan polymerization. These antibiotics include vancomycin,

lantibiotics such as nisin and mersacidin, mannopeptimycin, katanosin B, plusbacin A_3 and ramoplanin (reviewed in Breukink and de Kruijff 2006). For the most part, these compounds have little activity against Gram-negative bacteria because they are excluded by the outer membrane, and activity may only be seen in organisms with LPS defects or in the naturally more permeable organisms such as *Helicobacter pylori*, *Campylobacter jejuni* and *Neisseria* species (Mota-Meira et al. 2000). Introduction of positively charged residues into nisin produced variants with increased activity against Enterobacteriaceae and *Pseudomonas* (Yuan et al. 2004). Bacitracin binds bactoprenol diphosphate released after the transglycosylation reaction and thus inhibits cell growth by depleting the pool of bactoprenol phosphate required for both LPS and peptidoglycan precursor biosynthesis (Stone and Strominger 1971). Like many other peptidic antibiotics, it is too large to cross the outer membrane of most Gram-negative bacteria.

The transglycosylases are targetted by the moenomycin group of antibiotics (reviewed in Ostash and Walker 2010). In *E. coli*, moenomycin (compound **25**) inhibits transglycosylation, causing accumulation of the peptidoglycan precursors and triggering lysis and cell death (Van Heijenoort et al. 1987). However, the activity against Gram-negative bacteria is usually not very potent and is really only exhibited in mutants or naturally more permeable organisms. There have been several attempts to find moenomycin derivatives with improved activity (Baizman et al. 2000; Goldman et al. 2000; Sofia et al. 1999) and a screen based on competition with a fluorescent moenomycin derivative has been reported (Cheng et al. 2007). Although compounds active against Gram-positive bacteria have been found, no activity against Gram-negative bacteria was reported (Derouaux et al. 2011).

The transpeptidases essential for cross-linking of the glycan chains of peptidoglycan is the well-known target of the β-lactam antibiotics (reviewed in Page 2007). Resistance is mediated by restricted influx through porins (see below), efflux (reviewed in Dreier 2007), β-lactamases (reviewed in Bush and Bradford 2007), or mutation of the transpeptidase (reviewed in Page 2007). Among the Gram-negative bacteria, combination of restricted permeation of the outer membrane with expression of one or more β-lactamases is the predominant mechanism of resistance. PBP-mediated resistance is most common in the naturally more permeable organisms such as *Neisseria* and *Haemophilus* species. Mutations in or alteration by genetic exchange between related genes of PBP1 and PBP2 have been identified in *N. gonorrhoeae*, *N. denitrificans* and *N. meningitidis*. Non-lactamase-mediated resistance to β-lactams in *Haemophilus influenzae* has been associated with accumulation of multiple mutations PBP 3. Alterations in the apparent affinity and expression levels of PBPs have been noted in clinical isolates of *P. aeruginosa*, although a causal relation with clinical resistance has yet to be demonstrated. Reduced expression of PBP 2 is one of the most frequently observed mechanisms of resistance to carbapenems in *Acinetobacter* species.

There are two new β-lactams in clinical development for treatment of Gram-negative infections. They both combine features that address the prevalent resistance mechanisms of Gram-negative bacteria. The cephalosporin CXA-101 (compound **26**) has excellent potency against *P. aeruginosa* (Takeda et al. 2007). It is stable towards

class C β-lactamases and is not a substrate for efflux systems of *P. aeruginosa*. It is being developed in combination with tazobactam, a β-lactamase inhibitor, to cover at least some of its lability towards the class A extended-spectrum β-lactamases. BAL30072 (compound **27**) is a siderophore monosulfactam that can exploit iron-uptake systems in some organisms to gain accelerated uptake into the periplasm (Page et al. 2010). The monocyclic beta-lactams are uniquely stable towards class B β-lactamases and act as inhibitors of class C β-lactamases, and BAL30072 appears to be quite resistant to hydrolysis by carbapenemases from class A and class D (Fig. 5).

4 Porins, Efflux and Other Transport Proteins of the Outer-Membrane

The proteins localized in the periplasm and outer membrane of Gram-negative bacteria have to be secreted across the cytoplasmic membrane (Fig. 7). Although some proteins, such as the components of the flagellum, have specific secretion pathways, all secreted proteins are ultimately dependent on the general secretion machine as the components of the specific secretion pathways are themselves secreted by this route. In addition, most nascent inner membrane proteins are recognized by the ubiquitous signal recognition particle and targeted to the general secretion machine. The secretory machine comprises SecYEG, which forms a protein-conducting channel (Osborne and Rapoport 2007), SecA, an ATPase that provides the energy for secretion (Hunt et al. 2002), accessory complexes comprising SecDFYajC and YidC, which work in conjunction with the canonical SecYEG pathway and also as a Sec-independent insertase for integral membrane proteins (Nouwen and Driessen 2002). Lipoproteins destined for the outer membrane are recognized by the LolCDE ATP-binding cassette transporter, delivered to the binding protein LolA, which carries it to LolB, located in the inner leaflet of the outer membrane where the nascent lipoprotein is inserted. The β-barrel outer membrane proteins, including porins, outer membrane components of LPS translatory machine and of efflux proteins and PagP, are recognized by periplasmic chaperones including Skp, DegP and SurA that help transfer the nascent protein the assembly complex formed from YaeT, NlpB, YfiO and YfgL (Ruiz et al. 2006).

The central role of the Sec secretory machine in the assembly of the outer membrane and secretion of resistance and virulence factors has made it the focus of considerable attention in the search for new antimicobial agents (Stephens and Shapiro 1997). A number of inhibitors of the signal peptidase have been identified (Kuo et al. 1993; Black and Bruton 1998; Hu et al. 2003; Roberts et al. 2007; Kaderbhai and Khan 2008), but so far lack significant activity against Gram-negative bacteria. Penetration across the outer membrane appears to be one of the limiting factors, but the chemical stability of some of the compounds may be another factor. It is not known how accessible the signal peptidase active site is during normal bacterial growth and it may be that the nascent secreted protein out

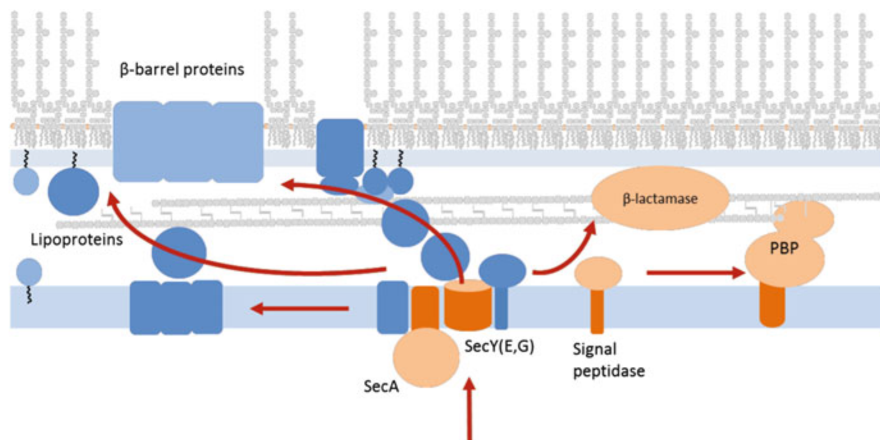


Fig. 7 Schematic representation of the secretory pathways for periplasmic and outer membrane proteins. Proteins that have been studied as potential targets for antimicrobials are named and highlighted in orange

competes the exogenous inhibitors. A number of SecA inhibitors have been reported but have shown only modest activity in *E. coli* strains with LPS defects (Li et al. 2008; Sugie et al. 2002; Chen et al. 2010).

Porins form water-filled channels through the outer membrane and thereby constitute the most important route by which hydrophilic antibiotics can pass the outer membrane barrier. Their role in restricting the influx of antibiotics and thus contributing to resistance has been described in many organisms (Parr et al. 1987; Ochs et al. 1999; Ruiz et al. 2003; Olesky et al. 2006; Vila et al. 2007). There is a delicate interplay between the role of porins, or their lack, and the active extrusion of the antibiotics by efflux pumps (Dreier 2007 and elsewhere in this book). This is compounded in the case of β-lactams by the active destruction of the antibiotic in the periplasmic space through the action of β-lactamases. When there is a significant decrease in the rate of entry, for example through the loss of a specific porin (Ochs et al. 1999) or significant extrusion by an efflux pump (Dreier 2007), relatively modest hydrolytic activity is sufficient to cause high-level resistance (Pai et al. 2001).

References

Baasov T, Belakov V (1999) Recent Res Develop Org Chem 3:195–206
Baasov T, Belakov V (2000) Drug Develop Res 50:416–424
Baasov V, Tkacz T, Sheffer-Dee-Noor S, Belakhov V (2001) Curr Org Chem 5:127–138
Baizman ER, Branstrom AA, Longley CB, Allanson N, Sofia MJ, Gange D, Goldman RC (2000) Microbiology 146:3129–3140
Banemann A, Deppisch H, Gross R (1998) Infect Immun 66:5607–5612
Banerjee DK (1989) J Biol Chem 264:2024–2028

Bastin DA, Brown PK, Haase A, Stevenson G, Reeves PR (1993) Mol Microbiol 7:725–734
Batchelor RA, Alifiano P, Biffali E, Hull SI, Hull RA (1992) J Bacteriol 174:5228–5236
Baum EZ, Crespo-Carbone SM, Klinger A, Foleno BD, Turchi I, Macielag M, Bush K (2007) Antimicrob Agents Chemother 51:4420–4426
Baum EZ, Crespo-Carbone SM, Foleno BD, Simon LD, Guillemont J, Macielag M, Bush K (2009) Antimicrob Agents Chemother 53:3240–3247
Benson RE, Gottlin EB, Christensen DJ, Hamilton PT (2003) Antimicrob Agents Chemother 47:2875–2881
Bigham EC, Gragg CE, Hall WR, Kelsey JE, Mallory WR, Richardson DC, Benedict C, Ray PH (1984) J Med Chem 27:717–726
Birck MR, Holler TP, Woodard RV (2000) J Am Chem Soc 122:9334–9335
Bishop RE (2005) Mol Microbiol 57:900–912
Black MT, Bruton G (1998) Curr Pharm Des 4:133–154
Bodewits K, Raetz CRH, Govan JR, Campopiano DJ (2010) Antimicrob Agents Chemother 54:3531–3533
Bozzoli A, Kazmierski W, Kennedy G, Paquarello A, Pecunioso A (2000) Bioorg Med Chem Lett 10:2759–2763
Bush K, Bradford P (2007) In: Bonomo RA, Tolmasky ME (eds) Enzyme-mediated resistance to antibiotics: mechanisms, dissemination, and prospects for inhibition. ASM Press, Washington, DC, pp 67–80
Brandish PE, Kimura K-I, Inukai M, Southgate R, Lonsdale JT, Bugg TDH (1996) Antimicrob Agents Chemother 40:1640–1644
Breukink E, de Kruijff B (2006) Nat Rev Drug Discov 5:321–332
Caroff M, Karibian D (2003) Carbohydr Res 338:2431–2447
Chen H, Gartner E, Rolfe BG (1993) Appl Environ Microbiol 59:1058–1064
Chen W, Huang YJ, Gundala SR, Yang H, Li M, Tai PC, Wang B (2010) Bioorg Med Chem 18:1617–25
Cheng TJ, Sung MT, Liao HY, Chang YF, Chen CW, Huang CY, Chou LY, Wu YD, Chen YH, Cheng YS, Wong CH, Ma C, Cheng WC (2008) Proc Natl Acad Sci USA 105:431–436
Claesson A, Luthman K, Gustafsson K, Bondesson G (1987) Biochem Biophys Res Commun 143:1063–1068
Chimalakonda G, Ruiz N, Chng S-S, Garner RA, Kahne D, Silhavy TJ (2011) Proc Natl Acad Sci USA 108:2492–2497
Clements JM, Coignard F, Johnson I, Chandler S, Palan S, Waller A, Wijkmans J, Hunter MG (2002) Antimicrob Agents Chemother 46:1793–1799
Clementz T, Raetz CR (1991) J Biol Chem 266:9687–9696
Cosloy SD (1973) J Bacteriol 114:679–684
Davies JA, Anderson GK, Beveridge TJ, Clark HC (1983) J Bacteriol 156:837–845
Davidson AL, Dassa E, Orelle C, Chen J (2008) Microbiol Mol Biol Rev 72:317–364
De Leon GP, Elowe NH, Koteva KP, Valvano MA, Wright GD (2006) Chem Biol 13:437–441
Derouaux A, Turk S, Olrichs NK, Gobec S, Breukink E, Amoroso A, Offant J, Bostock J, Mariner K, Chopra I, Vernet T, Zervosen A, Joris B, Frère JM, Nguyen-Distèche M, Terrak M (2011) Biochem Pharmacol 81:1098–1105
Doerrler WT, Reedy MC, Raetz CR (2001) J Biol Chem 276:11461–11464
Doerrler WT, Raetz CR (2002) J Biol Chem 277:36697–36705
Doerrler WT, Gibbons HS, Raetz CR (2004) J Biol Chem 279:45102–45109
Desroy N, Moreau F, Briet S, Le Fralliec G, Floquet S, Durant L, Vongsouthi V, Gerusz V, Denis A, Escaich S (2009) Bioorg Med Chem 17:1276–1289
Dreier J (2007) In: Bonomo RA, Tolmasky ME (eds) Enzyme-mediated resistance to antibiotics: mechanisms, dissemination, and prospects for inhibition. ASM Press, Washington DC, pp 235–264
Du S, Tsipori H, Baasov T (1997) Bioorg Med Chem Lett 7:2469–2472
Durka M, Tikad A, Périon R, Bosco M, Andaloussi M, Floquet S, Malacain E, Moreau F, Oxoby M, Gerusz V, Vincent SP (2011) Chemistry 17:11305–11313
Fletcher E, Fleiszig SMJ, Brannan NA (1993) Invest Ophthalmol Vis Sci 34:1930–1936

García P, Arca P, Evaristo Suárez J (1995) Antimicrob Agents Chemother 39:1569–1573
Gibbons HS, Kalb SR, Cotter RJ, Raetz CRH (2005) Mol Microbiol 55:425–440
Goldman RC, Baizman ER, Branstrom AA, Longley CB (2000) Bioorg Med Chem Lett 10:2251–2254
Goldman RC, Kohlbrenner WE, Lartey P, Pernet A (1987) Nature 329:162–164
Gotoh N, Murata T, Ozaki T, Kimura T, Kondo A, Nishino T (2003) J Infect Chemother 9:101–103
Gram HC (1884) Fortschr Med 2:185–189
Gronenberg LS, Kahne D (2010) J Am Chem Soc 132:2518–2519
Gronow S, Oertelt C, Ervela E, Zamyatina A, Kosma P, Skurnik M, Holst O (2001) J Endotoxin Res 7:263–270
Helena Mäkelä P, Valtonen VV, Valtonen M (1973) J Infect Dis 128; Supplement: Bacterial lipopolysaccharides: chemistry, biology, and clinical significance of endotoxins. pp S81–S85
Holtje JV (1998) Microbiol Mol Biol Rev 62:181–203
Hu XE, Kim NK, Grinius L, Morris CM, Wallace CD, Mieling GE, Demuth TP Jr (2003) Synthesis 2003(11):1732–1738
Hug I, Feldman MF (2011) Glycobiology 21:138–151
Hunt JF, Weinkauf S, Henry L, Fak JJ, McNicholas P, Oliver DB, Deisenhofer J (2002) Science 297:2018–26
Isono F, Katayama T, Inukai M, Haneishi T (1989) J Antibiot (Tokyo) 42:674–679
Istivan TS, Coloe PJ (2006) Microbiology 152:1263–1274
Jackman JE, Raetz CR, Fierke CA (1999) Biochemistry 38:1902–1911
Jackman JE, Fierke CA, Tumey LN, Pirrung M, Uchiyama T, Tahir SH, Hindsgaul O, Raetz CRH (2000) J Biol Chem 275:11002–11009
Kelly J, Jarrell H, Millar L, Tessier L, Fiori LM, Lau PC, Allan B, Szymanski CM (2006) J Bacteriol 188:2427–2434
Kaderbhai N, Khan T (2008) Int J Pept Res Ther 14:173–178
Kahan FM, Kahan JS, Cassidy PJ, Kropp H (1974) Ann N Y Acad Sci 235:364–86
Kenne L, Lindberg B, Söderholm E, Bundle DR, Griffith DW, Morris JG (1983) Carbohydr Res 111:289–296
Kido N, Torgov VI, Sugiyama T, Uchiya K, Sugihara H, Komatsu T, Kato N, Jann K (1995) J Bacteriol 177:2178–2187
Kintz E, Goldberg JB (2008) Future Microbiol 3:191–203
Kline T, Andersen NH, Harwood EA, Bowman J, Malanda A, Endsley S, Erwin A, Doyle M, Fong S, Harris A, Mendelsohn B, Mdluli K, Raetz CRH, Stover CK, Witte PR, Yabannavar A, Zhu S (2002) J Med Chem 45:3112–3129
Kneidinger B, Graninger M, Puchberger M, Kosma P, Messner P (2001) J Biol Chem 276:20935–20944
Kondo K, Doi H, Adachi H, Nishimura Y (2004) Bioorg Med Chem Lett 14:467–470
Kuo DW, Chan K, Wilson CJ, Griffin PP, Williams H, Knight WB (1993) Arch Biochem Biophys 303:274–280
Lee H, Hsu FF, Turk J, Groisman EA (2004) J Bacteriol 186:4124–4133
Li M, Huang YJ, Tai PC, Wang B (2008) Biochem Biophys Res Commun 368:839–845
Lindberg B, Lindh F, Lönngren J, Lindberg AA, Svenson SB (1981) Carbohydr Res 97:105–112
Lior H (1994) Classification of *Escherichia coli*. In: Gyles CL (ed) Escherichia coli in domestic animals and humans. CAB International, UK, pp 31–72
Liu D, Cole R, Reeves PR (1996) J Bacteriol 178:2102–2107
Luthman K, Claesson A (1987) Carbohydr Res 166:233–251
Malinverni JC, Silhavy TJ (2009) Proc Natl Acad Sci USA 106:8009–8014
Mansoor UF, Vitharana D, Reddy PA, Daubaras DL, McNicholas P, Orth P, Black T, Siddiqui MA (2011) Bioorg Med Chem Lett 21:1155–1161
Martinić M, Hoare A, Contreras I, Alvarez SA (2011) PLoS One 6:e25557, Epub 2011 Oct 3
McDonald LA, Barbieri LR, Carter GT, Lenoy E, Lotvin J, Petersen PJ, Siegel MM, Singh G, Williamson RT (2001) J Am Chem Soc 124:10260–10261
McGowan CC, Necheva A, Thompson SA, Cover TL, Blaser MJ (1998) Mol Microbiol 30:19–31

Mochalkin I, Knaefels JD, Lightle S (2008) Protein Sci 17:450–457
Mohammadi T, van Dam V, Sijbrandi R, Vernet T, Zapun A, Bouhss A, Diepeveen-de Bruin M, Nguyen-Disteche M, de Kruijff B, Breukink E (2011) EMBO J 30:1425–1432
Mota-Meira M, LaPointe G, Lacroix C, Lavoie MC (2000) Antimicrob Agents Chemother 44:24–29
Narita S, Tokuda H (2009) FEBS Lett 583:2160–2164
Navas J, León J, Arroyo M, García Lobo JM (1990) Antimicrob Agents Chemother 34:2016–2018
Nikaido H (1976) Biochim Biophys Acta 433:118–132
Nikaido H, Vaara M (1985) Microbiol Rev 49:1–32
Nikaido H (2003) Microbiol Mol Biol Rev 67:593–656
Norbeck DW, Rosenbrook W, Kramer JB, Grampovnik DJ, Lartey PA (1989) J Med Chem 32:625–629
Nouwen N, Driessen AJ (2002) Mol Microbiol 44:1397–1405
Ochs MM, McCusker MP, Bains M, Hancock REW (1999) Antimicrob Agents Chemother 43:1085–1090
Onishi HR, Pelak BA, Gerckens LS, Silver LL, Kahan FM, Chen MH, Patchett AA, Galloway SM, Hyland SA, Anderson MA, Raetz CRH (1996) Science 274:980–982
Olesky M, Zhao S, Rosenberg RL, Nicholas RA (2006) J Bacteriol 188:2300–2308
Osborne AR, Rapoport TA (2007) Cell 129:97–110
Ostash B, Walker S (2010) Nat Prod Rep 27:1594–1617
Page MGP (2007) In: Bonomo RA, Tolmasky M (eds) Enzyme-mediated resistance to antibiotics: mechanisms, dissemination and prospects for inhibition. ASM Press, Washington DC, pp 81–100
Page MGP, Dantier C, Desarbre E (2010) Antimicrob Agents Chemother 54:2291–2302
Pai H, Kim J-W, Kim J, Lee JH, Choe KW, Gotoh N (2001) Antimicrob Agents Chemother 45:480–484
Palomar J, Puig M, Montilla R, Loren JG, Vinas M (1995) Microbios 82:21–26
Parr TR Jr, Moore RA, Moore LV, Hancock RE (1987) Antimicrob Agents Chemother 31:121–123
Perry MB, MacLean L, Griffith DW (1986) Biochem Cell Biol 64:21–28
Popoff MY, Minor LL (1997) Antigenic formulas of the *Salmonella serovars*, 7th revision. WHO Collaborating Centre for Reference and Research on *Salmonella*. Institut Pasteur.
Raetz CRH (1993) J Bacteriol 175:5745–5753
Raymond CK, Sims EH, Kas A, Spencer DH, Kutyavin TV, Ivey RG, Zhou Y, Kaul R, Clendenning JB, Olson MV (2002) J Bacteriol 184:3614–3622
Rees DC, Johnson E, Lewinson O (2009) Nat Rev Mol Cell Biol 10:218–227
Reeves PR, Hobbs M, Valvano M, Skurnik M, Whitfield C, Coplin D, Kido N, Klena J, Maskell D, Raetz C, Rick P (1996) Trends Microbiol 4:495–503
Reizer J, Reizer A, Saier MH (1992) Protein Sci 1:1326–1332
Rigsby R, Fillgrove K, Beihoffer L, Armstrong R (2005) Methods Enzymol 401:367–379
Roberts TC, Smith PA, Cirz RT, Romesberg FE (2007) J Am Chem Soc 129:15830–15838
Ruiz N, Gronenberg LS, Kahne D, Silhavy TJ (2008) Proc Natl Acad Sci USA 105:5537–5542
Ruiz N, Kahne D, Silhavy TJ (2006) Nat Rev Microbiol 4:57–64
Ruiz N, Montero T, Hernandez-Borrell J, Viñas M (2003) Microb Drug Resist 9:257–264
Russell RRB (1972) J Bacteriol 111:622–624
Samuel G, Reeves P (2003) Carbohydr Res 338:2503–2519
Sheffer-Dee-Noor S, Belakhov V, Baasov T (1993) Biorg Med Chem Lett 3:1583–1588
Sofia MJ, Allanson N, Hatzenbuhler NT, Jain R, Kakarla R, Kogan N, Liang R, Liu D, Silva DJ, Wang H, Gange D, Anderson J, Chen A, Chi F, Dulina R, Huang B, Kamau M, Wang C, Baizman E, Branstrom A, Bristo N, Goldman R, Han K, Longley C, Midha S, Axelrod HR (1999) J Med Chem 42:3193–3198
Sperandeo P, Villa R, Martorana AM, Šamalikova M, Grandori R, Dehò G, Polissi A (2011) J Bacteriol 193:1042–1053

Srinivars N, Jetter P, Ueberbacher BJ, Werneburg M, Zerbe K, Steinmann J, Van der Meijden B, Bernadini F, Lederer A, Dias RLA, Misson PE, Henze H, Zumbrunn J, Gombert FO, Obrecht D, Hunziker P, Schauer S, Ziegler U, Käch A, Eberl L, Riedel K, DeMarco SJ, Robinson JA (2010) Science 327:1010–1013

Stachyra T, Dini C, Ferrari P, Bouhss A, Heijenoort J, Mengin-Lecreulx D, Blanot D, Biton J, Le Beller D (2004) Antimicrob Agents Chemother 48:897–902

Stephens C, Shapiro L (1997) Chem Biol 4:637–641

Stone KJ, Strominger JL (1971) Proc Natl Acad Sci 68:3223–3227

Sugie Y, Inagaki S, Kato Y, Nishida H, Pang CH, Saito T, Sakemi S, Dib-Hajj F, Mueller JP, Sutcliffe J, Kojima Y (2002) J Antibiot (Tokyo) 55:25–9

Takeda S, Nakai T, Wakai Y, Ikeda F, Hatano K (2007) Antimicrob Agents Chemother 51:826–830

Thomsen LE, Chadfield MS, Bispham J, Wallis TS, Olsen JE (2003) FEMS Microbiol Lett 228:225–231

Thuruthyil SJ, Zhu H, Willcox MD (2001) Clin Experiment Opthalmol 29:147–149

Touzé T, Tran AX, Hankins JV, Mengin-Lecreuix D, Trent MS (2008) Mol Microbiol 67:264–277

Trent MS, Raetz CRH (2002) J Endotoxin Res 8:158

Ulaganathan V, Buetow L, Hunter WN (2007) J Mol Biol 369:305–312

Vaara M (1993) Antimicrob Agents Chemother 37:354–356

Valvano MA, Messner P, Kosma P (2002) Microbiology 148:1979–1989

Van Heijenoort Y, Leduc M, Singer H, Van Heijenoort J (1987) J Gen Microbiol 133:667–674

Vila J, Marti S, Sánchez-Céspedes J (2007) J Antimicrob Chemother 59:1210–1215, jac.oxfordjournals.org

Vuorio R, Vaara M (1992) Antimicrob Agents Chemother 36:826–829

Whitfield C, Amor PA, Köplin R (1997) Mol Microbiol 23:629–638

Whittington DA, Rusche KM, Shin H, Fierke CA, Christianson DW (2003) Proc Natl Acad Sci 100:8146–8150

Wilkinson SG (1996) Prog Lipid Res 35:283–343

Williams AH, Immormino RM, Gewirth DT, Raetz CRH (2006) Proc Natl Acad Sci USA 103:10877–10882

Wyckoff TJO, Raetz CRH, Jackman JE (1998) Trends Microbiol 6:154–159

Yamamoto S, Miyake K, Koike Y, Watanabe M, Machida Y, Ohta M, Iijima S (1999) J Bacteriol 181:5176–5184

Yan A, Guan Z, Raetz CRH (2007) J Biol Chem 282:36077–36089

Yuan J, Zhang ZZ, Chen XZ, Yang W, Huan LD (2004) Appl Microbiol Biotechnol 67:444–452

Zamyatina A, Gronow S, Oertelt C, Puchberger M, Brade H, Kosma P (2000) Angew Chem Int 39:4150–4153

Prevention of Drug Resistance by Combined Drug Treatment of Tuberculosis

Denis A. Mitchison

Contents

1 Principle of Combined Drug Treatment 88
1.1 History of Combined Drug Treatment for Tuberculosis 88
1.2 Combined Drug Treatment for Other Diseases 90
2 Conditions Necessary for the Success of Combined Drug Treatment 90
2.1 Pure Population of the Pathogen 91
2.2 Mutations to Resistance Are Chromosomal and Not Carried by Plasmids 91
2.3 Each Antibacterial Drug Is Sufficiently Active 91
2.4 Special Bacterial Populations 93
2.5 Post-antibiotic Effects 94
3 Emergence of Drug Resistance 95
References 95

Abstract Treatment with a combination of anti-tuberculosis drugs is thought to work by the first drug killing mutants resistant to the second drug, while the second drug kills those resistant to the first drug. Combined treatment has been remarkably successful in preventing the emergence of resistance during the treatment of tuberculosis. This success has led to the introduction of multi-drug treatment for leprosy, HIV infections and cancer. Its success in tuberculosis depends on a number of conditions such as the chromosomal nature of drug resistance in *Mycobacterium tuberculosis* and the absence of plasmids carrying resistance factors as well as the manner in which the bacterial population in tuberculosis does not come into contact with other potentially resistant bacteria. For multi-drug treatment to be effective in preventing resistance, the drugs must be sufficiently active so that each can inhibit all the bacteria in lesions. There must also be effective post-antibiotic lags in growth restarting to prevent growth

D.A. Mitchison (✉)
Centre for Infection, St George's University of London, Cranmer Terrace,
London SW17 0RE, UK
e-mail: dmitchis@sgul.ac.uk

A.R.M. Coates (ed.), *Antibiotic Resistance*, Handbook of Experimental Pharmacology 211, DOI 10.1007/978-3-642-28951-4_6,

© Springer-Verlag Berlin Heidelberg 2012

between doses. Special bacterial populations that are drug tolerant or survive drug action unusually successfully are also a potential source of resistance.

Keywords Mycobacterium tuberculosis • Chromosomal mutation • Drug resistance • Drug activity • Post-antibiotic effect

1 Principle of Combined Drug Treatment

The use of combined drug treatment to prevent the emergence of drug resistance was first explored in the treatment of tuberculosis with antibacterial drugs. Resistance is thought to arise because of the presence of mutations to resistance occurring in a low proportion of bacilli in all sensitive strains. These mutant bacilli multiply when the drug concerned is given alone, eventually replacing the sensitive bacilli. When two drugs are given together, the second drug kills the mutants resistant to the first drug and conversely the first drug kills the mutants resistant to the second drug so that resistance to either drug is prevented (Mitchison 1954).

1.1 History of Combined Drug Treatment for Tuberculosis

The results of the first clinical trial of streptomycin for the treatment of pulmonary tuberculosis were published in 1948 (Medical Research Council 1948). This trial, the first in any aspect of medicine with a randomized intake, compared the standard available treatment with bed rest and good diet to the same treatment with the addition of daily injections of streptomycin, an antibiotic shown to be active against *Mycobacterium tuberculosis* in culture and in experimental tuberculosis of the guinea pig. While the initial response of those patients that received streptomycin was better, in terms of mortality, radiographic improvement and sputum culture, a high proportion of the patients developed streptomycin-resistant strains and the 5-year follow-up results indicated that there was little long-term benefit over those that received no streptomycin (Fox et al. 1954). This result caused those working on improving treatment to concentrate on preventing the emergence of resistance as a principle aim in further clinical trials. The next step forward was a trial in which there were three treatment series that received either (1) streptomycin alone, (2) the weak antibacterial *p*-aminosalicylic acid (PAS) or (3) streptomycin plus PAS (Medical Research Council 1950). A comparison of the emergence of streptomycin resistance in the groups that were given streptomycin alone and those given streptomycin plus PAS indicated that the emergence of streptomycin resistance was to a large extent prevented by giving PAS with the streptomycin, though it was not completely eliminated (Fig. 1). Survival long term was improved (Fox and Sutherland 1956). In 1952, isoniazid became available and proved to be a more active drug than either streptomycin or PAS, being effective in preventing resistance when used in 2-drug combinations with streptomycin (Fox 1953; Medical

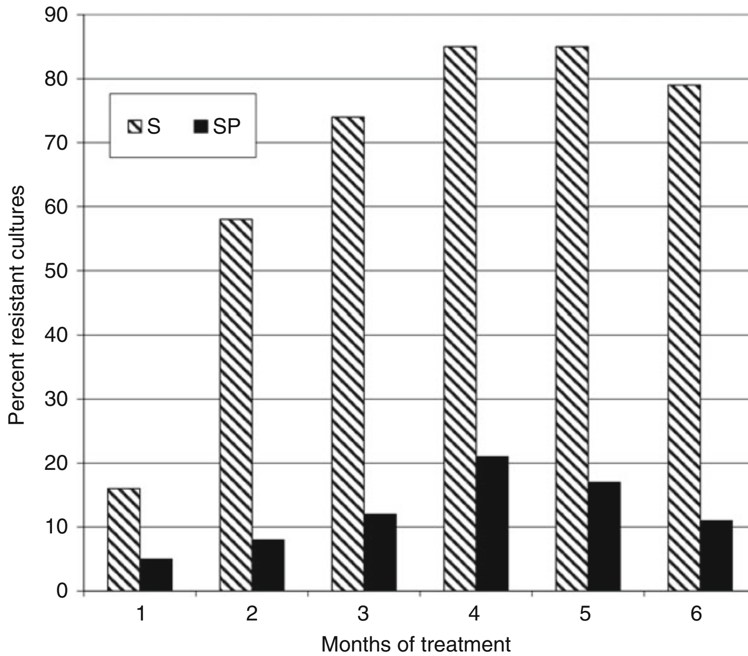


Fig. 1 The percentages of cultures with streptomycin-resistant strains at each month of treatment of patients with either streptomycin alone (S) or with streptomycin + PAS (SP)

Research Council 1953a, b). A survey of the occurrence of drug resistance in the initial cultures of patients attending a sample of British chest clinics showed that drug-resistant strains were mainly resistant to only one of the three drugs, isoniazid, streptomycin or PAS (Fox et al. 1957; Mitchison and Selkon 1957), a finding that encouraged Crofton to explore treatment starting with all three drugs so that even those with resistance to one drug would be successfully treated (Crofton 1958). This led to the regimen of isoniazid, streptomycin and PAS for 2–3 months followed by isoniazid and PAS, which became standard treatment in Europe from about 1960. While the emergence of drug resistance was almost unknown if patients were prepared to take their treatment in hospital for at least 12 months, an international study of this regimen showed that almost half of the patients who were admitted failed to complete their treatment (International Union against Tuberculosis 1964). Following the demonstration in Madras that domiciliary treatment could be given successfully (Andrews et al. 1960; Dawson et al. 1966; Tuberculosis Chemotherapy Centre, Madras 1959), and the introduction of rifampicin and pyrazinamide into the regimen (East African/British Medical Research Council 1972) in 1970, the modern short-course treatment regimen, now accepted as the world standard, was developed in British Medical Research Council trials (Fox et al. 1999). This regimen gives four drugs, isoniazid, rifampicin, pyrazinamide and ethambutol, for 2 months

followed by a further 4 months of isoniazid and rifampicin. The principle of giving combined drugs to prevent the emergence of resistance thus evolved from the initial use of two or three drugs to four drugs during the early phase of treatment when bacterial populations were largest. In the later clinical trials, drug resistance never emerged during treatments that included rifampicin for 6 months, except in four patients in one trial (Hong Kong Chest Service/British Medical Research Council 1991). The great majority of the relapses after the end of treatment were with drug-sensitive strains, which responded well to repeat treatment with the same regimen (Hong Kong Tuberculosis Treatment Services and East African/British Medical Research Councils 1976), but resistance of some sort had emerged in a small proportion of patients in a few studies (East and Central African/British Medical Research Council 1986). It is apparent that the simultaneous use of several drugs in treatment was remarkably successful in preventing the emergence of resistance. Increasingly prevalent drug resistance has become a more important problem in recent years because the standards of treatment were inadequate in some countries, and the multiple drug-resistant (MDR) strains that were created have been spread in local epidemics and by travel to other regions.

1.2 Combined Drug Treatment for Other Diseases

The prevention of drug resistance by the use of combined drugs in the treatment of tuberculosis is not only of vital importance for the effective treatment of tuberculosis but also as a model for the treatment of other important diseases as it has led to a similar use of combined drugs for treating them. Thus, dapsone alone was used starting in the 1950s for the treatment of leprosy. When it became apparent in the 1970s that dapsone-resistant strains of *Mycobacterium leprae* were being created, treatment was started with the drug combination of rifampicin and clofazimine as well as dapsone (Ji 1985; Matsuoka 2010; Waters 1983; WHO Study Group 1982; WHO Expert Committee on Leprosy 1998; World Health Organization Regional Office for South-East Asia New Delhi 2006), drawing from the experience in tuberculosis, with great improvements in results. Combined drug treatment based on its success in tuberculosis is also widely used in the chemotherapy of carcinoma and in the HAART 3-drug treatment of HIV infections (Vandamme et al. 1998).

2 Conditions Necessary for the Success of Combined Drug Treatment

Certain conditions are necessary or desirable for combined drug treatment to be effective in preventing the emergence of resistance. We will consider the nature of these conditions in the treatment of tuberculosis.

Table 1 Efficacy of anti-tuberculosis drugs in preventing the emergence of resistance to isoniazid in 2-drug combinations. Data from (Mitchison 1980)

Isoniazid with	No. of patients	Failures of treatment (%)
Rifampicin	183	0.5
Streptomycin	96	2
Ethambutol	105	4
PAS	309	12
Thiacetazone	425	14

2.1 Pure Population of the Pathogen

M. tuberculosis is usually the sole bacterial pathogen in tuberculous lesions. If other potential pathogens are present, as in infections of the upper respiratory tract, treatment with a single antibacterial drug results in killing of the sensitive bacterial population but its replacement with other naturally resistant species. Under these conditions, combined drug treatment fails to be effective. Lesions of tuberculosis are sometimes caused by more than one separately identifiable strain of *M. tuberculosis* (Cohen et al. 2011; Huang et al. 2010; Mallard et al. 2010; Shamputa et al. 2006), and patients may be freshly infected with another resistant strain during treatment (Rustomjee et al. 2008). Invasions of old tuberculous lesions by non-tuberculous mycobacteria or by fungi also happen but are rare.

2.2 Mutations to Resistance Are Chromosomal and Not Carried by Plasmids

Mutations to drug resistance in *M. tuberculosis* arise in chromosomal genes. If they had been carried by plasmids as resistance transfer factors, resistance to the different drug used in treatment would accumulate in the plasmids and would be transferred (horizontally) with ease to other strains when they met in lesions. However, plasmids do not occur naturally in *M. tuberculosis.*

2.3 Each Antibacterial Drug Is Sufficiently Active

In early days, the "strength" of an anti-tuberculosis drug was understood to mean its efficacy in treatment as found in clinical trials. More conveniently, it was defined in terms of its ability to prevent the emergence of acquired resistance, in most instances when given in 2-drug combinations with isoniazid. (Table 1) (Mitchison 1980). Thus, PAS and thiacetazone allowed isoniazid resistance to emerge in 12–16% of patients, while streptomycin and ethambutol were more effective, allowing resistance in 2–4% of patients, and rifampicin was the most effective

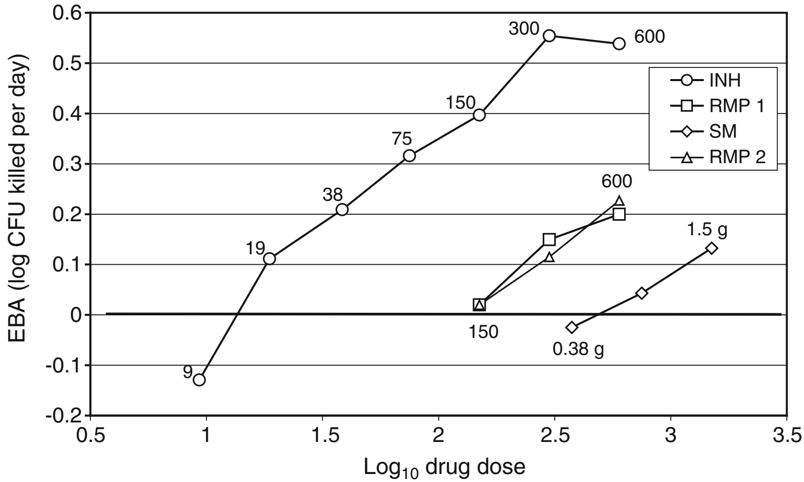


Fig. 2 The early bactericidal activity (EBA) during the first 2 days of treatment with decreasing dose sizes of isoniazid (INH), rifampicin (RMP) or streptomycin (SM). The data from two separate studies with rifampicin are shown. Reprinted with permission of the American Thoracic Society. Copyright © American Thoracic Society (Sirgel et al. 1993)

allowing resistance in only 0.5%. One important characteristic of drug activity relevant to activity is the "therapeutic margin", defined as the ratio between the usual size of the drug dose and its minimal effective dose obtained in a study of its early bactericidal activity (EBA) (Donald et al. 1997). In such a study, the drug is given in a series of decreasing dose sizes to groups of patients for the first 2 days of their treatment (Fig. 2). The content of viable *M. tuberculosis* in their sputum is measured before treatment and at 2 days by plating on selective culture medium. The EBA 0–2 is defined as equal to the fall in log cfu/ml sputum/day during the 2 days of treatment. The results of EBA studies with isoniazid (Donald et al. 1997), rifampicin (Sirgel et al. 1993, 2005) and streptomycin (Donald et al. 2002) show that the therapeutic margin for isoniazid is about 20 since the usual dose size is 300 mg and the dose that just has no effect is about 15 mg. This is unusually high. The therapeutic margin for rifampicin is 4 and for streptomycin about 3 (Fig. 2). Why does the therapeutic margin measure efficacy? It seems likely that a good drug inhibits all of the bacteria in a lesion while less effective drugs only inhibit some of them. As a drug penetrates into avascular or necrotic tissue, the peak concentration sinks lower and lower. There may therefore be parts of the lesion where the peak falls below the MIC, and the drug is therefore inactive. An extreme example of this mechanism was a failure of therapy due to low drug concentrations in a patient with a tuberculous empyema for whom resistance developed during 12 months of therapy to isoniazid (high therapeutic margin) but not to rifampicin or ethambutol (low therapeutic margins) (Elliott et al. 1995). Thus, only isoniazid, with its exceptionally high therapeutic margin, was active against all of the bacterial population in the empyema. While inadequate dosage and consequent low

therapeutic margins are likely to be an important cause of poor drug efficacy, it is not the only cause. For instance, PAS has a moderately high EBA0-2 of 0.26 log cfu/ml sputum/day (Jindani et al. 1980) about the same as 0.21 log cfu/ml sputum/day for rifampicin (Fig. 2) but is undoubtedly a very poor drug when tested in clinical trials (Lehmann 1946).

2.4 *Special Bacterial Populations*

The existence of special bacterial populations within tuberculous lesions that respond differently to the anti-tuberculosis drugs has been postulated (Mitchison 1979; Mitchison and Coates 2004). It is thought that there are considerable differences in growth rates, on the one hand, with a multiplying population that is susceptible to isoniazid and whose killing is marked by a rapid fall in counts of viable bacilli in the sputum during the first week of treatment and, on the other, by one or more static populations that are tolerant to the action of isoniazid and may not be easily killed by other drugs (Jindani et al. 2003). Examples of such static populations are found in the Hu/Coates models where an initially drug-sensitive culture held static for 100 days is found to contain an appreciable proportion of bacilli tolerant to isoniazid and high rifampicin concentrations (Hu et al. 2000, 2003). Another example is the bacilli requiring resuscitation-promoting factors for their growth which are found to constitute the majority of bacilli in sputum (Mukamolova et al. 2010). These tolerant populations are not genetically resistant, but their tolerance might allow resistance to develop.

Again, it is difficult to determine an acid pH that allows bacterial multiplication of all strains but is also sufficiently acid to allow the considerable bactericidal action of pyrazinamide to occur from about day 2 of treatment with pyrazinamide alone (Gumbo et al. 2009; Sulochana et al. 2009). It is therefore reasonable to suppose that the pH of lesions may vary with more acid conditions in regions of active inflammation while the pH would tend towards normality in areas of healing. The existence of these special populations usually makes combined drug treatment less effective than might be supposed and accounts in part at least for the use of four drugs (isoniazid, rifampicin, pyrazinamide, ethambutol) rather than just two in the initial intensive phase of treatment. Because pyrazinamide may not be effective in healing parts of the lesions, it cannot be relied upon as a companion drug, but it does work well on static tolerant populations and so is a good companion for rifampicin. Some drug combinations may provide greater or more complete bactericidal activity than the individual drugs. Thus, pyrazinamide and the diarylquinoline TMC207 have synergistic activity partly because TMC 207 inhibits ATP synthase and so stops the creation of bacterial ATP while a low ATP level promotes the intracellular accumulation of pyrazinoic acid, the active moiety of the prodrug pyrazinamide, and partly because pyrazinoic acid may damage the ATP synthase (Ibrahim et al. 2007). Thus, we have to examine the action of different drugs in multiple therapies to understand why certain combinations are better than others.

2.5 Post-antibiotic Effects

Each dose of most anti-tuberculosis drugs is given once daily, and the pulse where the drug plasma concentration is above the MIC usually lasts for only a few hours. The issue of what happens in the period between the end of the pulse and the arrival of the next dose, while the concentration is lower than the MIC, is largely determined by (1) host immunity which will hinder further growth but may be defective in states of immunosuppression such as HIV infection and (2) the post-antibiotic effect (PAE) of the drug (Mitchison and Dickinson 1971). Of the four drugs given in the initial phase, the PAEs of isoniazid (Dickinson and Mitchison 1966; Mitchison and Dickinson 1971) and rifampicin (Dickinson and Mitchison 1970) have received most study, while the PAE of pyrazinamide is obscure and the dose of ethambutol is probably too low to contribute appreciably to the overall PAE. When a pulse of either isoniazid or rifampicin lasting for about 4 h (as happens during treatment) is given to a culture of *M. tuberculosis*, growth starts again without appreciable delay after the drug is removed. If a longer pulse of isoniazid is given, there is an appreciable delay, lasting up to 6 days, before growth suddenly starts again. Furthermore, if several short (4 h) pulses of isoniazid are given on successive days, they are then followed by a similar long delay in the restart of growth (Awaness and Mitchison 1973). The mechanism involved with this cumulative effect is unknown, but it is of major importance in preventing regrowth between daily doses of isoniazid. On the other hand, no such cumulative effect has been found with rifampicin so that there is little lag in regrowth after each daily dose even when successive doses have been given. The poor effect of the rifampicin PAE in inhibiting regrowth is also due to the way that rifampicin has a much smaller therapeutic margin of 4 than the corresponding value of about 20 for isoniazid despite similar pharmacokinetic data for both drugs. This poor activity of rifampicin has been attributed to plasma binding, but there is no proof that this is the actual cause. A consequence of this difference between the PAEs of isoniazid and rifampicin was found in recent set of experiments with SCID and other immunosuppressed mice treated with isoniazid and rifampicin (Zhang et al. 2011). These mice rapidly developed resistance to isoniazid but not to rifampicin, presumably because the effective isoniazid PAE prevented growth of rifampicin-resistant mutants while the defective rifampicin PAE allowed growth of isoniazid-resistant mutants. Normal mice presumably have enough immunity to prevent the regrowth of any resistant mutants between daily doses of drugs so that the phenomenon is only apparent in the absence of immunity.

Most of the early work on PAEs of anti-tuberculosis drugs was done by Dickinson in the 1960s and only one important contribution of the PAEs of moxifloxacin has been done recently (Ginsburg et al. 2005). The existence of effective PAEs is one of the most important mechanisms for the prevention of drug resistance during combined drug therapy and deserves much further study. In particular, we use drugs in combinations for treatment, but there is no experimental evidence whatsoever on the PAEs that arise after exposure to drug combinations.

3 Emergence of Drug Resistance

In the first instance, drug resistance almost always emerges during the treatment as a result of irregularity in taking drugs, as a result of mechanisms described elsewhere (Mitchison 1998).

As remarked previously, the emergence of resistance during a trial was an extremely rare event, and when it did happen, we cannot exclude the possibility that it was due to a superinfection with a resistant strain since strain identification by fingerprinting methods were not then available. Very rarely, it may arise as a result of resistance appearing for the first time in a relapse culture presumably because of slow selection pressures occurring during the preceding therapy. Once resistance to one drug has appeared, the strain is likely to develop resistance to other drugs if the same treatment is continued, so a cardinal rule in treatment is never just to add one additional drug if treatment is failing but redesign the whole regimen. Strains that are multiple resistant, particularly if they are resistant to isoniazid and rifampicin (MDR), are particularly difficult to treat. Furthermore, such strains are often transmitted to other patients including those attending the same clinic so that new infections may occur with the resistant strain or they may appear as superinfections in patients who started treatment with pan-sensitive strains (Rustomjee et al. 2008). Since the existence of these superinfections is hard to detect except in clinical trials where cultures and susceptibility tests are done as a routine during treatment, it is difficult to assess the frequency of such infections in new case relative to those occurring during treatment. There is certainly a current tendency for multiple drug resistance to be spread increasingly frequently as new infections, and it is also likely that their spread at clinics is also increasing. New effective drugs are essential to meet these problems, and the delays in testing them that arise from rapidly rising costs of clinical trials and over-regulation of the trials themselves are causing huge delays (Jindani and Griffin 2010). The same problems of multiple drug resistance seem also to be arising in leprosy (Ji 1985) and will also probably occur in other diseases where multiple drug treatment is used, but this is still in the future.

References

Andrews RH, Devadatta S, Fox W, Radhakrishna S, Ramakrishnan CV, Velu S (1960) Prevalence of tuberculosis among close family contacts of tuberculous patients in South India, and influence of segregation of the patient on the early attack rate. Bull World Health Organ 23:463–510

Awaness AM, Mitchison DA (1973) Cumulative effects of pulsed exposures of *Mycobacterium tuberculosis* to isoniazid. Tubercle 54:153–158

Cohen T, Wilson D, Wallengren K, Samuel EY, Murray M (2011) Mixed-strain *Mycobacterium tuberculosis* infections among patients dying in a hospital in KwaZulu-Natal, South Africa. J Clin Microbiol 49:385–388

Crofton J (1958) Sputum conversion and the metabolism of isoniazid. Am Rev Tuberc 77:869–871

Dawson JJY, Devadatta S, Fox W, Radhakrishna S, Ramakrishnan CV, Somasundaram PR, Stott H, Tripathy SP, Velu S (1966) A 5-year study of patients with pulmonary tuberculosis in a concurrent comparison of home and sanatorium treatment for one year with isoniazid plus PAS. Bull World Health Organ 34:533–551

Dickinson JM, Mitchison DA (1966) In vitro studies on the choice of drugs for intermittent chemotherapy of tuberculosis. Tubercle 47:370–380

Dickinson JM, Mitchison DA (1970) Suitability of rifampicin for intermittent administration in the treatment of tuberculosis. Tubercle 51:82–94

Donald PR, Sirgel FA, Botha FJ, Seifart HI, Parkin DP, Vandenplas ML, Van de Wal BW, Maritz JS, Mitchison DA (1997) The early bactericidal activity of isoniazid related to its dose size in pulmonary tuberculosis. Am J Respir Crit Care Med 156:895–900

Donald PR, Sirgel FA, Venter A, Smit E, Parkin DP, Van de Wal BW, Dore CJ, Mitchison DA (2002) The early bactericidal activity of streptomycin. Int J Tuberc Lung Dis 6:693–698

East African/British Medical Research Council (1972) Controlled clinical trial of short-course (6-month) regimens of chemotherapy for treatment of pulmonary tuberculosis. Lancet 299: 1079–1085

East and Central African/British Medical Research Council (fifth Collaborative Study) (1986) Controlled clinical trial of 4 short-course regimens of chemotherapy (three 6-month and one 8-month) for pulmonary tuberculosis: final report. Tubercle 67:5–15

Elliott AM, Berning SE, Iseman MD, Peloquin CA (1995) Failure of drug penetration and acquisition of drug resistance in chronic tuberculous empyema. Tuber Lung Dis 76:463–467

Fox W (1953) The medical research council trials of isoniazid. Recent results of combined chemotherapy. Bull Int Union Tuberc Lung Dis 23:292–307

Fox W, Ellard GA, Mitchison DA (1999) Studies on the treatment of tuberculosis undertaken by the British Medical Research Council Tuberculosis Units, 1946–1986, with relevant subsequent publications. Int J Tuberc Lung Dis 3:S231–S279

Fox W, Sutherland I (1956) A five-year assessment of patients in a controlled trial of streptomycin, para-aminosalicylic acid, and streptomycin plus para-aminosalicylic acid, in pulmonary tuberculosis. Q J Med 25:221–243

Fox W, Sutherland I, Daniels M (1954) A five-year assessment of patients in a controlled trial of streptomycin in pulmonary tuberculosis. Q J Med 23:347–366

Fox W, Wiener A, Mitchison DA, Selkon JB, Sutherland I (1957) The prevalence of drug-resistant tubercle bacilli in untreated patients with pulmonary tuberculosis: A national survey, 1955–56. Tubercle 38:71–84

Ginsburg AS, Lee J, Woolwine SC, Grosset JH, Hamzeh FM, Bishai WR (2005) Modeling in vivo pharmacokinetics and pharmacodynamics of moxifloxacin therapy for *Mycobacterium tuberculosis* infection by using a novel cartridge system. Antimicrob Agents Chemother 49:853–856

Gumbo T, Dona CS, Meek C, Leff R (2009) Pharmacokinetics-pharmacodynamics of pyrazinamide in a novel in vitro model of tuberculosis for sterilizing effect: a paradigm for faster assessment of new antituberculosis drugs. Antimicrob Agents Chemother 53:3197–3204

Hong Kong Tuberculosis Treatment Services and East African/British Medical Research Councils (1976) First-line chemotherapy in the retreatment of bacteriological relapses of pulmonary tuberculosis following a short-course regimen. Lancet 1:162–163

Hong Kong Chest Service/British Medical Research Council (1991) Controlled trial of 2, 4 & 6 months of pyrazinamide in 6-month, 3× weekly regimens for smear- positive pulmonary tuberculosis, including an assessment of a combined preparation of isoniazid, rifampin and pyrazinamide. Am Rev Respir Dis 143:700–706

Hu Y, Mangan JA, Dhillon J, Sole KM, Mitchison DA, Pd B, Coates ARM (2000) Detection of mRNA transcripts and active transcription in persistent Mycobacterium tuberculosis induced by exposure to rifampin or pyrazinamide. J Bacteriol 182:6358–6365

Hu Y, Coates ARM, Mitchison DA (2003) Sterilizing activities of fluoroquinolones against rifampin-tolerant populations of Mycobacterium tuberculosis. Antimicrob Agents Chemother 47:653–657

Huang HY, Tsai YS, Lee JJ, Chiang MC, Chen YH, Chiang CY, Lin NT, Tsai PJ (2010) Mixed infection with Beijing and non-Beijing strains and drug resistance pattern of *Mycobacterium tuberculosis*. J Clin Microbiol 48:4474–4480

Ibrahim M, Andries K, Lounis N, Chauffour A, Truffot-Pernot C, Jarlier V, Veziris N (2007) Synergistic activity of R207910 combined with pyrazinamide against murine tuberculosis. Antimicrob Agents Chemother 51:1011–1015

International Union against Tuberculosis (1964) An international investigation of the efficacy of chemotherapy in previously untreated patients with pulmonary tuberculosis. Bull Int Union Tuberc 34:80–191

Ji BH (1985) Drug resistance in leprosy—a review. Lepr Rev 56:265–278

Jindani A, Aber VR, Edwards EA, Mitchison DA (1980) The early bactericidal activity of drugs in patients with pulmonary tuberculosis. Am Rev Respir Dis 121:939–949

Jindani A, Doré CJ, Mitchison DA (2003) The bactericidal and sterilising activities of antituberculosis drugs during the first 14 days. Am J Respir Crit Care Med 167:1348–1354

Jindani A, Griffin GE (2010) Challenges to the development of new drugs and regimens for tuberculosis. Tuberculosis (Edinb) 90:168–170

Lehmann J (1946) Para-aminosalicylic acid in the treatment of tuberculosis. Lancet 5(1):15

Mallard K, McNerney R, Crampin AC, Houben R, Ndlovu R, Munthali L, Warren RM, French N, Glynn JR (2010) Molecular detection of mixed infections of *Mycobacterium tuberculosis* strains in sputum samples from patients in Karonga District, Malawi. J Clin Microbiol 48:4512–4518

Matsuoka M (2010) Drug resistance in leprosy. Jpn J Infect Dis 63:1–7

Medical Research Council (1948) Streptomycin treatment of pulmonary tuberculosis. Br Med J 2:769–782

Medical Research Council (1950) Treatment of pulmonary tuberculosis with streptomycin and para-amino-salicylic acid. Br Med J 2:1073–1085

Medical Research Council (1953a) Isoniazid in the treatment of pulmonary tuberculosis. Second report. Br Med J 1:521–536

Medical Research Council (1953b) Emergence of bacterial resistance in pulmonary tuberculosis under treatment with isoniazid, streptomycin plus PAS, and streptomycin plus isoniazid. Lancet 262:217–223

Mitchison DA (1954) Problems of drug resistance. Br Med Bull 69:640–641

Mitchison DA (1979) Basic mechanisms of chemotherapy. Chest 76S:771–781S

Mitchison DA (1980) Treatment of tuberculosis. J R Coll Physicians Lond 14:91–99

Mitchison DA (1998) How drug resistance emerges as a result of poor compliance during short course chemotherapy for tuberculosis. Int J Tuberc Lung Dis 2:10–15

Mitchison DA, Coates ARM (2004) Predictive in vitro models of the sterilizing activity of anti-tuberculosis drugs. Curr Pharm Des 10:3285–3295

Mitchison DA, Dickinson JM (1971) Laboratory aspects of intermittent drug therapy. Postgrad Med J 47:737–741

Mitchison DA, Selkon JB (1957) Bacteriological aspects of a survey of the incidence of drug-resistant tubercle bacilli among untreated patients. Tubercle 38:85–98

Mukamolova GV, Turapov O, Malkin J, Woltmann G, Barer M (2010) Resuscitation-promoting factors reveal an occult population of tubercle bacilli in sputum. Am J Respir Crit Care Med 181:174–180

Rustomjee R, Lienhardt C, Kanyok T, Davies GR, Levin J, Mthiyane T, Reddy C, Sturm AW, Sirgel FA, Allen J, Coleman DJ, Fourie B, Mitchison DA, the Gatifloxacin for TB (OFLOTUB) study team (2008) A phase II study of the sterilizing activities of ofloxacin, gatifloxacin and moxifloxacin in pulmonary tuberculosis. Int J Tuberc Lung Dis 12:128–138

Shamputa IC, Jugheli L, Sadradze N, Willery E, Portaels F, Supply P, Rigouts L (2006) Mixed infection and clonal representativeness of a single sputum sample in tuberculosis patients from a penitentiary hospital in Georgia. Respir Res 7:99

Sirgel FA, Botha FJH, Parkin DP, Van de Wal BW, Donald PR, Clark PK, Mitchison DA (1993) The early bactericidal activity of rifabutin in patients with pulmonary tuberculosis measured by sputum viable counts. A new method of drug assessment. J Antimicrob Chemother 32:867–875

Sirgel FA, Fourie PB, Donald PR, Padayatchi N, Rustomjee R, Levin J, Roscigno G, Norman J, McIllernon H, Mitchison DA, and the Rifapentine EBA Collaborative Study Group (2005) The early bactericidal activities of rifampicin and rifapentine in pulmonary tuberculosis. Am J Respir Crit Care Med 172:128–135

Sulochana S, Mitchison DA, Kubendiren G, Venkatesan P, Paramasivan CN (2009) Bactericidal activity of moxifloxacin on exponential and stationary phase cultures of *Mycobacterium tuberculosis*. J Chemother 21:127–134

Tuberculosis Chemotherapy Centre, Madras (1959) A concurrent comparison of home and sanatorium treatment of pulmonary tuberculosis in South India. Bull World Health Organ 21:51–144

Vandamme AM, Van Vaerenbergh K, De Clercq E (1998) Anti-human immunodeficiency virus drug combination strategies. Antivir Chem Chemother 9:187–203

Waters MF (1983) The treatment of leprosy. Tubercle 64:221–32

WHO Study Group (1982) Chemotherapy of leprosy for control programs. WHO Tech rep Ser 675

WHO Expert Committee on Leprosy (1998) Seventh Report. World Health Organ Tech Rep Ser 874:1–43

World Health Organization Regional Office for South-East Asia New Delhi (2006) Global strategy for further reducing the leprosy burden and sustaining leprosy control activities 2006–2010. Operational guidelines. Lepr Rev 77:1–50

Zhang M, Li SY, Rosenthal IM, Almeida DV, Ahmad Z, Converse PJ, Peloquin CA, Nuermberger EL, Grosset JH (2011) Treatment of tuberculosis with rifamycin-containing regimens in immune deficient mice. Am J Respir Crit Care Med 183:1254–1261

Nonmultiplying Bacteria are Profoundly Tolerant to Antibiotics

Yanmin Hu and Anthony Coates

Contents

1 Nonmultiplying Bacteria and Persisters ... 100
1.1 Nonmultiplying Stationary-Phase Bacteria ... 101
1.2 Biofilms: Another Form of Nonmultiplying Bacteria ... 102
1.3 Persisters ... 103
1.4 Dormant Bacteria ... 105
2 Clinical Importance of Persistent Bacteria ... 105
3 Persistent *M. tuberculosis* ... 106
3.1 Different Populations of *M. tuberculosis* in Human Lesions ... 107
3.2 Dormant *M. tuberculosis* in Animal Models ... 107
3.3 Subpopulations of Nonmultiplying *M. tuberculosis* In Vitro ... 108
4 Antibiotics Kill Nonmultiplying Bacteria ... 109
4.1 Bactericidal and Sterilizing Antibiotics ... 109
4.2 Antimicrobials Targeting Cell Membrane and Cell Wall ... 110
4.3 Antipersister Formation and Waking up Dormancy ... 111
5 Conclusion ... 112
References ... 113

Abstract Bacteria survive treatments with antimicrobial agents; they achieve this in two ways. Firstly, bacteria quickly become tolerant to these agents. This tolerance is temporary, reversible, and associated with slowing of the multiplication rate. Secondly, bacteria can undergo genetic mutations leading to permanent clonal resistance to antimicrobial agents. In patients with infections, nonmultiplying bacteria, some of which may be viable but nonculturable, exist side by side with multiplying bacteria. Current antibiotics capable of killing actively multiplying bacteria have very limited or no effect against nonmultiplying bacteria. Treatment

Y. Hu (✉) • A. Coates
Medical Microbiology, Division of Clinical Sciences, Centre for Infection, St George's University of London, Cranmer Terrace, London SW17 ORE, UK
e-mail: ymhu@sgul.ac.uk

A.R.M. Coates (ed.), *Antibiotic Resistance*, Handbook of Experimental Pharmacology 211, DOI 10.1007/978-3-642-28951-4_7,
© Springer-Verlag Berlin Heidelberg 2012

of such infections requires a regimen of multiple antimicrobial agents in order to control nonmultiplying persistent bacteria. This is especially important in tuberculosis where there is co-existence of slowly multiplying tolerant bacteria with fast growing sensitive organisms. For this reason, a prolonged length of chemotherapy, lasting 6 months, is necessary to achieve cure. This long duration of treatment is due to the slow, inadequate effect of antibiotics on nonmultiplying persistent bacteria. Similar problems with eradication of persistent bacteria are evident in the treatment of biofilms. These bacteria serve as a pool for recurrent infections. Extended courses of antibiotics increase the likelihood of genetic resistance, raise the cost of treatments, and lead to more side effects.

Keywords Antibiotic tolerance • Nonmultiplying bacteria • Stationary phase • Persisters • Dormant bacteria

1 Nonmultiplying Bacteria and Persisters

In modern medicine, antibiotics are important in the fight against infections (Ball et al. 2004) such as bacteremia (Austrian and Gold 1964) and tuberculosis (Dineeen et al. 1976) which used to have a poor survival rate. Today, bacterial resistance to antimicrobial agents is emerging at a rate which outpaces the discovery of new antibiotics (Vashishtha 2010) and multiple-drug-resistant (MDR) bacterial pathogens (Gootz 2010) are common. This resistance profile is due to genomic modifications of bacteria by point mutations and horizontal gene transfer which lead to permanent clonal resistance to antimicrobial agents. There is another mechanism of antibiotic resistance or tolerance which is primarily dependent on the bacterial physiological state. The efficacy of the majority of antibiotics depends on the target bacteria exhibiting a high level of metabolic activity. However, once growth rate of bacteria slows down, they become insensitive to antibiotic treatment. In infectious diseases, slow or nonmultiplying tolerant bacteria coexist with fast growing sensitive organisms (Seguin et al. 2003). Antibiotics are capable of killing actively multiplying bacteria, but are almost always only partially active against slowly multiplying, or are inactive against nonmultiplying persistent bacteria (Hu et al. 2010; Coates and Hu 2006; Hu and Coates 2005). Also more than 60% of all microbial infections are caused by nonmultiplying bacteria such as those present in biofilms (Lewis 2001). The persistent bacteria are responsible for recurrent infections as seen in tuberculosis. With the currently available antibiotics, most persistent infections cannot be eradicated and are therefore associated with poor clinical outcomes (Kyd et al. 2011; Wood and Douglas 2010). Antibiotic tolerance is an important problem in patients with infections because prolonged treatment with multiple doses of antimicrobial agents is required for most bacterial infections. Compared to shorter courses with a single agent, this prolonged treatment with multiple antibiotic regimens can increase the frequency of genetic resistance associated with poor patient compliance (Coates and Hu 2006; Pechere et al. 2007), increase the cost of the treatment, and cause more side effects.

1.1 Nonmultiplying Stationary-Phase Bacteria

Bacteria grow and divide exponentially in cell suspension when growth conditions are favorable. If a certain nutrient in a medium is reduced below a threshold level, it halts key metabolic processes, such as DNA replication, and growth is arrested (Navarro Llorens et al. 2010). In order to survive nutrient deprivation, the bacteria are able to make an orderly transition from exponential growth phase to stationary phase. Cells in stationary-phase culture can survive long periods of starvation (Hu and Coates 2005) in the nonmultiplying stage (Roostalu et al. 2008). The stationary-phase nonmultiplying bacteria are able to resume growth rapidly when nutrients again become available (Siegele and Kolter 1992, 1993). Kolter et al. (1993) reported that entry into stationary phase involves a process of transition which starts at a time point in the exponential phase when DNA, proteins, and total cell mass stop increasing and continues until no further increase in cell numbers is detected. Cells are then in the stationary phase. Many factors can be responsible for this phenomenon. The most clearly defined one is starvation for a single nutrient required for growth. Some bacteria form endospores and myxospores in response to starvation (Kaiser 1986; Losick et al. 1986), but most bacterial species do not generate such differentiated cells. Only nondifferentiating bacteria are discussed in this chapter.

The stationary-phase response gives rise to dramatic changes in cell morphology, physiology, and gene expression (Smith 1995; Wortinger et al. 1998; Navarro Llorens et al. 2010). Lange and Hengge-Aronis examined *Escherichia coli* cells by light microscopy (Lange and Hengge-Aronis 1991). They found that *E. coli* cells became much smaller and almost spherical by reductive division when they entered into stationary phase. Some bacteria greatly reduce their size during starvation and form ultramicrocells which are less than 0.4 μm in diameter (Yourassowsky et al. 1979; Lee and Veeranagouda 2009). Changes in chromosomal topology accompanied with reduced cell division were also observed. After a few hours in stationary phase, changes in the negative superhelical density of plasmids can be detected in *E. coli* (Balke and Gralla 1987). The stationary-phase cultures of *S. aureus* produce small-colony variants (Fig. 1) which are very tolerant to antibiotics and are responsible for recurrent infections in humans (Proctor et al. 1998, 2006; Singh et al. 2009).

The changes in cell envelope and cell membrane of starved cells mirror those which insulate and protect bacteria from stress (Martinez-Rodriguez and Mackey 2005; Siegele and Kolter 1992; Tuomanen and Tomasz 1990). For example, many stationary-phase marine bacteria coat their surfaces with hydrophobic materials that lead to cell adhesion and aggregation. Alterations in membrane composition such as fatty acids result in less fluid and less permeable membranes (Siegele and Kolter 1992; Cronan 1968).

As bacteria enter into stationary phase, their overall metabolic rate decreases but a low level of endogenous metabolism is retained (Hu et al. 2000), which enables the bacteria to take substrates into the cell during starvation and resume growth when nutrients become available (Siegele and Kolter 1992). At the same time

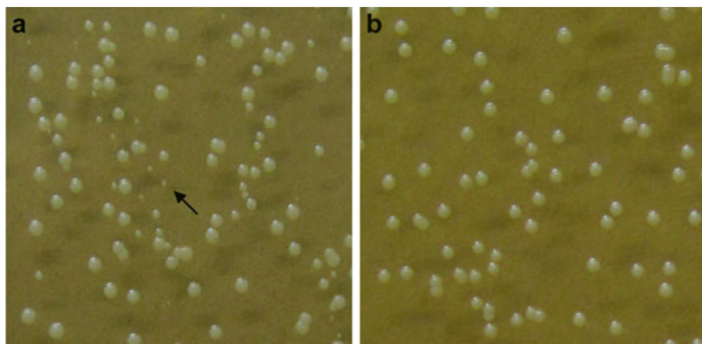


Fig. 1 Colony morphology of *S. aureus* in stationary-phase cultures. *S. aureus* was grown in broth culture for 6 days. The stationary-phase culture was diluted and plated onto a nutrient agar plate. There are many small colony variants formed in the stationary-phase culture indicated by the *arrow* (**a**) compared to the colonies formed by an exponential growth phase culture (**b**)

stationary-phase bacteria synthesize and accumulate storage compounds, such as glycogen and polyphosphates and protective substances, such as trehalose (Hengge-Aronis et al. 1991).

Stationary-phase bacteria are more resistant to stress conditions such as heat shock, osmotic challenge, acidic and oxidative stress than exponential-phase bacteria (Soares et al. 2010; Hengge-Aronis 1993; Jenkins et al. 1988, 1990; Cornet et al. 2010; Davis et al. 1996; Steels et al. 1994; Arnold and Kaspar 1995; Navarro Llorens et al. 2010; Zech et al. 2009). Particularly, stationary-phase bacteria are insensitive to conventional antibiotic treatment and can only be killed by multiple doses of antibiotics at high concentration (McLeod and Spector 1996; Tuomanen and Tomasz 1990; Hu et al. 2000; Dorr et al. 2009). This is called antibiotic indifference (Jayaraman 2008).

1.2 Biofilms: Another Form of Nonmultiplying Bacteria

In nature, apart from growing in a planktonic form which is suspended or growing in a fluid environment, bacteria will also grow on surfaces to form biofilms (Trautner and Darouiche 2004). A biofilm is highly organized. It is a compact multicellular community which is on a liquid–surface interface and is embedded in a self-produced exopolysaccharide matrix. Multiplying cells occur superficially and slow or nonmultipliers live in the deeper layers. Biofilm formation is an inevitable key step in the life cycle of most microorganisms and found on many biological and nonbiological surfaces. They are associated with many infectious diseases, such as infective endocarditis, chronic skin wounds, osteomyelitis, dental plaques, infective cystic fibrosis, and infections due to indwelling medical devices such as catheters, prosthetic heart valves, and shunts (Fux et al. 2005; Mittal et al. 2009). Antibiotic therapy of infections which are associated with biofilms often lead to a poor clinical

outcome, because the bacteria within the biofilms are extremely tolerant to antimicrobial agents (Lewis 2008). Successful treatment of biofilm-associated diseases requires multiple doses of high concentration antibiotic regimens or the removal of foreign body devices (Trautner and Darouiche 2004). Bacteria within biofilms are not genetically resistant strains per say. When the same cells are spread out and re-cultured in a planktonic culture, they retain sensitivity to the antibiotics (Spoering and Lewis 2001). The physiological states of the bacteria in the depth of a biofilm are very similar to those in stationary-phase planktonic culture. They are induced by nutrient starvation, high density of cell population, and accumulation of metabolic waste (Spoering and Lewis 2001; Fux et al. 2005). Biofilms are resistant to environmental stresses such as altered pH, osmolarity, and nutrient limitation. Furthermore, biofilm formation enables the bacteria to become resistant to host immune defenses (Vuong et al. 2004; Fux et al. 2005).

It has been suggested that biofilm tolerance to antibiotics and host immunity is due to the following (Lewis 2001; Fux et al. 2005): Firstly, slime matrix limits penetration of the antibiotic (Renslow et al. 2010). The exopolysaccharide matrix in which bacteria are embedded plays a key role in preventing large or small antimicrobial agents from binding to and penetrating into a biofilm. The slime matrix also protects bacteria from being engulfed during phagocytosis (Rohde et al. 2005; Vuong et al. 2004) and enhances bacterial virulence (Begun et al. 2007). Antibodies bind to the matrix and are not able to penetrate into bioflims (de Beer et al. 1997). Bacterial proteins in the biofilm matrix render them more resistant to attacks by complement (Simmons and Dybvig 2007). The exopolysaccharide matrix is likely to prevent smaller antibiotics, such as glycopeptides, crossing the diffusion barrier (Lewis 2001; Singh et al. 2010). In addition, the negatively charged exopolysaccharide binds to the positively charged antibiotics such as aminoglycosides, which effectively blocks antibiotic activity (Walters et al. 2003). Secondly, different physiological states of bacteria determine antibiotic tolerance. Biofilm formation undergoes highly regulated processes including surface attachment, cellular proliferation by cell–cell interactions, maturation by producing matrix, antibiotic tolerance development and detachment which results in bacteria regaining planktonic growth mode (O'Toole et al. 2000). In the early stage of biofilm formation, when bacteria are actively replicating, the cells are sensitive to eradication by antimicrobial agents (Gunther et al. 2009). In a mature bioflim, bacteria slow down or terminate replication and become tolerant to antibiotics. A small proportion of nonmultiplying bacteria in a biofilm cannot be eradicated by any antimicrobial agents and human immune clearance (Lewis 2008). Once the antimicrobial agents become unavailable, these persistent bacteria reform the biofilm and this leads to the relapse of infection.

1.3 Persisters

In the 1940s, Bigger (Bigger 1944) noticed that a culture of *S. aureus* could not be completely killed by penicillin, even at high concentration. He called these surviving bacterial cells "persisters." Persistent cells are also present in the

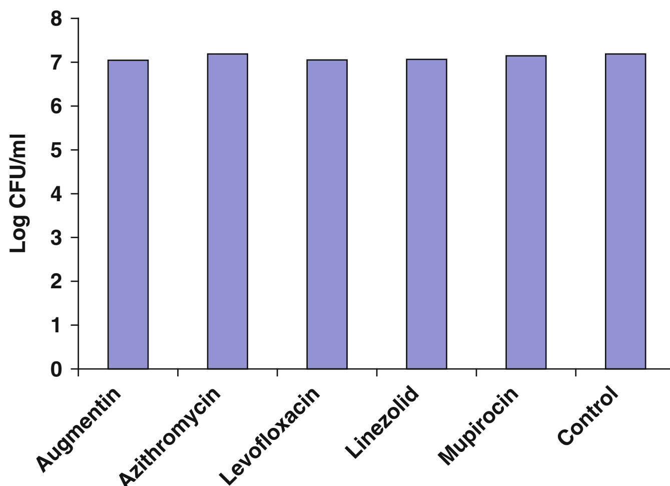


Fig. 2 Activities of currently marketed antibiotics against stationary-phase nonmultiplying *S. aureus*. The 6-day stationary-phase culture was incubated with augmentin (amoxicillin/clavulanate), azithromycin, levofloxacin, linezolid, and mupirocin at 100 μg/ml for 24 h. Viability of the bacteria was determined by colony forming unit counts. There were no antimicrobial activities observed against the stationary-phase nonmultiplying bacteria for any the tested antibiotics

populations of "old" stationary-phase bacteria in batch cultures (Kaprelyants and Kell 1993; Kaprelyants et al. 1993). Old persisters can survive for a long time—it has been demonstrated that a 38-year-old stationary-phase *E. coli* culture contained more than 10^5 viable bacteria/ml in the nonmultiplying persistent stage (Eisenstark et al. 1992). Generally, persistent bacteria can be isolated by antibiotic treatment which eliminates the multiplying bacterial population (Hu et al. 2000). Persisters can be found in exponential growth culture of *E. coli* (Balaban et al. 2004), and this has been observed in single cells using a microfluidic device. So, persisters preexist the addition of an antibiotic to a culture and are not actually produced by such treatment. The proportion of the persistent bacteria increases upon entry into stationary phase and during biofilm formation. Persisters are nonmultiplying (Roostalu et al. 2008) and constitute 1% of the bacterial population in stationary-phase cultures and biofilms (Shah et al. 2006; Balaban et al. 2004; Spoering and Lewis 2001). These persisters are not genetically modified mutants, but are transient phenotypic variants of their parental population (Wiuff et al. 2005). They are profoundly tolerant to most marketed antibiotics (Wiuff et al. 2005; Hu et al. 2010; Keren et al. 2004a, b). If *E. coli* or *S. aureus* cells in a late stationary-phase culture are resuspended in phosphate buffered saline, the nonmultiplying persistent bacteria are very tolerant to high doses of the current antibiotics (Hu et al. 2010) as seen in Fig 2. After removal of antibiotic pressure, the small fraction of the persistent cells restore growth and become sensitive to antibiotics again (Bigger 1944).

1.4 Dormant Bacteria

A number of studies have indicated that there is a subgroup of dormant microbes among nonsporulating bacterial species which are unable to divide or form colonies on agar plates but are able to produce daughter cells under appropriate conditions (Kaprelyants et al. 1993). They are formed as a result of adaptation to starvation or other unfavorable conditions. The term "viable but nonculturable" (VBNC) was introduced by Colwell et al. (1985) and includes the dormant bacteria which are present in populations of some Gram-negative bacteria such as *E. coli* (Darcan et al. 2009), *Salmonella* (Turner et al. 2000), and *Vibrio* spp. (Chaiyanan et al. 2007; Baffone et al. 2003) starved in an aquatic environment. VBNC cells are also evident in many human pathogens such as S. *enteritidis, V. cholerase, Shigella sonnei, S. flexneri* (Colwell et al. 1985; Roszak et al. 1984), *Listeria monocytogenes* (Lindback et al. 2010; Cappelier et al. 2005, 2007), *Enterococcus* spp. (Lleo et al. 2001), *Campylobacter jejuni* (Jackson et al. 2009; Klancnik et al. 2009; Baffone et al. 2006), *Helicobacter pylori* (Saito et al. 2003), *Staphylococcus aureus* (Masmoudi et al. 2010), *Mycobacterium tuberculosis* (Hu et al. 2000), and *Pseudomonas aeruginosa* (Moore et al. 2007).

VBNC cells are usually smaller than the normal cells (Roszak and Colwell 1987). Torrellar and Morita (1981) observed very small cells of marine bacteria (ultramicrocells) which had reduced growth rates. Nilsson and colleagues (1991) also found that nonculturable *V. vulnificus* formed small cocci of 1.0 μm in diameter. In contrast, their rod-shaped (3.0 μm in length) vegetative cells are much larger. In general, most ultramicrobacteria of marine bacteria are unable to form colonies on agar plates (Morita 1988) but can exist as VBNCs. These cells still exhibit low metabolic activity (Barcina et al. 1989). Also, it has been found that Gram-negative and Gram-positive VBNC cells alter their cell wall components which results in robust resistance to environmental stress (Signoretto et al. 2000, 2002; Costa et al. 1999). VBNC cells can be resuscitated. Successful in vitro resuscitation and growth of *S. enteritidis* has been reported by Roszak et al. (1984). After addition of nutrients to microcosms (sterile river water cultures) for 25 h, the nonculturable cells produce colonies on solid agar plates.

2 Clinical Importance of Persistent Bacteria

Persistent bacteria including the VBNC forms are present in many chronic human infectious diseases (Rihl et al. 2006; Coates et al. 2008). Although persisters such as VBNC bacteria cannot cause overt disease, they form an important reservoir of latent survivors and are responsible for persistent and recurrent infections (Velazquez and Feirtag 1999). Persistent infections almost always need multiple antibiotic regimens and long periods of treatment (El Solh et al. 2008). As these persistent bacteria are nonmultiplying and cannot be cultured by traditional

microbiological methods, it has proved to be difficult to detect the presence of these populations in an infection (Lleo et al. 2007; Signoretto et al. 2004). Persisters retain their pathogenicity as evidenced by maintaining their ability to adhere to human tissues and to produce toxins (Oliver 1995; Fischer-Le Saux et al. 2002; Pruzzo et al. 2002, 2003; Pommepuy et al. 1996). In chronic urinary tract infections (UTIs), a nonculturable form of *E. coli* with a round shape has been detected in bladder epithelial cells, and this may serve as a pool for recurrent UTIs (Anderson et al. 2003, 2004; Rivers and Steck 2001; Mulvey et al. 2001). Persistent bacteria which are present in UTIs are extremely tolerant to the highest achievable serum concentrations of all antibiotics commonly prescribed for this disease (Seguin et al. 2003). This means that eradication of persisters needs long-term antibiotic treatment, and under certain circumstances, such as catheter-associated infections, persisters cannot be eradicated by antibiotics at all, so the infected catheter has to be physically removed from the patient. This situation also applies to many other implant-associated infections. It has also been found that VBNC *H. pylori* is present in the gastric mucosa. These bacteria change shape from vegetative rods to an unusual round form (Kusters et al. 2006). Reactive arthritis is associated with bacterial infections which are caused by pathogens such as *Chlamydia* spp., *Salmonella* spp., *Yersinia* spp., *Shigella* spp., *Campylobacter* spp., and *Clostridium* spp. It is believed that there may be small numbers of VBNC persisters present in the joint. These bacteria cannot be cultured from synovial specimens, but their existence can be confirmed by the presence of bacterial antigens which are synthesized by metabolically active bacteria (Colmegna et al. 2004; Rihl et al. 2006). Prolonged antibiotic therapy of 12–50 weeks duration is required to cure the disease (Rihl et al. 2006) due to these persistent nonculturable bacteria.

3 Persistent *M. tuberculosis*

One of the most important characteristics of *M. tuberculosis* in the pathogenesis of the disease is its ability to persist in human tissue for the life span of the human host. During *M. tuberculosis* infection, the acquired immune response inhibits the replication of tubercle bacilli, but not all bacilli are destroyed. A small proportion of the bacilli remain in a dormant or persistent form (Dannebery and Rook 1994) causing latent infections. Dormant *M. tuberculosis* is important not only because it can survive attacks by the immune response but also because, when compared to actively growing bacteria, it is more tolerant to antibacterial agents. This means prolonged chemotherapy for 6 months is required to produce a cure (Mitchison 2004). These persistent bacteria are usually drug sensitive at relapse, so their resistance to chemotherapy is phenotypic tolerance (Hu et al. 2000) rather than genetic mutation. It is suggested that an altered physiological state of persistent *M. tuberculosis* accounts for its tolerance to drugs as well as the ability to survive in the host for many years. Persistence is likely to be a combined effect of both the immune system and bacterial physiology, resulting in what is generally referred to as a dormant or a latent state (Bloom and McKinney 1999).

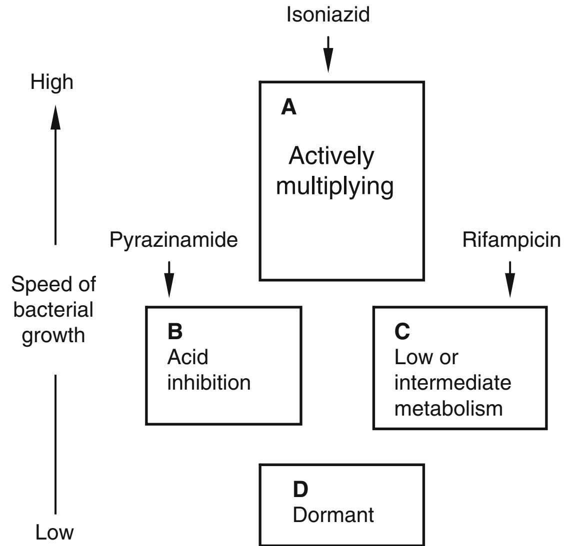


Fig. 3 Hypothesis of special populations of tubercle bacilli within lesions killed by the bactericidal and sterilizing drugs (Mitchison 1979)

3.1 *Different Populations of* M. tuberculosis *in Human Lesions*

It has been suggested (Mitchison 1979) that there are four different components of the bacterial population in tuberculosis lesions. As shown in Fig. 3, the majority of bacilli in the lesions of an untreated patient with tuberculosis are actively multiplying, probably at a rate similar to that in a log phase culture (population A). These bacilli may be present in the lining of open cavities with an abundant of oxygen supply, which favors the bacilli to grow rapidly. There is also a small proportion of dormant bacilli (population D) whose metabolism and growth is almost completely inhibited by an unfavorable environment, such as in closed lesions where oxygen becomes unavailable. In addition there are semidormant bacilli: Population B consists of those organisms which are inhibited by an acid environment, such as in early acute inflammatory lesions or within the phagolysosomes of macrophages. Population C is assumed to be those bacilli which contain brief or intermittent metabolic activity.

3.2 *Dormant* M. tuberculosis *in Animal Models*

The existence of dormant or persistent *M. tuberculosis* after chemotherapy was shown for the first time in an animal model by McCune and colleagues more than half century ago (McCune and Tompsett 1956; McCune et al. 1966b). The “Cornell model” which was named after Cornell University where the work was performed is considered to be the most useful model for demonstrating and studying mycobacterial dormancy. In this model, a large number of mice are infected

intravenously with 10^5–10^6 CFU of *M. tuberculosis* H37Rv. Therapy with isoniazid and pyrazinamide for 12 weeks is started immediately after infection. After termination of treatment, a “sterile” state is achieved. At this stage, no tubercle bacilli are detected from homogenates of lungs or spleens of the mice by bacteriological culture and microscopy. However, the disappearance of the bacilli does not mean that the organisms have been eliminated from the tissues. About 3 months after the end of drug treatment, culturable bacilli are detected from about one-third of the mice. The reappearance of the tubercle bacilli in another two-thirds of the animals occurs within 9 months after termination of the chemotherapy. Administration of large doses of cortisone, which suppresses the immune system of the mice, accelerates the reactivation of the tubercle bacilli. The persistence of the tubercle bacilli after 12 weeks of chemotherapy is not due to the emergence of drug-resistant mutants since the bacilli which are recovered from the mice remain essentially drug sensitive (McCune et al. 1956). Some similar studies were performed later with a combination of isoniazid and rifampicin. Isoniazid and rifampicin in combination were given to *M. tuberculosis* infected mice for 12 months. A high relapse rate of 60% was observed by giving the mice 2 months of high dose of cortisone (Grosset 1978). The original Cornell model and the later studies show that human tuberculosis can be converted from active disease to a latent infection with chemotherapy. The dormant bacilli which survive the drug treatment can be reactivated and can cause active disease later in life. So, latent infection in animal models is characterized by the presence of the tubercle bacilli which cannot be detected by microscopy or by culture and can only be demonstrated by the emergence of active disease (McCune et al. 1966a).

3.3 Subpopulations of Nonmultiplying M. tuberculosis *In Vitro*

Subpopulations of nonmultiplying bacteria have been modeled in experimental cultures. *M. tuberculosis* is allowed to grow in a culture without agitation. The initial growth of bacilli occurs at an exponential rate with a doubling time of 16–18 h (population A) until the cell density reaches 4×10^8 CFU/ml. At this stage, dissolved oxygen becomes limiting, and the growth rate decreases while the bacilli settle to the bottom of the container (Wayne 1976, 1994). The organisms in the sediment adapt to microaerophilic conditions and enter a homogenous physiological state of dormancy. The bacilli in the deposit of the settling culture can persist for long periods after transfer to anaerobic conditions (Wayne 1977). When an old, micro-aerophilically adapted culture is treated with a high dose of rifampicin, the antibiotic kills most of the bacilli which actively replicate (A) or shows intermittent metabolic activity (population B and C), but fails to remove a small proportion of a bacterial population (D) (Hu et al. 2000) which are rifampicin tolerant persisters. These persisters rapidly lose the ability to grow on solid medium

plates, but are alive and able to grow in liquid medium which contains no rifampicin (Hu et al. 2000). In such liquid subcultures, they regain the ability to grow on plates after 7 days of incubation and are then fully susceptible to rifampicin. During chemotherapy with rifampicin, tolerant bacilli that persist during treatment with rifampicin are likely to be the most difficult to kill, so that the in vitro models should reflect the likely sterilizing activity during human treatment of the drug under test, in combination with rifampicin.

4 Antibiotics Kill Nonmultiplying Bacteria

4.1 Bactericidal and Sterilizing Antibiotics

Currently, although certain antibiotics are capable of killing nonmultiplying bacteria, a very limited number of antibiotics can kill persistent or dormant bacteria. It is well established that antibiotics are ineffective against bacteria whose growth or metabolic activity has been almost completely inhibited. For example, antibiotics in the penicillin family such as penicillin, ampicillin, and amoxicillin only kill rapidly growing cells, but are ineffective against nonmultiplying cells. Although some of the antibiotics such as aminoglycosides and fluoroquinolone have activities against nongrowing bacteria such as those in biofilms, they are more effective in killing growing bacteria. In tuberculosis, antituberculous drugs have been shown to be both bactericidal and sterilizing (Fig. 3). Bactericidal activity of a drug is defined as the ability to kill rapidly replicating bacilli (population A) and is mostly effective at the beginning of treatment, such as isoniazid. The sterilizing activity of drugs such as rifampicin and pyrazinamide depends on their ability to kill semidormant bacilli (population B and C) which can persist for long periods during chemotherapy and give rise to relapses. Sterilizing activity may start early in treatment but is more evident in the later stages of treatment after bactericidal activity has declined (Mitchison and Fourie 2010). The sterilizing activity of drugs is of great clinical importance, since it determines the length of the chemotherapy. Neither bactericidal nor sterilizing drugs are currently available to kill the population D of dormant bacilli. The success of a combination of isoniazid, rifampicin, streptomycin, and pyraziamide or some other drugs in the short-course chemotherapy lies in their capacity to attack the semidormant as well as the actively replicating bacilli. In a nonmultiplying culture of *M. tuberculosis*, addition of fluoroquinolones such as moxifloxacin and gatifloxacin to the drug regimen of isoniazid, rifampicin, and pyraziamide reduces the numbers of persisters (Hu et al. 2003, 2006a). This indicates a need for new antibiotics which target nonmultiplying bacteria (Coates and Hu 2006, 2008) in order to shorten the length of chemotherapy.

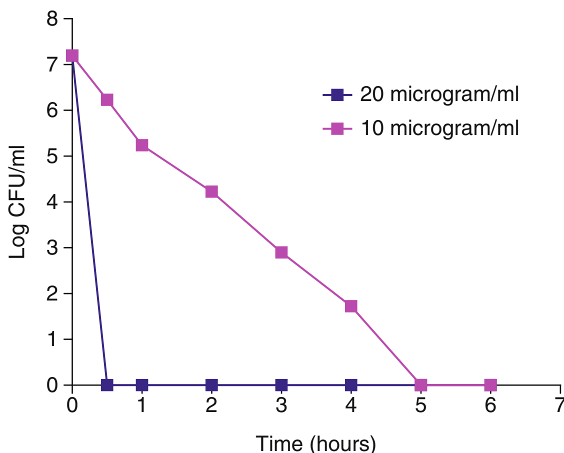


Fig. 4 Fast speed of kill by HT61 against nonmultiplying *S. aureus*. HT61 was incubated with nonmultiplying *S. aureus* at different concentrations for 6 h. At different time points, CFU counts were performed

4.2 Antimicrobials Targeting Cell Membrane and Cell Wall

There is growing evidence indicating that antimicrobial drugs against nonmultiplying bacteria are likely to target the bacterial cell membrane (Hurdle et al. 2011). The recently approved lipopeptide antibiotic, daptomycin, exhibits activity against stationary-phase nonmultiplying bacteria (Mascio et al. 2007). The mode of action of this drug is associated with modification of cell membrane potential by depolarization of the bacterial membrane (Silverman et al. 2003). Telavancin is a lipoglycopeptide derivative of vancomycin. Its mechanism of bactericidal action against nonmultiplying bacteria (Gander et al. 2005) is dependent on its ability to depolarize the bacterial cell membrane and to increase membrane permeability as well as inhibiting cell wall synthesis (Lunde et al. 2009; Nannini et al. 2010; Higgins et al. 2005). There are some antimicrobial drugs either in preclinical or clinical development targeting bacterial cell membrane or cell walls. For example, other lipoglycopeptide antibiotics such as oritavancin and dalbavancin are able to kill nonmultiplying *S. aureus* in stationary-phase cultures and biofilms (Belley et al. 2009; Darouiche and Mansouri 2005). The mechanism of action is to disrupt membrane potentials and to enhance the permeability of cell membranes (Belley et al. 2009). A small quinoline-derived compound HT61 was developed in an antibiotic discovery program which targets nonmultiplying bacteria from the onset of antibiotic development process (Hu et al. 2010). This is the first time that nonmultiplying cells have been targeted during the initial phases of antibiotic discovery. HT61 is very potent against nonmultiplying Gram-positive bacteria, including those that are methicillin sensitive and resistant, as well as Panton-Valentine leukocidin-carrying *S. aureus*. It also kills mupirocin-resistant MRSA. The action of the drug is fast, showing a complete kill within 0.5–1 h (Fig. 4). The mechanism of action of the drug is depolarization of the cell membrane and destruction of the cell wall (Hu et al. 2010). HT61 is in clinical trials

with aim of decolonizing the nose of *S. aureus* including MRSA. Other examples of these membrane acting drugs are XF-70 and XF-73, which are porphyrin-derived compounds (Ooi et al. 2009). XF-70 and XF-73 show significant potency against nonmultiplying or slowly multiplying bacteria (Ooi et al. 2010). Ceragenins such as CSA-13 are derived from bile acid which kills bacteria by depolarization of bacterial cell membrane (Epand et al. 2010).

Potential advantages of these membrane targeting antibiotics are firstly, they are not only active against actively multiplying bacterial but most importantly they are active against nonmultiplying bacteria. Treatment using single-drug regimens may remove all populations of bacteria in different physiological states which may lead to shortened or efficient antibiotic therapy. Secondly, experimental data demonstrates that there is a very low frequency of resistance development (Van Bambeke et al. 2008; Hu et al. 2010) against this class of antibiotics. After 50 passages of *S. aureus* with sub-MIC concentrations of HT61, no HT61 resistant strains were selected (Hu et al. 2010). The cell membrane is essential. In order for bacteria to develop resistance to membrane active drugs, they need to modify the charges on their membrane lipids or cell membrane components. This almost always leads to death (Van Bambeke et al. 2008). Thirdly, membrane active agents normally possess rapid bactericidal activities (Hu et al. 2010) which remove the persistent bacteria prior to the drug serum level dropping below their therapeutic concentrations. In other words, these agents destroy the bacteria before resistance has a chance to occur. Mutations which are capable of conferring drug resistance cannot occur in dead bacteria.

4.3 Antipersister Formation and Waking up Dormancy

The presence of persisters in human infections is the main reason for prolonged antibiotic treatment and recurrent infection. The mechanisms in which a bacterial community forms persisters are largely unknown. Obviously, a global metabolic shut down and a program of differential gene expression play very important roles. Protein and RNA synthesis associated with stationary phase has been intensively studied in bacteria such as *E. coli*, *S. typhimurium,* and *M. tuberculosis* (Hu et al. 1998; Ward et al. 2010; Soares et al. 2010; Chaussee et al. 2008; Spector and Cubitt 1992; Spector et al. 1988). The stationary-phase response involves the synthesis of a characteristic set of proteins (Dong and Schellhorn 2009) which is accompanied by a gradual decrease in total protein synthesis (Groat et al. 1986; Hu et al. 1998). Most of these proteins are unique to stationary phase (De Groote et al. 2009). This essential de novo protein synthesis, accompanied by the metabolic shutdown, affords the bacteria a remarkable degree of resistance to many stress conditions. Furthermore, these processes enable the bacteria to withstand prolonged periods of

stationary phase. Using transposon mutagenesis to search for persistent genes, it has been demonstrated that an intergenic region of *E. coli* genome is responsible for the persisters' tolerance to high dose antibiotics (Hu and Coates 2005). In *M. tuberculosis*, genes which control bacterial growth have been found (Hu et al. 2006b; Parish et al. 2003). *hspX* gene which encodes an alpha-crystallin-like protein acts as a growth suppresser (Hu et al. 2006b; Stewart et al. 2006). Deletion of the gene results in a hyper-virulent mutant which grows faster than its parental strain (Hu et al. 2006b). This indicates that if the *hspX* gene product was inhibited by targeting with novel drugs, it might be possible to prevent the transition of the bacilli from log-phase growth to the nonmultiplying stage or reduce the speed of growth shifting. If persisters no longer existed or are formed at a significantly reduced speed, conventional antibiotics could be more effective.

VBNC cells can be resuscitated to normal vegetative cells when growth conditions become favorable again. Also bacterial cells are capable of producing growth promoting factors or resuscitation promoting factors such as proteins which stimulate the VBNC cells to regain growth (Mukamolova et al. 1998). If dormant bacteria in human infection can be woken up on a regular basis followed by treatments with the current antibiotic arsenal, it might be possible to effectively achieve complete sterilization and shorten the duration of chemotherapy.

5 Conclusion

Nonmultiplying persistent bacteria were discovered more than 67 years ago. It is commonly accepted that after initial exponential growth in nutrient-rich conditions, bacteria slow their metabolic activities and gradually reach a nonmultiplying stage. When bacteria grow on a solid surface, the growth mimics the profiles of planktonic culture except that biofilms are formed with a self-generated matrix. Nonmultiplying persisters, for example, VBNC cells are present in both planktonic cultures and biofilms and are also present in almost all bacterial infections. The clinical significance of these persisters is that they are profoundly tolerant to antibiotics, which leads to the need to prolong the duration of antibiotic therapy, to use high doses and to employ drug combinations. It is therefore critically important to search for antimicrobials which target nonmultiplying persisters. The most promising antibiotics discovered to date are those acting on structures such as the cell membrane or cell wall. Also, using novel agents to slow down persister formation or waking up dormant cells will be beneficial to potentiate the activities of current antibiotics. In addition, combination therapy with bactericidal and sterilizing antibiotics such as tuberculosis treatment may be able to achieve shorter and more effective antibiotic therapy with improved clinical outcomes and better patient compliance.

References

Anderson GG, Palermo JJ, Schilling JD, Roth R, Heuser J, Hultgren SJ (2003) Intracellular bacterial biofilm-like pods in urinary tract infections. Science 301:105–107

Anderson M, Bollinger D, Hagler A, Hartwell H, Rivers B, Ward K, Steck TR (2004) Viable but nonculturable bacteria are present in mouse and human urine specimens. J Clin Microbiol 42:753–758

Arnold KW, Kaspar CW (1995) Starvation- and stationary-phase-induced acid tolerance in *Escherichia coli* O157:H7. Appl Environ Microbiol 61:2037–2039

Austrian R, Gold J (1964) Pneumococcal bacteremia with especial reference to bacteremic *Pneumococcal pneumonia*. Ann Intern Med 60:759–776

Baffone W, Citterio B, Vittoria E, Casaroli A, Campana R, Falzano L, Donelli G (2003) Retention of virulence in viable but non-culturable halophilic *Vibrio* spp. Int J Food Microbiol 89:31–39

Baffone W, Casaroli A, Citterio B, Pierfelici L, Campana R, Vittoria E, Guaglianone E, Donelli G (2006) Campylobacter jejuni loss of culturability in aqueous microcosms and ability to resuscitate in a mouse model. Int J Food Microbiol 107:83–91

Balaban NQ, Merrin J, Chait R, Kowalik L, Leibler S (2004) Bacterial persistence as a phenotypic switch. Science 305:1622–1625

Balke VL, Gralla JD (1987) Changes in the linking number of supercoiled DNA accompany growth transitions in *Escherichia coli*. J Bacteriol 169:4499–4506

Ball AP, Bartlett JG, Craig WA, Drusano GL, Felmingham D, Garau JA, Klugman KP, Low DE, Mandell LA, Rubinstein E, Tillotson GS (2004) Future trends in antimicrobial chemotherapy: expert opinion on the 43rd ICAAC. J Chemother 16:419–436

Barcina I, Gonzalez J, Iriberri J, Egea L (1989) Effect of visible light on progressive dormancy of *Escherichia coli* cells during the survival process in natural fresh water. Appl Environ Microbiol 55:6–251

Begun J, Gaiani JM, Rohde H, Mack D, Calderwood SB, Ausubel FM, Sifri CD (2007) Staphylococcal biofilm exopolysaccharide protects against Caenorhabditis elegans immune defenses. PLoS Pathog 3:e57

Belley A, Neesham-Grenon E, McKay G, Arhin FF, Harris R, Beveridge T, Parr TR Jr, Moeck G (2009) Oritavancin kills stationary-phase and biofilm *Staphylococcus aureus* cells in vitro. Antimicrob Agents Chemother 53:918–925

Bigger JW (1944) Treatment of staphylococcal infections with penicillin. Lancet II:497–500

Bloom BR, McKinney JD (1999) The death and resurrection of tuberculosis. Nat Med 5:872–874

Cappelier JM, Besnard V, Roche S, Garrec N, Zundel E, Velge P, Federighi M (2005) Avirulence of viable but non-culturable Listeria monocytogenes cells demonstrated by in vitro and in vivo models. Vet Res 36:589–599

Cappelier JM, Besnard V, Roche SM, Velge P, Federighi M (2007) Avirulent viable but non culturable cells of Listeria monocytogenes need the presence of an embryo to be recovered in egg yolk and regain virulence after recovery. Vet Res 38:573–583

Chaiyanan S, Grim C, Maugel T, Huq A, Colwell RR (2007) Ultrastructure of coccoid viable but non-culturable Vibrio cholerae. Environ Microbiol 9:393–402

Chaussee MA, Dmitriev AV, Callegari EA, Chaussee MS (2008) Growth phase-associated changes in the transcriptome and proteome of *Streptococcus pyogenes*. Arch Microbiol 189:27–41

Coates AR, Hu Y (2006) New strategies for antibacterial drug design: targeting non-multiplying latent bacteria. Drugs R&D 7:133–151

Coates AR, Hu Y (2008) Targeting non-multiplying organisms as a way to develop novel antimicrobials. Trends Pharmacol Sci 29:143–150

Coates H, Thornton R, Langlands J, Filion P, Keil AD, Vijayasekaran S, Richmond P (2008) The role of chronic infection in children with otitis media with effusion: evidence for intracellular persistence of bacteria. Otolaryngol Head Neck Surg 138:778–781

Colmegna I, Cuchacovich R, Espinoza LR (2004) HLA-B27-associated reactive arthritis: pathogenetic and clinical considerations. Clin Microbiol Rev 17:348–369

Colwell RR, Brayton BR, Grimes DJ, Roszak DB, Hug SA, Palmer LM (1985) Viable but non-culturable *Vibrio cholerae* and related pathogens in the environment: implication for release of genetically engineered microorganisms. BioTechnology 3:817–820

Cornet I, Van Derlinden E, Cappuyns AM, Van Impe JF (2010) Heat stress adaptation of *Escherichia coli* under dynamic conditions: effect of inoculum size. Lett Appl Microbiol 51:450–455

Costa K, Bacher G, Allmaier G, Dominguez-Bello MG, Engstrand L, Falk P, de Pedro MA, Garcia-del Portillo F (1999) The morphological transition of *Helicobacter pylori* cells from spiral to coccoid is preceded by a substantial modification of the cell wall. J Bacteriol 181:3710–3715

Cronan JE Jr (1968) Phospholipid alterations during growth of *Escherichia coli*. J Bacteriol 95:2054–2061

Dannebery AM, Rook GAW (1994) Pathogenesis of pulmonary tuberculosis: an interplay of tissue-damaging and macrophage-activating immune responses-dual mechanisms that control bacillary multiplication. In: Bloom BR (ed) Tuberculosis: pathogenesis, protection, and control. America Society for Microbiology press, Washington DC, pp 459–501

Darcan C, Ozkanca R, Idil O, Flint KP (2009) Viable but non-culturable state (VBNC) of *Escherichia coli* related to EnvZ under the effect of pH, starvation and osmotic stress in sea water. Pol J Microbiol 58:307–317

Darouiche RO, Mansouri MD (2005) Dalbavancin compared with vancomycin for prevention of *Staphylococcus aureus* colonization of devices in vivo. J Infect 50:206–209

Davis MJ, Coote PJ, O'Byrne CP (1996) Acid tolerance in Listeria monocytogenes: the adaptive acid tolerance response (ATR) and growth-phase-dependent acid resistance. Microbiology 142 (10):2975–2982

de Beer D, Stoodley P, Lewandowski Z (1997) Measurement of local diffusion coefficients in biofilms by microinjection and confocal microscopy. Biotechnol Bioeng 53:151–158

De Groote VN, Verstraeten N, Fauvart M, Kint CI, Verbeeck AM, Beullens S, Cornelis P, Michiels J (2009) Novel persistence genes in *Pseudomonas aeruginosa* identified by high-throughput screening. FEMS Microbiol Lett 297:73–79

Dineeen P, Homan WP, Grafe WR (1976) Tuberculous peritonitis: 43 years' expereince in diagnosis and treatment. Ann Surg 184:717–722

Dong T, Schellhorn HE (2009) Global effect of RpoS on gene expression in pathogenic *Escherichia coli* O157:H7 strain EDL933. BMC Genomics 10:349

Dorr T, Lewis K, Vulic M (2009) SOS response induces persistence to fluoroquinolones in Escherichia coli. PLoS Genet 5:e1000760

Eisenstark A, Miller C, Jones J, Leven S (1992) *Escherichia coli* genes involved in cell survival during dormancy: role of oxidative stress. Biochem Biophys Res Commun 188:1054–1059

El Solh AA, Akinnusi ME, Wiener-Kronish JP, Lynch SV, Pineda LA, Szarpa K (2008) Persistent infection with *Pseudomonas aeruginosa* in ventilator-associated pneumonia. Am J Respir Crit Care Med 178:513–519

Epand RF, Pollard JE, Wright JO, Savage PB, Epand RM (2010) Depolarization, bacterial membrane composition, and the antimicrobial action of ceragenins. Antimicrob Agents Chemother 54:3708–3713

Fischer-Le Saux M, Hervio-Heath D, Loaec S, Colwell RR, Pommepuy M (2002) Detection of cytotoxin-hemolysin mRNA in nonculturable populations of environmental and clinical Vibrio vulnificus strains in artificial seawater. Appl Environ Microbiol 68:5641–5646

Fux CA, Costerton JW, Stewart PS, Stoodley P (2005) Survival strategies of infectious biofilms. Trends Microbiol 13:34–40

Gander S, Kinnaird A, Finch R (2005) Telavancin: in vitro activity against staphylococci in a biofilm model. J Antimicrob Chemother 56:337–343

Gootz TD (2010) The global problem of antibiotic resistance. Crit Rev Immunol 30:79–93

Groat RG, Schultz JE, Zychlinsky E, Bockman A, Matin A (1986) Starvation proteins in *Escherichia coli*: kinetics of synthesis and role in starvation survival. J Bacteriol 168:486–493

Grosset J (1978) The sterilizing value of rifampicin and pyrazinamide in experimental short-course chemotherapy. Bull Int Union Tuberc 53:5–12

Gunther F, Wabnitz GH, Stroh P, Prior B, Obst U, Samstag Y, Wagner C, Hansch GM (2009) Host defence against *Staphylococcus aureus* biofilms infection: phagocytosis of biofilms by polymorphonuclear neutrophils (PMN). Mol Immunol 46:1805–1813

Hengge-Aronis R (1993) Survival of hunger and stress: the role of rpoS in early stationary phase gene regulation in *E. coli*. Cell 72:165–168

Hengge-Aronis R, Klein W, Lange R, Rimmele M, Boos W (1991) Trehalose synthesis genes are controlled by the putative sigma factor encoded by rpoS and are involved in stationary-phase thermotolerance in Escherichia coli. J Bacteriol 173:7918–7924

Higgins DL, Chang R, Debabov DV, Leung J, Wu T, Krause KM, Sandvik E, Hubbard JM, Kaniga K, Schmidt DE Jr, Gao Q, Cass RT, Karr DE, Benton BM, Humphrey PP (2005) Telavancin, a multifunctional lipoglycopeptide, disrupts both cell wall synthesis and cell membrane integrity in methicillin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother 49:1127–1134

Hu Y, Coates AR (2005) Transposon mutagenesis identifies genes which control antimicrobial drug tolerance in stationary-phase *Escherichia coli*. FEMS Microbiol Lett 243:117–124

Hu YM, Butcher PD, Sole K, Mitchison DA, Coates AR (1998) Protein synthesis is shutdown in dormant *Mycobacterium tuberculosis* and is reversed by oxygen or heat shock. FEMS Microbiol Lett 158:139–145

Hu Y, Mangan JA, Dhillon J, Sole KM, Mitchison DA, Butcher PD, Coates AR (2000) Detection of mRNA transcripts and active transcription in persistent *Mycobacterium tuberculosis* induced by exposure to rifampin or pyrazinamide. J Bacteriol 182:6358–6365

Hu Y, Coates AR, Mitchison DA (2003) Sterilizing activities of fluoroquinolones against rifampin-tolerant populations of *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 47:653–657

Hu Y, Coates AR, Mitchison DA (2006a) Sterilising action of pyrazinamide in models of dormant and rifampicin-tolerant *Mycobacterium tuberculosis*. Int J Tuberc Lung Dis 10:317–322

Hu Y, Movahedzadeh F, Stoker NG, Coates AR (2006b) Deletion of the *Mycobacterium tuberculosis* alpha-crystallin-like hspX gene causes increased bacterial growth in vivo. Infect Immun 74:861–868

Hu Y, Shamaei-Tousi A, Liu Y, Coates A (2010) A new approach for the discovery of antibiotics by targeting non-multiplying bacteria: a novel topical antibiotic for staphylococcal infections. PLoS One 5:e11818

Hurdle JG, O'Neill AJ, Chopra I, Lee RE (2011) Targeting bacterial membrane function: an underexploited mechanism for treating persistent infections. Nat Rev Microbiol 9:62–75

Jackson DN, Davis B, Tirado SM, Duggal M, van Frankenhuyzen JK, Deaville D, Wijesinghe MA, Tessaro M, Trevors JT (2009) Survival mechanisms and culturability of Campylobacter jejuni under stress conditions. Antonie Van Leeuwenhoek 96:377–394

Jayaraman R (2008) Bacterial persistence: some new insights into an old phenomenon. J Biosci 33:795–805

Jenkins DE, Schultz JE, Matin A (1988) Starvation-induced cross protection against heat or H2O2 challenge in *Escherichia coli*. J Bacteriol 170:3910–3914

Jenkins DE, Chaisson SA, Matin A (1990) Starvation-induced cross protection against osmotic challenge in *Escherichia coli*. J Bacteriol 172:2779–2781

Kaiser D (1986) Control of multicellular development: Dictyostelium and Myxococcus. Annu Rev Genet 20:539–566

Kaprelyants AS, Kell DB (1993) Dormancy in stationary-phase cultures of *Micrococcus luteus*: flow cytometric analysis of starvation and resuscitation. Appl Environ Microbiol 59:3187–3196

Kaprelyants AS, Gottschal JC, Kell DB (1993) Dormancy in non-sporulating bacteria. FEMS Microbiol Rev 10:271–285

Keren I, Kaldalu N, Spoering A, Wang Y, Lewis K (2004a) Persister cells and tolerance to antimicrobials. FEMS Microbiol Lett 230:13–18

Keren I, Shah D, Spoering A, Kaldalu N, Lewis K (2004b) Specialized persister cells and the mechanism of multidrug tolerance in *Escherichia coli*. J Bacteriol 186:8172–8180

Klancnik A, Guzej B, Jamnik P, Vuckovic D, Abram M, Mozina SS (2009) Stress response and pathogenic potential of *Campylobacter jejuni* cells exposed to starvation. Res Microbiol 160:345–352

Kolter R, Siegele DA, Tormo A (1993) The stationary phase of the bacterial life cycle. Annu Rev Microbiol 47:855–874

Kusters JG, van Vliet AH, Kuipers EJ (2006) Pathogenesis of *Helicobacter pylori* infection. Clin Microbiol Rev 19:449–490

Kyd JM, McGrath J, Krishnamurthy A (2011) Mechanisms of bacterial resistance to antibiotics in infections of COPD patients. Curr Drug Targets 12(4):521–530

Lange R, Hengge-Aronis R (1991) Identification of a central regulator of stationary-phase gene expression in *Escherichia coli*. Mol Microbiol 5:49–59

Lee K, Veeranagouda Y (2009) Ultramicrocells form by reductive division in macroscopic *Pseudomonas aerial* structures. Environ Microbiol 11:1117–1125

Lewis K (2001) Riddle of biofilm resistance. Antimicrob Agents Chemother 45:999–1007

Lewis K (2008) Multidrug tolerance of biofilms and persister cells. Curr Top Microbiol Immunol 322:107–131

Lindback T, Rottenberg ME, Roche SM, Rorvik LM (2010) The ability to enter into an avirulent viable but non-culturable (VBNC) form is widespread among *Listeria monocytogenes* isolates from salmon, patients and environment. Vet Res 41:8

Lleo MM, Bonato B, Tafi MC, Signoretto C, Boaretti M, Canepari P (2001) Resuscitation rate in different enterococcal species in the viable but non-culturable state. J Appl Microbiol 91:1095–1102

Lleo MM, Benedetti D, Tafi MC, Signoretto C, Canepari P (2007) Inhibition of the resuscitation from the viable but non-culturable state in *Enterococcus faecalis*. Environ Microbiol 9:2313–2320

Losick R, Youngman P, Piggot PJ (1986) Genetics of endospore formation in Bacillus subtilis. Annu Rev Genet 20:625–669

Lunde CS, Hartouni SR, Janc JW, Mammen M, Humphrey PP, Benton BM (2009) Telavancin disrupts the functional integrity of the bacterial membrane through targeted interaction with the cell wall precursor lipid II. Antimicrob Agents Chemother 53:3375–3383

Martinez-Rodriguez A, Mackey BM (2005) Physiological changes in *Campylobacter jejuni* on entry into stationary phase. Int J Food Microbiol 101:1–8

Mascio CT, Alder JD, Silverman JA (2007) Bactericidal action of daptomycin against stationary-phase and nondividing *Staphylococcus aureus* cells. Antimicrob Agents Chemother 51:4255–4260

Masmoudi S, Denis M, Maalej S (2010) Inactivation of the gene katA or sodA affects the transient entry into the viable but non-culturable response of *Staphylococcus aureus* in natural seawater at low temperature. Mar Pollut Bull 60:2209–2214

McCune RM Jr, Tompsett R (1956) Fate of *Mycobacterium tuberculosis* in mouse tissues as determined by the microbial enumeration technique. I. The persistence of drug-susceptible tubercle bacilli in the tissues despite prolonged antimicrobial therapy. J Exp Med 104:737–762

McCune RM Jr, McDermott W, Tompsett R (1956) The fate of *Mycobacterium tuberculosis* in mouse tissues as determined by the microbial enumeration technique. II. The conversion of tuberculous infection to the latent state by the administration of pyrazinamide and a companion drug. J Exp Med 104:763–802

McCune RM, Feldmann FM, Lambert HP, McDermott W (1966a) Microbial persistence. I. The capacity of tubercle bacilli to survive sterilization in mouse tissues. J Exp Med 123:445–468

McCune RM, Feldmann FM, McDermott W (1966b) Microbial persistence. II. Characteristics of the sterile state of tubercle bacilli. J Exp Med 123:469–486

McLeod GI, Spector MP (1996) Starvation- and Stationary-phase-induced resistance to the antimicrobial peptide polymyxin B in *Salmonella typhimurium* is RpoS (sigma(S)) independent and occurs through both phoP-dependent and -independent pathways. J Bacteriol 178:3683–3688

Mitchison DA (1979) Basic mechanisms of chemotherapy. Chest 76:771–781

Mitchison DA (2004) Antimicrobial therapy of tuberculosis: justification for currently recommended treatment regimens. Semin Respir Crit Care Med 25:307–315

Mitchison DA, Fourie PB (2010) The near future: improving the activity of rifamycins and pyrazinamide. Tuberculosis (Edinb) 90:177–181

Mittal R, Aggarwal S, Sharma S, Chhibber S, Harjai K (2009) Urinary tract infections caused by *Pseudomonas aeruginosa*: a minireview. J Infect Public Health 2:101–111

Moore JE, Nagano Y, Millar BC, McCalmont M, Elborn JS, Rendall J, Pattison S, Dooley JS, Goldsmith CE (2007) Environmental persistence of *Pseudomonas aeruginosa* and *Burkholderia multivorans* in sea water: preliminary evidence of a viable but non-culturable state. Br J Biomed Sci 64:129–131

Morita RY (1988) Bioavailability of energy and its relationship to growth and starvation survival in nature. Can J Microbiol 34:436–441

Mukamolova GV, Kaprelyants AS, Young DI, Young M, Kell DB (1998) A bacterial cytokine. Proc Natl Acad Sci USA 95:8916–8921

Mulvey MA, Schilling JD, Hultgren SJ (2001) Establishment of a persistent *Escherichia coli* reservoir during the acute phase of a bladder infection. Infect Immun 69:4572–4579

Nannini EC, Stryjewski ME, Corey GR (2010) Telavancin's interactions with the bacterial cell membrane. Future Microbiol 5:355–358

Navarro Llorens JM, Tormo A, Martinez-Garcia E (2010) Stationary phase in gram-negative bacteria. FEMS Microbiol Rev 34:476–495

Nilsson L, Oliver JD, Kjelleberg S (1991) Resuscitation of *Vibrio vulnificus* from the viable but nonculturable state. J Bacteriol 173:5054–5059

Oliver JD (1995) The viable but non-culturable state in the human pathogen *Vibrio vulnificus*. FEMS Microbiol Lett 133:203–208

Ooi N, Miller K, Hobbs J, Rhys-Williams W, Love W, Chopra I (2009) XF-73, a novel antistaphylococcal membrane-active agent with rapid bactericidal activity. J Antimicrob Chemother 64:735–740

Ooi N, Miller K, Randall C, Rhys-Williams W, Love W, Chopra I (2010) XF-70 and XF-73, novel antibacterial agents active against slow-growing and non-dividing cultures of *Staphylococcus aureus* including biofilms. J Antimicrob Chemother 65:72–78

O'Toole G, Kaplan HB, Kolter R (2000) Biofilm formation as microbial development. Annu Rev Microbiol 54:49–79

Parish T, Smith DA, Kendall S, Casali N, Bancroft GJ, Stoker NG (2003) Deletion of two-component regulatory systems increases the virulence of *Mycobacterium tuberculosis*. Infect Immun 71:1134–1140

Pechere JC, Hughes D, Kardas P, Cornaglia G (2007) Non-compliance with antibiotic therapy for acute community infections: a global survey. Int J Antimicrob Agents 29:245–253

Pommepuy M, Butin M, Derrien A, Gourmelon M, Colwell RR, Cormier M (1996) Retention of enteropathogenicity by viable but nonculturable *Escherichia coli* exposed to seawater and sunlight. Appl Environ Microbiol 62:4621–4626

Proctor RA, Kahl B, von Eiff C, Vaudaux PE, Lew DP, Peters G (1998) Staphylococcal small colony variants have novel mechanisms for antibiotic resistance. Clin Infect Dis 27(Suppl 1): S68–S74

Proctor RA, von Eiff C, Kahl BC, Becker K, McNamara P, Herrmann M, Peters G (2006) Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections. Nat Rev Microbiol 4:295–305

Pruzzo C, Tarsi R, Lleo MM, Signoretto C, Zampini M, Colwell RR, Canepari P (2002) In vitro adhesion to human cells by viable but nonculturable *Enterococcus faecalis*. Curr Microbiol 45:105–110

Pruzzo C, Tarsi R, Lleo MM, Signoretto C, Zampini M, Pane L, Colwell RR, Canepari P (2003) Persistence of adhesive properties in *Vibrio cholerae* after long-term exposure to sea water. Environ Microbiol 5:850–858

Renslow RS, Majors PD, McLean JS, Fredrickson JK, Ahmed B, Beyenal H (2010) In situ effective diffusion coefficient profiles in live biofilms using pulsed-field gradient nuclear magnetic resonance. Biotechnol Bioeng 106:928–937

Rihl M, Klos A, Kohler L, Kuipers JG (2006) Infection and musculoskeletal conditions: reactive arthritis. Best Pract Res Clin Rheumatol 20:1119–1137

Rivers B, Steck TR (2001) Viable but nonculturable uropathogenic bacteria are present in the mouse urinary tract following urinary tract infection and antibiotic therapy. Urol Res 29:60–66

Rohde H, Burdelski C, Bartscht K, Hussain M, Buck F, Horstkotte MA, Knobloch JK, Heilmann C, Herrmann M, Mack D (2005) Induction of *Staphylococcus epidermidis* biofilm formation via proteolytic processing of the accumulation-associated protein by staphylococcal and host proteases. Mol Microbiol 55:1883–1895

Roostalu J, Joers A, Luidalepp H, Kaldalu N, Tenson T (2008) Cell division in *Escherichia coli* cultures monitored at single cell resolution. BMC Microbiol 8:68

Roszak DB, Colwell RR (1987) Metabolic activity of bacterial cells enumerated by direct viable count. Appl Environ Microbiol 53:2889–2983

Roszak DB, Grimes DJ, Colwell RR (1984) Viable but nonculturable stage of *Salmonella enteritidis* in aquatic systems. Can J Microbiol 30:334–338

Saito N, Konishi K, Sato F, Kato M, Takeda H, Sugiyama T, Asaka M (2003) Plural transformation-processes from spiral to coccoid *Helicobacter pylori* and its viability. J Infect 46:49–55

Seguin MA, Vaden SL, Altier C, Stone E, Levine JF (2003) Persistent urinary tract infections and reinfections in 100 dogs (1989–1999). J Vet Intern Med 17:622–631

Shah D, Zhang Z, Khodursky A, Kaldalu N, Kurg K, Lewis K (2006) Persisters: a distinct physiological state of *E. coli*. BMC Microbiol 6:53

Siegele DA, Kolter R (1992) Life after log. J Bacteriol 174:345–348

Siegele DA, Kolter R (1993) Isolation and characterization of an *Escherichia coli* mutant defective in resuming growth after starvation. Genes Dev 7:2629–2640

Signoretto C, Lleo MM, Tafi MC, Canepari P (2000) Cell wall chemical composition of *Enterococcus faecalis* in the viable but nonculturable state. Appl Environ Microbiol 66:1953–1959

Signoretto C, Lleo MM, Canepari P (2002) Modification of the peptidoglycan of *Escherichia coli* in the viable but nonculturable state. Curr Microbiol 44:125–131

Signoretto C, Burlacchini G, Lleo MM, Pruzzo C, Zampini M, Pane L, Franzini G, Canepari P (2004) Adhesion of *Enterococcus faecalis* in the nonculturable state to plankton is the main mechanism responsible for persistence of this bacterium in both lake and seawater. Appl Environ Microbiol 70:6892–6896

Silverman JA, Perlmutter NG, Shapiro HM (2003) Correlation of daptomycin bactericidal activity and membrane depolarization in *Staphylococcus aureus*. Antimicrob Agents Chemother 47:2538–2544

Simmons WL, Dybvig K (2007) Biofilms protect Mycoplasma pulmonis cells from lytic effects of complement and gramicidin. Infect Immun 75:3696–3699

Singh R, Ray P, Das A, Sharma M (2009) Role of persisters and small-colony variants in antibiotic resistance of planktonic and biofilm-associated *Staphylococcus aureus*: an in vitro study. J Med Microbiol 58:1067–1073

Singh R, Ray P, Das A, Sharma M (2010) Penetration of antibiotics through Staphylococcus aureus and *Staphylococcus epidermidis* biofilms. J Antimicrob Chemother 65:1955–1958

Smith AW (1995) Stationary phase induction in Escherichia coli – new targets for antimicrobial therapy? J Antimicrob Chemother 35:359–361

Soares NC, Cabral MP, Gayoso C, Mallo S, Rodriguez-Velo P, Fernandez-Moreira E, Bou G (2010) Associating growth-phase-related changes in the proteome of *Acinetobacter baumannii* with increased resistance to oxidative stress. J Proteome Res 9:1951–1964

Spector MP, Cubitt CL (1992) Starvation-inducible loci of *Salmonella typhimurium*: regulation and roles in starvation-survival. Mol Microbiol 6:1467–1476

Spector MP, Park YK, Tirgari S, Gonzalez T, Foster JW (1988) Identification and characterization of starvation-regulated genetic loci in *Salmonella typhimurium* by using Mu d-directed lacZ operon fusions. J Bacteriol 170:345–351

Spoering AL, Lewis K (2001) Biofilms and planktonic cells of *Pseudomonas aeruginosa* have similar resistance to killing by antimicrobials. J Bacteriol 183:6746–6751

Steels EL, Learmonth RP, Watson K (1994) Stress tolerance and membrane lipid unsaturation in *Saccharomyces cerevisiae* grown aerobically or anaerobically. Microbiology 140(3):569–576

Stewart JN, Rivera HN, Karls R, Quinn FD, Roman J, Rivera-Marrero CA (2006) Increased pathology in lungs of mice after infection with an alpha-crystallin mutant of *Mycobacterium tuberculosis*: changes in cathepsin proteases and certain cytokines. Microbiology 152:233–244

Torrella F, Morita RY (1981) Microcultural study of bacterial size changes and microcolony and ultramicrocolony formation by heterotrophic bacteria in seawater. Appl Environ Microbiol 41:518–527

Trautner BW, Darouiche RO (2004) Role of biofilm in catheter-associated urinary tract infection. Am J Infect Control 32:177–183

Tuomanen E, Tomasz A (1990) Mechanism of phenotypic tolerance of nongrowing pneumococci to beta-lactam antibiotics. Scand J Infect Dis Suppl 74:102–112

Turner K, Porter J, Pickup R, Edwards C (2000) Changes in viability and macromolecular content of long-term batch cultures of *Salmonella typhimurium* measured by flow cytometry. J Appl Microbiol 89:90–99

Van Bambeke F, Mingeot-Leclercq MP, Struelens MJ, Tulkens PM (2008) The bacterial envelope as a target for novel anti-MRSA antibiotics. Trends Pharmacol Sci 29:124–134

Vashishtha VM (2010) Growing antibiotics resistance and the need for new antibiotics. Indian Pediatr 47:505–506

Velazquez M, Feirtag JM (1999) *Helicobacter pylori*: characteristics, pathogenicity, detection methods and mode of transmission implicating foods and water. Int J Food Microbiol 53:95–104

Vuong C, Voyich JM, Fischer ER, Braughton KR, Whitney AR, DeLeo FR, Otto M (2004) Polysaccharide intercellular adhesin (PIA) protects *Staphylococcus epidermidis* against major components of the human innate immune system. Cell Microbiol 6:269–275

Walters MC 3rd, Roe F, Bugnicourt A, Franklin MJ, Stewart PS (2003) Contributions of antibiotic penetration, oxygen limitation, and low metabolic activity to tolerance of *Pseudomonas aeruginosa* biofilms to ciprofloxacin and tobramycin. Antimicrob Agents Chemother 47:317–323

Ward WO, Swartz CD, Hanley NM, DeMarini DM (2010) Transcriptional characterization of Salmonella TA100 in log and stationary phase: influence of growth phase on mutagenicity of MX. Mutat Res 692:19–25

Wayne LG (1976) Dynamics of submerged growth of *Mycobacterium tuberculosis* under aerobic and microaerophilic conditions. Am Rev Respir Dis 114:807–811

Wayne LG (1977) Synchronized replication of *Mycobacterium tuberculosis*. Infect Immun 17:528–530

Wayne LG (1994) Dormancy of *Mycobacterium tuberculosis* and latency of disease. Eur J Clin Microbiol Infect Dis 13:908–914

Wiuff C, Zappala RM, Regoes RR, Garner KN, Baquero F, Levin BR (2005) Phenotypic tolerance: antibiotic enrichment of noninherited resistance in bacterial populations. Antimicrob Agents Chemother 49:1483–1494

Wood AJ, Douglas RG (2010) Pathogenesis and treatment of chronic rhinosinusitis. Postgrad Med J 86:359–364

Wortinger MA, Quardokus EM, Brun YV (1998) Morphological adaptation and inhibition of cell division during stationary phase in Caulobacter crescentus. Mol Microbiol 29:963–973

Yourassowsky E, Van Der Linden MP, Lismont MJ (1979) Growth curves, microscopic morphology, and subcultures of beta-lactamase-positive and -negative *Haemophilus influenzae* under the influence of ampicillin and cefamandole. Antimicrob Agents Chemother 15:325–331

Zech H, Thole S, Schreiber K, Kalhofer D, Voget S, Brinkhoff T, Simon M, Schomburg D, Rabus R (2009) Growth phase-dependent global protein and metabolite profiles of *Phaeobacter gallaeciensis* strain DSM 17395, a member of the marine Roseobacter-clade. Proteomics 9:3677–3697

Persister Cells: Molecular Mechanisms Related to Antibiotic Tolerance

Kim Lewis

Contents

1 Clinical Significance of Persister Cells 122
2 Molecular Mechanisms of Dormancy 126
References 130

Abstract It is a given that new antibiotics are needed to combat drug-resistant pathogens. However, this is only a part of the need—we actually never had antibiotics capable of eradicating an infection. All pathogens produce a small subpopulation of dormant persister cells that are highly tolerant to killing by antibiotics. Once an antibiotic concentration drops, surviving persisters re-establish the population, causing a relapsing chronic infection. Persisters are especially significant when the pathogen is shielded from the immune system by biofilms, or in sites where the immune components are limited—in the nervous system, the stomach, or inside macrophages.

Antibiotic treatment during a prolonged chronic infection of *P*. *aeruginosa* in the lungs of patients with cystic fibrosis selects for high-persister (*hip*) mutants. Similarly, treatment of oral thrush infection selects for *hip* mutants of *C*. *albicans*. These observations suggest a direct causality between persisters and recalcitrance of the disease. It appears that tolerance of persisters plays a leading role in chronic infections, while resistance is the leading cause of recalcitrance to therapy in acute infections. Studies of persister formation in E. coli show that mechanisms of dormancy are highly redundant. Isolation of persisters produced a transcriptome which suggests a dormant phenotype characterized by downregulation of energy-producing and biosynthetic functions. Toxin–antitoxin modules represent a major mechanism of

K. Lewis (✉)
Antimicrobial Discovery Center and Department of Biology, Northeastern University,
360 Huntingdon Avenue, Boston, MA 02115, USA
e-mail: k.lewis@neu.edu

A.R.M. Coates (ed.), *Antibiotic Resistance*, Handbook of Experimental Pharmacology 211, DOI 10.1007/978-3-642-28951-4_8,
© Springer-Verlag Berlin Heidelberg 2012

persister formation. The RelE toxin causes dormancy by cleaving mRNA; the HipA toxin inhibits translation by phosphorylating elongation factor Ef-Tu, and the TisB toxin forms a membrane pore, leading to a decrease in pmf and ATP.

Keywords Biofilm • Drug tolerance • High-persister mutants • Persister • Toxin/antitoxins

1 Clinical Significance of Persister Cells

Persisters represent a small subpopulation of cells that spontaneously go into a dormant, non-dividing state. When a population is treated with a bactericidal antibiotic, regular cells die, while persisters survive (Fig. 1). In order to kill, antibiotics require active targets, which explains tolerance of persisters. Taking samples and plating them for colony counts over time from a culture treated with antibiotic produces a biphasic pattern, with a distinct plateau of surviving persisters. By contrast, resistance mechanisms prevent antibiotics from binding to their targets (Fig. 2).

Infectious disease is often untreatable, even when caused by a pathogen that is not resistant to antibiotics. This is the essential paradox of chronic infections. In most cases, chronic infections are accompanied by the formation of biofilms, which seems to point to the source of the problem (Costerton et al. 1999; Del Pozo and Patel 2007). Biofilms have been linked to dental disease, endocarditis, cystitis, UTI, deep-seated infections, indwelling device and catheter infections, and the incurable disease of cystic fibrosis. In the case of indwelling devices such as prostheses and heart valves, reoperation is the method of choice for treating the infection. Biofilms do not generally restrict penetration of antibiotics (Walters et al. 2003), but do form a barrier for the larger components of the immune system (Jesaitis et al. 2003; Leid et al. 2002; Vuong et al. 2004). The presence of biofilm-specific resistance mechanisms was suggested to account for the recalcitrance of infectious diseases (Stewart and Costerton 2001). However, the bulk of cells in the biofilm are actually highly susceptible to killing by antibiotics; only a small fraction of persisters remains alive (Spoering and Lewis 2001). Based on these findings, we proposed a simple model of a relapsing chronic infection—antibiotics kill the majority of cells, and the immune system eliminates both regular cells and persisters from the bloodstream (Lewis 2001) (Fig. 3). The only remaining live cells are then persisters in the biofilm. Once the level of antibiotic drops, persisters repopulate the biofilm, and the infection relapses. While this is a plausible model, it is not the only one. A simpler possibility is that antibiotics fail to effectively reach at least some cells in vivo, resulting in a relapsing infection.

Establishing potential causality between persisters and therapy failure is not trivial, since these cells form a small subpopulation with a temporary phenotype, which precludes introducing them into an animal model of infection. We reasoned that causality can be tested based on what we know about selection for high persister (*hip*) mutants in vitro. Periodic application of high doses of bactericidal

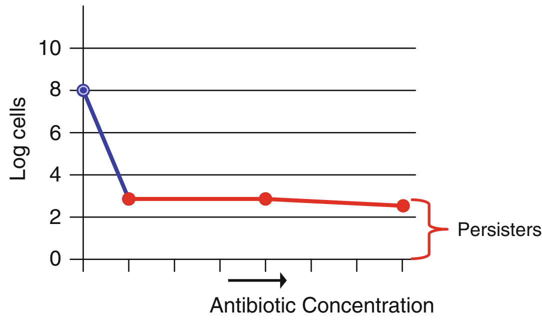


Fig. 1 Dose-dependent killing with a bactericidal antibiotic reveals a small subpopulation of tolerant cells, persisters

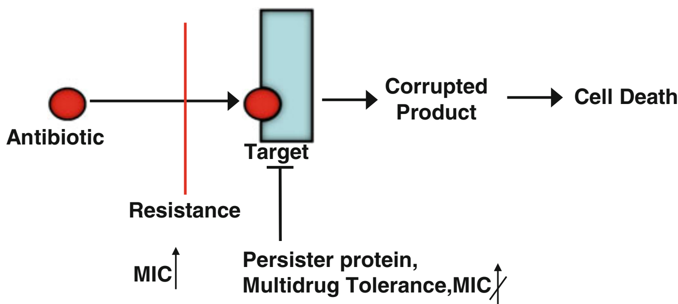


Fig. 2 Resistance and tolerance. Bactericidal antibiotics kill cells by forcing the active target to produce corrupted products. Persister proteins act by blocking the target, so no corrupted product can be produced. By contrast, all resistance mechanisms prevent the antibiotic from binding to the target

antibiotics leads to the selection of strains that produce increased levels of persisters (Moyed and Bertrand 1983; Wolfson et al. 1990). This is precisely what happens in the course of treating chronic infections—the patient is periodically exposed to high doses of antibiotics, which may select for *hip* mutants. But *hip* mutants would only gain advantage if the drugs effectively reach and kill the regular cells of the pathogen.

Patients with cystic fibrosis (CF) are treated for decades for an incurable *P. aeruginosa* infection to which they eventually succumb (Gibson et al. 2003). The periodic application of high doses of antibiotics provides some relief by decreasing the pathogen burden, but does not clear the infection. If *hip* strains of pathogens were selected in vivo, they would most likely be present in a CF patient. We took advantage of a set of longitudinal *P. aeruginosa* isolates from a single patient, collected over the course of many years (Smith et al. 2006). Testing persister levels by monitoring survival after challenge with a high dose of ofloxacin showed a dramatic, 100-fold increase in surviving cells in the last four isolates (Mulcahy et al. 2010). Testing paired strains from additional patients showed that in most cases, there was a considerable increase in persister levels in the late isolate

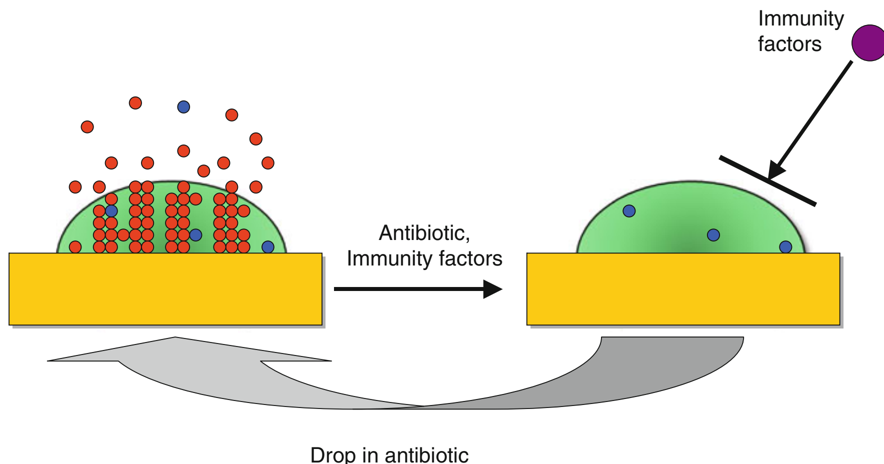


Fig. 3 A model of relapsing biofilm infections. Regular cells (*red*) and persisters (*blue*) form in the biofilm and are shed off into surrounding tissue and bloodstream. Antibiotics kill regular cells, and the immune system eliminates escaping persisters. The matrix protects persisters from the immune system, and when the concentration of the antibiotic drops, they repopulate the biofilm, causing a relapse

from a patient. Interestingly, most of the *hip* isolates had no increase in MIC compared to their clonal parent strain to ofloxacin, carbenicillin, and tobramycin, suggesting that classical acquired resistance plays little to no role in the recalcitrance of CF infection. These experiments directly link persisters to the clinical manifestation of the disease and suggest that persisters are responsible for the therapy failure of chronic CF infection. But why have the *hip* mutants with their striking survival phenotype evaded detection for such a long time?

The main focus of research in antimicrobials has been on drug resistance, and the basic starting experiment is to test a clinical isolate for its ability to grow in the presence of elevated levels of different antibiotics, and record any increases in the MIC. This is also the standard test employed by clinical microbiology laboratories. *hip* mutants are of course missed by this test, which explains why they had remained undetected, in spite of a major effort aimed at understanding pathogen survival to antimicrobial chemotherapy. Given that *hip* mutants are the likely main culprit responsible for morbidity and mortality of the CF infection, it makes sense to test for their presence. Testing for persister levels is not that much more difficult as compared to an MIC test.

Is selection for *hip* mutants a general feature of chronic infections? We recently examined patients with chronic oral thrush caused by *Candida albicans* (Lafleur et al. 2010). These were cancer patients undergoing chemotherapy, and suppression of the immune system caused the fungal infection. In patients where the disease did not resolve, the *C. albicans* isolates were almost invariably *hip* mutants, as compared to patients where the disease cleared within 3 weeks of treatment with chlorhexidine. The eukaryotic *C. albicans* forms persisters (Al-Dhaheri and

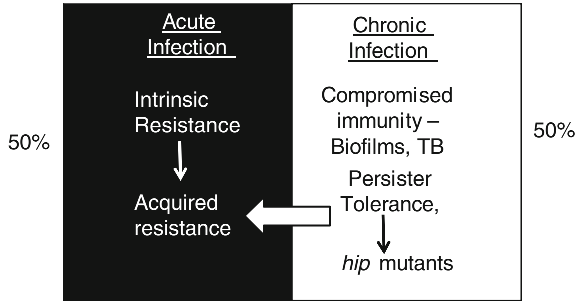


Fig. 4 The two faces of threat. Drug resistance plays an important role in recalcitrance of acute infections, while drug tolerance is largely responsible for failures of chemotherapy in chronic infections. Tolerance allows a large population of the pathogen to linger, which increases the probability of acquiring resistance

Douglas 2008; Harrison et al. 2007; LaFleur et al. 2006) through mechanisms that are probably analogous, rather than homologous to that of their bacterial counterparts. Given the similar life styles of the unrelated *P. aeruginosa* and *C. albicans*, we may expect that the survival advantage of a *hip* mutation is universal. Just as multidrug resistance has become the prevalent danger in acute infections, multidrug tolerance of persisters and *hip* mutants may be the main, but largely overlooked culprit of chronic infectious disease.

Biofilms apparently serve as a protective habitat for persisters (Harrison et al. 2005a, b; Harrison et al. 2009; LaFleur et al. 2006; Spoering and Lewis 2001), allowing them to evade the immune response. However, a more general paradigm is that persisters will be critical for pathogens to survive antimicrobial chemotherapy whenever the immune response is limited. Such cases would include disseminating infections in immunocompromised patients undergoing cancer chemotherapy or infected with HIV. Persisters are also likely to play an important role in immunocompetent individuals in cases where the pathogen is located at sites poorly accessible by components of the immune system. These include the central nervous system, where pathogens cause debilitating meningitis and brain abscesses (Honda and Warren 2009), and the gastrointestinal tract, where a hard-to-eradicate *H. pylori* causes gastroduodenal ulcers and gastric carcinoma (Peterson et al. 2000). Tuberculosis is perhaps the most prominent case of a chronic infection by a pathogen evading the immune system. The acute infection may resolve spontaneously or as a result of antimicrobial therapy, but a large reservoir of the pathogen is preserved in a dormant state in latent asymptomatic carriers (Barry et al. 2009). It is estimated that 1 in every 3 people carry latent *M. tuberculosis*, and 10% of them develop an acute infection at some stage in their lives. Virtually nothing is known about this dormant form. One simple possibility is that persisters are equivalent to the dormant form of the pathogen in a latent infection.

The above analysis underscores the significance of drug tolerance as a barrier to effective antimicrobial chemotherapy. Given its significance—roughly half of all cases of infection (Fig. 4)—the number of studies dedicated to tolerance is tiny as

compared to publications on resistance. The difficulty in pinpointing the mechanism of biofilm recalcitrance and the formidable barriers to study persister cells accounts for the lack of parity between these two comparably significant fields. Hopefully, a better balance will be achieved, and the following discussion summarizes recent advances in understanding the mechanism of tolerance.

2 Molecular Mechanisms of Dormancy

Persisters were initially discovered in 1944 (Bigger 1944), but the mechanism of their formation eluded us for a very long time. Only recently the molecular mechanism of dormancy began to emerge.

The most straightforward approach to finding an underlying mechanism of a complex function is by screening a library of transposon insertion mutants. This produces a set of candidate genes, and subsequent analysis leads to a pathway and a mechanism. This is indeed how the basic mechanisms of sporulation, flagellation, chemotaxis, virulence, and many other functions have been established. However, screening a Tn insertion library of *E. coli* for an ability to tolerate high doses of antibiotics produced no mutants completely lacking persisters (Hu and Coates 2005; Spoering 2006). With the development of the complete, ordered *E. coli* gene knockout library by the Mori group (Baba et al. 2006, the Keio collection), it seemed reasonable to revisit the screening approach. Indeed, there always remains a possibility that transposons missed a critical gene, or the library was not large enough. The use of the Keio collection largely resolves this uncertainty.

This advanced screen (Hansen et al. 2008), similarly to previous efforts, did not produce a single mutant lacking persisters, suggesting a high degree of redundancy. The screen did identify a number of interesting genes, with knockouts showing about a tenfold decrease in persister formation. The majority of hits were in global regulators, DksA, DnaKJ, HupAB, and IhfAB. This is an independent indication of redundancy—a global regulator can affect expression of several persister genes simultaneously, resulting in a phenotype (Fig. 5). The screen also produced two interesting candidate genes that may be more directly involved in persister formation—YgfA that can inhibit nucleotide synthesis, and YigB, which may block metabolism by depleting the pool of FMN.

A similar screen of a *P. aeruginosa* mutant library was recently reported (De Groote et al. 2009). As in *E. coli*, no persisterless mutant was identified, pointing to the similar redundancy theme.

The main conclusion from the screens is that persister formation does not follow the main design theme of complex cellular functions—a single linear regulatory pathway controlling an execution mechanism. By contrast, persisters are apparently formed through a number of independent parallel mechanisms (Fig. 5). There is a considerable adaptive advantage in this redundant design—no single compound will disable persister formation.

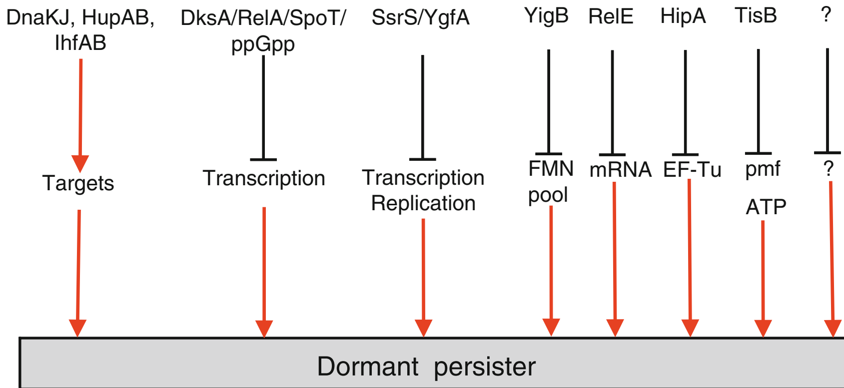


Fig. 5 Candidate persister genes. Persisters are formed through parallel redundant pathways

Screens for persister genes were useful in finding some possible candidates and pointing to redundancy of function. It seemed that a method better suited to uncover redundant elements would be transcriptome analysis. For this, persisters had to be isolated.

Persisters form a small and temporary population, making isolation challenging. The simplest approach is to lyse a population of growing cells with a β-lactam antibiotic, and collect surviving persisters (Keren et al. 2004). This allowed to isolate enough *E. coli* cells to perform a transcriptome analysis. A more advanced method aimed at isolating native persisters was developed, based on a guess that these are dormant cells with diminished protein synthesis (Shah et al. 2006). If the strain expressed degradable GFP, then cells that stochastically enter into dormancy will become dim. In a population of *E. coli* expressing degradable GFP under the control of a ribosomal promoter that is only active in dividing cells, a small number of cells indeed appeared to be dim. The difference in fluorescence allowed for the sorting of the two subpopulations. The dim cells were tolerant to ofloxacin, confirming that they are persisters.

Transcriptomes obtained by both methods pointed to downregulation of biosynthesis genes, and indicated increased expression of several toxin/antitoxin modules (RelBE, MazEF, DinJYafQ, YgiU). TA modules are found on plasmids where they constitute a maintenance mechanism (Gerdes et al. 1986b; Hayes 2003). Typically, the toxin is a protein that inhibits an important cellular function such as translation or replication, and forms an inactive complex with the antitoxin. The toxin is stable, while the antitoxin is degradable. If a daughter cell does not receive a plasmid after segregation, the antitoxin level decreases due to proteolysis, leaving a toxin that either kills the cell or inhibits propagation. TA modules are also commonly found on bacterial chromosomes, but their role is largely unknown. In *E. coli*, MazF and an unrelated toxin RelE induce stasis by cleaving mRNA which of course inhibits translation, a condition that can be reversed by expression of corresponding antitoxins (Christensen and Gerdes 2004; Pedersen et al. 2002). This property of toxins makes them into excellent candidates for persister genes.

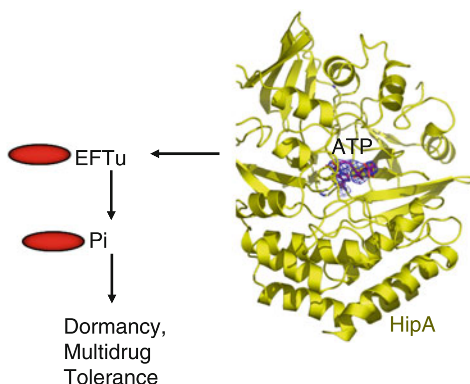


Fig. 6 The HipA toxin causes dormancy in *E. coli* by phosphorylating elongation factor Tu which inhibits protein synthesis

Ectopic expression of RelE (Keren et al. 2004) or MazF (Vazquez-Laslop et al. 2006) strongly increased tolerance to antibiotics. The first gene linked to persisters, *hipA* (Moyed and Bertrand 1983), is also a toxin, and its ectopic expression causes multidrug tolerance as well (Correia et al. 2006; Falla and Chopra 1998). Interestingly, a bioinformatic analysis indicates that HipA is a member of the Tor family of kinases, which have been extensively studied in eukaryotes (Schmelzle and Hall 2000), but have not been previously identified in bacteria. HipA is indeed a kinase, it autophosphorylates on ser150, and site-directed mutagenesis replacing it, or other conserved amino acids in the catalytic and Mg^{2+}-binding sites abolishes its ability to stop cell growth and confer drug tolerance (Correia et al. 2006). The crystal structure of HipA in complex with its antitoxin HipB was recently resolved, and a pull-down experiment showed that the substrate of HipA is elongation factor EF-Tu (Schumacher et al. 2009). Phosphorylated EF-Tu is inactive, which leads to a block in translation and dormancy (Fig. 6).

Deletion of potential candidates of persister genes noted above does not produce a discernible phenotype affecting persister production, possibly due to the high degree of redundancy of these elements. In *E. coli*, there are at least 15 toxin–antitoxin (TA) modules (Alix and Blanc-Potard 2009; Pandey and Gerdes 2005; Pedersen and Gerdes 1999), and more than 80 in *M. tuberculosis* (Ramage et al. 2009).

High redundancy of TA genes would explain the lack of a multidrug tolerance phenotype in knockout mutants, and therefore it seemed useful to search for conditions where a particular toxin would be highly expressed in a wild-type strain, and then examine a possible link to persister formation.

Several TA modules contain the Lex box and are induced by the SOS response. These are *symER, hokE, yafN/yafO* and *tisAB/istr1* (Courcelle et al. 2001; Fernandez De Henestrosa et al. 2000; Kawano et al. 2007; McKenzie et al. 2003; Motiejunaite et al. 2007; Pedersen and Gerdes 1999; Singletary et al. 2009; Vogel et al. 2004). Fluoroquinolones induce the SOS response (Phillips et al. 1987), and we tested the ability of ciprofloxacin to induce persister formation (Dörr et al. 2009, 2010).

Examination of deletion strains showed that the level of persisters dropped dramatically, 10–100 fold, in a Δ*tisAB* mutant. This suggests that TisB was responsible for the formation of the majority of persisters under conditions of

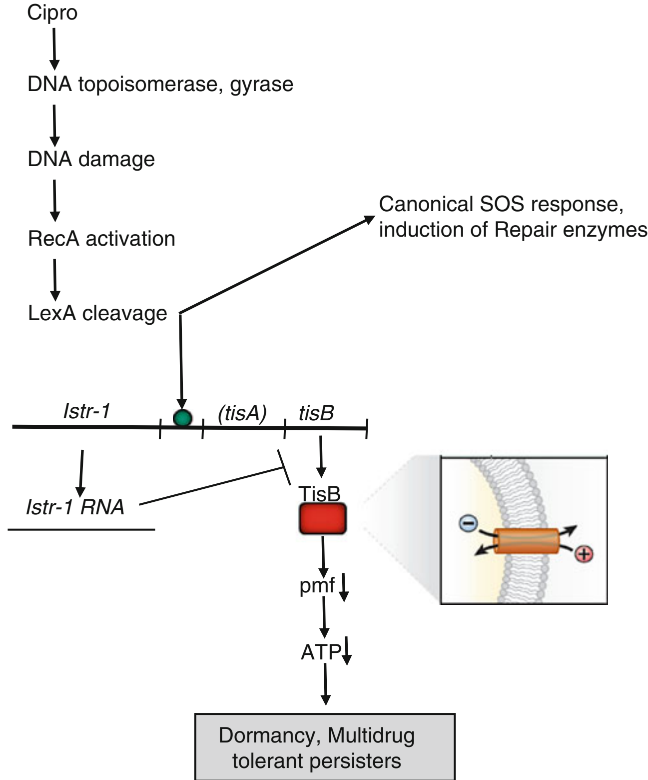


Fig. 7 Persister induction by antibiotic. The common antibiotic ciprofloxacin causes DNA damage by converting its targets, DNA gyrase and topoisomerase, into endonucleases. This activates the canonical SOS response, leading to increased expression of DNA repair enzymes. At the same time, the LexA repressor that regulates expression of all SOS genes also controls transcription of the TisAB toxin/antitoxin module. The TisB toxin is an antimicrobial peptide, which binds to the membrane, causing an increase in pmf and ATP. This produces a systems shutdown, blocking antibiotic targets, which ensures multidrug tolerance

SOS induction. The level of persisters was unaffected in strains deleted in the other Lex box containing TA modules. Persister levels observed in time-dependent killing experiments with ampicillin or streptomycin that do not cause DNA damage were unchanged in the Δ*tisAB* strain. TisB only had a phenotype in the presence of a functional RecA protein, confirming the dependence on the SOS pathway.

Ectopic overexpression of *tisB* sharply increased the level of persisters. A drop in persisters in a deletion strain and increase upon overexpression gives reasonable confidence in functionality of a persister gene. The dependence of TisB-induced persisters on a particular regulatory pathway, the SOS response, further strengthens the case for TisB as a specialized persister protein (Fig. 7). Incidentally, a *tisB* mutant is not present in the otherwise fairly complete Keio knockout library, and the small ORF might have been easily missed by Tn mutagenesis as well, evading detection by the generalized screens for persister genes.

The role of TisB in persister formation is unexpected based on what we know about this type of proteins. TisB is a small, 29 amino acid hydrophobic peptide that binds to the membrane and disrupts the proton motive force (pmf), which leads to a drop in ATP levels (Unoson and Wagner 2008). Bacteria, plants, and animals all produce antimicrobial membrane-acting peptides (Garcia-Olmedo et al. 1998; Sahl and Bierbaum 1998; Zasloff 2002). Toxins of many TA loci found on plasmids belong to this type as well. If a daughter cell does not inherit a plasmid, the concentration of a labile antitoxin decreases, and the toxin such as the membrane-acting *hok* kills the cell (Gerdes et al. 1986a). High-level artificial overexpression of TisB also causes cell death (Unoson and Wagner 2008). It is remarkable from this perspective that the membrane-acting TisB under conditions of natural (mild) expression has the exact opposite effect of protecting the cell from antibiotics.

Fluoroquinolones such as ciprofloxacin are widely used broad-spectrum antibiotics, and their ability to induce multidrug tolerant cells is unexpected and a cause of considerable concern. Induction of persister formation by fluoroquinolones may contribute to the ineffectiveness of antibiotics in eradicating infections. Indeed, pre-exposure with a low dose of ciprofloxacin drastically increased tolerance to subsequent exposure with a high dose, and TisB persisters are multidrug tolerant.

The finding of the role of TisB in tolerance opens an intriguing possibility of a wider link between other stress responses and persister formation. Pathogens are exposed to many stress factors in the host environment apart from DNA damaging agents—oxidants, high temperature, low pH, membrane-acting agents. It is possible that all stress responses induce the formation of surviving persisters.

While resistance and tolerance are mechanistically distinct, there is sufficient reason to believe that tolerance may be a major cause for developing resistance. Indeed, the probability of resistance development is proportional to the size of the pathogen population, and a lingering chronic infection that cannot be eradicated due to tolerance will go on to produce resistant mutants and strains acquiring resistant determinants by transmission from other bacteria (Levin and Rozen 2006) (Fig. 4). Combating tolerance then becomes a major component in preventing resistance.

References

Al-Dhaheri RS, Douglas LJ (2008) Absence of amphotericin B-tolerant persister cells in biofilms of some *Candida* species. Antimicrob Agents Chemother 52:1884–7

Alix E, Blanc-Potard A (2009) Hydrophobic peptides: novel regulators within bacterial membranes. Mol Microbiol 72:5–11

Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, Datsenko KA, Tomita M, Wanner BL, Mori H (2006) Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol Syst Biol 2:2006.0008

Barry CE 3rd, Boshoff HI, Dartois V, Dick T, Ehrt S, Flynn J, Schnappinger D, Wilkinson RJ, Young D (2009) The spectrum of latent tuberculosis: rethinking the biology and intervention strategies. Nat Rev Microbiol 7:845–55

Bigger JW (1944) Treatment of staphylococcal infections with penicillin. Lancet II:497–500

Christensen SK, Gerdes K (2004) Delayed-relaxed response explained by hyperactivation of RelE. Mol Microbiol 53:587–97

Correia FF, D'Onofrio A, Rejtar T, Li L, Karger BL, Makarova K, Koonin EV, Lewis K (2006) Kinase activity of overexpressed *HipA* is required for growth arrest and multidrug tolerance in *Escherichia coli*. J Bacteriol 188:8360–7
Costerton JW, Stewart PS, Greenberg EP (1999) Bacterial biofilms: a common cause of persistent infections. Science 284:1318–22
Courcelle J, Khodursky A, Peter B, Brown P, Hanawalt P (2001) Comparative gene expression profiles following UV exposure in wild type and SOS-deficient Escherichia coli. Genetics 158:41–64
De Groote VN, Verstraeten N, Fauvart M, Kint CI, Verbeeck AM, Beullens S, Cornelis P, Michiels J (2009) Novel persistence genes in *Pseudomonas aeruginosa* identified by high-throughput screening. FEMS Microbiol Lett 297:73–9
Del Pozo J, Patel R (2007) The challenge of treating biofilm-associated bacterial infections. Clinical Pharmacol Ther 82:204–9
Dörr T, Lewis K, Vulic M (2009) SOS response induces persistence to fluoroquinolones in *Escherichia coli*. PLoS Genet 5:e1000760
Dorr T, Vulic M, Lewis K (2010) Ciprofloxacin causes persister formation by inducing the TisB toxin in *Escherichia coli*. PLoS Biol 8:e1000317
Falla TJ, Chopra I (1998) Joint tolerance to beta-lactam and fluoroquinolone antibiotics in *Escherichia coli* results from overexpression of *hipA*. Antimicrob Agents Chemother 42:3282–4
Fernandez De Henestrosa AR, Ogi T, Aoyagi S, Chafin D, Hayes JJ, Ohmori H, Woodgate R (2000) Identification of additional genes belonging to the LexA regulon in Escherichia coli. Mol Microbiol 35:1560–72
Garcia-Olmedo F, Molina A, Alamillo JM, Rodriguez-Palenzuela P (1998) Plant defense peptides. Biopolymers 47:479–91
Gerdes K, Bech FW, Jorgensen ST, Lobner-Olesen A, Rasmussen PB, Atlung T, Boe L, Karlstrom O, Molin S, von Meyenburg K (1986a) Mechanism of postsegregational killing by the hok gene product of the parB system of plasmid R1 and its homology with the relF gene product of the E. coli relB operon. EMBO J 5:2023–9
Gerdes K, Rasmussen PB, Molin S (1986b) Unique type of plasmid maintenance function: postsegregational killing of plasmid-free cells. Proc Natl Acad Sci USA 83:3116–20
Gibson RL, Burns JL, Ramsey BW (2003) Pathophysiology and management of pulmonary infections in cystic fibrosis. Am J Respir Crit Care Med 168:918–51
Hansen S, Lewis K, Vulić M (2008) The role of global regulators and nucleotide metabolism in antibiotic tolerance in *Escherichia coli*. Antimicrob Agents Chemother 52:2718–2726
Harrison JJ, Ceri H, Roper NJ, Badry EA, Sproule KM, Turner RJ (2005a) Persister cells mediate tolerance to metal oxyanions in *Escherichia coli*. Microbiology 151:3181–95
Harrison JJ, Turner RJ, Ceri H (2005b) Persister cells, the biofilm matrix and tolerance to metal cations in biofilm and planktonic *Pseudomonas aeruginosa*. Environ Microbiol 7:981–94
Harrison JJ, Turner RJ, Ceri H (2007) A subpopulation of Candida albicans and Candida tropicalis biofilm cells are highly tolerant to chelating agents. FEMS Microbiol Lett 272:172–81
Harrison JJ, Wade WD, Akierman S, Vacchi-Suzzi C, Stremick CA, Turner RJ, Ceri H (2009) The chromosomal toxin gene *yafQ* is a determinant of multidrug tolerance for *Escherichia coli* growing in a biofilm. Antimicrob Agents Chemother 53:2253–8
Hayes F (2003) Toxins-antitoxins: plasmid maintenance, programmed cell death, and cell cycle arrest. Science 301:1496–9
Honda H, Warren DK (2009) Central nervous system infections: meningitis and brain abscess. Infect Dis Clin North Am 23:609–23
Hu Y, Coates AR (2005) Transposon mutagenesis identifies genes which control antimicrobial drug tolerance in stationary-phase *Escherichia coli*. FEMS Microbiol Lett 243:117–24
Jesaitis AJ, Franklin MJ, Berglund D, Sasaki M, Lord CI, Bleazard JB, Duffy JE, Beyenal H, Lewandowski Z (2003) Compromised host defense on *Pseudomonas aeruginosa* biofilms: characterization of neutrophil and biofilm interactions. J Immunol 171:4329–39

Kawano M, Aravind L, Storz G (2007) An antisense RNA controls synthesis of an SOS-induced toxin evolved from an antitoxin. Mol Microbiol 64:738–54

Keren I, Shah D, Spoering A, Kaldalu N, Lewis K (2004) Specialized persister cells and the mechanism of multidrug tolerance in *Escherichia coli*. J Bacteriol 186:8172–80

LaFleur MD, Kumamoto CA, Lewis K (2006) *Candida albicans* biofilms produce antifungal-tolerant persister cells. Antimicrob Agents Chemother 50:3839–46

Lafleur MD, Qi Q, Lewis K (2010) Patients with long-term oral carriage harbor high-persister mutants of *Candida albicans*. Antimicrob Agents Chemother 54:39–44

Leid JG, Shirtliff ME, Costerton JW, Stoodley AP (2002) Human leukocytes adhere to, penetrate, and respond to *Staphylococcus aureus* biofilms. Infect Immun 70:6339–45

Levin BR, Rozen DE (2006) Non-inherited antibiotic resistance. Nat Rev Microbiol 4:556–62

Lewis K (2001) Riddle of biofilm resistance. Antimicrob Agents Chemother 45:999–1007

McKenzie MD, Lee PL, Rosenberg SM (2003) The dinB operon and spontaneous mutation in Escherichia coli. J Bacteriol 185:3972–7

Motiejunaite R, Armalyte J, Markuckas A, Suziedeliene E (2007) Escherichia coli dinJ-yafQ genes act as a toxin-antitoxin module. FEMS Microbiol Lett 268:112–9

Moyed HS, Bertrand KP (1983) *hipA*, a newly recognized gene of *Escherichia coli* K-12 that affects frequency of persistence after inhibition of murein synthesis. J Bacteriol 155:768–75

Mulcahy LR, Burns JL, Lory S, Lewis K (2010) Emergence of *Pseudomonas aeruginosa* strains producing high levels of persister cells in patients with cystic fibrosis. J Bacteriol 192: 6191–6199

Pandey DP, Gerdes K (2005) Toxin-antitoxin loci are highly abundant in free-living but lost from host-associated prokaryotes. Nucleic Acids Res 33:966–76

Pedersen K, Christensen SK, Gerdes K (2002) Rapid induction and reversal of a bacteriostatic condition by controlled expression of toxins and antitoxins. Mol Microbiol 45:501–10

Pedersen K, Gerdes K (1999) Multiple *hok* genes on the chromosome of *Escherichia coli*. Mol Microbiol 32:1090–102

Peterson WL, Fendrick AM, Cave DR, Peura DA, Garabedian-Ruffalo SM, Laine L (2000) Helicobacter pylori-related disease: guidelines for testing and treatment. Arch Intern Med 160:1285–1291

Phillips I, Culebras E, Moreno F, Baquero F (1987) Induction of the SOS response by new 4-quinolones. J Antimicrob Chemother 20:631–8

Ramage HR, Connolly LE, Cox JS (2009) Comprehensive functional analysis of *Mycobacterium tuberculosis* toxin-antitoxin systems: implications for pathogenesis, stress responses, and evolution. PLoS Genet 5:e1000767

Sahl HG, Bierbaum G (1998) Lantibiotics: biosynthesis and biological activities of uniquely modified peptides from gram-positive bacteria. Annu Rev Microbiol 52:41–79

Schmelzle T, Hall MN (2000) TOR, a central controller of cell growth. Cell 103:253–62

Schumacher MA, Piro KM, Xu W, Hansen S, Lewis K, Brennan RG (2009) Molecular mechanisms of *HipA*-mediated multidrug tolerance and its neutralization by *HipB*. Science 323:396–401

Shah D, Zhang Z, Khodursky A, Kaldalu N, Kurg K, Lewis K (2006) Persisters: a distinct physiological state of *E. coli*. BMC Microbiol 6:53–61

Singletary LA, Gibson JL, Tanner EJ, McKenzie GJ, Lee PL, Gonzalez C, Rosenberg SM (2009) An SOS-regulated type 2 toxin-antitoxin system. J Bacteriol 191:7456–7465

Smith EE, Buckley DG, Wu Z, Saenphimmachak C, Hoffman LR, D'Argenio DA, Miller SI, Ramsey BW, Speert DP, Moskowitz SM, Burns JL, Kaul R, Olson MV (2006) Genetic adaptation by Pseudomonas aeruginosa to the airways of cystic fibrosis patients. Proc Natl Acad Sci USA 103:8487–92

Spoering A (2006) GlpD and PlsB participate in persister cell formation in Escherichia coli. J Bacteriol 188:5136–5144

Spoering AL, Lewis K (2001) Biofilms and planktonic cells of Pseudomonas aeruginosa have similar resistance to killing by antimicrobials. J Bacteriol 183:6746–6751

Stewart PS, Costerton JW (2001) Antibiotic resistance of bacteria in biofilms. Lancet 358:135–8
Unoson C, Wagner E (2008) A small SOS-induced toxin is targeted against the inner membrane in Escherichia coli. Mol Microbiol 70:258–70
Vazquez-Laslop N, Lee H, Neyfakh AA (2006) Increased persistence in *Escherichia coli* caused by controlled expression of toxins or other unrelated proteins. J Bacteriol 188:3494–7
Vogel J, Argaman L, Wagner EG, Altuvia S (2004) The small RNA Istr inhibits synthesis of an SOS-induced toxic peptide. Curr Biol 14:2271–2276
Vuong C, Voyich JM, Fischer ER, Braughton KR, Whitney AR, DeLeo FR, Otto M (2004) Polysaccharide intercellular adhesin (PIA) protects *Staphylococcus epidermidis* against major components of the human innate immune system. Cell Microbiol 6:269–75
Walters MC 3rd, Roe F, Bugnicourt A, Franklin MJ, Stewart PS (2003) Contributions of antibiotic penetration, oxygen limitation, and low metabolic activity to tolerance of Pseudomonas aeruginosa biofilms to ciprofloxacin and tobramycin. Antimicrob Agents Chemother 47:317–23
Wolfson JS, Hooper DC, McHugh GL, Bozza MA, Swartz MN (1990) Mutants of *Escherichia coli* K-12 exhibiting reduced killing by both quinolone and beta-lactam antimicrobial agents. Antimicrob Agents Chemother 34:1938–43
Zasloff M (2002) Antimicrobial peptides of multicellular organisms. Nature 415:389–95

Antimicrobial Textiles

J. Vaun McArthur, R.C. Tuckfield, and C. Baker-Austin

Contents

1 Introduction 136
2 Use of Metals as Bactericides 138
3 Nanoparticles 138
4 Antimicrobial Textiles 139
5 Cross-Selection and Co-selection of Metal Resistance Traits in Nature 141
6 Resistance to Ag and Other Metals Used in Microbial Textiles 143
7 Problems with Nanoparticles on Textiles 145
8 Evolutionary Arms Races 146
9 Is There a Reason for Concern? 147
10 Where Do We Go from Here? 148
References 149

Abstract Bacteria have evolved unique mechanisms that allow them survive in the presence of strong selection pressures. Included in these mechanisms is the ability to share genetic determinants among and between species of bacteria thus spreading metal or antibiotic resistance traits quickly. The textile industry in response to demand has developed antimicrobial fabrics by the addition of bactericidal compounds during production. Some of these antimicrobials include metal nanoparticles, quaternary ammonia compounds, and broad spectrum compounds like triclosan. Bacteria have already expressed resistance to each of these bactericides. Here we discuss the evolutionary and ecological consequences of antimicrobial textiles in terms of co-selection. We predict that continued use of such materials could result in increased and widespread resistance to specific

J.V. McArthur (✉) • R.C. Tuckfield
Savannah River Ecology Laboratory, University of Georgia, Aiken, SC 29803, USA
e-mail: mcarthur@srel.edu

C. Baker-Austin
Cefas, Weymouth Laboratory, Weymouth, Dorset DT4 8UB, UK

A.R.M. Coates (ed.), *Antibiotic Resistance*, Handbook of Experimental Pharmacology 211, DOI 10.1007/978-3-642-28951-4_9,
© Springer-Verlag Berlin Heidelberg 2012

antimicrobials, especially metals, with an increased resistance to antibiotics. Such increases have the potential to find their way into other bacterial populations of human pathogens leading to serious and unintended public health consequences.

Keywords Antimicrobial textiles • Resistance • Nanoparticles • Evolutionary arms race • Heavy metals

1 Introduction

Colonizing all known areas of the biosphere, there are approximately five nonillion (5×10^{30}) bacteria on Earth, forming much of the world's biomass (Whitman et al. 1998). Bacteria arose 3.8 billion years ago and, as a group, have survived longer than all other organisms combined. More importantly, they are still here. During this incredible evolutionary history microbial diversity has evolved to such an extent that, based on 16SRNA and metagenome sequencing, there is significantly far more genetic diversity among bacteria and archaea than among all other organisms.

Undoubtedly the first bacteria evolved in rich toxic metal environments (Silver 1998) or other similarly harsh environments. Survival required the ability to circumvent the toxic nature of these inhospitable extreme conditions. Continued existence and perpetuation of a species is contingent on the genetic repertoire. Consequently, it is difficult to avoid the conclusion that metal resistance genes were among the very first gene systems to evolve, and the genetic basis for metal homeostasis is evident in both recently diverged bacterial phyla as well as ancient crenarchaeota clades (Baker-Austin et al. 2005). Over time as organisms modified their environments, niches arose that did not require metal resistance and such traits were presumably lost or culled from the genome of certain species. However, in metal-rich environments these genes quite certainly persisted (Haefeli et al. 1984).

Bacteria have extraordinary adaptive genetic capacities and these capacities have been primarily shaped by horizontal gene transfer of mobile genetic elements (MGE) (Szczepanowski et al. 2008; O'Brien 2002; Ochman et al. 2000; Yurieva et al. 1997). Interestingly, the genes for regulation, resistance, and biosynthesis are often found linked together on the same continuous strand of DNA (Clardy et al. 2009). Thus, the evolutionary ecological history of bacterial genes is driven, under many circumstances, by horizontal gene transfer events suggesting that the survival capabilities of specific microbes are more dependent on the promiscuity and plasticity of its genome than the genetic characteristics of its ancestors.

The ability of MGE to interact with bacterial genomic DNA across multiple species and environmental barriers creates a unique evolutionary problem. Bacterial cells in ecological proximity to each other may be more important in microbial adaptations and gene exchange than genetic relatedness alone. Microbes that are able to draw from the collective genetic resistome (D'Costa et al. 2007) should have higher chances of survival than those individuals that lack such ability. However because of reproduction by binary fission, bacterial cells closest are often copies of

each other—and that fact frequently colors our collective interpretation regarding bacterial evolution.

Bacterial MGE spread not only vertically, by inheritance, but also horizontally by involving phylogenetically distant cells (Davison 1999; Lorenz and Wackernagel 1994; Sobecky 1999). The horizontal dissemination of MGEs can be accomplished via plasmid exchange (conjugation), the uptake of naked DNA from the environment (transformation), or via viral infection mechanisms (transduction). The metagenome of a bacterial community should indicate past and present selective pressures in that environment (Turner et al. 2002). It has been well documented that genes conferring antibiotic resistance are often more plentiful in bacterial communities exposed to antibiotic contamination (Heuer and Smalla 2007; Pei et al. 2006). For example, mercury resistance genes are more abundant in mercury-contaminated sediments (Smalla et al. 2006). However, in contrast to classic selection theory, previous studies have documented that additional genotypes and phenotypes can be co-selected along with traits under direct selection (Alonso et al. 2001; Baker-Austin et al. 2006; Summers et al. 1993).

There are no known examples of extinction of a bacterial lineage; although such may be the case. A number of reasons have been identified for why extinctions in bacteria may be very low (Dykhuizen 1998). Firstly, bacteria rarely starve to death because they can lay dormant until environmental conditions improve. Secondly, bacteria readily share genes between and within “species,” conferring a selective advantage in many instances to just a small subset of the overall population that can thrive even under highly inhospitable conditions. Thirdly, microbes do not require sex to reproduce, eliminating the limiting factor of finding a suitable mate to continue a genetic lineage. Finally, they, as a group, are able to live in a range of environments under extremes of physical and chemical conditions. The premise that bacteria rarely undergo extinction is important in understanding the ability of man to alter bacterial communities and populations through various technologies and methods.

Perhaps more important than low extinction rates are the high speciation rates found among bacteria (Dykhuizen 1998). It is this ability to rapidly adapt and evolve to novel or changing conditions that allows bacteria to exploit extreme habitats and to withstand strong selection that, for more genetically and phenotypically “advanced” organisms, results in mass extinctions. Such exploitation of the environment is primarily facilitated by horizontal gene transfer.

Resistance to antibiotics is a prime example of this rapid evolution. The word antibiotic was first used as noun by Selman Waksman in 1941 as a description of molecules produced by one microbe that has a negative effect on the growth of another (Clardy et al. 2009). Large-scale production of antibiotics began in the early 1940s with the expectation that infectious diseases might become relegated to the past. In fact the use of antibiotics dropped the rate of death from infectious disease from nearly 800/100,000 in 1900 to $<$40/100,000 in 1980 (Walsh and Wright 2005). Unfortunately, by the mid-1940s antibiotic-resistant bacteria had emerged. It is this ever expanding resistance to antibiotics, both old and new generation, that has motivated the emergence of novel delivery or bactericidal methods. The discovery of antibiotics has been considered one of the defining events in medicine and science

during the twentieth century (Davies and Davies 2010). However, the ever increasing levels of antibiotic resistance in agriculture, hospitals, and in environmental reservoirs seemingly in step with their use are equally or of greater importance than their discovery. Microbes relying on the strong selection imposed by the misuse and overuse of antibiotics have been able to exploit mechanisms of gene exchange to spread every source of resistance genes among close and unrelated taxa. In the process they have developed multiple mechanisms of resistance and many species harbor multiple resistance genes. Some species have been screened that are resistant to almost every class of antibiotic, including recently developed drugs (Stepanauskas et al. 2005, 2006).

Genotypes may persist in a bacterial community even after a given selective pressure no longer exists (Enne et al. 2001). This observation is counter intuitive as there should be a fitness cost associated with maintaining extraneous DNA when there is no immediate benefit. However, several studies have shown that bacteria carrying these extra-genetic elements do not seem to show decreased fitness when compared to strains of the same species without the traits (Andersson and Levin 1999; Schrag et al. 1997). Therefore, various gene combinations can and do remain in the bacterial community and are available for transfer via horizontal gene transfer mechanisms or selective increase under changing environmental conditions.

2 Use of Metals as Bactericides

Although various metals are necessary for bacterial survival as micronutrients, e.g., Cr, Co, Cu, Mn, Mo, Ni, Se, W, V, Zn, and Fe, many of these elements are toxic at higher concentrations. The antibacterial effects of some of these and certain other metals have been known since antiquity (Silver and Phung 1996) prompting their use in dentistry and medicine (Kim et al. 2007; Catauro et al. 2004; Crabtree et al. 2003). The efficacy of a metal biocide is dependent on the concentrations used and the exposure time. While many studies report dramatic decreases in bacterial numbers (see discussion below) very few studies have shown complete elimination of bacteria. This observation is critical and drives the remaining discussion.

3 Nanoparticles

The bioavailability of particles is enhanced as the size decreases. Indeed the science of nanotechnology is focused on making materials with significantly improved physical, chemical, and biological properties (Wang 2000) with increased functionality because of the nanosize. However, there are a number of problems with bringing nanotechnology into large-scale commercial use (Mazzola 2003; Serov et al. 2003; Ohshima 2003) not the least of which is significant differences between batches using the same protocols to produce standard reference materials for use in experiments.

One area where nanotechnology may have an impact is drug delivery and therapeutics. The use of nanoparticles as an antimicrobial allows the delivery of metals to the actual cell. This increase in the efficacy of the metal as a biocide has brought about what has been called a “new generation of antimicrobials” (Rai et al. 2009). Shrivastava et al. (2008) presented what they described as the enhanced antimicrobial effect of novel silver nanoparticles. Interestingly, their methods were more pronounced against gram-negative bacteria than gram-positive organisms. In fact none of the doses of silver nanoparticles they used were effective against *Staphylococcus aureus*.

There is a general consensus regarding the modes of antimicrobial activity of metal and carbon nanoparticles. Most studies indicate that nanoparticles cause disruption of bacterial cell membranes in probable response to various oxygen species (Neal 2008). For this to occur there needs to be contact between the bacteria and the nanoparticle with interfacial process such as electrostatic interactions. Toxicity of the nanoparticle may also be important and while unlikely, accumulation does in some instances occur after disruption of the membrane. With the discovery of the mode of action of nanoparticles there seems to be a similar sense of wonder to that expressed when the first “wonder drugs” were developed, i.e., an assurance that we can beat the processes of bacterial evolution. We will discuss the evolutionary arms race between man and bacteria below.

4 Antimicrobial Textiles

Textiles, both natural fiber and engineered fibers, provide surfaces for microbial growth. Given optimal conditions of temperature, water, minimal nutrients, electron donors and acceptors, and a carbon source, microbes associated with textiles can multiply rapidly. In general, engineered fibers are more resistant to microbial degradation than naturally derived fibers. Textiles thus become an excellent media for microbial growth with the resulting consequences such as odor, discoloration, and increased likelihood of infections, etc. Cultural evolution of human societies, especially in developed countries, has placed an ever increasing demand for clothing that is hygienic. Gao and Cranston (2008) indicate that sportswear, socks, shoe linings, and lingerie account for 85% of the total production of antimicrobial textiles. Production of these textiles in Western Europe has increased more than 15% per year between 2001 and 2005 (Gao and Cranston 2008). Other industries have increased the demand for antimicrobial fibers for use in air filters, automotive and outdoor textiles, home furnishings, and textiles used in medicine.

Manufacturers have met this increasing demand imposed by a public concern about comfort, hygiene, and physical well-being by incorporating a wide range of broad spectrum biocides. Various antimicrobials have been used including silver, quaternary ammonium compounds, and triclosan on most of the products of the textile industry (Table 1). These substances are applied at various stages of the

Table 1 Some commercial biocides used in textiles with comments and bacterial resistance potential. Modified from Gao and Cranston (2008)

Biocide	Fiber	Comments
Silver	Polyester Wool Nylon Regenerated cellulose	Slow release, Ag can be depleted, bacterial resistance
QACs (e.g., *AEM 5700)*	Cotton Polyester Nylon Wool	Covalent bonding, very durable, possible bacterial resistance
PHMB	Cotton Polyester Nylon	Requires large amounts, possible bacterial resistance
Triclosan	Polyester Nylon Polypropylene Cellulose acetate Acrylic fibers	Requires large amounts, breakdown products toxic, bacterial resistance

textile process such as finishing or through incorporation into synthetic fibers during extrusion.

The effectiveness of these treatments has been varied. For example, Tomšič et al. (2009) examined the antimicrobial activity of AgCl that had been embedded in a silica matrix on cotton and found that the biocidal effects were strongly influenced by the concentration of the silver present in the coatings. Saengkiettiyut et al. (2008) demonstrated that silver nanoparticles on cotton fabrics have excellent antibacterial activity against *Staphylococcus aureus, Staphylococcus aureus* methicillin resistance strain (MRSA)*, Escherichia coli,* and *Pseudomonas aeruginosa*. In another study (Dubas et al. 2006) antimicrobial silver nanoparticles were immobilized on nylon and silk fibers using a layer-by-layer deposition method. This procedure showed an 80% reduction in *Staphylococcus aureus* on silk fibers and a 50% reduction on the nylon fibers. While this procedure did demonstrate a decrease in bacterial activity there were significant numbers of bacteria remaining on both fibers.

Conversely, a study (Li et al. 2006) designed to assess the antimicrobial activity of silver nitrate and titanium dioxide nanoparticle coated facemasks in an effort to protect against infectious agents found unequivocal results. After 48 h of exposure and incubation the authors found a 100% reduction in viable *Escherichia coli* and *S. aureus* whereas there was a 20% and 50% respective increase in the viable counts of these two species on untreated masks. A study that compared the effect of Ag nanoparticles on microbial activity on cotton and wool showed variable results for both fibers and species of microorganism examined (Falletta et al. 2008). When applied to cotton and polyester fibers there was complete inhibition of bacterial and fungal growth; however, when applied to wool there was only inhibition against fungi.

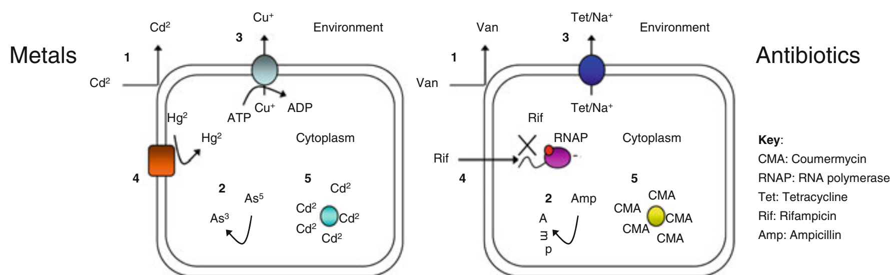
While numerous other studies can be reviewed similar results are obtained, i.e., high variability between and within studies on the effectiveness of a nanoparticle treatment. This variability ranges from complete inhibition to no inhibition and seems to be dependent on dose, exposure, and type of fiber under consideration. Most importantly is the observation that the efficacy of this treatment regime is, in many cases, not completely successful against a wide variety of microorganisms.

5 Cross-Selection and Co-selection of Metal Resistance Traits in Nature

Strikingly, the structural basis of microbial resistance to metals and antibiotics is highly similar (Fig. 1). Bacteria use just a handful of mechanisms to detoxify metals and antimicrobials, such as active extrusion from the cell, sequestration, and exclusion of toxin by the cell membrane, among others. Of further evidence of this symmetry, recent studies have implicated the role metal contamination has as a selective agent in the proliferation of antibiotic resistance (See Baker-Austin et al. 2006 for review). These studies have documented that various associations between types and levels of metal contamination and environmental patterns of antibiotic resistance exist in nature. Such patterns suggest that various mechanisms underlie the co-selection of metal and antibiotic resistance. There are at least two co-selection mechanisms that include co-resistance and cross-resistance. Co-resistance occurs when different resistance genes are found on the same genetic element, e.g., resistance to aminoglycosides and Hg resistance genes on the same mobile genetic element such as a plasmid or integrase. In cross-resistance the same genetic determinant is responsible for more than one type of resistance, e.g., Cd and tetracycline efflux pumps where the same pump is used to rid the cell of either Cd or tetracycline.

Metal contamination of the environment is different from the release of antibiotics because antibiotics can, over time, be degraded. Metals cannot be degraded although their mobility through soil matrices can be altered by changes in valence state and the chemical composition of soil particles. Depending on the valence state of the metal the bioavailability similarly changes. If the metal is tightly bound to a surface it is less likely to have an impact on the microbial communities than the same metal that is in solution. The toxic impact of metals on bacteria is dependent on how much the microorganism interacts with the metal as well as the chemical state of the metal upon contact (e.g., reduced, oxidized, methylated, etc.).

In controlled laboratory experiments, it has been shown that exposure of bacteria to either metals or antibiotics results in an increase in the proportion of bacteria resistant to both metals and antibiotics. In these experiments, naïve bacteria in river water were exposed to either Cd or Ni chloride at increasing concentrations or to the antibiotics tetracycline or ampicillin at increasing concentrations. Both the metals



Mechanism

1. Exclusion of toxin by cell membrane
2. Intracellular modification of toxin
3. Extrusion from the cell
4. Reduction in sensitivity of cellular target
5. Intracellular sequestration

Fig. 1 Structural similarities between metal and antibiotic resistance

and antibiotics had significant effects on the fraction of cultivable bacteria in the microcosms. Total numbers of bacteria decreased nearly one order of magnitude after being exposed to the metals. Of those bacteria remaining the proportions of resistant bacteria increased dramatically. Where less than 20% of cultivable bacteria obtained from the control microcosms (river water) were resistant to any of the tested antibiotics between 50 and 100% of isolates from the microcosms amended with either Cd or Ni were resistant to at least one antibiotic (Stepanauskas et al. 2006). Of equal interest while only 3% of isolates obtained from the control microcosms were resistant to Cd or Ni, 50–60% of isolates obtained from the microcosms amended with tetracycline and ampicillin were resistant to at least one of the two metals. Of greater concern when river water microbial assemblages were individually exposed to each of the four toxicants, the frequency of microorganisms with multiple resistances (metal and antibiotic resistances) increased. This study is important for at least three reasons:

1. It demonstrated that bacteria exposed to metals increase the frequency of antibiotic-resistant bacteria.
2. It demonstrated that bacteria exposed to antibiotics increase the frequency of metal-resistant bacteria.
3. It provided the first experimental evidence that the exposure of freshwater microbial assemblages to individual metals and antibiotics selects for multiresistant microorganisms (Stepanauskas et al. 2006).

These laboratory studies as well as numerous observational studies from the environment confirm that exposure of bacteria to metals increases the proportion of bacteria resistant to the metals but more importantly the number of bacteria resistant to antibiotics. Even without sequencing it is apparent that co-selection is

occurring. Whether the mechanisms in these multiresistant phenotypes are cross-resistance or co-resistance is interesting from a basic science perspective but the most important outcomes have significant practical importance and may directly affect public health.

6 Resistance to Ag and Other Metals Used in Microbial Textiles

Silver and colleagues (Silver 2003; Silver and Phung 1996; Silver et al. 2006) have for some time been warning of increased resistance to metals used in biocides and to silver specifically. Basing their predictions on models explaining the rapid and widespread increase in antibiotic resistance that has been carefully documented, they suggest that horizontal gene transfer, under strong selection of metal exposure, will move metal resistance traits quickly through microbial assemblages and populations. A restrained word of caution has been offered by Percival et al. (2005) who suggest that the risks of antibacterial resistance developing from the use of metal containing biocides have been overstated. They feel that there have been too few studies that have effectively documented the prevalence of resistance. Where Silver (2006) argues the possibility that widespread use of Ag may promote the development of resistance, Percival et al. (2005) citing numerous studies to support their conjecture, counter that the probability of transferring silver resistance genes is low, unstable, difficult to maintain and to transfer. Interestingly, Percival et al. (2005) suggest that the occurrence of silver resistance can be variable under different conditions of exposure and that it is difficult to distinguish between sensitive and resistant bacteria. They then provide a list of different bacteria that have been shown to be silver resistant or from which silver-resistance conferring plasmids have been isolated. While the list is not exhaustive it is suggestive that the trait can occur in many different types of bacteria and should be considered indicative of the possibility of many other bacteria showing the trait.

The greatest possibility of finding silver-resistant bacteria is in environments where usage or occurrence of metals is highest such as dentistry where amalgams contain 35% silver (Brunne 1986), in hospital burn units where silver is used in dressings (Klasen 2000), or on catheters (Sampath et al. 1995). Several controlled laboratory studies have shown a relationship between bacterial resistance to biocides and cross-resistance to antibiotics. While these studies create various levels of contention among researchers the results seem to be following the same paths trod in the early days of studies on antibiotic resistance.

Following Percival et al. (2005) publication, Silver et al. (2006) provided a new review of silver resistance studies. From this review we find that bacterial resistance to Ag^{+} has been observed repeatedly (Silver et al. 2006) but that until recently the genetic mechanisms were not understood. Genes located on plasmids frequently confer resistance to toxic metals including silver (Davis et al. 2005; Gupta et al. 2001). The plasmid

pMG101, which was originally found in *Salmonella,* encodes resistance to several antibiotics as well as Hg^{2+}, Ag^{+}, and tellurite (Gupta et al. 1999, 2001; McHugh et al. 1975). However, genes conferring resistance to Ag are not restricted to plasmids. In *Salmonella* and *Escherichia coli*, a chromosomal Ag^{+} resistance determinant has been described (Gupta et al. 2001; Franke et al. 2001; Silver 2003). Selection imposed by the widespread, often indiscriminate use of antiseptics, has resulted in increased resistance to Ag^{+} in clinical and hospital settings (Davis et al. 2005). For example, (Silver 2003) found in a random sampling of enteric bacteria from a Chicago hospital that more than 10% had resistance genes for Ag^{+} resistance. These observations suggest that the potential for wide-scale horizontal gene transfer of metal resistance genes, similar to antibiotic resistance genes, could occur under such strong selection. Such strong selection is clearly employed in hospital settings but is also used in the application to textiles. However, Chopra (2007) followed with a review which concluded the variability in results from various studies is probably due to a failure to establish standard procedures for determining MIC values, a lack of recognized breakpoints, differences in the way products and media release silver, and finally that there is no standard way to measure inhibitory activity. Chopra (2007) suggests that because the clinical incidence of silver resistance remains low the emergence of resistance can be contained or minimized if silver ion concentrations are high and bactericidal activity rapid. Gao and Cranston (2008) counter that because large quantities of biocides are frequently required on textiles to achieve an adequate durability and effect there should be increased concern. Most biocides used in textiles can and do induce bacterial resistance to the biocide (Table 1) but more importantly this selection increases resistance to antibiotics through co-selection. Gao and Cranston (2008) suggest that the long-term benefits and potential problems of antimicrobial textiles need to be closely monitored.

What is curious about these review articles is that many fail to discuss the other side of resistance, i.e., naturally occurring resistance and the use of bacteria to synthesize nanoparticles of various metals. Nanoparticle scientists rely on the ability of various bacteria to not only resist metal toxicity but to sequester metals within the cells (Mandal et al. 2006). As early as 1979, bioaccumulation of silver in bacteria had been described in a multispecies community of bacteria (Charley and Bull 1979). In this study, an isolated stable community of three species had an unusually high tolerance to silver where tolerance is defined as remaining viable under the imposed conditions but not necessarily reproducing. The community was composed of *Pseudomonas maltophilia, Staphylococcus aureus,* and an unidentified species. The effect of exposure to Ag was a change in the relative abundance of the three species. Tolerance of high silver concentrations was reduced when the community was grown in the absence of silver suggesting that these resistance-bearing bacteria were less able to compete in the nonselective environment. Considering that in several recent studies silver did not affect or had minimal effect of *S. aureus* it seems surprising that these earlier studies on environmental bacteria did not help design experiments or to define the hypotheses and predictions regarding antimicrobial textile studies.

The most important observation that needs to be made in studies on the efficacy of antimicrobial textiles is how many of the bacteria that remain are metal resistant. If we decrease the total viable bacteria to ranges found in various studies (92–99.99%) that means that there are still between 1,000 and hundreds of thousands of bacteria remaining. It is likely that the remaining bacteria are simply tolerant and not necessarily resistant. The same is true of bacteria exposed to an initial dose of antibiotics, i.e., the initial low dose does not kill all bacteria at once and hence the need to take the full regimen of antibiotics to increase the likelihood that all disease-causing bacteria are eliminated. Failure to do so means that these tolerant or minimally resistant bacteria are free to proliferate either in the host or in the environment resulting in an increase in both resistant pathogens and their genes that can be transferred to other species of bacteria. Since bacterial growth rates are high these remaining bacteria can increase in density or pass their genes to bacteria that are adapted for the ambient conditions and thus increase the number of resistant genes in the assemblage.

7 Problems with Nanoparticles on Textiles

One very important observation regarding antimicrobial textiles is that the effective particles or applications do not necessarily remain on the textile but can be released by repeated washings and enter into the environment. Unfortunately, there has not been sufficient research to mimic natural systems in laboratory settings or to actual controlled releases into complex natural systems (Neal 2008). Failure to perform carefully designed experiments has resulted in a poor understanding of likely consequences from the release of these substances into the environment. Neal (2008) in reviewing what the then current studies were, states that there is a general failure to identify any significant effects at the microbial level of nanoparticles in complex systems. Does this means that there are no effects or that we have not yet observed or measured the component of the system where an effect has occurred?

There is a cause for concern about the toxicity and bioavailability of these substances to bacteria involved in critical ecosystem level processes especially biogeochemical cycles such as carbon and nitrogen cycling. Chronic low level release of these antimicrobials into the environment will increase tolerance and over time result in increased numbers of diverse species carry resistance genes. More importantly, the chronic input of metal-rich nanoparticles into the environment may have unforeseen consequences. A clear understanding of effects on critical players in important biogeochemical processes must be explored under various natural conditions. Only then can we make intelligent predictions of future impacts or lack thereof.

The pathways to increased exposure of environmental bacteria are similar between antibiotics in the environment and nanoparticles. Washing of metal-coated textiles will distribute released substances into waterways. Increased antibiotic resistance traits can be found at much higher levels below water treatment facilities

and below intensive agricultural areas. We would predict similar increases in metal-resistant bacteria below such facilities due to co-selection caused by increased selection by the antibiotics present in the water column (Baquero et al. 2008). Because nanoparticles and antibiotics will have the same vectors of inputs into waterways it will be difficult to ascertain whether increased metal resistance found downstream is due to direct selection or via co-selection mechanisms. The same problem is true in clinical situations. Because of the strong selection from the various biocidal agents used it may be difficult to determine the exact mechanism of increased metal and antibiotic resistance. Nevertheless, such studies are imperative.

8 Evolutionary Arms Races

An evolutionary arms race occurs between two organisms when one is antagonistic or parasitic towards the other. Selection should favor traits in the host or antagonized species that prevent or decrease the incidence of infection or antagonism. However, this change in the host then provides selection for traits in the antagonizing species which can overcome the acquired traits of the host. This tit-for-tat cycle continues as a spiral of ever increasing point and counter-point between the two species with each evolving to unique characteristics that promote survival.

The limits of this spiral are determined by the genetic resources of the two organisms. Researchers have investigated such an arms race between bacteria and bacteriophage (Weitz et al. 2005). In this system the bacteria evolve to change the binding site(s) that are used by the phage to gain entry into the bacterial cell. This in turn results in selection of phage that are able to bind to novel sites. It is clear that these arms races never eliminate either the host or the infectious agent but the interaction controls densities.

Given that bacteria evolved in a metal-rich environment we should expect that various gene combinations can be found that confer resistance to metals. Indeed, Silver (1998) has stated that there are genes for all metals. In other words, there are no toxic metals for which there are no genes or gene systems that can confer resistance. Furthermore, there are genes that actually allow bacteria to accumulate or sequester metals within the cytoplasm, such as metallochaperones. As man continues to attempt to modify his environment, especially in the realm of clinical, strong selection is imposed on potential disease-causing organisms. The result is a general decrease in bacterial numbers and in many cases what appears as an initial elimination of the disease-causing bacterial agents. However, over time resistant bacteria emerge with traits that allow them to survive in ever increasing concentrations of the bactericidal substances. Thus, an evolutionary arms race is occurring between the capacity of bacteria to evolve and the creative ability of man to make new substances that are effective against disease-causing bacteria. This is a new area of evolution that, although the mechanisms used by bacteria have been in

existence for millennia, is not between one species evolving in response to the evolution of another species. Rather the selection is imposed by the technological innovation of man and the genetic evolution of bacteria. The only question is which species has the greatest probability to counter the other. Given the evolutionary history of microorganisms and their impressive ability to develop resistances to all known classes of naturally occurring and synthetic drug agents, bacteria represent formidable opponents.

Novel or what seem to be novel methods to treat bacterial infections, regardless of whether the substance used is newly synthesized and thus never been found before in nature, has only a limited life expectancy or efficacy. Time and again, bacteria have overcome these barriers and continue to infect man with decreasing options for effective clinical intervention.

9 Is There a Reason for Concern?

One additional finding first reported in Baker-Austin et al. (2009) raises the specter of unwitting artificial selection for so-called superbugs. They showed that with the increasing number of antibiotics to which a bacterial isolate is resistant, there appeared to be a concomitant increase in the concentration of the antibiotic that was minimally inhibitive to those isolates. We refer to this as the "multiresistance effect." The bacteria in Baker-Austin et al. (2009) were shellfish pathogens *Vibrio vulnificus*, collected from two known metal contaminated estuaries and from a third nearly pristine estuary along the southeastern Atlantic coast. Figure 2 shows a similar pattern among *Escherichia coli* bacteria from the same collection events (unpublished). The obvious implication is that constant exposure to heavy metals, in naturally occurring or anthropogenic elevated concentrations, co-selects for multiple antibiotic resistance traits. More importantly, the more traits acquired, the higher the concentration (on average) of the corresponding antibiotics required to inhibit growth. This is a somewhat puzzling outcome given the prevailing hypothesis that the acquisition of new traits/genes comes with a fitness cost that isolates without such traits do not have to bear. Traditionally, a trade-off is expected between the costs of carrying and replicating new genes in the genome and the conferred fitness benefit in the presence of the selective agent. However, MacLean et al. (2010) point out that

> Once a population is dominated by resistant cells, mutants that have second-site compensatory mutations that recover the cost of resistance have higher fitness than resistant genotypes lacking the compensatory mutations, and natural selection ultimately results in the fixation of compensatory mutations,

which benefits remain, they point out, in the absences of antibiotics. Perhaps, this explains the "multiresistance effect," but to do so completely requires us to acknowledge conveniently high second-site mutation rates given metal contaminant selection pressure in only ecological time. We suspect the more likely explanation still has to do with HGT of those genes which recover fitness costs from

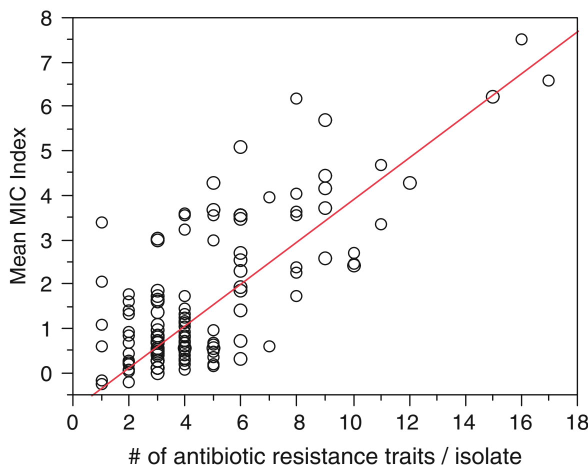


Fig. 2 An illustration among estuarine *E. Coli* isolates of the unexpected "multiresistance effect." This is a positive and statistically significant ($p < 0.05$) relationship between the number of antibiotics to which an isolate is resistant and the mean minimum Inhibitory concentration (MIC) among only those traits acquired per isolate, expressed on a standard normal deviate scale

carrying antibiotic resistance genes. After all, as we have discussed above, such genes are likely to have been around for a very long time.

This research finding of a "multiresistance effect," combined with evidence provided earlier in this chapter should be sufficient cause for concern over the widespread use of antimicrobials including metals on textiles. Co-selected bacteria with resistance traits are likely to find their way into public water treatment facilities and become gene nurseries for multiply resistant strains. Furthermore, HGT will only add to this concern from the acquisition of antibiotic resistance genes by human pathogens in such public works settings. Note that our discussion here does not technically qualify as an ugly prediction, but should give pause for the assessment of current research on these potential biological innovations that may have unintended consequences.

10 Where Do We Go from Here?

Modern culture imposes economic incentives for the development of products that meet perceived needs. Included in these needs are fabrics that help to eliminate odors, that resist degradation, and that are capable of killing or greatly reducing potential disease-causing organisms. Prior to the development of broad-spectrum bactericides washing clothes was sufficient for the effective removal or temporary inactivation of bacteria present in clothing. However, medicine and dentistry require materials that can fight off infections. The development of these materials opened the door for the development of textiles to be used by the general public.

Many products are available to kill 99.99% of microbes on contact such as hand washes and wipes. Progression towards fabrics and materials with antibacterial substances embedded or attached is a continued step towards trying to rid humans of possible infection-causing agents.

Given the seemingly unlimited capacity of bacteria to respond to and exploit novel environments it is not surprising that resistance to metals, biocides, and antibiotics occurs. Both metals and antibiotic are found in nature although the role of antibiotics in nature is not fully elucidated. It is our contention that the use of bactericidal products will over time select for resistant bacteria. Bacteria resistant to the bactericide probably carry with it other co-selected traits. Bacteria can live in the most inhospitable places on the planet, e.g., high level radioactive waste tanks, thus it is unlikely that even high concentrations of metal nanoparticles would be effective for long periods of time. In addition, the effects of metal nanoparticles, released from antimicrobial textiles into the environment, are not understood and could have serious consequences. Similar concerns have been raised about the long- and short-term effects of human-derived antibiotics on natural systems. While there may have been short-term effects such as the reduction in the abundance of specific groups of bacteria, overall biogeochemical cycles have not been significantly impacted by the release of antibiotics into the environment. However, as noted above, antibiotics can be degraded over time either by microbial activity or through natural weathering whereas metal nanoparticles cannot. Thus, while the effect of antibiotics on natural processes has not been shown to be a major problem the effect of metal nanoparticles is less sure. Regardless of whether metal resistance increases in the community, some species are less capable of obtaining new genetic traits and identifying and characterizing which bacteria are impacted is the question of concern. For example if nitrogen fixing bacteria are negatively impacted we might expect significant changes in nitrogen cycling over time.

The arms race will continue with man and his array of technologies and ability to perceive, conceive, and achieve being matched by the long evolutionary history of microbes. Microbes who first colonized the planet and continue to do so 3.8 billion years later.

Acknowledgements Preparation of this chapter was partially supported by the US Department of Energy under Award Number DE-FC09-07SR225056 to the University of Georgia Research Foundation.

References

Alonso A, Sanchez P, Martinez JL (2001) Environmental selection of antibiotic resistance genes. Environ Microbiol 3:1–9

Andersson D, Levin BR (1999) The biological cost of antibiotic resistance. Curr Opin Microbiol 2:489–493

Baker-Austin C, Dopson M, Wexler M, Sawers G, Bond PL (2005) Molecular insight into extreme copper resistance in the acidophilic archaeon '*Ferroplasma acidarmanus*' Fer1. Microbiology 151:2637–2646

Baker-Austin C, Wright MS, Stepanauskas R, McArthur JV (2006) Co-selection of antibiotic and metal resistance. Trends Microbiol 14:176–182

Baker-Austin C, McArthur JV, Lindell AH, Wright MS, Tuckfield RC, Gooch J, Warner L, Oliver J, Stepanauskas R (2009) Multi-site analysis reveals widespread antibiotic resistance in the marine pathogen *Vibrio vulnificus*. Microb Ecol 57:151–159

Baquero F, Martinez JL, Canton R (2008) Antibiotics and antibiotic resistance in water environments. Curr Opin Biotechnol 19:260–265

Brunne D (1986) Metal release from dental biomaterials. Biomaterials 7:163–175

Catauro M, Raucci MG, De Gaetano FD, Marotta A (2004) Antibacterial and bioactive silver-containing $Na_2O \times CaO \times 2SiO_2$ glass prepared by sol-gel method. J Mater Sci Mater Med 15:831–837

Charley RC, Bull AT (1979) Bioaccumulation of silver by a multispecies community of bacteria. Arch Microbiol 123:239–244

Chopra I (2007) The increasing use of silver-based products as antimicrobial agents: a useful development or a cause for concern? J Antimicrob Chemother 59:587–59

Clardy J, Fischbach M, Currie C (2009) The natural history of antibiotics. Curr Biol 19: R437–R441

Crabtree JH, Burchette RJ, Siddiqi RA, Huen IT, Handott LL, Fishman A (2003) The efficacy of silver-ion implanted catheters in reducing peritoneal dialysis-related infections. Perit Dial Int 23:368–74

D'Costa VM, Griffiths E, Wright GD (2007) Expanding the soil antibiotic resistome: exploring environmental diversity. Curr Opin Microbiol 2007(10):481–489

Davies J, Davies D (2010) Evolution of antibiotic resistance. Microbiol Mol Biol Rev 74:417–433

Davis IJ, Richard H, Mullany P (2005) Isolation of silver- and antibiotic-resistant *Enterobacter cloacae* from teeth. Oral Microbiol Immunol 20:191–194

Davison J (1999) Genetic exchange between bacteria in the environment. Plasmid 42:73–91

Dubas ST, Kumlangdudsana P, Potiyaraj P (2006) Layer-by-layer deposition of antimicrobial silver nanoparticles on textile fibers. Colloid Surf A Physicochem Eng Asp 289:105–109

Dykhuizen DE (1998) Santa Rosalia revisited: why are there so many species of bacteria? Antonie Van Leeuwenhoek 73(25–33):1998

Enne VI, Livermore DM, Stephens P, Hall LMC (2001) Persistence of sulphonamide resistance in Escherichia coli in the UK despite national prescribing restriction. Lancet 357:1325–1328

Falletta E, Bonini M, Fratini E, Nostro AL, Pesavento G, Becheri A, Nostro PL, Canton P, Baglioni P (2008) Clusters of poly(acrylates) and silver nanoparticles: structure and applications for antimicrobial fabrics. J Phys Chem 112:11758–11766

Franke S, Gras G, Nies DH (2001) The product of the *ybdE* gene of the *Escherichia coli* chromosome is involved in detoxification of silver ions. Microbiology 147:965–972

Gao Y, Cranston R (2008) Recent advances in antimicrobial treatments of textiles. Tex Res J 2008 (78):60–72

Gupta A, Matsui K, Lo JF, Silver S (1999) Molecular basis for resistance to silver cations in *Salmonella*. Nat Med 5:183–188

Gupta A, Phung LT, Taylor DE, Silver S (2001) Silver resistance genes in plasmids of the IncHII incompatibility group and on the *Escherichia coli* chromosome. Microbiology 147:3393–3402

Haefeli C, Franklin C, Hardy K (1984) Plasmid-determined silver resistance in *Pseudomonas stutzeri* isolated from a silver mine. J Bacteriol 158:389–392

Heuer H, Smalla K (2007) Manure and sulfadiazine synergistically increased bacterial antibiotic resistance in soil over at least two months. Environ Microbiol 9:657–666

Kim JS, Kuk E, Yu KN, Kim J, Park SJ, Lee HJ, Kim SH, Park YK, Park YH, Hwang C, Kim YK, Lee YS, Jeong DH, Cho M (2007) Antimicrobial effects of silver nanoparticles. Nanomedicine 3:95–101

Klasen HJ (2000) Historical review of the use of silver in the treatment of burns. Part I. Early uses. Burns 26:117–130

Li Y, Leung P, Yao L, Song QW, Newton E (2006) Antimicrobial effect of surgical masks coated with nanoparticles. J Hosp Infect 62:58–63

Lorenz MG, Wackernagel W (1994) Bacterial gene-transfer by natural genetic-transformation in the environment. Microbiol Rev 58:563–602

MacLean RC, Hall AR, Perron GG, Buckling A (2010) The evolution of antibiotic resistance: Insight into the roles of molecular mechanisms of resistance and treatment context. Discovery Medicine. http://www.discoverymedicine.com/R-Craig-MacLean/2010/08/04/the-evolution-of-antibiotic-resistance-insight-into-the-roles-of-molecular-mechanisms-of-resistance-and-treatment-context/

Mahendra Rai M, Yadav A, Gade A (2009) Toxic Silver nanoparticles as a new generation of antimicrobials. Biotechnol Adv 27:76–83

Mandal DME, Bolander D, Mukhopadhyay GS, Mukherjee P (2006) The use of microorganisms for the formation of metal nanoparticles and their application. Appl Microbiol Biotechnol 69:485–492

Mazzola L (2003) Commercializing nanotechnology. Nat Biotechnol 21:1127–1143

McHugh SL, Moellering RC, Hopkins CC, Swartz MN (1975) Salmonella typhimurium resistant to silver nitrate, chloramphenicol, and ampicillin. Lancet 1:235–240

Neal AL (2008) What can be inferred from bacterium–nanoparticle interactions about the potential consequences of environmental exposure to nanoparticles? Ecotoxicology 17:362–371

O'Brien T (2002) Emergence, spread, and environmental effect of antimicrobial resistance: how use of an antimicrobial anywhere can increase resistance to any antimicrobial anywhere else. Clin Infect Dis 34:S78–84

Ochman H, Lawrence JG, Groisman EA (2000) Lateral gene transfer and the nature of bacterial innovation. Nature 405:299–304

Ohshima M (2003) Control and design problems in material processing—how can process systems engineers contribute to material processing? J Process Cont 7:599–605

Pei RT, Kim SC, Carlson KH, Pruden A (2006) Effect of River Landscape on the sediment concentrations of antibiotics and corresponding antibiotic resistance genes (ARG). Water Res 40:2427–2435

Percival SL, Bowlera PG, Russell D (2005) Bacterial resistance to silver in wound care. J Hosp Infect 60:1–7

Sampath LA, Chowdhury N, Caraos L, Modak SM (1995) Infection resistance of surface modified catheters with either shortlived or prolonged activity. J Hosp Infect 30:201–210

Saengkiettiyut K, Rattanawaleedirojn P, Sangsuk S (2008) A study on the antimicrobial efficacy of nano silver containing textile. CMU J Nat Sci Special Issues on Nanotechnology 7:33–36

Schrag SJ, Perrot VR, Levin BR (1997) Adaptation to the fitness costs of antibiotic resistance in *Escherichia coli*. Proc R Soc Lond B 264:1287–1291

Serov IN, Zhabrev VA, Margolin VI (2003) Problems of nanotechnology in modern materials science. Glass Phys Chem 29:169–178

Shrivastava S, Bera T, Roy A, Singh G, Ramachandrarao P, Dash D (2008) Characterization of enhanced antibacterial effects of novel silver nanoparticles. Nanotechnology 18:103–112

Silver S, Phung LT (1996) Bacterial heavy metal resistance: new surprises. Annu Rev Microbiol 50:753–789

Silver S (1998) Genes for all metals—a bacterial view of the periodic table: The 1996 Thom Award Lecture. J Ind Microbiol Biotechnol 20:1–12

Silver S (2003) Bacterial silver resistance: molecular biology and uses and misuses of silver compounds. FEMS Microbiol Rev 27:341–353

Silver S, Phung LT (1996) Bacterial heavy metal resistance: new surprises. Annu Rev Microbiol 50:753–89

Silver S, Phung LT, Silver G (2006) Silver as biocides in burn and wound dressings and bacterial resistance to silver compounds. J Ind Microbiol Biotechnol 33:627–634

Smalla K, Haines AS, Jones K, Krogerrecklenfort E, Heuer H, Schloter M, Thomas CM (2006) Increased abundance of IncP-1 beta plasmids and mercury resistance genes in mercury-polluted river sediments: first discovery of IncP-1 beta plasmids with a complex mer transposon as the sole accessory element. Appl Environ Microbiol 72:7253–7259

Sobecky PA (1999) Plasmid ecology of marine sediment microbial communities. Hydrobiologia 401:9–18

Stepanauskas R, Glenn TC, Jagoe CH, Tuckfield RC, Lindell AH, McArthur JV (2005) Elevated microbial tolerance to metals and antibiotics in metal-contaminated industrial environments. Environ Sci Technol 39:3671–3678

Stepanauskas R, Glenn TC, Jagoe CH, Tuckfield RC, Lindell AH, King CJ, McArthur JV (2006) Coselection for microbial resistance to metals and antibiotics in freshwater microcosms. Environ Microbiol 8:1510–1514

Summers A, Wireman J, Vimy MJ, Lorscheider FL, Marshall B, Levy SB, Bennett S, Billard L (1993) Mercury released from dental silver fillings provokes an increase in mercury-resistant and antibiotic-resistant bacteria in oral and intestinal floras of Primates. Antimicrob Agents Chemother 37:825–834

Szczepanowski R, Bekel T, Goesmann A, Krause L, Krömeke H, Kaiser O, Eichler W, Pühler A, Schlüter A (2008) Insight into the plasmid metagenome of wastewater treatment plant bacteria showing reduced susceptibility to antimicrobial drugs analysed by the 454-pyrosequencing technology. J Biotechnol 136:54–64

Tomšič B, Simončič B, Orel B, Žerjav M, Schroers H, Simončič A, Samardžija Z (2009) Antimicrobial activity of AgCl embedded in a silica matrix on cotton fabric. Polymers 75:618–626

Turner SL, Bailey MJ, Lilley AK, Thomas CM (2002) Ecological and molecular maintenance strategies of mobile genetic elements. FEMS Microbiol Ecol 42:177–185

Walsh CT, Wright G (2005) Introduction: antibiotic resistance. Chem Rev 105:391–394

Wang ZL (2000) Characterizing the structure and properties of individual wire-like nanoentities. Adv Mater 12:1295–1298

Weitz JS, Hartman H, Levin SA (2005) Coevolutionary arms races between bacteria and bacteriophage. PNAS 102:9535–9540

Whitman WB, Coleman DC, Wiebe WJ (1998) Prokaryotes: the unseen majority. Proc Natl Acad Sci USA 95(12):6578–83

Yurieva O, Kholodii G, Minakhin L, Gorlenko Z, Kalyaeva E, Mindlin S, Nikiforov V (1997) Intercontinental spread of promiscuous mercury-resistance transposons in environmental bacteria. Mol Microbiol 24:321–329

Efflux: How Bacteria Use Pumps to Control Their Microenvironment

E. David G. McIntosh

Contents

1 Introduction 154
2 Efflux Pumps 154
2.1 Pseudomonas aeruginosa 157
2.2 Acinetobacter baumannii 159
3 Tigecycline 160
4 Conflict of Interest 161
References 161

Abstract Efflux pumps are a potent and clinically important cause of antibiotic resistance. The particular focus of this chapter is on the efflux pump as a target for antimicrobial therapy and the development of new antibacterials to address the efflux problem.

Tigecycline is an example of how old antibiotics, in this case tetracyclines, which have become substrates for efflux pumps, can be extensively modified to restore antimicrobial activity and clinical efficacy.

Keywords Antibacterials • Antibiotic resistance mechanisms • Efflux pumps

E.D.G. McIntosh (✉)
Faculty of Medicine, Imperial College, London SW7 2AZ, UK

Scientific Center for Children's Health, Russian Academy of Medical Science, Moscow, Russia
e-mail: e.mcintosh@imperial.ac.uk

A.R.M. Coates (ed.), *Antibiotic Resistance*, Handbook of Experimental Pharmacology 211, DOI 10.1007/978-3-642-28951-4_10,
© Springer-Verlag Berlin Heidelberg 2012

1 Introduction

The inventiveness of academic institutions and the pharmaceutical industry alike in bringing new antibiotics to the healthcare system is stimulated by the diversity of resistance mechanisms exhibited by microorganisms. Such mechanisms include the production of enzymes and biofilms, drug inactivation, target alteration and efflux (Poole 2002; Wright 2003; McDermott et al. 2003). For the microbial biotechnologists, the challenges are to use microbial genomics and X-ray crystallography to identify novel antibacterial targets and to design more potent antimicrobial compounds respectively (Yoneyama and Katsumata 2006), including efflux pump inhibitors (Lomovskaya and Bostian, 2006; Lynch 2006; Schumacher et al. 2006).

2 Efflux Pumps

With the discovery of microorganisms came the scientific curiosity as to how they control their microenvironment, how they absorb the substances necessary for their function (Bryskier 2005). With the discovery of antibiotics came a hope that they would be consistently absorbed directly into the bacteria. This hope was dashed with the identification of resistance mechanisms, a prime example of which is efflux. This was epitomised by the resistance of *Escherichia coli* to tetracyclines (McMurry et al. 1980; Ball et al. 1980). Uptake of tetracyclines was decreased and efflux increased, in otherwise metabolically functioning but resistant strains. For a long time, efflux was considered of importance only for Gram-negative organisms and in relation to tetracyclines. It is now known that the problem of efflux also affects Gram-positive organisms and relates to antibiotics other than tetracyclines (Poole 2005). They also affect other organisms such as fungi and mycobacteria (Table 1), and are potential targets for new antifungal therapies (Kolaczkowski et al. 2009).

Efflux pumps are "transport proteins involved in the extrusion of toxic substances (including virtually all classes of clinically relevant antibiotics) from within cells into the external environment" (Webber and Pidock 2003). According to Webber and Piddock (2003), there are five major families of efflux transporter in the prokaryotic kingdom:

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

Table 1 shows examples of recent resistance problems related to efflux pumps while recent particular examples of RND efflux pumps affecting Gram-negative organisms are shown in Table 2. What is evident is the variety of efflux mechanisms available to organisms. When combined with other resistance mechanisms,

Table 1 Recent examples of resistance problems related to efflux pumps

Organism	Mechanism	Proposed consequence	Reference
Dermatophytes/fungi			
Trichophyton rubrum	ABC transporter TruMDR2	Resistance to terbinafine, 4-nitroquinoline *N*-oxide and ethidium bromide	Fachin et al. (2006)
Candida albicans	CDR1	Resistance despite repression by serum	Yang et al. (2006)
Candida albicans	PK/PD-related modulation of CDR1	Resistance to fluconazole	Andes et al. (2006)
Candida albicans	Multidrug efflux pump of major facilitator superfamily	Resistance to fluconazole	Hiller et al. (2006)
Mycobacteria			
Mycobacterium fortuitum	Tap	Tetracycline resistance	Ramón-García et al. (2006)
Mycobacterium tuberculosis	Overexpression of genes following exposure to anti-tuberculous drugs	Isoniazid, ethambutol	Gupta et al. (2010), Srivastava et al. (2010)
Mycobacteria	Mycobacterial efflux pumps	Tetracycline, fluoroquinolones, aminoglycosides and isoniazid resistance	De Rossi et al. (2006)
Gram-negative bacteria			
Gram-negative bacteria	For RND types: tripartite complex of inner membrane pump, outer membrane pore and periplasmic adaptor protein	Hinge-bending motion and rotation of the alpha-helical hairpin of MexA	Vaccaro et al. (2006)
Burkholderia cenocepacia	RND	Resistance to fluoroquinolones, tetraphenylphosphonium, streptomycin and ethidium bromide	Guglierame et al. (2006)
Escherichia coli	Plasmid-mediated efflux pump Qep	Fluoroquinolone resistance	Yamame et al. (2006)
Escherichia coli	Tet	Tetracycline and minocycline resistance	Tuckman et al. (2007)
Escherichia coli	Multiple changes in proteome	Piperacillin-tazobactam	dos Santos et al. (2010)
Vibrio fluvialis	Class 1 integrons and SXT integrases	Quinolone resistance	Srinivasan et al. (2006)
Salmonella enterica serovar Typhimurium	AcrAB-TolC multidrug efflux pump	Fluoroquinolone resistance	Quinn et al. (2006)
Enterobacter aerogenes	AcrAB-TolC multidrug efflux pump	Quinolone resistance	Mahamoud et al. (2006)
Enterobacter cloacae	EmrF SMR efflux pump	Multidrug	He and Varela (2006)
Haemophilus influenzae	*AcrAB*	Telithromycin and macrolide resistance	Bogdanovich et al. (2006)
Neisseria gonorrhoeae	MacA–MacB ABC transporter and Mtr-C-MtrD-MtrE	Macrolide resistance	Rouquette-Loughlin et al. (2005)
Helicobacter pylori	Addition of efflux pump inhibitors	Partial restoration of antibiotic activity against otherwise resistant strains	Zhang et al. (2010)

(continued)

Table 1 (continued)

Organism	Mechanism	Proposed consequence	Reference
Gram-positive bacteria			
Streptococcus pneumoniae	Macrolide efflux pump	Macrolide resistance	Ambrose et al. (2005)
Streptococcus pneumoniae	Quinolone efflux pump	Fluoroquinolone resistance	Jumbe et al. (2006)
Anaerobes			
Bacteroides fragilis	RND transporter *BmeRABC*-5	Metronidazole resistance	Pumbwe et al. (2006)

(see Poole 2005 for a full listing of efflux pumps)

Table 2 Recent examples of RND efflux pumps affecting Gram-negative organisms

AcrB multidrug efflux pump of *Escherichia coli* regulated by the global transcription activator MarA and the local transcriptional repressor AcrR (Su et al. 2007)
"Super-resistant" *Pseudomonas aeruginosa* with overexpression of the MexAB–OprM pump and the presence of the metallo-ß-lactamase gene, conferring resistance to imipenem (Ohara et al. 2007)
Plasmid-encoded OqxAB in *E. coli* of porcine origin with wide substrate specificity (Hansen et al. 2007)
Multiple high-level carbapenem resistance in an isolate of *P. aeruginosa* with overexpressed MexAB–OprM and MexXY–OprM, ß-lactamase genes and deficiency of porin OprD (Maniati et al. 2007)
Overexpression of MexB and deficiency of porin OprD in multiple *P. aeruginosa* isolates resistant to carbapenems (Tam et al. 2007)
CmeABC mediated resistance to macrolides and fluoroquinolones in *Campylobacter jejuni* and *Campylobacter coli* (Quinn et al. 2007)
YhiV (MdtF) mediated multidrug resistance (fluoroquinolones, novobiocin, macrolides/ketolides, ethidium bromide, oxacillin, linezolid, tetracycline) associated with a single point mutation in *Escherichia coli* but which can also cause a decreased MIC to azithromycin and telithromycin (Bohnert et al. 2007)
Linezolid resistance in *Escherichia coli*, *Citrobacter freundii* and *Enterobacter aerogenes* (Schumacher et al. 2007)

a formidable defence system can be built. A picture of the genetic functional relationships forming the basis of the structure of efflux pumps is now emerging, as shown with the work on the bacterial efflux pump AcrAB–ToIC (Kim et al. 2010). Preferential sites are being discovered whereby efflux pump inhibitors of AcrB of *Escherichia coli* are found to be either "cave-binders" or "groove-binders" (Takatsuka et al. 2010). New work on old compounds such as trimethoprim is rediscovering them as potential efflux pump inhibitors (Piddock et al. 2010).

An excellent review of efflux-mediated antimicrobial resistance is available (Poole 2005). The present chapter focuses on recent examples with a particular emphasis on two difficult-to-treat organisms: *Pseudomonas aeruginosa* and *Acinetobacter baumannii*.

Antibiotic resistance in the non-lactose-fermenting Gram-negative pathogens such as *Pseudomonas aeruginosa* and *Acinetobacter* species poses a particular challenge to

Table 3 Antimicrobial activity against multidrug resistant *Pseudomonas aeruginosa* in the SENTRY Surveillance Program (1997 1999) (Gales et al. 2001)

MIC$_{50}$ µg/mL (% of isolates)				
Antibiotic	Asia-Pacific $n = 15$	Europe $n = 78$	Latin America $n = 90$	USA $n = 30$
Meropenem	>8 (0)	>8 (0)	>8 (3.3)	>8 (0)
Tobramycin	16 (0)	>16 (0)	>16 (3.8)	>16 (20)
Amikacin	32 (33.3)	>32 (19)	>32 (12.2)	16 (50)
Ciprofloxacin	>2 (13.3)	>2 (2.5)	>2 (6.6)	>2 (3.3)
Tetracycline	>8 (0)	>8 (0)	>8 (0)	>8 (6.6)

clinicians wishing to treat patients with infections caused by them. Both organisms are responsible for serious infections of the respiratory, skin and soft tissue, and other systems in both immunocompetent and immunocompromised hosts, especially in causing hospital-acquired pneumonia. Their widespread presence in the environment enables their ready transmission to humans.

2.1 Pseudomonas aeruginosa

For *Pseudomonas aeruginosa*, multidrug resistance related to efflux pumps is a topical problem (Livermore 2002). Four multidrug efflux pump systems have been well characterised in *Pseudomonas aeruginosa* (Aeschlimann 2003):

- MexA–MexB–OprM
- MexC–MexD–OprJ
- MexE–MexF–OprN
- MexX–MexY–OprM

These efflux pumps have different substrate specificities. Their production and activity can be increased by many factors commonly present in infections, for example:

- High inocula of bacteria
- Low pH
- Stationary-phase growth

During 1997–1999, a total of 6,631 *Pseudomonas aeruginosa* isolates were analysed in the global SENTRY Program by geographic region and body site of infection (Gales et al. 2001). *Pseudomonas aeruginosa* isolates were defined as multiresistant if they were resistant to:

- Piperacillin (MIC ≥128 µg/mL)
- Ceftazidime (MIC >16 µg/mL)
- Imipenem (MIC >8 µg/mL)
- Gentamicin (MIC >8 µg/mL)

Multidrug-resistant *Pseudomonas aeruginosa* was present in up to 10% of all strains analysed. No agent routinely tested inhibited >50% of multidrug-resistant strains (Table 3).

Aeschlimann (2003) proposed the following structure/function of the MexAB–OprM and related efflux pumps of *Pseudomonas aeruginosa*. Antibiotics can be captured by MexB, D, F or Y (RND exporter proteins) from the periplasmic space, the cytoplasmic membrane, and/or the cytoplasmic space. MexA, C, E or X, which are MFP proteins, act as conduits between the cytoplasmic and outer membranes. OprM, J or N, which are gated outer membrane porin proteins, serve as the final step in removal of the antibiotic from the cell. Yoshihara and Eda (2007) further proposed the oligomeric channel structure of MexAB–OprM as a trimer of OprM, OprJ and OprN. A study has been performed to elucidate the role of each protein in the formation of the intact functional MexAB–OprM complex (Welch et al. 2010). This showed that, in the absence of MexA and OprM, MexB can recruit AcrA and TolC from *Escherichia coli* to form a functional complex. MexB can also transport a toxic compound in an organism where the periplasmic space and outer membrane are absent as well as catalysing the transmembrane chemical proton gradient. Also, regulation of MexA–MexB–OprM by the mexO operon through the MarR protein MexR appears to be strongly affected by mutations responsible for multidrug resistance (Andrésen et al. 2010).

Among the common substrates for *Pseudomonas aeruginosa* efflux pumps are fluoroquinolones, aminoglycosides, β-lactams, tetracyclines, choramphenicol and macrolides (Rice 2006). The impact of efflux pumps on the activity of fluoroquinolones against *Pseudomonas aeruginosa* is large (King et al. 2006). Overexpression of MexXY–OprM, MexAB–OprM, MexCD–OprJ or MexEF–OprN increased fluoroquinolone MICs from 16 to 64-fold, 32 to 128-fold, 32 to 512-fold and 64 to 256-fold, respectively. In addition, the impact of single, double and triple target mutations ranged from 2 to 16-fold, 8 to 512-fold and 32 to 1024-fold respectively, and was the same regardless of the level of expression of efflux pumps. Overexpression of efflux pumps in the presence of target mutations had a multiplicative effect on resistance to fluoroquinolones. This observation that overexpression in addition to multiple efflux regulatory gene mutations or efflux protein expression is associated with multidrug resistance in *Pseudomonas aeruginosa* is supported by work on 180 pulmonary and blood isolates from the patients in the intensive care of Colorado Hospital between 1 January 1999 and 31 December 2004 (Kiser et al. 2010). And the simple addition of an efflux pump inhibitor to a fluoroquinolone would not be sufficient to overcome resistance in *Pseudomonas aeruginosa* (Dunham et al. 2010).

Although no pump yet identified uses imipenem as a substrate, increased expression of MexEF–OprD does confer resistance to imipenem through its action on OprD2 (Ochs et al. 1999). There is evidence that the combination of imipenem plus levofloxacin can prevent the emergence of this type of resistance (Lister and Wolter 2005). Carbapenem resistance in bloodstream isolates of *Pseudomonas aeruginosa* was found to be commonly associated with efflux pump overexpression dealt in a large study from Houston (Tam et al. 2007). One new antibiotic,

doripenem, shows promise for the treatment of patients with infections due to resistant *Pseudomonas aeruginosa* infections (Jones et al. 2004; Ge et al. 2004), although it is unlikely to work against imipenem-resistant strains.

2.2 Acinetobacter baumannii

As a threat to the bio-hygiene of hospitals, *Acinetobacter baumannii*, and occasionally other related species, rank with methicillin-resistant *Staphylococcus aureus* (MRSA) and *Clostridium difficile* as being not only difficult to treat but also very difficult to eradicate. The phenomenon of efflux-pump-mediated resistance in *Acinetobacter baumannii* has been well described in a review by Vila et al. (2007), who note a sixth multidrug efflux family, the drug/metabolite transporter (DMT) superfamily. Vila et al. (2007) note that, in *A. baumannii*, efflux-pump-mediated resistance is generally associated with the MFS (Tet(A), Tet(B) and CmlA) and RND (AdeABC) families. For *Acinetobacter baumannii* the efflux determinant Tet(A) confers resistance to tetracycline and Tet(B) confers resistance to tetracycline and minocycline (Martí et al. 2006). The novel Tet(39) efflux protein has been reported in *Acinetobacter baumannii* (Agersø and Guardabassi, 2005). There appears to be horizontal transfer of *tet* efflux genes among different genera of Gram-negative bacteria.

Overexpression of the *AdeABC* efflux pump, of the RND family, confers resistance to aminoglycosides, ß-lactams, chloramphenicol, erythromycin, tetracyclines and ethidium bromide and, in addition, AdeB has been associated with the acquisition of reduced susceptibility to fluoroquinolones (Vila et al. 2007). AdeABC takes the form of a three-component efflux pump. In addition to MFS and RND, the MATE efflux pump *AbeM* is also able to confer *Acinetobacter baumannii* with resistant properties.

The other mechanisms of antibiotic resistance utilised by *Acinetobacter baumannii* include AmpC cephalosporinases and other β-lactamases, serine and metallo-β-lactamases (carbapenemases), outer-membrane porin changes, aminoglycoside-modifying enzymes, plasmid-mediated quinolone resistance, as well as efflux pumps (Bonomo and Szabo 2006), although efflux is the major mechanism of tetracycline resistance in this organism (Guardabassi et al. 2000). An RND-type efflux pump, AdeABC, is responsible for aminoglycoside resistance and is also associated with resistance to quinolones, tetracyclines, chloramphenicol, erythromycin, trimethoprim and ethidium bromide (Magnet et al. 2001; Marchand et al. 2004). A classical feedback mechanism upregulates the expression of the efflux pump in response to the prevailing concentration of antibiotic.

3 Tigecycline

Tigecycline is an expanded broad-spectrum antibiotic derived from tetracyclines. The explanation for its mechanism of action lies in the fact that tigecycline specifically inhibits bacterial protein synthesis with a potency 3- and 20-fold greater than that of minocycline and tetracycline, respectively (Olson et al. 2006). Whilst efflux pumps are a well-known mechanism of resistance deployed against tetracycline (McMurry et al. 1980), tigecycline is a poor substrate for tetracycline-specific efflux pumps (Petersen et al. 1999). Tigecycline exhibits antibacterial activity against a wide spectrum of aerobic and anaerobic bacteria (Stein and Craig 2006). Tigecycline has potent in vitro activity against *Acinetobacter baumannii* (Gales and Jones 2000; Betriu et al. 2002; Henwood et al. 2002; Hoban et al. 2005) including strains resistant to imipenem (Pachón-Ibáñez et al. 2004). Tigecycline is also active against Burkholderia such as *Burkholderia pseudomallei* and *Burkholderia thailandensis* (Thamlikitkul and Trakulsomboon, 2006), but is not active against *Pseudomonas aeruginosa*.

The existence of two RND-type pumps in *Acinetobacter baumannii* raises the possibility that tigecycline resistance could emerge during therapy (Rice, 2006). A group in Pittsburgh (Peleg et al. 2007a) obtained the following strains of *Acinetobacter baumannii*: three clinical isolates from separate patients with *A. baumannii* bloodstream infections, two isolates (with reduced susceptibility) from patients receiving intravenous tigecycline for indications other than *A. baumannii* bloodstream infection (Peleg et al. 2007b) and a tigecycline-susceptible laboratory isolate. The two isolates with reduced susceptibility were exposed to the efflux pump inhibitor phenyl-arginine-ß-naphthylamide and a four-fold reduction in tigecycline MIC was observed. A gene coding for the transmembrane component of the AdeABC pump *adeB*, as well as the two-component regulatory system *adeS* and *adeR*, was found to be carried by both tigecycline-susceptible and tigecycline-nonsusceptible isolates. A 25-fold increase in *adeB* expression was observed in a comparison between a tigecycline-susceptible isolate and its isogenic tigecycline-nonsusceptible mutant. The results indicated that an efflux-based mechanism was playing a role in the reduced susceptibility to tigecycline.

The role of AdeABC has recently been reported, in a larger number of clinical isolates ($n = 106$), from the *Acinetobacter calcoaceticus–Acinetobacter baumannii* complex (Ruzin et al. 2010). The results indicate that the overexpression of the AdeABC efflux pump is a prevalent mechanism in those isolates that had developed decreased susceptibility of tigecycline.

Resistance nodulation cell division family pumps (AcrAB and MexAB–OprM) are responsible for the intrinsic tigecycline resistance of *Pseudomonas aeruginosa, Morganella morganii* and *Proteus mirabilis* (Dean et al. 2003; Visalli et al. 2003; Ruzin et al. 2005a). Tigecycline may function as a substrate for MepA, a novel multidrug and toxin extrusion (MATE) efflux pump overexpressed in mutated *Staphylococcus aureus*, but this is unlikely to result in a high-level resistance to

tigecycline by *Staphylococcus aureus* (McAleese et al. 2005). It is unlikely that clinically significant resistance to tigecycline will emerge due to this mechanism.

Whilst the majority of *Klebsiella pneumoniae* isolates are fully susceptible to tigecycline, some have decreased susceptibility; it appears that the transcriptional activator *ramA* is associated with this, due to its role in the expression of the AcrAB multidrug efflux pump (Ruzin et al. 2005b). Similar mechanisms are responsible for decreased susceptibility of some *Enterobacter cloacae* strains to tigecycline (Keeney et al. 2007). And the up-regulation of the SdeXY–HasF efflux system, an RND-type efflux pump, is responsible for tigecycline resistance in *Serratia marcescens* (Hornsey et al. 2010).

For an extensive review of the use of inhibitors of antibiotic efflux pumps in Gram-negative bacteria see Mahamoud et al. (2007), and for a review of bacterial efflux pump inhibitors from natural sources see Stavri et al. (2007).

4 Conflict of Interest

None declared

References

Aeschlimann JR (2003) The role of multidrug resistance pumps in the antibiotic resistance or *Pseudomonas aeruginosa* and other gram-negative bacteria. Pharmacotherapy 23:916–924

Agersø Y, Guardabassi L (2005) Identification of Tet39, a novel class of tetracycline resistance determinant in *Acinetobacter* spp. of environmental and clinical origin. J Antimicrob Chemother 55:566–569

Ambrose KD, Nisbet R, Stephens DS (2005) Macrolide efflux in *Streptococcus pneumonia* is mediated by a dual efflux pump (*mel* and *mef*) and is erythromycin inducible. Antimicrob Agents Chemother 49:4203–4209

Andes D, Lepak A, Nett J, Lincoln L, Marchillo K (2006) In vivo fluconazole pharmacodynamics and resistance development in a previously susceptible *Candida albicans* population examined by microbiologic and transcriptional profiling. Antimicrob Agents Chemother 50:2384–2394

Andrésen C, Jalal S, Aili D, Wang Y, Islam S, Jarl A, Liedberg B, Wretlind B, Martensson LG, Sunnerhagen M (2010) Critical biophysical properties in the *Pseudomonas aeruginosa* efflux gene regulator MexR are targeted by mutations conferring multidrug resistance. Protein Sci 19:680–692

Ball PR, Shales SW, Chopra I (1980) Plasmid-mediated tetracycline resistance in *Escherichia coli*. Biochem Biophys Commun 93:74–81

Betriu C, Rodríguez-Avial I, Sánchez BA, Gómez M, Álvarez J, Picazo J, and the Spanish Group of tigecycline (2002) In vitro activities of tigecycline (GAR-936) against recently isolated clinical bacteria in Spain. Antimicrob Agents Chemother 46:892–895

Bogdanovich T, Bozdogan B, Appelbaum PC (2006) Effect of efflux on telithromycin and macrolide susceptibility in *Haemophilus influenzae*. Antimicrob Agents Chemother 50:893–898

Bohnert JA, Schuster S, Fähnrich E, Trittler R, Kern WV (2007) Altered spectrum of multidrug resistance associated with a single point mutation in the *Escherichia coli* RND-type MDR efflux pump YhiV (MdtF). J Antimicrob Chemother 59:1216–1222

Bonomo RA, Szabo D (2006) Mechanisms of multidrug resistance in *Acinetobacter* species and *Pseudomonas aeruginosa*. Clin Infect Dis 43:S49–S56

Bryskier A (2005) Microbial efflux of antibiotics and inhibitors of efflux pumps. In: Bryskier A (ed) Antimicrobial agents. ASM, Washington, DC

Dean CR, Visalli MA, Projan SJ, Sum PI, Bradford PA (2003) Efflux-mediated resistance to tigecycline (GAR-936) in *Pseudomonas aeruginosa* PAO1. Antimicrob Agents Chemother 47:972–978

De Rossi E, Aínsa JA, Riccardi G (2006) Role of mycobacterial efflux transporters in drug resistance: an unresolved question. FEMS Microbiol Rev 30:36–52

dos Santos KV, Diniz CG, Veloso Lde C, de Andrade HM, Giusta Mda S, Pires Sda F, Santos AV, Apolonio AC, de Carvalho MA, Farias Lde M (2010) Proteomic analysis of *Escherichia coli* with experimentally induced resistance to piperacillin/tazobactam. Res Microbiol 161:268–275

Dunham SA, McPherson CJ, Miller AA (2010) The relative contribution of efflux and target gene mutations to fluoroquinolone resistance in recent clinical isolates of *Pseudomonas aeruginosa*. Eur J Clin Microbiol Infect Dis 29:279–288

Fachin AL, Ferreira-Nozawa MS, Maccheroni W Jr, Martinez-Rossi NM (2006) Role of the ABC transporter TruMDR2 in terbinafine, 4-nitroquinolone N-oxide and ethidium bromide susceptibility in *Trichophyton rubrum*. J Med Microbiol 55(Pt 8):1093–1099

Gales AC, Jones RN (2000) Antimicrobial activity and spectrum of the new glycylcycline GAR-936 tested against 1,203 recent clinical bacterial isolates. Diagn Microbiol Infect Dis 36:19–36

Gales AC, Jones RN, Turnidge J, Rennie R, Ramphal R (2001) Characterization of *Pseudomonas aeruginosa* isolates: occurrence rates, antimicrobial susceptibility patterns, and molecular typing in the global SENTRY antimicrobial surveillance program, 1997–1999. Clin Infect Dis 32:S146–55

Ge Y, Wikler MA, Sahm DF, Blosser-Middleton RS, Karlowsky JA (2004) In vitro antimicrobial activity of doripenem: a new carbapenem. Antimicrob Agents Chemother 48:1384–1396

Guardabassi L, Dijkshoom L, Collard JM et al (2000) Distribution and in vitro transfer of tetracycline resistance determinants in clinical and aquatic *Acinetobacter* strains. J Med Microbiol 49:929–936

Guglierame P, Pasca ME, De Rossi E, Buroni S, Arrigo P, Manina G, Riccardi G (2006) Efflux pump genes of the resistance-nodulation-division family in *Burkholderia cenocepacia* genome. BMC Microbiol 6:66

Gupta AK, Katoch VM, Chauhan DS, Sharma R, Singh M, Venkatesan K, Sharma VD (2010) Microarray analysis of efflux pump genes in multidrug-resistant *Mycobacterium tuberculosis* during stress induced by common anti-tuberculous drugs. Microb Drug Resist 16:21–28

Hansen LH, Jensen LB, Sørensen HI, Sørensen SJ (2007) Substrate specificity of the OqxAB multidrug resistance pump in *Escherichia coli* and selected enteric bacteria. J Anitmicrob Chemother 60:145–147

He GX, Varela MF. EmrF, a novel SMR family multidrug efflux pump in *Enterobacter cloacae*. Abstracts of the 46th Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC), 27 to 30 September 2006, San Francisco, California. Abstract C1-1488 (p87)

Henwood CJ, Gatward T, Warner M, James D, Stockdale MW, Spence RP, Towner KJ, Livermore DM, Woodford N (2002) Antibiotic resistance among clinical isolates of *Acinetobacter* in the UK, and in vitro evaluation of tigecycline (GAR-936). Antimicrob Agents Chemother 49:479–487

Hiller D, Stahl S, Morschhauser J (2006) Multiple cis-acting sequences mediate upregulation of the MDR1 efflux pump in a fluconazole-resistant *Candida albicans* isolate. Antimicrob Agents Chemother 50:2300–2308

Hoban DJ, Bouchillon SK, Johnson BM, Johnson JL, Dowzicky MJ (2005) In vitro activity of tigecycline against 6792 Gram-negative and Gram-positive clinical isolates from the global Tigecycline Evaluation and Surveillance Trial (TEST Program, 2004). Diagn Microbiol Infect Dis 52:215–227

Hornsey M, Ellington MJ, Doumith M, Hudson S, Livermore DM, Woodford N (2010) Tigecycline resistance in *Serratia marcescens* associated with up-regulation of the Sde-XY-HasF efflux system also active against ciprofloxacin and cefpirome. J Antimicrob Chemother 65:479–482

Jones RN, Huynh HK, Biedenbach DJ, Fritische TR, Sader HS (2004) Doripenem (S-4461), a novel carbapenem: comparative activity against contemporary pathogens including bactericidal action and preliminary in vitro methods evaluations. J Antimicrob Chemother 54:144–154

Jumbe NL, Louie A, Miller MH, Liu W, Deziel MR, Tam VH, Bachhawat R, Drusano GL (2006) Quinolone efflux pumps play a central role in emergence of fluoroquinolone resistance in *Streptococcus pneumoniae*. Antimicrob Agents Chemother 50:310–317

Keeney D, Ruzin A, Bradford PA (2007) RamA, a transcriptional regulator, and AcrAB, a RND-type efflux pump, are associated with decreased susceptibility to tigecycline in *Enterobacter cloacae*. Microb Drug Resist Mech Epidemiol Dis 13:1–6

Kim HM, Xu Y, Lee M, Piao S, Sim SH, Ha NC, Lee K (2010) Functional relationships between the AcrA hairpin tip region and the ToIC aperture tip region for the formation of the bacterial tripartite efflux pump AcrAB-ToIC. J Bacteriol 192:4498–4503

King P, Gentile A, Saechao B, Myers M, Lokovskaya O. Impact of efflux pumps and target mutations on activity of multiple fluoroquinolones (FQs) against *Pseudomonas aeruginosa* (PA). Abstracts of the 46th Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC), 27 to 30 September 2006, San Francisco, California. Abstract C1-44 (p67)

Kiser TH, Obritsch MD, Jung R, MacLaren R, Fish DN (2010) Efflux pump contribution to multidrug resistance in clinical isolates of *Pseudomonas aeruginosa*. Pharmacotherapy 30:632–638

Kolaczkowski M, Kolaczkowska A, Stermitz FR (2009) Modulation of the antifungal activity of new medicinal plant extracts active on *Candida glabrata* by the major transporters and regulators of the pleiotropic drug-resistance network in *Saccharomyces cerevisiae*. Microb Drug Resist 15:11–17

Lister PD, Wolter DJ (2005) Levofloxacin-imipenem combination prevents the emergence of resistance among clinical isolates of *Pseudomonas aeruginosa*. Clin Infect Dis 40:S105–S114

Livermore DM (2002) Multiple mechanisms of antimicrobial resistance in *Pseudomonas aeruginosa*: our worst nightmare? Clin Infect Dis 34:634–640

Lomovskaya O, Bostian KA (2006) Practical applications and feasibility of efflux pump inhibitors in the clinic – a vision for applied use. Biochem Pharmacol 71:910–918

Lynch AS (2006) Efflux systems in bacterial pathogens: an opportunity for therapeutic intervention? An industry view. Biochem Pharmacol 71:949–956

Magnet S, Courvalin P, Lambert T (2001) Resistant-nodulation-cell-division-type efflux pump involved in aminoglycoside resistance in *Acinetobacter baumannii* strain BM4454. Antimicrob Agents Chemother 45:3375–3380

Mahamoud A, Chevalier J, Davin-Regli A, Barbe J, Pages JM (2006) Quinolone derivatives as promising inhibitors of antibiotic efflux pump in multidrug resistant *Enterobacter aerogenes* isolates. Curr Drug Targets 7:843–847

Mahamoud A, Chevalier J, Alibert-Franco S, Kern WV, Pagès J-M (2007) Antibiotic efflux pumps in Gram-negative bacteria: the inhibitor response strategy. J Antimicrob Chemother 59:1223–1229

Maniati M, Ikonomidis A, Mantzana P, Daponte A, Maniatis AN, Pournaras S (2007) A highly carbapenem-resistant *Pseudomonas aeruginosa* isolate with a novel $bla_{\mathrm{VIM\text{-}4}}/bla_{\mathrm{P1b}}$ integron overexpresses two efflux pumps and lacks OprD. J Antimicrob Chemother 60:132–135

Marchand I, Damier-Piolle L, Courvalin P, Lambert T (2004) Expression of RND-type efflux pump AdeABC in *Acinetobacter baumannii* is regulated by the AdeRS two-component system. Antimicrob Agents Chemother 48:3298–3304

Martí S, Fernández-Cuenca F, Pascual A et al (2006) Prevalence of the *tetA* and *tetB* genes as mechanisms of resistance to tetracycline and minocycline in *Acinetobacter baumannii* clinical isolates. Enferm Infecc Microbiol Clin 24:77–80

McAleese F, Petersen P, Ruzin A, Dunman PM, Murphy E, Projan SJ, Bradford PA (2005) A novel MATE family efflux pump contributes to the reduced susceptibility of laboratory-derived *Staphylococcus aureus* mutants to tigecycline. Antimicrob Agents Chemother 49:1865–1871

McDermott PF, Walker RD, White DG (2003) Antimicrobials: modes of action and mechanisms of resistance. Int J Toxicol 22:135–143

McMurry LM, Petrucci RE Jr, Levy SB (1980) Active efflux of tetracycline encoded by four genetically different tetracycline resistance determinants in *Escherichia coli*. Proc Natl Acad Sci USA 77:3974–3977

Ochs MM, McCusker MP, Bains M, Hancock RE (1999) Negative regulation of the *Pseudomonas aeruginosa* outer membrane porin OprD selective for imipenem and basic amino acids. Antimicrob Agents Chemother 43:1085–1090

Ohara M, Kouda S, Onodera M, Fujiue Y, Sasaki M, Kohara T et al (2007) Molecular characterization of imipenem-resistant *Pseudomonas aeruginosa* in Hiroshima, Japan. Microbiol Immunol 51:271–277

Olson MW, Ruzin A, Feyfant E, Rush TS III, O'Connell J, Bradford PA (2006) Functional, biophysical, and structural bases for antibacterial activity of tigecycline. Antimicrob Agents Chemother 50:2156–2166

Pachón-Ibáñez ME, Jiménez-Mejías ME, Pichardo C, Llanos AC, Pachón J (2004) Activity of tigecycline (GAR-936) against *Acinetobacter baumannii* strains, including those resistant to imipenem. Antimicrob Agents Chemother 48:4479–4481

Peleg AY, Adams J, Paterson DL (2007a) Tigecycline efflux as a mechanism for nonsusceptibility in *Acinetobacter baumannii*. Antimicrob Agents Chemother 51:2065–2069

Peleg AY, Potoski BA, Rea R, Adams J, Sethi J, Capitano B, Husain S, Kwak EJ, Bhat SV, Paterson DL (2007b) *Acinetobacter baumannii* bloodstream infection while receiving tigecycline: a cautionary report. J Antimicrob Chemother 59:128–131

Petersen PJ, Jacobus NV, Weiss WJ et al (1999) In vitro and in vivo anti-bacterial activities of a novel glycylcycline, the 9-*t*-butylglycylamido derivative of minocycline (GAR-936). Antimicrob Agents Chemother 43:738–744

Poole K (2002) Mechanisms of bacterial biocide and antibiotic resistance. J Appl Microbiol 92 (Suppl 1):55S–64S

Poole K (2005) Efflux-mediated antimicrobial resistance. J Antimicrob Chemother 56:20–51

Piddock LJ, Garvey MI, Rahman MM, Gibbons S (2010) Natural and synthetic compounds such as trimethoprim behave as inhibitors of efflux in Gram-negative bacteria. J Antimicrob Chemother 65:1215–1223

Pumbwe L, Smith R, Chang A, Wexler HM. The RND transporter, *BmeRABC*-5, is a metronidazole efflux system in *Bacteroides fragilis*. Abstracts of the 46th Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC), 27 to 30 September 2006, San Francisco, California. Abstract C1-1485 (p86)

Quinn T, O'Mahony R, Baird AW, Drudy D, Whyte P, Fanning S (2006) Multi-drug resistance in *Salmonella enterica*: efflux mechanisms and their relationship with the development of chromosomal resistance gene clusters. Curr Drug Targets 7:849–860

Quinn T, Bolla J-M, Pagès J-M, Fanning S (2007) Antibiotic-resistant *Campylobacter*: could efflux pump inhibitors control infection? J Antimicrob Chemother 59:1230–1236

Ramón-García S, Martín C, Aínsa JA, De Rossi E (2006) Characterization of tetracycline resistance mediated by the efflux pump Tap from *Mycobacterium fortuitum*. J Antimicrob Chemother 57:252–259

Rice LB (2006) Challenges in identifying new antimicrobial agents effective for treating infections with *Acinetobacter baumannii* and *Pseudomonas aeruginosa*. Clin Infect Dis 43:S100–S105

Rouquette-Loughlin C, Balthazar JT, Shafer WM (2005) Characterization of the MacA-MacB efflux system in *Neisseria gonorrhoeae*. J Antimicrob Chemother 56:856–860

Ruzin A, Keeney D, Bradford PA (2005a) AcrAB efflux pump plays a role in decreased susceptibility to tigecycline in *Morganella morganii*. Antimicrob Agents Chemother 49:791–793

Ruzin A, Visalli MA, Keeney D, Bradford PA (2005b) Influence of transcriptional activator RamA on expression of multidrug effluz pump AcrAB and tigecycline susceptibility in *Klebsiella pneumoniae*. Antimicrob Agents Chemother 49:1017–1022

Ruzin A, Immermann FW, Bradford PA (2010) RT-PCR and statistical analyses of *adeABC* expression in clinical isolates of *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex. Microb Drug Resist 16:87–89

Schumacher A, Steinke P, Bohnert JA, Akova M, Jonas D, Kern WV (2006) Effect of 1-(1-naphthylmethyl)-piperazine, a novel putative efflux pump inhibitor, on antimicrobial drug susceptibility in clinical isolates of Enterobacteriaceae other than *Escherichia coli*. J Antimicrob Chemother 57:344–348

Schumacher A, Trittler R, Bohnert J, Kümmerer K, Pagès J-M, Kern WV (2007) Intracellular accumulation of linezolid in *Escherichia coli*, *Citrobacter freundii* and *Enterobacter aerogenes*: role of enhanced efflux pump activity and inactivation. J Antimicrob Chemother 59:1261–1264

Srinivasan VB, Virk RK, Kaundal A, Chakraborty R, Datta B, Ramamurthy T, Mukhopadhyay AK, Ghosh A (2006) Mechanism of drug resistance in clonally related clinical isolates of *Vibrio fluvialis* isolated in Kolkata, India. Antimicrob Agents Chemother 50:2428–2432

Srivastava S, Musuka S, Sherman C, Meek C, Leff R, Gumbo T (2010) Efflux-pump-derived multiple drug resistance to ethambutol monotherapy in *Mycobacterium tuberculosis* and the pharmacokinetics and pharmacodynamics of ethambutol. J Infect Dis 201:1225–1231

Stavri M, Piddock LJV, Gibbons S (2007) Bacterial efflux pump inhibitors from natural sources. J Antimicrob Chemother 59:1247–1260

Stein GE, Craig WA (2006) Tigecycline: a critical analysis. Clin Infect Dis 43:518–524

Su C-C, Rutherford DJ, Yu EW (2007) Characterization of the multidrug efflux regulator AcrR from *Escherichia coli*. Biochem Biophys Res Commun 361:85–90

Takatsuka Y, Chen C, Nikaido H (2010) Mechanism of recognition of compounds of diverse structures by the multidrug efflux pump AcrB of *Escherichia coli*. Proc Natl Acad Sci USA 107:6559–6565

Tam VH, Chang K-T, LaRocco MT, An S, McCauley SK, Poole K, Garey KW (2007) Prevalence, mechanisms, and risk factors of carbapenem resistance in blood stream isolates of *Pseudomonas aeruginosa*. Diagn Microbiol Infect Dis 58:309–314

Thamlikitkul V, Trakulsomboon S (2006) In vitro activity of tigecycline against *Burkholderia pseudomallei* and *Burkholderia thailandensis*. Antimicrob Agents Chemother 50:1555–1557

Tuckman M, Petersen PJ, Howe A, Orlowski M, Mullen S, Chan K, Bradford PA, Jones CH (2007) Occurrence of tetracycline resistance genes among *Escherichia coli* isolates from Phase 3 clinical trials for tigecycline. Antimicrob Agents Chemother 51:3205–3211

Vaccaro L, Koronakis V, Sansom MS (2006) Flexibility in a drug transport accessory protein: molecular dynamics simulations of MexA. Biophys J 91:558–564

Vila J, Martí S, Sánchez-Céspedes J (2007) Porins, efflux pumps and multidrug resistance in *Acinetobacter baumannii*. J Antimicrob Chemother 59:1210–1215

Visalli MA, Murphy E, Projan SJ, Bradford PA (2003) AcrAB multidrug efflux pump is associated with reduced levels of susceptibility to tigecycline (GAR-936) in *Proteus mirabilis*. Antimicrob Agents Chemother 47:665–669

Webber MA, Pidock LJV (2003) The importance of efflux pumps in bacterial antibiotic resistance. J Antimicrob Chemother 51:9–11

Welch A, Awah CU, Jing S, van Heen HW, Venter H (2010) Promiscuous partnering and independent activity of MexB, the multidrug transporter protein from *Pseudomonas aeruginosa*. Biochem J 430:355–364

Wright GD (2003) Mechanisms of resistance to antibiotics. Curr Opin Chem Biol 7:563–569

Yamame K, Wachino J, Kimura K, Suzuki S, Shibata N, Arakawa Y. Novel plasmid-mediated fluoroquinolone efflux pump, Qep, identified in *Escherichia coli*. Abstracts of the 46th

Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC), 27 to 30 September 2006, San Francisco, California. Abstract C1-586 (p73)

Yang YL, Lin YH, Tsao MY, Chen CG, Shih HI, Fan JC, Wang JS, Lo HJ (2006) Serum repressing efflux pump CFR1 in *Candida albicans*. BMC Mol Biol 7:22

Yoneyama H, Katsumata R (2006) Antibiotic resistance in bacteria and its future for novel antibiotic development. Biosci Biotechnol Biochem 70:1060–1075

Yoshihara E, Eda S (2007) Diversity in the oligomeric channel structure of the multidrug efflux pumps in *Pseudomonas aeruginosa*. Microbiol Immunol 51:47–52

Zhang Z, Liu ZQ, Zheng PY, Tang FA, Yang PC (2010) Influence of efflux pump inhibitors on the multidrug resistance of *Helicobacter pylori*. World J Gastroenterol 16:1279–1284

Antibiotics in Phase II and III Clinical Trials

Anthony R.M. Coates and Gerry Halls

Contents

1 Overview 168
2 Antibiotics for Gram-Positives 169
2.1 Phase II 169
2.2 Phase III 171
3 Broad-Spectrum Antibiotics 172
3.1 Phase II 172
4 Antibiotics for Gram-Negatives 174
4.1 Phase II 174
4.2 Phase III 176
5 The Future 177
5.1 Combination Therapy 177
5.2 Low Resistance Inducer Antibiotics 177
5.3 Regulation 178
5.4 Pipeline 178
6 Conclusions for the Future 179
References 179

Abstract There are 19 compounds in late-stage clinical trials, of which ten may be suitable for Gram-positive infections. However, there are only five compounds in development for Gram-negative infections, in addition to four broad-spectrum ones. There are two new classes in late-stage clinical development. This chapter discusses in some detail each of the antibiotics in Phase II and Phase III clinical trials. Only those that appear in the literature are covered. The shortage of compounds in

A.R.M. Coates (✉)
Medical Microbiology, Division of Clinical Sciences, Centre for Infection, St George's University of London, Cranmer Terrace, London SW17 ORE, UK
e-mail: acoates@sgul.ac.uk

G. Halls
Medical Marketing Services, Beaconsfield, Buckinghamshire, UK

A.R.M. Coates (ed.), *Antibiotic Resistance*, Handbook of Experimental Pharmacology 211, DOI 10.1007/978-3-642-28951-4_11,
© Springer-Verlag Berlin Heidelberg 2012

development for Gram-negatives and the small number of new classes in the pipeline is of serious concern; this matter needs to be addressed by governments, the regulatory authorities, the pharmaceutical industry and academia urgently.

Keywords Antibiotics • Phase II • Phase III • clinical trials • Gram-positives • Gram-negatives • classes • combinations • resistance • regulation • pipeline

1 Overview

Consideration of the current pipeline of antibiotics in the later stages of clinical development is a guide to the antibiotics which may enter the market. However, not all of those compounds in Phase II and III clinical trials will reach the market. It is estimated (CMR International 2003) that of those antibiotics which enter into Phase II, 25% will enter the market, 50 % of those which complete Phase II trials will do so, and 75% of those which complete Phase III trials will be marketed. In this chapter, only those antibiotics which are in the public domain are described. Examination of the data suggests that there are six anti-Gram-positive compounds in Phase II and four in Phase III. There are only four antibacterials with both anti-Gram-positive and Gram-negative activity in Phase II, but none in Phase III. Of the five antibiotics which are active against Gram-negatives, three are in Phase II and two in Phase III. There are only two new classes in Phase II or III, a leucyl-tRNA synthase inhibitor anti-Gram-negative in Phase II and a PDF (Peptide deformylase) inhibitor with activity against staphylococci and streptococci and respiratory tract infection (RTI) pathogens. The paucity of new classes in development is a long-term problem for antibiotic discovery because the constant erosion of efficacy of antibiotics by the emergence of resistance means many new classes are required (Coates et al. 2011). Whilst analogues will supply the needs of the world for the time being, at least for Gram-positives, eventually new classes will be required because the ingenuity of the medicinal chemists will become exhausted due to restraints surrounding the chemistry of old classes. Hybrid antibacterials have potential, but, in my view, bacteria may come to recognize the individual components and become resistant to them in a similar fashion to their response to the parent molecules. Also of note is that there are more anti-Gram-positive antibacterials (10) in development than anti-Gram-negatives (5). In addition, there are four in Phase III Gram-positive development but only two in the Phase III Gram-negative stage. Assuming the chances of reaching the market are in the same ratio as previously described (CMR 2003), it is estimated that of these antibiotics in Phase II and Phase III clinical trials, 3–4 anti-Gram-positives, one broad-spectrum and two anti-Gram-negatives will reach the market over the next few years. It is unlikely that either of the new classes will reach the market because they are both in Phase II, with only a 25% chance of being marketed. These data emphasize the acute shortage in development of new classes and anti-Gram-negative antibiotics, an observation which has been highlighted in many previous publications (Abandeh et al. 2012; Meyer 2005; Butler and Cooper 2011; Coates et al. 2011; Kumarasamy et al. 2010; Boucher et al. 2009; Peirano and Pitout 2010).

2 Antibiotics for Gram-Positives

2.1 Phase II

Of the six compounds which are in Phase II, TD1792 inhibits cell wall biosynthesis of Gram-positives by simultaneously targeting the main targets of glycopeptides and beta-lactams. TD-1792 is a glycopeptide-cephalosporin hybrid. It has completed Phase IIa clinical trials in skin and soft tissue infections (SSTI) (Stryjewski et al. 2007). It is active in vitro (Leuthner et al. 2010) against methicillin-sensitive and methicillin-resistant *Staphylococcus aureus* including isolates with reduced susceptibility to vancomycin. This compound has the potential to be more potent than its individual components, but it remains to be seen whether or not it will induce resistance as readily as glycopeptides and cephalosporins. Development is on hold waiting a partner. EDP-420 (modithromycin) is another compound in Phase II clinical trials. It is a bridged bicyclolide which is active against Gram-positive and respiratory pathogens (Furuie et al. 2010) with improved antibacterial activity against Gram-positive cocci, including telithromycin-resistant streptococci and intracellular Gram-negative bacteria of the *Legionella* species (Sato et al. 2011). It is being developed for community-acquired respiratory tract infection. The drug is well absorbed after oral administration. It is slowly distributed to the alveoli where its concentration is 244 times higher than in plasma. These data suggest that the compound is concentrated in alveolar macrophages. In a Phase II clinical trial, it showed good efficacy (Kohno et al. 2007). Another compound, solithromycin, is a fluoroketolide which is active against Gram-positive bacteria and respiratory tract infection pathogens. It is being developed for the intravenous and oral routes. Its indication is community-acquired RTIs and biothreat pathogens, and it is in Phase I/II clinical trials. The compound's in vitro activity has been studied in detail (Farrell et al. 2010). It is active against organisms which are resistant to other ketolide compounds and has been shown not to inhibit the α3β4 nicotinic acetylcholine receptors associated with the visual side effects and muscle weakness of telithromycin and the α7 receptors in the vagus nerve which are critical in protecting the liver from inflammation (Abstracts: A2-588 ICAAC 2011; 252 IDSA 2010; A1-580 ICAAC 2009). BC-3781 is a systemic pleuromutilin which is active against Gram-positive and respiratory tract pathogens, especially multidrug-resistant (MDR) bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA), MDR *Streptococcus pneumoniae* and vancomycin-resistant *Enterococcus faecium*. In clinical trials, it is being developed for both oral and IV administration. In Phase I trials, it has been shown to be safe, well tolerated and to have no clinically significant adverse drug interactions. Nabriva recently completed a Phase II clinical trial with this compound; intravenously, it was comparable to vancomycin in SSTI (Abstract: L966 ICAAC 2011). It is being developed with a view to oral/intravenous delivery for community-acquired RTIs and SSTIs (ICAAC 2010 An Age and Gender Study Investigating the Safety, Tolerance and

Pharmacokinetics of BC-3781; Activity of BC-3781, a Novel Pleuromutilin Compound, Tested against Clinical Isolates of MRSA, Including Molecularly Characterized Community-Acquired and Hospital-Associated Strains; Antimicrobial Activity of the Investigational Pleuromutilin BC-3781 against Organisms Responsible for CA-RTI; ECCMID 2010 Safety, Tolerance and Pharmacokinetics of Single and Repeat Doses of BC-3781, a Novel Antimicrobial). Another compound, a new oxazolidinone, radezolid, is being developed by Rib-X against Gram-positives. It has activity against linezolid- and daptomycin-resistant bacteria. It is currently in Phase II as part of systemically (IV and oral) developed SSTI and community-acquired pneumonia (CAP). The company is claiming an improved safety profile. GSK1322322 is a hydrazine tight-binding time-dependent reversible inhibitor of peptide deformylase in development for hospitalized CABP and ABSSSIs. It is active against *S. pneumoniae*, *H. influenzae*, *M. catarrhalis*, *S. aureus, S. pyogenes* and the atypical RTI pathogens. Finally, actinonin was published as a new class-a PDF inhibitor in 2000 (Chen et al. 2000). It is the first hydrazinopyrimidine with in vivo efficacy against MRSA. GSK1322322 was selected from this class for development in 2007. Many companies have explored this class, two compounds reaching Phase I trials (Johnson et al. 2005; Bell et al. 2005; Rolan et al. 2011), but have abandoned development on finding the rapid emergence of *fmt* mutants. However, *fmt*-resistant mutants reported by GlaxoSmithKline have a fitness cost, lack production of virulence factors and cannot establish an infection in immunocompetent animals. Phase I was reported at ECCMID 2011 (Abstract: 1512), and the Phase IIa study in ABSSSI against linezolid completed in December 2010 and was expected to report end 2011. A Phase IIb in CABP is planned.

Class	Product	Spectrum	IV/oral	Indications	Phase	Company (licensor/ originator)
Glycopeptide-cephalosporin hybrid	TD-1792	Gram-positive	IV	SSTI, HAP	Phase IIa	Theravance
Ketolide	EDP-420	Gram-positive and respiratory tract infection pathogens	Oral	Community-acquired RTIs	Phase II	Enanta (Shionogi)
Fluoroketolide	Solithromycin	Gram-positive and respiratory tract infection pathogens	IV/oral	Community-acquired RTIs Biothreat pathogens	IV Phase I/ oral Phase II	Cempra (Optimer)
Pleuromutilin	BC-3781	Gram-positive and respiratory tract infection pathogens	Oral/IV	Community-acquired RTIs and SSTIs	IV Phase II/ oral Phase I	Nabriva
Oxazolidinone	Radezolid	Gram-positive (including linezolid- and	IV/oral	SSTI, CAP	Oral Phase IIa/IV Phase I	Rib-X

(continued)

Class	Product	Spectrum	IV/oral	Indications	Phase	Company (licensor/ originator)
		daptomycin-resistant strains)				
PDF inhibitor	GSK1322322	Gram-positive and respiratory tract infection pathogens	IV/oral	SSTI, CAP	Oral Phase II IV Phase I	GlaxoSmithKline

2.2 Phase III

The four compounds for Gram-positives which are in Phase III clinical trials include dalbavancin, oritavancin, iclaprim and torezolid (formerly torezolid). All of them are being developed for cSSTI; dalbavancin (Busse et al. 2010) is a lipoglycopeptide which has activity against Gram-positives, but not vancomycin-resistant enterococci. Durata Therapeutics (Pfizer) is developing this antibiotic for once weekly intravenous cSSTI. It has a long half-life which means that it can be administered once per week. It has completed a Phase III clinical trial for cSSTI, but after feedback from FDA, a further Phase III trial is under way.

Oritavancin (Targanta 2007; http://www.farm.ucl.ac.be/Full-texts-FARM/Domenech-2009-1.pdf; Scheinfeld 2007; Belley 2010) is another glycopeptide which is active against Gram-positives but has an advantage over televancin in that it is also active against vancomycin-resistant enterococci. The Medicines Company (Lilly) is developing the drug for cSSTI as a single intravenous dose. Iclaprim (Peppard and Schuenke 2008; Morgan et al. 2009) is a diaminopyrimidine dihydrofolate reductase inhibitor (DHFR) and is active against Gram-positives. It is being developed by Acino for cSSTI for the intravenous and oral route. An NDA for this drug was submitted to the FDA in 2008 but was not progressed due to new draft guidance emerging from FDA in 2010. This guidance recommends new clinical endpoints and study populations in a newly defined indication, ABSSSI. It is likely that Acino will need to undertake more clinical trials for both the USA and the EU.

An oxazolidinone called torezolid is under development by Trius and Bayer for cSSTI as an oral and/or intravenous treatment. It is active against Gram-positives and is also active against linezolid- and daptomycin-resistant bacteria (Locke et al. 2010) and against *S. aureus* strains harbouring the *cfr* resistance mechanism with or without ribosomal L3-resistance mutations; activity against strains with the rRNA mutation, especially G2576T, is more variable (Abstracts: C2-945; E-1324 ICAAC 2011). A Phase III trial in SSTI completed enrolment in September 2011 comparing 6-day 200-mg q24h oral treatment with 10 days of linezolid 600 mg q12h and was reported on the company website to be noninferior to linezolid with a lower

incidence of adverse events (http://investor.triusrx.com/releasedetail.cfm?ReleaseID=634111 accessed January 12, 2012).

Class	Product	Spectrum	IV/oral	Indications	Phase	Company (licensor/ originator)
Glycopeptide	Dalbavancin	Gram-positive (excluding VRE)	IV; once weekly	cSSTI	Phase III	Durata Therapeutics (Pfizer)
Glycopeptide	Oritavancin	Gram-positive (including VRE)	IV; single-dose treatment	cSSTI	Phase III	The Medicines Company (Lilly)
DHFR inhibitor	Iclaprim	Gram-positive and respiratory tract infection pathogens	IV/oral	cSSTI	Phase III	Acino (Arpida)
Oxazolidinone	Torezolid	Gram-positive (including linezolid- and daptomycin-resistant strains)	IV/oral	cSSTI	Phase III	Trius (Dong-A)

3 Broad-Spectrum Antibiotics

3.1 Phase II

There are relatively few broad-spectrum antibiotics in late-stage clinical development, only four in Phase II clinical trials and none in Phase III. Of those in development, three are fluoroquinolones and one is a cephalosporin in combination with a β-lactamase inhibitor. The shortage of broad-spectrum antibiotics may be due to a lack of available bacterial metabolic pathways which are so far unexploited (Becker et al. 2006).

In other words, all the tractable metabolic pathways may already be targeted by existing antibiotics. Alternatively, this situation may reflect the increased difficulty in discovering antibiotics which are active against Gram-negative bacteria. (see Sect. 1.4)

Of the broad-spectrum fluoroquinolones in clinical development, delafloxacin is active against fluoroquinolone-resistant MRSA. It is being progressed by Rib-X for cSSTI and community-acquired pneumonia. Pharmacokinetic-pharmacodynamic (PK-PD) studies with delafloxacin suggest that a dose of 300 mg intravenously twice a day is optimal and is also safe and effective in a Phase II study of cSSTI (Rubino et al. 2010). In a Phase IIb trial, delafloxacin 300 mg IV q12h was reported

to be statistically significantly superior to vancomycin ± aztreonam [though not to linezolid ± aztreonam] in ABSSSI (cSSTI) on Investigators' Global Assessment of Cure. The company also stressed that delafloxacin did not demonstrate evidence for the toxicities that have been common in the fluoroquinolone class of antibiotics, such as phototoxicity, dysglycaemia and QT prolongation (http://www.rib-x.com/investors/press-release_2011_12_15.php; accessed January 12, 2012). Finafloxacin is another broad-spectrum fluoroquinolone in Phase II clinical development. It is being developed by Merlion as an oral drug for urinary tract infection and for *Helicobacter pylori*. The antibiotic is more potent at low pH and retains bactericidal activity in artificial urine (Dalhoff et al. 2011). A single dose of 200 mg or 800 mg was administered to six healthy volunteers (Wagenlehner et al. 2011), and for 48 h urinary concentrations and urinary bactericidal titres were measured for a reference strain and nine selected bacterial urinary pathogens. For those administered 200 mg and 800 mg of the drug, the mean maximum urinary concentration was 69.3 mg/l (0–2 h) and 150 mg/l (4–8 h), respectively. The median urinary bactericidal concentrations were in general agreement with minimal inhibitory concentrations of strains. Furiex is developing another broad-spectrum fluoroquinolone, JNJ-Q2 as an oral treatment for cSSTI. Against 511 clinical *Staphylococcus aureus* isolates, JNJ-Q2 was active against MRSA and fluoroquinolone-resistant strains (Farrell et al. 2011). It is 16-fold or more potent than moxifloxacin, levofloxacin and ciprofloxacin. A Phase II clinical double-blind noninferiority study (Covington et al. 2011) was undertaken with 161 patients with acute bacterial skin and skin structure infections (ABSSSI) treated with JNJ-Q2 250 mg twice per day for 7–14 days versus linezolid. Primary intent-to-treat analysis was unable to declare noninferiority. Using prespecified clinical cure rates 2–14 days after the end of treatment revealed similar rates between JNJ-Q2 and linezolid. These data suggest that JNJ-Q2 is a promising candidate for the treatment of ABSSSI.

A combination of ceftaroline and a β-lactamase inhibitor avibactam (formerly NXL104) is being developed by Astra Zeneca/Forest as an intravenous treatment for complicated urinary tract infection, SSTI and community-acquired pneumonia. The combination is active against ceftriaxone-resistant *S. pneumoniae*, MRSA and MDR Enterobacteriaceae, including KPC- and OXA-48 producers but not against metallo-β-lactamases (Mushtaq et al. 2010). Whilst the concept of combining a β-lactam antibiotic with a β-lactamase inhibitor is not new, it is a proven route for improving the efficacy of old β-lactam antibiotics and for bringing new combinations to the market. A potential disadvantage of this approach is that it takes so long to get a combination to market that new β-lactamases have arisen during the development phase against which the compound is inactive. If the time to market these combinations could be shortened, it would, to some extent, help but would not prevent the underlying problem of regular emergence of new β-lactamases.

Class	Product	Spectrum	IV/oral	Indications	Phase	Company (licensor/ originator)
Fluoroquinolone	Delafloxacin	Broad-spectrum including fluoroquinolone-resistant MRSA	IV/oral	cSSTI, CAP	IV Phase IIb	Rib-X (Abbott)
Fluoroquinolone	Finafloxacin	Broad-spectrum; enhanced activity at acid pH	Oral	UTI, H. pylori	Phase II	Merlion
Fluoroquinolone	JNJ-Q2	Enhanced Gram-positive activity including fluoroquinolone-resistant MRSA	Oral	cSSTI	Phase II	Furiex (J&J)
Cephalosporin/ β-lactamase inhibitor	Ceftaroline/ avibactam (NXL104)	MRSA and MDR Enterobacteriaceae, excluding metallo-ß-lactamases	IV	cUTI, SSTI, CAP	Phase II ready	AstraZeneca/ Forest

4 Antibiotics for Gram-Negatives

4.1 *Phase II*

A group of three potential antibiotics which is at the Phase II stage of clinical development is most important, because it provides hope of some which might treat intractable Gram-negative infections. However, this hope must be tempered with the realization that it is likely that not all of them will reach the market due to toxicity and other problems that may arise during development. The group includes one new class, a leucyl-tRNA synthase inhibitor. This is not sufficient to provide the 20 or so new classes which the world needs for the next 50 years (Coates et al. 2011). Also, not all of them are effective against metallo-β-lactamase-producing bacteria, which is a growing problem in the treatment of Gram-negative infections.

The group contains an aminoglycoside, plazomicin ACHN-490, which is active against MDR Enterobacteriaceae and *S. aureus*, including aminoglycoside-resistant and metallo-ß-lactamase producers. It is being developed by Achaogen as an intravenous treatment for complicated urinary tract infections and complicated intra-abdominal infection (cIAI). Livermore et al. (2011a) tested ACHN-490 against 82 carbapenem-resistant Enterobacteriaceae isolates. Although the drug was active at $\leq$2 mg/l against 65 carbapenem-resistant strains, it was inactive against 16 of 17 NDM-1-carrying isolates. This resistance profile was associated with ArmA and RmtC 16S rRNA methylases. Interestingly, they observed that a veterinary antibiotic apramycin was active against most strains which harbour armA or rmtC and suggested that this could provide a starting point for future aminoglycoside development. Safety, tolerability and pharmacokinetics of

intravenous ACHN-490 have been investigated in healthy subjects in two randomized, double-blind, placebo-controlled clinical trials (Cass et al. 2011). Mild to moderate adverse events which rapidly resolved were observed with no renal or ear toxicity. The drug showed linear and dose-proportional PK.

Another compound in Phase II clinical trials, GSK2251052, which is a leucyl-tRNA synthase inhibitor (new class), is being developed by GlaxoSmithKline as an intravenous treatment for complicated urinary tract infections, complicated intra-abdominal infections, hospital-acquired and ventilator-associated pneumonia. It is active against MDR Enterobacteriaceae and *Pseudomonas aeruginosa* (Sutcliffe 2011). In Phase I, it showed dose linearity up to 2 g daily for 14 d, but there was a reversible effect on reticulocytes which appeared after 4 days of 8-d dosing (Abstract: P1521) The mechanism of the reticulocyte toxicity is unknown; it does not inhibit mitochondrial protein synthesis (Abstract: O90 ECCMID 2011).

A combination of the carbapenem, imipenem/cilastatin and a β-lactamase inhibitor, MK-7655, a diazabicyclic-octane, the same class as avibactam (Mangion et al. 2011), is being developed by Merck. It is active against MDR *P. aeruginosa* and Enterobacteriaceae, excluding metallo-ß-lactamases. It is being used as an intravenous treatment for complicated urinary tract infections, complicated intra-abdominal infections, hospital-acquired pneumonia and ventilator-associated pneumonia. It has been suggested (http://antibiotics-theperfectstorm.blogspot.com/2010/09/superbug-ndm-1.html) that aztreonam should be combined with MK-7655 or avibactam on the grounds that such a combination would be active against NDM-1- and other metallo-ß-lactamase-carrying bacteria.

Class	Product	Spectrum	IV/ oral	Indications	Phase	Company (licensor/ originator)
Aminoglycoside	Plazomicin (ACHN-490)	MDR Enterobacteriaceae and *S. aureus*, including aminoglycoside-resistant and metallo-ß-lactamase producers	IV	cUTI, cIAI	Phase II	Achaogen
Leucyl-tRNA synthase inhibitor (new class)	GSK2251052	MDR Enterobacteriaceae and *P. aeruginosa*	IV	cUTI, cIAI, HAP/ VAP	Phase II	Anacor/GSK
Carbapenem/ β-lactamase-inhibitor	Imipenem/ cilastatin/ MK-7655	MDR *P. aeruginosa* and Enterobacteriaceae, excluding metallo-ß-lactamases; acinetobacter	IV	cUTI, cIAI, HAP/ VAP	Phase II ready	Merck & Co

4.2 Phase III

Although the need for new antibiotics against MDR Gram-negatives is perhaps more than in any other area of antibacterial discovery, there is documentation of only two compounds in Phase III clinical trials. Ceftolozane (CXA-101) is an oxyimino-cephalosporin which is being developed by Cubist. It has more activity than ceftazidime against *Pseudomonas spp*. (Livermore et al. 2010). It is also active against Enterobacteriaceae, but not effective against extended-spectrum, AmpC and carbapenem-hydrolyzing beta-lactamases. Combination with tazobactam increases the proportion of extended-spectrum beta-lactamase-carrying bacteria against which it is active. This combination has improved performance in vitro against 90% of those extended-spectrum beta-lactamase producers, AmpC hyperproducers and K1 hyperproducers. However, most isolates with KPC or other carbapenemases are likely to be resistant, even to the combination (Livermore et al. 2010). Population PK/PD modelling showed target attainment [40% T > MIC] of 98.2% based on MIC of 8 μg/ml for pathogens in a surveillance programme (Abstract: P1520 ECCMID 2011). Its safety, tolerability and pharmacokinetics were studied in a Phase I clinical trial (Ge et al. 2010) in which it was shown to be safe, well tolerated and have a predictable pharmacokinetic profile. Ceftolozane completed a Phase II trial in complicated urinary tract infections (Abstract: L1-361a ICAAC 2010), and the combination with tazobactam (CXA-201) started Phase III trials in cUTI in July 2011.

One compound is Phase III ready. This is a combination of the cephalosporin, ceftazidime and the beta-lactamase inhibitor avibactam. It is active against MDR *Pseudomonas aeruginosa* and Enterobacteriaceae (Stachyra et al. 2009). Avibactam inhibits the activity of class A extended-spectrum beta-lactamases, class C enzymes and class A carbapenemases and some class D β-lactamases such as OXA-48. However, it is not active against bacteria which harbour metallo-ß-lactamases (Livermore et al. 2011b). Livermore and colleagues (2011) have found that a combination of aztreonam and NXL104 is active against all carbapenemase producers, including Enterobacteriaceae which carry metallo-beta-lactamases. It seems that new MDR bacteria, such as NDM-1, are appearing faster than the pharmaceutical industry can bring antibiotics which are effective against them, to the market. AstraZeneca is developing the ceftazidime/avibactam combination as an intravenous preparation for community-acquired urinary tract infection, SSTI and ventilator-associated pneumonia.

Class	Product	Spectrum	IV/ oral	Indications	Phase	Company (licensor/ originator)
Cephalosporin/ β-lactamase inhibitor	Ceftolozane/ tazobactam (CXA-201)	MDR *P. aeruginosa* and susceptible Enterobacteriaceae	IV	cUTI/cIAI/ HAP/VAP	Phase III Phase II	Cubist
Cephalosporin/ β-lactamase inhibitor	Ceftazidime/ avibactam (NXL104)	MDR *P. aeruginosa* and Enterobacteriaceae, excluding metallo-ß-lactamases	IV	cUTI, SSTI, VAP	Phase III ready	AstraZeneca/ Forest

5 The Future

The main problem with antibiotics is that resistance has existed for thousands of years (D'Costa et al. 2011), and so, after entry of a new antibiotic into the market, bacteria quickly generate resistant mutants which eventually renders them ineffective. How might we address this problem?

5.1 *Combination Therapy*

This is one possible way forward. For example, tuberculosis is treated with combinations because resistance arises quickly when one drug is used on its own (Mitchison 1954; MRC 1948, 1950; Fox et al. 1954, 1999; Crofton 1958). The chapter by Mitchison in this book describes this in more detail. Whilst combination therapy prevents the emergence of resistance during tuberculosis treatment, poor compliance and inadequate treatment regimens have led to the emergence of resistant mutants (Mitchison 1998). It should also be noted (Haas 2000) that *Mycobacterium tuberculosis* does not usually exist in a community of other species of bacteria and so is not exposed to the transfer of plasmid-mediated resistance which is common amongst Gram-negative bacteria in, for example, the colon (Shoemaker et al. 2001; Potron et al. 2011). This means that it is less likely to become resistant as a result of acquisition of resistance genes in exogenous DNA. There are few data (Traugott et al. 2011; D'Agata et al. 2008) which support the use of combinations in the treatment of Gram-negative infections with the specific aim of preventing the emergence of resistance. In my view, what is needed is a new antibiotic which can be added to an old antibiotic, and the combination reduces the mutation prevention concentration (Cantón and Morosini 2011) of the old antibiotic. This would lead to the old antibiotic concentration in the serum of a patient being above the mutation prevention window for longer, and so could reduce the emergence of resistance.

The combination of a beta-lactam antibiotic with a beta-lactamase inhibitor is a well-trodden path which holds some hope, for example, for the treatment of existing extended-spectrum beta-lactamase-, carbapenemase- and metallo-ß-lactamase-carrying Gram-negative bacteria. Whether such a combination could prevent the emergence of resistance remains to be seen.

5.2 *Low Resistance Inducer Antibiotics*

Some antibiotics induce resistance more readily than others. For example, antibiotics such as rifampicin target single genes. This is associated with a high level of resistance emergence (Cirz et al. 2005). Other antibiotics induce resistance

more slowly, and membrane-targeting drugs are thought to offer hope in this regard (Hurdle et al. 2011). So, the development of membrane-active drugs may be a potential way forward.

5.3 Regulation

The expense and time-consuming nature of Phase III clinical trials is considerable. This is a significant impediment to the development of new antibiotics. Large pharmaceutical companies are reducing their research capacity and relying more upon small research companies and universities to produce new compounds. However, once a product is ready to enter Phase III clinical trials, the cost is so high that, traditionally, large pharmaceutical companies have to fund these trials. However, the amount of money which is currently available is unlikely to match the demand for new antibiotics and new combinations which are needed for the future (Coates et al. 2011). For example, 20 new classes were marketed between 1940 and 1962. Since then, only two new classes have been produced (Butler and Buss 2006; Hair and Keam 2007). The efficacy of the current set of antibiotics, including analogues, is being eroded by resistance, and so it is quite likely that the world needs another 20 classes of antibiotics to see it through the next 50 years (Coates et al. 2011). The way forward, in my opinion, is for the regulatory authorities to allow companies to proceed to market with a drug that has satisfied the safety requirements and also kills or inhibits bacteria in patients. In other words, regulators should allow new antibiotics to enter the market on the basis of microbiological endpoints which are fully justified as being associated with the appropriate clinical endpoint for a specific indication. In epidemic situations, influenza vaccines can be licensed rapidly, without extensive efficacy testing (Scheifele et al. 2011). In epidemic MDR situations, perhaps a similar rapid system, microbiological endpoints could be used, at least for some clinical indications. For example, decolonization of the nose, urinary tract infections, genital infections such as gonorrhoea and tuberculosis relapse may be suitable for microbiological endpoints. Other clinical indications may also be suitable for microbiological endpoints. The advantage of this approach is that microbiological endpoints are statistically more precise than clinical endpoints such as death, so fewer patients are required, which reduces the costs of and time required for Phase III clinical trials. The net effect of the introduction of microbiological endpoints would be faster development of antibiotics and potentially more drugs reaching the market if the pipeline could be increased in size (see below).

5.4 Pipeline

The current pipeline is not sufficient to provide the world with enough antibiotics which are effective against a broad range of bacteria, particularly Gram-negatives (Freire-Moran et al. 2011). It is possible that the Fleming method of drug discovery

itself which targets multiplying bacteria is exhausted (Becker et al. 2006) because of its Achilles heel, namely, resistance and because all available metabolic pathways are already targeted by existing antibiotics. On the other hand, it is possible that there are insufficient incentives for companies to develop new antibiotics. One way forward would be for the world's governments and large pharmaceutical companies to rebuild the infrastructure which led to the golden age of antibiotic discovery in the middle of the last century. Meanwhile, new methods of antibiotic discovery are needed, for example, genome based (Payne et al. 2007), targeting non-multiplying bacteria (Coates and Hu 2007), exploring non-culturable species (Piel 2011) or bacteriophage development (Burrowes et al. 2011). As yet, none of these new methods has produced antibiotics which have been marketed in developed countries.

6 Conclusions for the Future

Resistance to antibiotics threatens to reduce the efficacy of antibiotics to such an extent that the practice of medicine will have to change radically (Chan 2011). Possible ways forward are the development of combinations of antibiotics which lower the mutation prevention concentration and the development of low resistance inducer antibiotics. The traditional way of dealing with the resistance problem, namely, develop more and more antibiotics with the Fleming method, is not fit for purpose. The pipeline of antibiotics is insufficient for the world's needs over the next 50 years. A new method of antibiotic discovery is required. Finally, a new way of bringing antibiotics to market is needed. Phase III clinical trials are too expensive and too slow. With the current regulatory hurdles, by the time a new antibiotic or a new combination against, for example, NDM-1, has been brought to market, it is likely that further resistance mechanisms will have emerged which makes the new antibiotic redundant. A sensible way to reduce this cost and time of development could be to base market authorization on microbiological rather than clinical endpoints. If this could be linked to a special regulatory programme, such as that currently used for influenza, many more new antibiotics which address current resistant bacteria could reach the market in a time which is sufficiently short to be useful.

References

Abandeh FI, Drew ME, Sopirala MM (2012) Carbapenem-hydrolyzing gram-negative bacteria: current options for treatment and review of drugs in development. Recent Pat Antiinfect Drug Discov 7(1):19–27

Becker D, Selbach M, Rollenhagen C, Ballmaier M, Meyer TF, Mann M, Bumann D (2006) Robust salmonella metabolism limits possibilities for new antimicrobials. Nature 16:303–307

Bell JM, Turnidge JD, Inoue M, Kohno S, Hirakata Y, Ono Y, Jones RN (2005) Activity of a peptide deformylase inhibitor LBM415 (NVP PDF-713) tested against recent clinical isolates from Japan. J Antimicrob Chemother 55(2):276–278

Belley A (2010) Oritavancin disrupts membrane integrity of *Staphylococcus aureus* and vancomycin-resistant enterococci to effect rapid bacterial killing. Antimicrob Agents Chemother 54:5369–5371

Boucher HW, Talbot GH et al (2009) Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America. Clin Infect Dis 48(1):1–12

Burrowes B, Harper DR, Anderson J, McConville M, Enright MC (2011) Bacteriophage therapy: potential uses in the control of antibiotic-resistant pathogens. Expert Rev Anti Infect Ther 9(9):775–85

Busse KH, Oltrogge KM, Oxencis CJ, Peppard WJ (2010) Dalbavancin: a review of its use in the treatment of gram-positive infections. Clin Med Insights Ther 2:7–13

Butler MS, Buss AD (2006) Natural products–the future scaffolds for novel antibiotics? Biochem Pharmacol 71(7):919–29

Butler MS, Cooper MA (2011) Antibiotics in the clinical pipeline in 2011. J Antibiot (Tokyo) 64(6):413–25

Cantón R, Morosini MI (2011) Emergence and spread of antibiotic resistance following exposure to antibiotics. FEMS Microbiol Rev 35(5):977–91

Cass RT, Brooks CD, Havrilla NA, Tack KJ, Borin MT, Young D, Bruss JB (2011) Pharmacokinetics and safety of single and multiple doses of ACHN-490 injection administered intravenously in healthy subjects. Antimicrob Agents Chemother 55(12):5874–80

Chen DZ, Patel DV, Hackbarth CJ, Wang W, Dreyer G, Young DC, Margolis PS, Wu C, Ni ZJ, Trias J, White RJ, Yuan Z (2000) Actinonin, a naturally occurring antibacterial agent, is a potent deformylase inhibitor. Biochemistry 39(6):1256–62

Chan M (2011) Combat drug resistance: no action today means no cure tomorrow. Statement by WHO Director-General, Dr Margaret Chan. World Health Day, 6 April 2011. http://www.who.int/mediacentre/news/statements/2011/whd_20110407/en/index.html. Accessed 2nd February 2012

Cirz RT, Chin JK, Andes DR, de Crécy-Lagard V, Craig WA, Romesberg FE (2005) Inhibition of mutation and combating the evolution of antibiotic resistance. PLoS Biol 3(6):176

CMR International Institute for Regulatory Science in April 2003 in Nutfield, Surrey, UK

Coates AR, Hu Y (2007) Novel approaches to developing new antibiotics for bacterial infections. Br J Pharmacol 152(8):1147–54

Coates ARM, Halls G, Hu Y (2011) Novel classes of antibiotics or more of the same? Brit J Pharmacol 163:184–194

Covington P, Davenport JM, Andrae D, O'Riordan W, Liverman L, McIntyre G, Almenoff J (2011) Randomized, double-blind, phase II, multicenter study evaluating the safety/tolerability and efficacy of JNJ-Q2, a novel fluoroquinolone, compared with linezolid for treatment of acute bacterial skin and skin structure infection. Antimicrob Agents Chemother 55(12):5790–7

Crofton J (1958) Sputum conversion and the metabolism of isoniazid. Am Rev Tuberc 77:869–871

D'Agata EM, Dupont-Rouzeyrol M, Magal P, Olivier D, Ruan S (2008) The impact of different antibiotic regimens on the emergence of antimicrobial-resistant bacteria. PLoS One 3(12):4036

D'Costa VM, King CE, Kalan L, Morar M, Sung WW, Schwarz C, Froese D, Zazula G, Calmels F, Debruyne R, Golding GB, Poinar HN, Wright GD (2011) Antibiotic resistance is ancient. Nature 477(7365):457–61

Dalhoff A, Stubbings W, Schubert S (2011) Comparative in vitro activities of the novel antibacterial finafloxacin against selected Gram-positive and Gram-negative bacteria tested in Mueller-Hinton broth and synthetic urine. Antimicrob Agents Chemother 55(4):1814–8

Farrell DJ, Sader HS, Castanheira M, Biedenbach DJ, Rhomberg PR, Jones RN (2010) Antimicrobial characterization of CEM-101 activity against respiratory tract pathogens including multidrug-resistant pneumococcal serogroup 19A isolates. Int J Antimicrob Agent 35:537–543

Farrell DJ, Liverman LC, Biedenbach DJ, Jones RN (2011) JNJ-Q2, a new fluoroquinolone with potent in vitro activity against Staphylococcus aureus, including methicillin- and fluoroquinolone-resistant strains. Antimicrob Agents Chemother 55(7):3631–4

Fox W, Sutherland I, Daniels M (1954) A five-year assessment of patients in a controlled trial of streptomycin in pulmonary tuberculosis. Q J Med 23:347–366

Fox W, Ellard GA, Mitchison DA (1999) Studies on the treatment of tuberculosis undertaken by the British Medical Research Council Tuberculosis Units, 1946–1986, with relevant subsequent publications. Int J Tuberc Lung Dis 3:S231–S279

Freire-Moran L, Aronsson B, Manz C, Gyssens IC, So AD, Monnet DL, Cars O (2011) ECDC-EMA Working Group. Critical shortage of new antibiotics in development against multidrug-resistant bacteria-time to react is now. Drug Resist Updat 14(2):118–24

Furuie H, Saisho Y, Yoshikawa T, Shimada J (2010) Intrapulmonary pharmacokinetics of S-013420, a novel bicyclolide antibacterial, in healthy Japanese subjects. Antimicrob Agents Chemother 54(2):866–70, Epub 2009 Nov 23

Ge Y, Whitehouse MJ, Friedland I, Talbot GH (2010) Pharmacokinetics and safety of CXA-101, a new antipseudomonal cephalosporin, in healthy adult male and female subjects receiving single- and multiple-dose intravenous infusions. Antimicrob Agents Chemother 54(8):3427–31

Haas DW (2000) Mycobacterial diseases, Chapter 240. In: Mandell GL, Bennett JE, Dolin R (eds) Principles and practice of infectious diseases, vol 2, 5th edn. Churchill Livingstone, Philadelphia, London, pp 2576–2607

Hair PI, Keam SJ (2007) Daptomycin: a review of its use in the management of complicated skin and soft-tissue infections and Staphylococcus aureus bacteraemia. Drugs 67(10):1483–512

http://www.farm.ucl.ac.be/Full-texts-FARM/Domenech-2009-1.pdf. “Interactions of oritavancin, a new lipoglycopeptide derived from vancomycin, with phospholipid bilayers: Effect on membrane permeability and nanoscale lipid membrane organization” 2009

Johnson KW, Lofland D, Moser HE (2005) PDF inhibitors: an emerging class of antibacterial drugs. Curr Drug Target Infect Disord 5:39–52, 1568-0053/05 © 2005 Bentham Science Publishers Ltd

Hurdle JG, O’Neill AJ, Chopra I, Lee RE (2011) Targeting bacterial membrane function: an underexploited mechanism for treating persistent infections. Nat Rev Microbiol 9(1):62–75

Kohno S, Yamaguchi K, Tanigawara Y, Watanabe A, Aoki A, Niki Y, Fujita J (2007) Abstr. 47th Intersci. Conf. Antimicrob. Agents Chemother., abstr. L-485

Kumarasamy KK, Toleman MA, Walsh TR, Bagaria J, Butt F, Balakrishnan R, Chaudhary U, Doumith M, Giske CG, Irfan S, Krishnan P, Kumar AV, Maharjan S, Mushtag S, Noorie T, Paterson DL, Pearson A, Perry C, Pike R, Rao B, Ray U, Sarma JB, Sharma M, Sheridan E, Thirunarayan MA, Turton J, Upadhyay S, Warner M, Welfare W, Livermore DM, Woodford N (2010) Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological and epidemiological study. Lancet Infect Dis 10:597–602

Leuthner KD, Vidaillac C, Cheung CM, Rybak MJ (2010) In vitro activity of the new multivalent glycopeptide-cephalosporin antibiotic TD-1792 against vancomycin-nonsusceptible Staphylococcus isolates. Antimicrob Agents Chemother 54(9):3799–803, Epub 2010 Jun 28

Livermore DM, Mushtaq S, Ge Y (2010) Chequerboard titration of cephalosporin CXA-101 (FR264205) and tazobactam versus beta-lactamase-producing Enterobacteriaceae. J Antimicrob Chemother 65(9):1972–4

Livermore DM, Warner MSM, Zhang J-C, Maharjan S, Doumith M, Woodford N (2011a) Activity of aminoglycosides, including ACHN-490, against carbapenem-resistant Enterobacteriaceae isolates. J Antimicrob Chemother 66(1):48–53

Livermore DM, Mushtaq S, Warner M, Zhang J, Maharjan S, Doumith M, Woodford N (2011b) Activities of NXL104 combinations with ceftazidime and aztreonam against carbapenemase-producing Enterobacteriaceae. Antimicrob Agents Chemother 55(1):390–4

Locke JB, Finn J, Hilgers M, Morales G, Rahawi S, Picazo JJ, Im W, Shaw KJ, Stein JL (2010) Structure-activity relationships of diverse oxazolidinones for linezolid-resistant Staphylococcus aureus strains possessing the cfr methyltransferase gene or ribosomal mutations. Agents Chemother 54(12):5337–43

Mangion IK, Ruck RT, Rivera N, Huffman MA, Shevlin M (2011) A concise synthesis of a β-lactamase inhibitor. Org Lett 13(20):5480–3, http://antibiotics-theperfectstorm.blogspot.com/2010/09/superbug-ndm-1.html

Medical Research Council (1948) Streptomycin treatment of pulmonary tuberculosis. Br Med J 2:769–782

Medical Research Council (1950) Treatment of pulmonary tuberculosis with streptomycin and para-amino-salicylic acid. Br Med J 2:1073–1085

Meyer AL (2005) Prospects and challenges of developing new agents for tough Gram-negatives. Curr Opin Pharmacol 5(5):490–4

Mitchison DA (1954) Problems of drug resistance. Br Med Bull 69:640–641

Mitchison DA (1998) How drug resistance emerges as a result of poor compliance during short course chemotherapy for tuberculosis. Int J Tuberc Lung Dis 2:10–15

Morgan A, Cofer C, Stevens DL (2009) Iclaprim: a novel dihydrofolate reductase inhibitor for skin and soft tissue infections. Future Microbiol 4(2):131–44

Mushtaq S, Warner M, Williams G, Critchley I, Livermore DM (2010) Activity of chequerboard combinations of ceftaroline and NXL104 versus beta-lactamase-producing Enterobacteriaceae. J Antimicrob Chemother 65(7):1428–32

No authors. (2009) Deal watch: Novartis acquires marketing rights for novel broad-spectrum antibiotic. Nat Rev Drug Discov 2009 (12):922

Payne DJ, Gwynn MN, Holmes DJ, Pompliano DL (2007) Drugs for bad bugs: confronting the challenges of antibacterial discovery. Nat Rev Drug Discov 6(1):29–40

Peirano G, Pitout JD (2010) Molecular epidemiology of *Escherichia coli* producing CTX-M beta-lactamases: the worldwide emergence of clone ST131 O25:H4. Int J Antimicrob Agents 35(4):316–21

Peppard WJ, Schuenke CD (2008) Iclaprim, a diaminopyrimidine dihydrofolate reductase inhibitor for the potential treatment of antibiotic-resistant staphylococcal infections. Curr Opin Investig Drugs 9(2):210–25

Piel J (2011) Approaches to capturing and designing biologically active small molecules produced by uncultured microbes. Annu Rev Microbiol 65:431–53

Potron A, Poirel L, Nordmann P (2011) Plasmid-mediated transfer of the bla(NDM-1) gene in Gram-negative rods. FEMS Microbiol Lett 324(2):111–6

Rolan P, Sun H, MacLeod C, Bracken K, Evans TG (2011) Pharmacokinetics and unexpected safety issues of LBM415, a novel oral peptide deformylase inhibitor. Clin Pharmacol Ther 90 (2):256–262

Rubino C, Bhavnani S, Burak E, Ambrose P (2010) Pharmacokinetic-pharmacodynamic (PK-PD) target attainment (TA) analyses supporting delafloxacin (DFX) Phase 3 dose regimen decisions. ICAAC. http://www.drugs.com/clinical_trials/rib-x-pharmaceuticals-presents-data-supporting-delafloxacin-potential-best-class-fluoroquinolone-10109.html

Sato T, Tateda K, Kimura S, Iwata M, Ishii Y, Yamaguchi K (2011) In vitro antibacterial activity of modithromycin, a novel 6,11-bridged bicyclolide, against respiratory pathogens, including macrolide-resistant Gram-positive cocci. Antimicrob Agents Chemother 55(4):1588–93, Epub 2011 Jan 10

Scheifele DW, Marty K, LaJeunesse C, Fan SY, Bjornson G, Langley JM, Halperin SA (2011) Strategies for successful rapid trials of influenza vaccine. Clin Trials 8(6):699–704

Scheinfeld N (2007) A comparison of available and investigational antibiotics for complicated skin infections and treatment-resistant *Staphylococcus aureus* and *enterococcus*. J Drugs Dermatol 6(4):97–103

Shoemaker NB, Vlamakis H, Hayes K, Salyers AA (2001) Evidence for extensive resistance gene transfer among Bacteroides spp. and among Bacteroides and other genera in the human colon. Appl Environ Microbiol 67(2):561–8

Stachyra T, Levasseur P, Péchereau MC, Girard AM, Claudon M, Miossec C, Black MT (2009) In vitro activity of the {beta}-lactamase inhibitor NXL104 against KPC-2 carbapenemase and Enterobacteriaceae expressing KPC carbapenemases. J Antimicrob Chemother 64(2):326–9

Stryjewski ME, Barriere SL, Kitt MM, Corey GR (2007) TD-1792 vs vancomycin (VAN) for treatment of complicated gram-positive skin and skin structure infections (cSSSIs), poster L-1147a. Abstract 47th Interscience Conference on Antimicrobial Agents and Chemotherapy. American Society for Microbiology, Washington, DC

Sutcliffe JA (2011) Antibiotics in development targeting protein synthesis. Ann N Y Acad Sci 1241(1):122–52

Targanta Revives Oritavancin: Next Weapon Against cSSSI? BioWorld Today, November 26, 2007

Traugott KA, Echevarria K, Maxwell P, Green K, Lewis JS 2nd (2011) Monotherapy or combination therapy? The Pseudomonas aeruginosa conundrum. Pharmacotherapy 31(6):598–608

Wagenlehner FM, Wagenlehner CM, Blenk B, Blenk H, Schubert S, Dalhoff A, Naber KG (2011) Urinary pharmacokinetics and bactericidal activity of finafloxacin (200 and 800 mg) in healthy volunteers receiving a single oral dose. Chemotherapy 57(2):97–107

Index

A

ABSSSI. *See* Acute bacterial skin and skin structure infections (ABSSSI)
Academics, 11
Actinonin, 170
Acute bacterial skin and skin structure infections (ABSSSI), 4, 171
Advisory Committee on Antimicrobial Resistance and Healthcare Associated Infection (ARHAI), 4–5
Aminoglycosides, 3, 174
Analogues, 168
Animal, mice, 107
Animal models
  Monte Carlo, 59
  pharmacokinetic/pharmacodynamic (PK/PD), 59
Antibiotic stress, 22
Antibiotic resistance, 1, 32–34
Antibiotic resistome, 15, 17
Antibiotics
  amikacin, 157
  aminoglycosides, 158, 159
  antibacterial, 160
  azithromycin, 156
  β–lactams, 158, 159
  carbapenem, 158
  ceftazidime, 157
  chloramphenicol, 159
  choramphenicol, 158
  ciprofloxacin, 157
  erythromycin, 159
  ethambutol, 155
  ethidium bromide, 155, 159
  fluoroquinolone, 155, 156, 158, 159
  gentamicin, 157
  imipenem, 156–160
  isoniazid, 155
  levofloxacin, 158
  macrolide, 155, 156, 158
  meropenem, 157
  methicillin, 159
  metronidazole, 156
  minocycline, 155, 159, 160
  piperacillin, 157
  piperacillin-tazobactam, 155
  quinolone, 155, 156
  streptomycin, 155
  telithromycin, 155, 156
  tetracycline, 154, 155, 157–160
  tetraphenylphosphonium, 155
  tigecycline, 153, 160–161
  tobramycin, 157
  trimethoprim, 159
  trime-thoprim, 154
Antifungal resistance
  amphotericin B, 54
  azole, 55
  echinocandins, 55
Antimicrobia, 157
Antimicrobial resistance
  carbapenemases (CREs), 46
  extended-spectrum beta lactamase (ESBL), 46
  methicillin-resistant *Staphylococcus aureus* (MRSA), 45
  penicillin-resistant *Streptococcus pneumoniae* (PRSP), 45
  vancomycin-resistant *Enterococci* (VRE), 45
Arms race, 149
  evolutionary, 139, 146–147

A.R.M. Coates (ed.), *Antibiotic Resistance*, Handbook of Experimental Pharmacology 211, DOI 10.1007/978-3-642-28951-4,

© Springer-Verlag Berlin Heidelberg 2012

ATP synthase, 93
Atypical RTI pathogens, 170
Avibactam, 173
Aztreonam, 173

B
Bacteraemia, 7
Bacteria
  bacilli, 107
  Campylobacter jejuni, 105
  Campylobacter spp., 106
  Chlamydia spp., 106
  Clostridium spp., 106
  endospores, 101
  Enterococcus spp., 105
  Escherichia coli, 101
  gram-negative, 105
  gram-positive, 105
  Helicobacter pylori, 105
  Mycobacterium tuberculosis, 105
  myxospores, 101
  Pseudomonas aeruginosa, 105
  Salmonella, 105
  Salmonella spp., 106
  S. enteritidis, 105
  S. flexneri, 105
  Shigella sonnei, 105
  Shigella spp., 106
  Staphylococcus aureus, 101
  tubercle bacilli, 108
  VBNC, 112
  V. cholerase, 105
  Vibrio spp., 105
  Vibrio vulnificus, 105
  Yersinia spp, 106
Bacterial challenge, 11
Bacterial resistance, 32–34
Bas 30072, 10
BC-3781, 169
Beta-lactams, 3
  methicillin/oxacillin, 48
  penicillin, 48
Bicyclolide, 169
Bioaccumulation, 144
Biofilm, 122, 124–126
Biothreat pathogens, 169
β-lactamase inhibitor, 172
β-lactamases, 20, 24
Blood cultures, 10
British Society for Antimicrobial Chemotherapy (BSAC), 8, 39

C
Candida albicans, 124
CAP. *See* Community-acquired pneumonia (CAP)
Carbapenem, 174
Carbapenemases
  etrapenem, 51
  imipenem, 52
  meropenem, 52
  NDM-1, 51
Ceftaroline, 173
Ceftazidime, 10, 176
Ceftolozane (CXA-101), 176
Centers for Disease Control and Prevention (CDC), 2
Cephalosporin, 172
cfr resistance, 171
Chromosomal genes, 91
Ciprofloxacin, 22, 130, 173
Classes, 168
Clinical trials, 2
Clofazimine, 90
Clostridium difficile
  fidaxomicin, 50
  metronidazole, 50
  vancomycin, 50
Combination antibacterials
  CBR-2092, 58
  CSA-13, 58
  EDP-420, 58
Community-acquired pneumonia (CAP), 170
Community-acquired respiratory tract infection., 169
Companies, 11
Co-selection, 141, 142, 144
Co-selects, 147–149
Critical path, 6

D
Dalbavancin, 171
Danish Integrated Antimicrobial Resistance Monitoring and Research Programme (DANMAP 2009), 35, 36
Dapsone, 90
Daptomycin, 2
Delafloxacin, 172
Development
  carbapenem, 57
  cefdinir, 57
  ceftaroline, 57
  colistin, 56

dalbavancin, 57
garenoxacin, 58
iclaprim, 58
penem, 57
plazomicin, 58
prulifioxacin, 58
retapamulin, 57
Diagnostics, 11
Diaminopyrimidine, 171
Diazabicyclic-octane, 175
Dihydrofolate reductase, 171
Diversity
genetic, 136
microbial, 136
Domiciliary treatment, 89
Dormancy mechanisms
β-lactam, 127
candidate persister genes, 126–127
persister induction, 129
phosphorylating elongation factor, 128
regulators, 126
TA modules, 127, 128
TisB role, 130
Drug resistance and drug tolerance, 122–123, 125–126

E
Early bactericidal activity (EBA), 92
Economic barriers, 9
EDP-420, 169
Efficacy Working Party (EWP), 6
Efflux, 22
multiresistant, 157
resistance, 153–157
Efflux pumps, 3
Enterobacteriaceae, 174
Enterococcus faecium, 35, 169
Erm, 19
Ertapenem, 4
Erythromycin, 19
Escherichia coli, 34, 36
ESKAPE, 4
Ethambutol, 89
European Antimicrobial Resistance network (EARS-NET), 8
European Centre for Disease Prevention and Control (ECDC), 7
European Federation of Pharmaceutical Industries and Associations (EFPIA), 7
European Medicines Agency (EMA), 6–7
Evidence base, 9
Extended-spectrum beta-lactamases (ESBLs), 3
cephalosporins, 51
cephamycins, 51
Extinction, 137

F
Febrile neutropenia, 7
Finafloxacin, 173
Fitness
cost, 138, 147
decreased, 138
Fluoroquinolones, 21, 130, 172
fmt mutants, 170
Food and Drug Administration (FDA), 2

G
Gemifloxacin, 4
Generic, 9
Gene transfer, horizontal, 136, 138
GlaxoSmithKline, 9
Governmental/Institutional Surveillance Programmes
Active Bacterial Core surveillance (ABCs), 37
Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS), 35
Danish Integrated Antimicrobial Resistance Monitoring and Research Programme (DANMAP 2009), 36
European Centre for Disease Prevention and Control (ECDC), 34
National Antimicrobial Resistance Monitoring System (NARMS), 36
Swedish Strategic Programme against Antibiotic Resistance (STRAMA), 35
Governments, 11
Gram-negative bacteria, 38
Burkholderia cepaecia, 75
Campylobacter jejuni, 71, 80
Escherichia coli, 70
Haemophilus, 80
Helicobacter pylori, 80
Klebsiella pneumoniae, 70
N. denitrificans, 80
Neisseria gonorrhoeae, 80
Neisseria meningitidis, 80
Pseudomonas, 80
Pseudomonas aeruginosa, 70, 80

Gram-negative bacteria (*cont.*)
  Salmonella enterica, 70
  Shigella, 70
Gram-negative multidrug-resistant pathogens, 1
Gram-positive bacteria, 38
Gram-positive community-acquired pneumonia (CAP), 4
Gram-positives, 169–172
Greece, 4
GSK1322322, 170
GSK2251052, 175
Guinea pigs, 88

H
HAART 3, 90
Haemophilus influenzae, 39, 170
Health Protection Agency (HPA), 4
High-risk clones, 10
hip mutants, 121–125
HIV, 90
Hu/Coates models, 93
Hybrid antibacterials, 168
Hydrazinopyrimidine, 170

I
Iclaprim, 171
ICUs, 4
Imipenem, 175
Individual benefit, 4
Industry/Pharmaceutical Surveillance Programmes
  British Society for Antimicrobial Chemotherapy (BSAC), 39
  SENTRY Antimicrobial Surveillance, 38
  Study for Monitoring Antimicrobial Resistance Trends (SMART), 38
  Tigecycline Evaluation and Surveillance Trial (T.E.S.T.), 40
Infection
  bacteremia, 100
  chronic skin wounds, 102
  dental plaques, 102
  infective cystic fibrosis, 102
  infective endocarditis, 102
  osteomyelitis, 102
  tuberculosis, 100
Infection control, 11
Institute of Medicine (IOM), 2
Intra-abdominal infection, 174
Intravenous, 169
Intrinsic resistome, 25
Isoniazid, 88

J
JNJ-Q2, 173

K
Ketolide, 169
Klebsiella pneumoniae, 34, 36
KPC, 10

L
Lab on a chip, 10
Leprosy, 90
Leucyl-tRNA synthase, 168, 174
Levofloxacin, 173
Linezolid, 173
Lipoglycopeptide, 171
Lipopolysaccharide (LPS)
  core saccharide, 68, 71
  3-deoxy-D-manno-oct-2-ulosonic acid, 71
  L-glycero-D-manno-heptose, 71
  lipid A, 68, 73, 77
  O-antigen polysaccharide, 68

M
Mass redundancies, 7
MDR. *See* Multidrug-resistant (MDR)
Medicine
  aminoglycosides, 109
  amoxicillin, 109
  ampicillin, 109
  antibiotics, 100
  antimicrobial agents, 100
  dalbavancin, 110
  daptomycin, 110
  fluoroquinolone, 109
  HT61, 110
  isoniazid, 108
  oritavancin, 110
  penicillin, 103
  pyrazinamide, 108
  streptomycin, 109
  telavancin, 110
  vancomycin, 110
Metallo-β-lactamase, 174
Methicillin-resistant *Staphylococci* (MRSA), 4, 8
MGE. *See* Mobile genetic elements (MGE)
MK-7655, 175
Mobile genetic elements (MGE), 136, 137
Moraxella catarrhalis, 39, 170
Moxifloxacin, 173

MRSA. *See* Methicillin-resistant *Staphylococci* (MRSA)
Multidrug-resistant (MDR), 90, 169
Multiresistance effect, 147, 148
Mycobacterium leprae, 90
Mycobacterium tuberculosis, 21, 125

N
Nalidixic acid, 2
Nanoparticles
  metal, 149
  silver, 139, 140
  silver nitrate, 140
  titanium dioxide, 140
  toxicity, 139
Nanotechnology, 138
Narrow-spectrum antibiotic, 10
National Institutes of Health (NIH), 2
National surveillance data, 2
NDM-1-carrying enterobacteriaceae, 5
Neisseria gonorrhoeae
  cefixime, 53
  ceftriaxone, 53
New Delhi metallo-beta-lactamase (NDM-1), 3
NIAID and public-private research efforts, 6
Non-government organisation, 11
Non-inferiority/superiority studies, 7
Nonmultiplying persistence bacteria
  biofilm, 102
  dormant, 105
  persisters, 103
  stationary-phase, 101
Novexel 104, 10
NXL104, 176

O
Oral, 169
Oritavancin, 171
Orphan drug route, 3
Oxazolidinone, 170
Oxyimino-cephalosporin, 176

P
p-aminosalicylic acid (PAS), 88
Panresistant gram-negative pathogens, 11
Pathogenicity
  adhere to human tissues, 106
  produce toxins, 106
Peptide deformylase, 168
Peptidoglycan
  β-lactam, 80
  moenomycin, 80
  transglycosylases, 80
  transpeptidases, 80
Persister cells
  biofilms, 125
  dose-dependent killing, 122–123
  drug resistance and drug tolerance, 122–123, 125–126
  hip mutants, 124–125
  Mycobacterium tuberculosis, 125
  patients with cystic fibrosis (CF), 123–124
Pfizer Wyeth, 9
pH, 93
Pharmacokinetic-pharmacodynamic (PK-PD) studies, 172
Physiological state, 103
  exponential growth phase, 101
  nonmultiplying persistent stage, 104
Plasmids, 3, 91
Plazomicin ACHN-490, 174
Pleuromutilin, 169
Pneumonia
  community-acquired, 176
  hospital-acquired, 175
  ventilator-associated, 175
Polymyxin, 3
Porin defects, alteration in cell wall, 3
Post-antibiotic effect (PAE), 94
Pseudomonas aeruginosa, 8, 33, 123, 125, 175
  aminoglycoside, 52
  biofilm, 53
  peptides, 53
  porin-mediated, 52
Public health, 4
Pyrazinamide, 89
Pyrazinoic acid, 93

Q
Quinolones, 3

R
Radezolid, 170
Rapid diagnostics, 3
React, 7–8
Regulatory guidelines, 9
Research and development (R&D), 6
Resistance
  antibiotic, 137, 138, 141–143
  bacterial, 143, 144
  biocoides, 149

Resistance (*cont.*)
  co-resistance, 141, 143
  cross, 141, 143
  development of, 143
  mercury, 137
  metal, 136, 141–143, 146, 149
  methicillin, 140
  microbial, 141
  multiple, 138, 142
  prevalence of, 143
  silver, 143, 144
Respiratory tract infection (RTI), 168
Resuscitation-promoting factors, 93
Rifampicin, 89
RNA, 3
RTI. *See* Respiratory tract infection (RTI)

S
Salmonella, 36
Salvarsan, 14
Sanofi-Aventis, 9
Scientific Advisory Group (SAG), 7
Selection, strong, 146
Skin and soft tissue infections (SSTI), 169
Solithromycin, 169
Speciation rates, 137
Staphylococci, 168
Staphylococcus aureus, 35, 37
  methicillin-resistant, 169
  methicillin-sensitive, 169
Stewardship
  cycle, 61
  restrict usage, 61
Strains, 90
Streptococcus pneumoniae, 34, 169
  macrolides, 49
  penicillin, 49
Streptococcus pyogenes, 170
Streptomycin, 88
Stress condition
  acidic and oxidative stress, 102
  altered pH, 103
  heat, 102
  nutrient limitation, 103
  osmolarity, 103
  osmotic challenge, 102
  shock, 102
  starvation, 105
Surveillance Data Link Network (SDLN)
Surveillance Programmes. *See* Governmental/ Institutional Surveillance Programmes; Industry/Pharmaceutical Surveillance Programmes
Survival
  amplification, 46
  efflux, 46
  inactivation, 46
Sweden, 4

T
TATFAR, 7
Tazobactam, 176
TD1792, 169
Tetracyclines, 3
Textiles, 139
  antimicrobial, 144, 149
Therapeutic margin, 92
Thiacetazone, 91
Tigecycline, 4
TMC207, 93
Tolerance, 144, 145
Tolerant, 93
Torezolid, 171
Toxin/antitoxins, 121, 127–129
Transduction, 137
Transformation, 137
Ttorezolid, 171

U
Urinary tract infection, 173
US Congress, 8

V
Vancomycin, 19, 173
Vancomycin resistance
  plasmid-mediated, 48
  vanA, 48
  vanB, 48
  vanC, 48
  vancomycin, 48

W
WHO Global Strategy, 5
Wonder drugs, 2