Vipin Chandra Kalia *Editor*

Quorum Sensing vs Quorum Quenching: A Battle with No End in Sight

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Preface

Human beings encounter scenarios where they develop severe symptoms of a disease. The discovery of antibiotics was thought to relieve human beings of their miseries caused by microbial infections. However, microbes have been evolving rapidly to become resistant to antibiotics. It has been realized that bacteria have a unique system of multiplying silently through the phenomenon termed Quorum Sensing (QS). QS operates through signal molecules, which enable bacteria to sense their population density. At high cell density, bacteria activate their arsenal of virulence factors. QS mediated biofilms formed by pathogenic bacteria allow them to with stand high doses of antibiotics. This provoked scientist to look for novel alternatives to antibiotics. It led to the discovery of QS inhibitors, both natural and synthetic. Recent studies have indicated that QSIs may meet the same fate as antibiotics. Although a lot of scientific literature is being published rapidly since the last few years, it is limited largely to scientific research journals. A compilation of these important findings is not available to students at graduate and post-graduate levels. The best thing about this book is that the various chapters have been written by experts in the respective areas. As it demands tremendous and dedicated effort, we are extremely thankful to all the authors for their prompt responses and their contributions. I was inspired by my parents (Mr. R.B. Kalia and Mrs. Kanta Kalia), wife (Amita), Sunita (Sister), Ravi and Satyendra (Brothers), children (Daksh and Bhrigu), teachers, and Rup and Hemant (friends) to write this book. Throughout the preparation of this work, I was supported by Mr. Prasun Kumar, my Ph.D. student.

Delhi, India   Vipin Chandra Kalia

Contents

About the Editor

Dr. Vipin Chandra Kalia is presently working as Chief Scientist, Microbial Biotechnology and Genomics, CSIR-Institute of Genomics and Integrative Biology, Delhi. He is a Professor, AcSIR who obtained his M.Sc. and Ph.D. in Genetics, from Indian Agricultural Research Institute, New Delhi. He has been elected as: (1) Fellow of the Association of Microbiologists of India (FAMI), and (2) Fellow of the National Academy of Sciences (FNASc). His main areas of research are microbial biodiversity, bioenergy, biopolymers, genomics, microbial evolution, quorum sensing, quorum quenching, drug discovery and antimicrobials. He has published 65 papers in scientific journals such as (1) Nature Biotechnology, (2) Biotechnology Advances, (3) Trends in Biotechnology, (4) Critical Reviews in Microbiology, (5) Bioresource Technology, (6) PLoS ONE, and (vii) BMC Genomics. His works have been cited 1750 times with an *h* index of 23 and an i10 index of 36. He is presently the editor in chief of the Indian Journal of Microbiology and editor of (1) Journal of Microbiology & Biotechnology (Korea), (2). Appl. Biochem. & Biotechnology (USA), (3) International Scholarly Res. Network Renewable Energy, (4) Dataset Papers in Microbiology, and (5) PLoS ONE. He is a life member of the following scientific societies: (1) Society of Biological Chemists of India (2) Society for Plant Biochemistry and Biotechnology, India; (3) Association of Microbiologists of India; (4) Indian Science Congress Association; (5) BioEnergy Society of India, and (6) the Biotech Research Society of India (BRSI). He is also a member of the American Society for Microbiology. He can be contacted at: vckalia@igib.res.in; vc_kalia@yahoo.co.in

Microbes: The Most Friendly Beings?

Vipin C. Kalia

Introduction

Microbes, plants, animals, and human beings are intricately related to each other. In quite a few scenarios, these organisms live in close association with each other, for example, in rhizosphere, phyllosphere, etc. (Lindow and Brandl 2003). Epiphytes harbor microbes which prove helpful to the host (Hempel et al. 2008). Endophytic fungi prevent diseases and provide drought tolerance to *Poaceae* (grasses), and mycorrhizal fungi form symbiosis with vascular plants and protect them from diseases and enable them to sequester phosphate from the soil (Khan 2006; Rodriguez et al. 2009). Among all the symbiotic relationships, nitrogen fixation by rhizobia-legume interaction can be listed as the most beneficial outcomes (Drevon et al. 1987, 1988; Zhuang et al. 2013; Gao et al. 2014).

Human beings are conscientiously accomplished to exploit others for their personal benefits. Associations of microbes and human beings are not viewed in the positive light. Most microbes with an ability to infect human beings are perceived to lead to an unhealthy situation (http://ca-biomed.org/csbr/pdf/connect. pdf). However, each organism has been bestowed with a mechanism to protect itself from attack

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(Peterson et al. 2014). All organisms have distinct genetic makeup that they are able to maintain their identity and integrity with high precision. Incidentally, recent developments in microbial sciences have revealed that microbes stay on and inside our body (Walter et al. 2011). It has been realized that we harbor ten times more bacterial cells than our own (10 trillion) (http://gotsomescienceonyou.com/2013/05/08/there-isnt-that-much-you-in-you/). Skin microbiota is dominated by members of Bacteroidetes, Corynebacteria, Propionibacterium, Proteobacteria, Staphylococcus, etc. A second major group of microbes (around 160 species) is present in the human gut (Qin et al. 2010). Intensive analysis of gut microbes has provided very interesting insights for human health. In fact, our well-being is dependent upon the composition of this community (Gerritsen et al. 2011). It may not be inappropriate to state that their meticulous functioning is important for our health and welfare. We feel happy and are able to enjoy life while bacteria inside the gut are working silently. Our physical fitness and healthy appearance can be assigned to Firmicutes and Bacteroidetes (Wexler 2007). When these two groups compose between 44 and 48 % of the total gut microbiota, our metabolic activities allow us to have a lean appearance. However, if these two dominant groups reach a threshold level of around, 82–86 % of the total population, our body loses track of its growth. Our body starts expanding outward like a half balloon and we become obese. Firmicutes grow rapidly if

the fat intake is high, whereas Bacteroidetes multiply on a diet rich in carbohydrates (David et al. 2014). Actinobacterial, Fusobacterial, and Verrucomicrobial members assist the gut bacteria to function normally. While bacterial activities are going on in full swing, we do not take any notice of their presence. In addition to these, two relatively gentle set of bacteria, our body is also inhabited by a third group. It is composed of the pathogenic bacteria, which infect different body parts and our body becomes diseased. This pathogenic association is caused by two kinds of bacteria. They "love" our body and stay there for short or long duration depending upon their nature. The first group infects the body, multiplies fast, and exits hurriedly. These rapidly flushing out bacteria cause diseases like diarrhea, cholera, septicemia, etc. These gastroenteric infections are caused by enterotoxigenic *Escherichia coli*, *Klebsiella, Pseudomonas aeruginosa*, *Salmonella typhi*, *Serratia*, *Shigella*, *Vibrio cholerae*, Bacteroides, and *Proteus* (http://www2. hawaii.edu/ johnb/micro/m130/eadings/Septic Shock.htm). The worst part is their high resistance to antibiotics (White et al. 2002). The second group is composed of those bacteria which seem to have permanently "migrated" to the human body. They settle in different body parts and feel "comfortable" as there is no crunch of nutrients. Bacteria like *Mycobacterium tuberculosis, P. aeruginosa, and Burkholderia cepacia* cause infections and multiply in an uninterrupted manner by constantly evading the human immune system (Costerton et al. 1999; Mahenthiralingam et al. 2002; Purohit et al. 2007; Bhushan et al. 2013). Hence, we take notice of the presence of bacteria in and on our body when they cause diseases which we can no longer afford to avoid.

The Rise and Fall of Antibiotics

In the last few centuries, human beings have encountered severe bacterial infections. In the absence of any effective mechanism to get rid of these pathogenic organisms, the mortality rates used to be skyrocketing (Schmid-Hempel 2009). Health departments worldwide are constantly

on their toes and making efforts to control the growth of these organisms and achieve a major reduction in human mortality and morbidity rate. During these epidemics, there were instances of entire human communities getting wiped out. It was realized that toxin-producing organisms such as *Staphylococcus aureus, Clostridium* spp.*, Yersinia enterocolitica, Streptococcus mutans, S. pneumoniae,* etc., were the major culprits. A great discovery came to the rescue of patients infected with chronic and acute diseases. Sir Alexander Fleming was honored with the Nobel Prize for his contribution – the discovery of the wonderful antibiotic, penicillin. It totally shifted the way people could envision the future – a world free from diseases. All the same, this notion was soon shattered as bacteria were quick to respond. Their arsenal of genetic reservoir came to their deliverance. By undergoing genetic mutations, bacteria became resistant to penicillin. *Staphylococcus* resistant to penicillin emerged in 1940, which was soon followed by *Shigella* showing resistance to tetracycline, and evolution of erythromycin-resistant *Streptococcus,* etc. This trend of evolution of bacterial resistance to the newly discovered antibiotics was seen in the last few decades (Davies and Davies 2010). Now, we are in an era where the prevalence of multiple drug resistant (MDR) and extremely drug resistant (XDR) bacteria seems to be the norm of the day (Bhardwaj et al. 2013). It reckons a very depressing picture for scientists and pharmaceutical companies. Industrial houses are ordained to think before investing in R&D for novel antibiotics. It appears that bacterial genetic makeup is quite subsistent after having been exposed to antibiotics produced by other organisms. Pharmaceutical companies find investing in scouting drugs for chronic diseases more lucrative than in short-term diseases, which can be treated with a few doses of an antibiotic (Kalia et al. 2007).

Biofilm: The Bacterial Shield

It has been realized that the biofilm-forming bacteria are responsible for 80 % of the infectious diseases. This biofilm formation happens only

under specific conditions and allows bacteria to survive in the presence of antibiotic doses up to 1,000 times more than those required to kill them than their free-living counterparts. Biofilm is a unique structure which is formed only when certain genes get expressed by a phenomenon called quorum sensing (QS) (Kalia and Purohit 2011). Bacteria grow and remain "silent" while their cell density is low. It enables them to evade the human immune system. Nevertheless, once above a threshold population density, they are able to sense it. This requisite cell density is paramount to survive under stress conditions, and they kick-start expressing genes which are responsible for biofilm formation, production of virulence factors, toxins, etc. This process of gene expression at high cell density is mediated by signal molecules produced by genes responsible for synthase enzymes. These signal molecules are sensed by the bacteria and form a complex with intended receptor molecules, which in turn transcribes genes for pathogenic behavior of the bacteria. The system was initially detected in *Vibrio fischeri*, which produced light and helped to eliminate the shadow of the host (squid) organism, cast by the moonlight and enables it to save itself from praying fishes (Nyholm et al. 2000). It was realized that if bacteria are not expressing bioluminescence, they are involved in phenomena such as antibiotic production, biofilm formation, nitrogen fixation, motility, sporulation, toxins, virulence, etc. (Nakayama et al. 2006; Kolodkin-Gal et al. 2007, Kolodkin-Gal and Engelberg-Kulka 2008; Rinaudi and Giordano 2010; Kalia and Purohit 2011, Kalia et al. 2011; Li and Tian 2012; Bogino et al. 2013). QS-regulated biofilm-forming bacteria – *Aeromonas, Vibrio,* and *Yersinia* spp. – are a major cause of diseases in fish (Bhargava et al. 2012; Chu et al. 2013). It affects the economies of Departments of Fishery and Aquacultures. Another adversely affected industrial sector that is involved in the reclamation of wastewater and producing drinking water. Biofilms formed on the membranes used for filtering water lead to inadequate recovery and add to economic losses (Bereschenko et al. 2010). The growth of bacteria on archaeological buildings and monuments leads to their rapid deterioration (Scheerer et al. 2009).

A New Era of Antibacterials

In view of the fact that we are losing a battle against bacteria, scientists have embarked upon a search as to how to circumvent this problem. It has been envisaged that the QSS can be interrupted at one or more stages, which consequently may enable us to inhibit their pathogenicity (Kalia and Purohit 2011, Kalia 2013). This strategy of employing quorum-sensing inhibitors (QSIs) can prove effective in designing novel antibacterials (Romero et al. 2012; LaSarre and Federle 2013). The presence of bioactive molecules with properties to act as QSIs have been detected in prokaryotes, animal, and plants (Huma et al. 2011; Kalia and Purohit 2011, Kalia et al. 2011; Desouky et al. 2013; Kalia 2013; Kumar et al. 2013; Nakayama et al. 2013). Chemists, with their specialty in synthesizing analogues of natural QSIs, have contributed significantly in improving the efficiency of these molecules (Mai et al. 2011; Klein et al. 2012; Kalia et al. 2013; Lu et al. 2012; Sahner et al. 2013). Only those molecules which exclusively affect QS without affecting bacterial growth are categorized as QSI. Thus, unlike antibiotics, which affect bacterial growth and eventually kill them, QSIs do not put much selective pressure (Kalia and Purohit 2011, Kalia 2013). This has become a strong basis for scientists to jump to the conclusion that bacteria will not evolve resistance to QSIs. It is no surprise that bacteria persistently tailor their genetic potential to blunt any attack on their survival. For bacteria the encounter with "novel" antibiotics is a situation of déjà vu. Questions are conjuring around the fate of QSIs. There are evidences which suggest that bacteria may evolve mechanisms to evade QSIs? (Defoirdt et al. 2013; Kalia et al. 2014). So the great query is: Are we doomed to drop dead at the hands of bacteria and lose the battle of life? Will we be the mute witnesses to human beings dying at an annual pace of millions of deaths by these pathogens (http://www.civilwar.org/education/pdfs/civil-was-curriculum-medicine.pdf)? How or will we be able to survive the onslaughts of this never-ending battle?

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Part I

Quorum Sensing Mediated Processes

Evolution of MDRs

Ashima Kushwaha Bhardwaj and Kittappa Vinothkumar

Introduction

With the evolution of multidrug-resistant bacteria at threatening rates, the mankind has witnessed the glorious rise and fall of antibiotics. Antibiotics, the miracle drugs and the magic bullets that appeared to have marked the end of the infectious diseases, are fast losing their charm and effectiveness in human medicine. The swift and untimely demise of these wonder molecules has been attributed chiefly to the resistance mounted by bacteria against them. The phenomenon of antibiotic resistance is inevitable and was something that was cautioned in the Noble Prize lecture by Sir Alexander Fleming in 1945. Dr. Joshua Lederberg very accurately fathomed the seriousness of these resistant bacteria whom he considered much more dangerous a threat as compared to Ebola and West Nile virus. Resistance to any molecule or drug intended to kill a target organism is a very natural phenomenon for the survival of that organism; a cancer cell being subjected to chemotherapeutic treatment, a fungal cell subjected to anti-fungals and, similarly, anti-parasitic and antibacterial compounds are all likely to face resistance from their target cells. Thus, all the popular drugs including antimalarials, anti-tuberculosis, anti-parasitic, antivirals, anti-fungals and antibacterial drugs are facing the risk of becoming obsolete. Consequently, the human race faces the risk of an apocalypse in the hands of these invincible bugs that no drug is able to kill. This chapter describes various genetic and some of the non-genetic factors such as environmental, social and political factors that have led to the evolution of a phenomenon called multidrug resistance (MDR). The threat of antibiotic resistance now spans a wide range of infectious agents including Gram-positive and Gram-negative bacteria, all the infectious diseases and all the geographical locations on this planet.

There has been an evolution of a myriad of resistant bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococci* (VRE), vancomycin-resistant *Staphylococcus aureus* (VRSA), extremely drug-resistant tuberculosis (XDR), totally drug-resistant tuberculosis (TDR), New Delhi metallo-β-lactamases (NDM)-carrying superbugs, extended spectrum β-lactamases (ESBLs)-carrying bugs and carbapenem-resistant *Klebsiella pneumoniae* (CRKP) to name a few. Having thrived in hospital settings at operation theatres and intensive care units or in community settings, these superbugs have wreaked havoc and led to the number of deaths spiralling high. This exhaustive list also deserves the mention of major threats posed by *Pseudomonas aeruginosa* and *Acinetobacter baumannii* in

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nosocomial infections. In addition to this, *Vibrio cholerae* actually stands at the top of the superbug list (Davies and Davies 2010). The world has witnessed seven pandemics due to this pathogen which has shown continuous evolution in terms of virulence factors and antibiotic resistance traits.

Causes of MDR

MDR is a complex phenomenon that arises due to interplay of large number of factors that work in synergy to manifest this problem. Some of the causes of MDR are described below.

Genetic Factors

There is a plethora of genes responsible for the evolution and dissemination of MDR. These genes could be chromosome borne or carried by molecular vehicles such as plasmids, viruses, integrons and transposons, some of which will be described in sections "General mechanisms involved in the evolution of antibiotic resistance" and "Role of MGEs in evolution of MDR".

Social and Political Factors

The underuse, overuse as well as misuse of antibiotics can lead to serious consequences in treatment of infectious diseases. Inappropriate prescriptions due to callousness of the medical doctors like prescribing drugs with improper dosage or prescribing wrong drugs could be one of the reasons. Using antibiotics to treat viral infections could lead to the development of resistance in bacteria residing in the human body. Poor compliance to the drug courses, sub-inhibitory concentrations or premature abrogation of antibiotic usage by humans serve as crucial factors for the development of resistant bugs. Profligate use of antibiotics in human medicine, aquaculture, agriculture and poultry adds to the reservoir of resistance-conferring genes from drug-resistant bacteria in environment, also termed as resistome. Through

the extensive and persistent use of antibiotics, the selective pressures continue to be exerted on the bacterial communities. In this scenario, the probability of infection of any individual with a drug-resistant bacterium is much higher as compared to infection with a drug-susceptible bacterium. Therefore, the problem that started with a single patient or a small group of people assumes the proportion of a public health problem. Lack of government policies for proper disease surveillance, antibiotic usage as well as containment of the infectious diseases often leads to the spread of MDR. Pharmaceutical industries are losing interest in the antibiotic development due to the lack of government policies that could give incentives to the pharmaceutical sector for the research and development of new antibiotics. Inability to detect the newer and more subtle antibiotic resistance phenotypes with the available laboratory diagnostic techniques may lead to longer survival and circulation of MDR pathogens in human populations often hindering successful treatment regimens. For example, pneumococcal resistance to β-lactams and staphylococcal resistance to vancomycin are the difficult phenotypes to detect.

Environmental Factors

Natural disasters or calamities like earthquakes, floods, tsunamis and famines and political situations like civil wars or unrest where the medical facilities are heavily impaired can also lead to the high case fatality rates due to the thriving MDR bacteria. Many drugs such as antibiotics, antidepressants, chemotherapeutics and their residues often escape purification by water treatment plants and, therefore, contaminate drinking water supplies. Considerable amounts of these antibiotics are released into the biosphere by hospitals, research laboratories, pharmaceutical industries and domestic use. It is not surprising that the microbial world in soil, water and food has to resort to myriad resistance determinants to avert the catastrophe due to these contaminants. The Environmental Protection Agency (EPA) and Food and Drug

Administration (FDA) have not yet formulated any rules and regulations on this aspect of drug contamination in drinking water. This has serious consequences not only for humans but also for the aquatic ecosystems. At many places such as the United States and India, drugs have been dumped by pharmaceutical companies in the rivers (http://www.purewaterfreedom.com/osc/pharma_water_contamination.php). In addition to the spurt in the appearance and dissemination of drug resistance genes, their toxicity to all the organisms in water or land or air is probably unfathomable.

Consequences of MDR

The dissemination of MDR traits in bacteria has different consequences for mankind as well as the pathogens.

For the human hosts that fall prey to the super bugs, it leads to:

- Treatment failure
- Prolonged stays in the hospitals escalating the health-care budgets
- Reduction in manpower that has both social as well as economical consequences

For the bacterial populations, this MDR translates into:

- Increased virulence of the bacterium. For example, the studies on *A. baumannii* have revealed that genomic islands in this organism also harbour virulence determinants in addition to the antibiotic resistance determinants (Barbe et al. 2004). Similarly, the community-acquired MRSA has equipped itself with a wide range of genes that endow the bacteria with pathogenicity genes as well as antibiotic resistance genes (DeLeo and Chambers 2009).
- More efficient transmission of bacterium.
- Dissemination of the resistance genes to all other pathogens in their vicinity leading to amplification of the resistance genes in nature.
- Transfer of resistance genes to the commensal organisms residing in the host affecting the microflora often leading to some other outcomes in the host health.

General Mechanisms Involved in the Evolution of Antibiotic Resistance

The evolutionary history of resistant bacteria predates the introduction of the antibiotic era. It is understandable that the antibiotic producers were actually the reservoirs of drug resistance genes. These antibiotic resistance genes were part of the paraphernalia involved in the production of antibiotics by the bacteria where they provided protection to the producers.

As antibiotics target the vital processes of a bacterial cell, they create a do-or-die situation for a bacterium. Hence, it is indispensable for the bugs to resist the action of antibiotics at any cost by devising various tactics. The molecular mechanisms of resistance exerted by bacteria to overcome drugs have been well studied, and they employ any one or a combination of the following strategies (Alekshun and Levy 2007).

Chromosomal Mutations at the Target Sites of Antibiotics

Mutations at the antibiotic target sites are the main mode of resistance to most of the antibiotics. Mutations occurring as a result of replication errors reduce the affinity of the antibiotics to their targets resulting in the resistant phenotype. For example, quinolone and fluoroquinolone resistance occurs through the mutations at the DNA gyrase and topoisomerase IV genes. Similarly, mutations in the gene encoding dihydropteroate synthase decrease the enzyme affinity to the sulphonamides. In *Mycobacterium tuberculosis*, resistance to the common drugs such as rifampin, streptomycin, ethambutol and fluoroquinolones used to treat the pathogen arises due to mutations in the genes that are involved in metabolic pathways or in housekeeping. Additional mutations in the already mutated genes result in increasing the minimum inhibitory concentration (MIC) of the antibiotic for the pathogen or extending the spectrum of resistance such as the development of extended spectrum β-lactamases (ESBLs) in the pneumococcus (Medeiros 1997).

Increased Efflux and Reduced Influx of Antibiotics in the Bacterial Cell

Efflux pumps play a major role in conferring resistance to antibiotics by efficiently recognising and throwing them out of the cells. Efflux pumps in bacteria can be classified into five different families, namely, the resistance nodulation cell division (RND), major facilitator super family (MFS), small multidrug resistance (SMR), ATP-binding cassette (ABC) and multidrug and toxic compound extrusion (MATE) families (Bhardwaj and Mohanty 2012). Among these five pumps, ABC pumps utilise ATP as their energy source, whereas others are driven by the proton-motive force (PMF). Generally efflux pumps are known to extrude out a wide range of substances including antibiotics, and therefore, this is a non-specific mechanism of resistance. However, few pumps are shown to have high specificity towards particular drugs. For example, TetA and NorM are found to be more specific towards tetracycline and norfloxacin, respectively, whereas AcrB, VcmA and MdfA have multiple substrate specificities. Efflux pumps confer only low level resistance to the bacteria towards drugs but their over-expression or cooperativity with other mechanisms could result in moderate to high-level resistance (Bhardwaj and Mohanty 2012).

Porins present in the cell membrane of bacteria are the passages which facilitate the entry and exit of antibiotics and other small organic molecules. Decrease in the expression of porins results in reduced uptake of antibiotics. For example, mutations that caused reduced expression of OprD porins contributed to imipenem resistance (Alekshun and Levy 2007).

Enzymatic Drug Modification or Degradation

This mechanism of resistance involves enzymes that either degrade or chemically modify the antibiotics so that they cannot exert their action. β-lactamases are the well-known examples for the enzymes that degrade β-lactam antibiotics. Few of them behave as extended spectrum β-lactamases (ESBLs) and as carbapenemases and show wider spectrum of resistance to newer generation β-lactam antibiotics (Alekshun and Levy 2007). There are a large number of aminoglycoside-modifying enzymes which chemically modify (acetylate or adenylate or phosphorylate) the aminoglycosides. Similarly, chloramphenicol is inhibited by chloramphenicol acetyltransferases and tetracycline by a flavin-dependent monooxygenase TetX (Alekshun and Levy 2007).

Protection and Alteration of Drug Target

Resistance to fluoroquinolones is mediated by a large number of pentapeptide repeat proteins, quinolone resistance (Qnr) proteins, which protect target DNA gyrase and topoisomerase IV from the antibiotic action. As these proteins mimic the structure of DNA, they occupy the DNA-binding portion of the topoisomerases and prevent the antibiotics from exerting their effect on these protected topoisomerase targets. The altered penicillin-binding protein (PBP) of methicillin-resistant *S. aureus*, PBP2a, confers resistance to most of the β-lactams by contributing the transpeptidase activity when exposed to methicillin (Fig. 1a).

Other Mechanisms

Sometimes resistance to different antibiotics can be conferred by a single determinant. For example, aminoglycoside acetyl transferase (aac (6′)-Ib) generally acetylates aminoglycosides like amikacin, kanamycin and tobramycin. But its mutant form aac (6′)-Ib-cr is known to acetylate quinolones like ciprofloxacin also (Robicsek et al. 2006). Therefore, a single protein renders resistance to aminoglycosides as well as quinolone class of antibiotics (Fig. 1b).

The tandem duplication of the resistance-conferring gene results in overexpression which
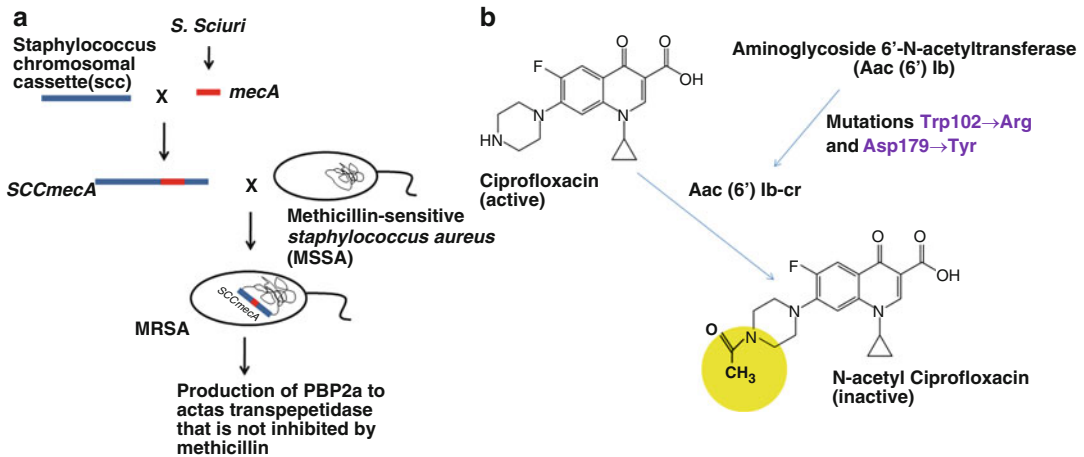
Fig. 1 Evolution of methicillin-resistant *Staphylococcus aureus* (MRSA) and Aac (6′) Ib-cr, an enzyme showing promiscuous drug resistance. (**a**). The evolution of MRSA by the successful acquisition and expression of *mecA* from *Staphylococcus sciuri*. The evolved *S. aureus* expresses *mecA*-derived PBP2a that acts as an alternate transpeptidase that is not inhibited by methicillin; (**b**). By acquiring mutations at the active sites, the modifying enzyme Aac (6′)-Ib evolves as Aac (6′)-Ib-cr with the additional ability to modify ciprofloxacin

eventually helps the bacteria to exhibit a high-level resistance to the antibiotics. In one instance, the overexpression of tandem duplicated genes of AcrAB drug efflux pumps in *E. coli* led to an MDR phenotype (Alekshun and Levy 2007).

Processes That Drive Evolution of MDR

There are chiefly two processes through which the mechanisms of resistance described in section "General mechanisms involved in the evolution of antibiotic resistance" lead to the evolution and persistence of MDR. These processes described below are either the horizontal gene transfers or the pressures due to environment. SOS responses mounted in a bacterium due to antibiotic exposure or HGT are also related to these processes and therefore deserve a special mention in this section (Fig. 2).

Horizontal Gene Transfer (HGT)

The process of HGT enables bacteria to exchange genetic material within themselves without the requirement of cell division. Different kinds of mobile genetic elements (MGEs) are transferred between bacteria through this process leading to the adaptation and evolution of bacteria/bacterial communities in tune with the changing environments. HGT is mediated by the processes of transformation, transduction or conjugation, and different types of MGEs could move through these processes of HGT (Fig. 2). These MGEs as agents of evolution will be described in section "Role of MGEs in evolution of MDR".

Selective Pressure due to Environment

Environment plays a vital role in the selection and spread of antibiotic resistance among bacterial communities that would be discussed at many places in this chapter with examples. These selective pressures lead to induction of mutations in the drug target genes conferring the mutant bacteria, a resistant phenotype (Fig. 2). The transmission dynamics of MDR is hugely responsive to the environmental factors at the hospitals or the communities.
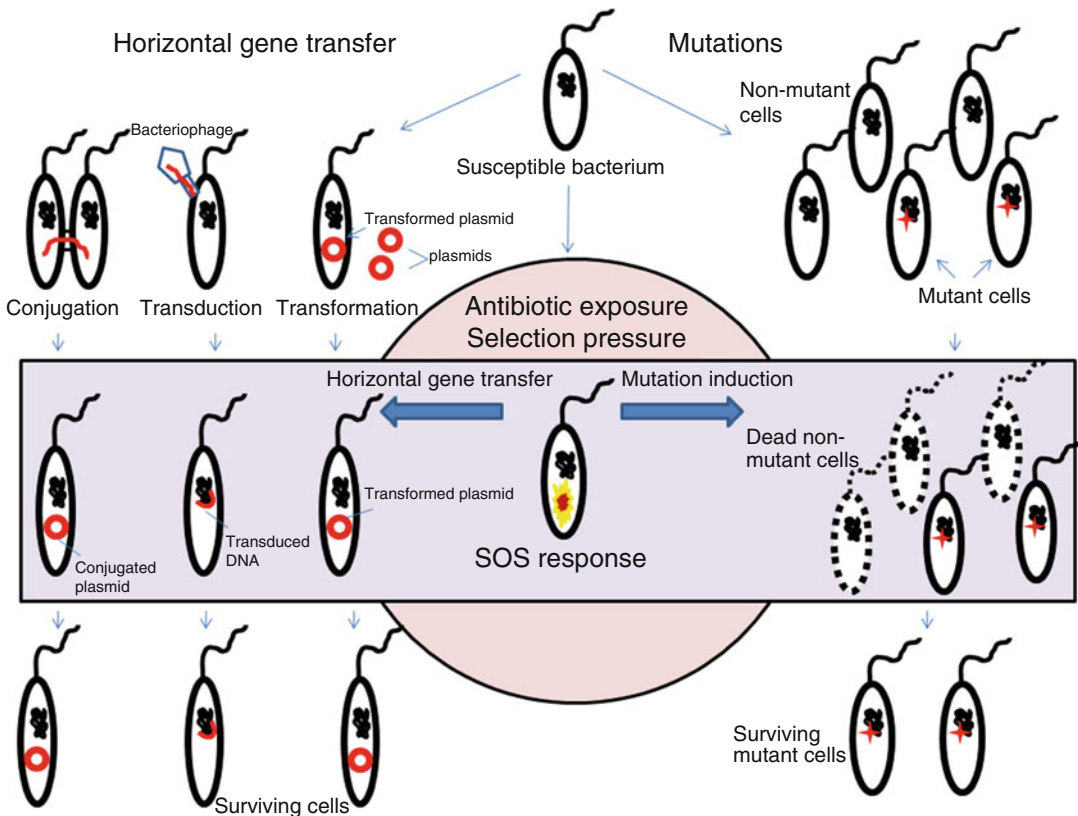
Fig. 2 The process of evolution of MDR bugs. The susceptible bacteria attain some mutations at their antibiotic target sites prior to the exposure of antibiotics. These mutants are selected under antibiotic pressure when they emerge, persist and disseminate as resistant bugs. The acquisition of resistance genes by the bacteria through horizontal gene transfer (HGT) also helps bacteria to resist antibiotics under selection pressure. Antibiotic exposure elicits SOS responses which facilitate both mutations and HGT processes. Therefore, evolution of MDR is an interplay of three processes: HGT, selection pressure and SOS responses

SOS Responses in Bacterium on HGT/Antibiotic Exposure

Any type of HGT through conjugation, transformation and transduction or any type of antibiotic challenge induces SOS response (Fig. 2) through events mediated by single-stranded DNA, RecA protein and LexA repressor (Baharoglu et al. 2013). On antibiotic exposure/HGT, RecA gets activated which leads to autoproteolysis of LexA repressor that keeps the SOS regulon in the repressed state under normal conditions. LexA inactivation thus leads to the expression of a diverse array of genes that were repressed by it.

Integrases associated with integrons and integrating conjugative elements (ICEs) are examples of the genes that are induced during SOS due to LexA inactivation (Baharoglu et al. 2013). This leads to the escape of integrons and ICEs from the bacterial cell under crisis. Similarly, the regulation of expression of *qnrB2* (a quinolone resistance determinant) through SOS response is induced by ciprofloxacin in LexA-/RecA-dependent manner. Even sub-inhibitory concentration of ciprofloxacin was found to cleave LexA repressor so that it was prevented from binding on the LexA binding site present in the promoter region of *qnrB2* gene (Da Re et al. 2009).

Therefore, under ciprofloxacin pressure, the bacterial cell expressed resistance gene for this antibiotic through SOS-mediated pathway.

Role of MGEs in Evolution of MDR

MDR evolves through a large spectrum of genetic elements that could either reside on the chromosomes of a bacterium or reside on the pieces of DNA that are mobile. The latter types are called mobile genetic elements (MGEs) and include a diverse class of genetic elements such as integrons, bacteriophages, integrating conjugative elements (ICE) and conjugative plasmids (Fig. 3). These MGEs play an important role in reshaping and in the evolution of the bacterial genomes enabling bacteria to thrive in a variety of ecological niches. In the subsequent sections, the MGEs that have resulted in fast acquisition and dissemination of MDR genes have been described.

Integrons

These MGEs are capable of capturing the gene cassettes by site-specific recombination, integrating them and expressing them using a common promoter (Stokes and Hall 1989; Recchia and Hall 1995). Integrons therefore convert the acquired open reading frames (ORFs) into their functional form. Integrons consist of an integrase gene (*intI*), a recombination site (*attI*) and a promoter Pc. There are numerous classes of integrons known that are classified based on the sequences of their integrase genes. Class 1 integrons have been studied most extensively, and these integrons have been characterised vis-à-vis their role in dispersal of the MDR genes in clinical isolates of Gram-negative bacteria. Integrons have been an important part of bacterial evolution, are widespread among all the bacteria and have a wider role to play in bacterial physiology and adaptation than simply antibiotic resistance (Rapa and Labbate 2013). The structure of a class
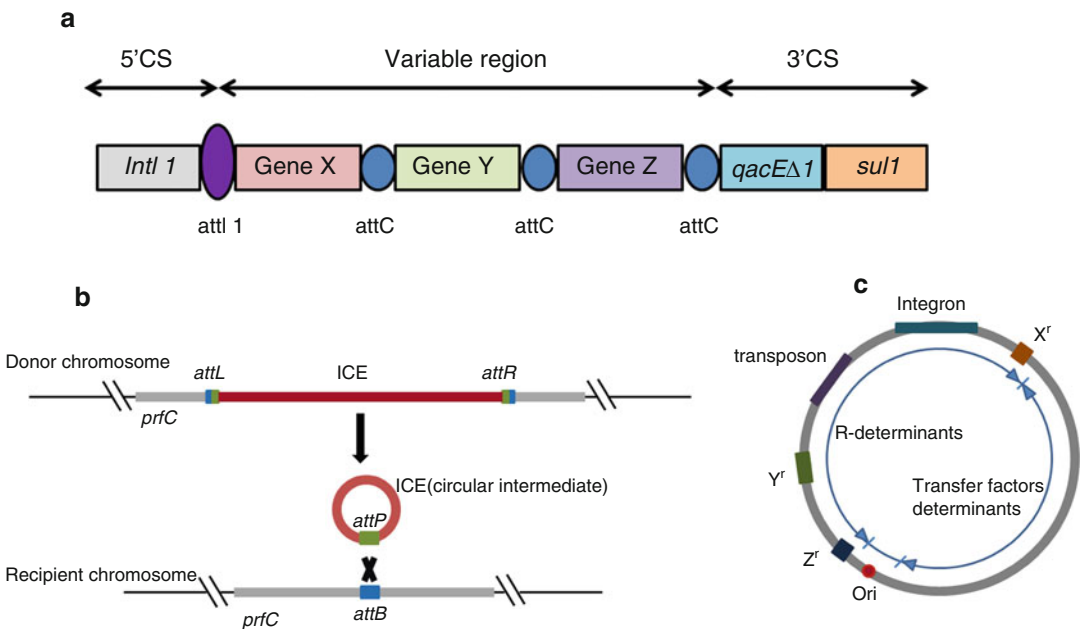
Fig. 3 MGEs that facilitate evolution of MDR. (**a**). Structure of a class 1 integron having conserved segments at its 5′ and 3′ ends (5′CS and 3′CS, respectively) and variable region consisting of extraneous gene cassettes that encode various functions including antibiotic resistance. *intI 1* encodes an integrase and *attI 1,* and *attC* sites are formed with the insertion of the extraneous gene cassettes. (**b**). Insertion and excision of integrating conjugative elements (ICEs) in the *prfC* region of a bacterial genome. The recombination process is mediated by a circular intermediate. (**c**). A conjugative R plasmid that may carry resistance genes, integrons and transposons along with transfer factor determinants

1 integron consists of the conserved segments at the 5′ and 3′ ends of the integron (5′ CS and 3′CS). These conserved segments encompass a variable region which varies with the number and nature of the gene cassettes captured by the integrons (Fig. 3a). The 5′ CS consists of *intI* gene, the *attI* site and the promoter, whereas the 3′CS consists of two genes that encode resistance for ethidium bromide and sulphonamides. The extraneous gene cassettes captured by integrons are usually promoterless, and they recombine with the *attI* site through a recombination site called *attC* or 59-base element (Fig. 3a). Each gene cassette captured in an integron is thus bound by the *attI* site on its 5′ end and an *attC* site on its 3′ end. The *attC* sites share a common set of characteristics that enable them to be identified despite the diversity of their sequences and sizes (Hall et al. 1991). They are characterised by a palindrome of variable length and sequence between the RYYYAAC (R = Purines; Y = Pyrimidine) inverse core site and the GTTR-RRY core site. The size of these recombination sites vary in length from 57 to 141 bp.

Integrons have been reported from a wide variety of bacteria such as *V. cholerae*, *V. fluvialis*, *V. parahaemolyticus*, *P. aeruginosa* and *K. pneumoniae*. They have been shown to harbour a diverse array of genes including antibiotic resistance genes. Resistance genes for chloramphenicol (*catB*, *cmlA*), trimethoprim (*dfrA*, *dfrB*), β-lactam antibiotics (*bla*, *oxa*), aminoglycosides (*aac* and *aad*) and many ORFs of unknown functions have been observed in integrons. Two types of integrons are known to exist: chromosomal integrons (CIs) or superintegrons (SIs) that are sedentary in nature and the mobile integrons (MIs) that are associated with mobile DNA elements and involved in the spread of antibiotic resistance genes (Rowe-Magnus et al. 2002). CIs are located on the chromosomes of a large number of bacteria. MIs usually contain less than 20, while SIs/CIs contain more than twenty-gene cassettes. The nature of gene cassettes harboured by MIs and SIs also varies. While MIs usually contain antibiotic resistance gene cassettes, majority of cassettes associated with SIs are of unknown functions.

Integrating Conjugative Elements (ICEs)

ICEs are a type of conjugative transposons that integrate and replicate with the chromosomal DNA of the host bacterium (Burrus and Waldor 2004). ICEs are not capable of autonomous replication, and therefore, they have to depend on the host cell machinery for its survival. They excise themselves from the host chromosome, form a circular intermediate and then get transferred to the recipient cell during conjugation (Fig. 3b). OriT, a *cis*-acting site, is required on ICEs for their translocation to the recipient through the mating bridge formed during conjugation. ICE known as SXT element was first reported from Madras, India, in 1992, in *V. cholerae* O139 strains where they imparted resistance to drugs like trimethoprim, sulphamethoxazole, streptomycin and chloramphenicol (Waldor et al. 1996). Since then, these elements have been reported from a large number of bacteria such as *V. cholerae*, *Providencia alcalifaciens* and *P. rettgeri* at many places as important vehicles for spreading of antibiotic resistance. The integration in the host genome is mediated by an integrase, and ICEs also encode other functions required for their maintenance. These functions include conjugative transfer of these elements, their excision and integration and regulation of the events related to ICE transfer and maintenance. ICEs harbour a wide array of genes for diverse functions such as antibiotic resistance, heavy metal resistance and complex degradation pathways for toxic compounds. Two different ICE elements can also recombine to produce a tandem array of ICE elements called hybrid ICEs. One such hybrid is an SXT/R391 family of ICEs which is the largest family of ICEs detected in clinical as well as environmental strains of many bacteria. Through the process of recombination mediated mainly by RecA protein, these hybrids are known to promote their own diversity resulting in the formation of novel mosaics with new combinations of antibiotic resistance genes (Garriss et al. 2009).

Plasmids

Plasmids are autonomously replicating extrachromosomal DNA molecules that are transferred from donor to the recipient bacterium through conjugation. Resistance plasmids also known as R plasmids, harbouring genes conferring antibiotic resistance, have been well known for their role in the transfer of resistance traits from a drug-resistant bacterium to a drug-sensitive bacterium (Fig. 3c). Plasmids appear to have a major contribution in the spread of drug resistance, and several pathogens have been reported that harbour plasmids with multiple resistance traits. Bacteria carry either conjugative plasmids that are large in size or non-conjugative small-sized plasmids. Non-conjugative plasmids can be mobilised with the help of other conjugative plasmids present in the same cell or by the process of transformation. In some cases, these plasmids may carry integrons or transposons on them facilitating the dissemination of antibiotic resistance gene cassettes in different species of bacteria (Fig. 3c). In enteric pathogens *V. cholerae* and *Shigella dysenteriae*, the multidrug resistance plasmids have been responsible for MDR thus complicating the treatment of diarrhoeal diseases (Ries et al. 1994; Sack et al. 2001). In another pathogen *V. fluvialis*, plasmids have been shown to confer resistance to a large number of drugs (Rajpara et al. 2009; Singh et al. 2012).

Role of Environmental Factors in Evolution of MDR

The presence of an antibiotic in the environment accentuates the appearance of bacteria resistant to this antibiotic. Often, there is a direct correlation between the antibiotic consumption and the appearance of strains resistant to that antibiotic. Antibiotics promote evolution of MDR by the random genetic drift or by induction of large mutational events selecting for the survival of resistant bacteria (Baquero et al. 1998). Random genetic drift occurs during the crisis situations where the random variations acquired by the bacteria may improve the chances of

bacterial survival. Apart from antibiotic usage, other environmental factors such as epidemiological features, other drugs being used at the time of study, host immunity and pollutants present in an environment also induce selective pressures for the development of MDR (Baquero et al. 1998). The resistant strain may have higher possibility of surviving in an immunocompromised host as compared to an immunocompetent host. Similarly, the presence of some other non-antibiotic drugs could alter the expression of porins or efflux pumps of bacteria eventually affecting the antibiotic concentrations inside the bacterial cell. This will lead to a change/evolution in the resistance phenotype of this bacterium. For example, drugs such as salicylate lead to the increase in efflux pump expression. Cumulative effect of all these environmental factors allows the survival of bacteria which have acquired the mutations to face the antibiotic pressure. These factors also promote the proliferation and dissemination of such novel bacterial mutants. Profligate use of antibiotics in all the spheres of life including human health, veterinary medicine, food industry and aquaculture actually seems to have provided selective pressures for the evolution of MDR in frightening proportions as we witness it today. Each new generation of antibiotics has spawned new generations of bacterial proteins/mechanism to thwart the effect of antibiotics. As described in Sect. "Extended spectrum β-lactamase (ESBL)-producing bacteria", when new β-lactams such as cefotaxime were produced to face the challenges imposed by early β-lactam-resistant bacteria, the β-lactamases acquired some additional mutations to inactivate these newer drugs. Higher mutated variants like TEM-10 of the β-lactamase TEM were evolved to provide higher resistance. Especially interesting is the scenario in intensive care units where multiple antibiotics are used at varying concentrations for different patients and different pathogens. This leads to selection of a large number of MDR bacteria due to the vast availability of resistome (the population of resistance genes in nature) with the potential to get incorporated into the genome of any bacterial cell and to express the trait.

Case Studies on the Evolution of MDR Bugs

As described in the earlier sections, the evolution of MDR bugs is mediated by the processes such as HGT, selective pressure and SOS response, and these processes are induced by several genetic and environmental factors. The antibiotic era has witnessed the evolution of several resistant bugs, and the following examples can explain the evolution of MDR bugs as a result of interplay of the above-mentioned factors.

Methicillin-Resistant *Staphylococcus aureus* (MRSA)

The rise of MRSA could explain the extraordinary ability of bacteria to evolve as a menace for public health. *S. aureus* is an omnipresent bacterium mostly found in the human nostrils and skin. They often cause respiratory diseases (e.g. nosocomial pneumonia) and skin diseases (e.g. impetigo) in humans. During early 1940s, the infections caused by this bacterium were treated using penicillin as they were extremely sensitive to these wonder drugs at that time. But soon the upsurge of penicillinase-mediated penicillin-resistant strains of *S. aureus* led to the arrival of an alternative drug, methicillin, a semisynthetic penicillin. Methicillin with a power to resist the penicillinase action came to therapeutic use in 1959. But within a short time span, the first case of MRSA was reported. The spectacular mechanism of resistance exhibited by MRSA to fight methicillin was a new penicillin-binding protein, PBP2a. PBPs, the targets of penicillin, methicillin and other β-lactams, are transpeptidases which are responsible for the cross-linking of the cell wall of bacteria. But the new variant of PBP, PBP2a, has low affinity for methicillin and other β-lactams and could substitute the role of native PBPs for cell wall formation (Fig. 1a). PBP2a was encoded by *mecA* gene which is a distinctive feature of MRSA and hence the methicillin resistance. The *mecA* gene was found in the chromosome of

MRSA but associated with a large mobile genetic element called staphylococcal chromosome cassette [SCC] (Pantosti and Venditti 2009). The *mecA* gene seemed to have originated from *Staphylococcus sciuri* and then got incorporated into SCC to become SCC*mec* (Fig. 1a). The successful acquisition and expression of SCC*mec* in *S. aureus* gave rise to the strain of MRSA (de Lencastre et al. 2007). The first MRSA clone appeared in the 1960s, spread widely in the hospitals and clinical settings for about 17 years and new clonal types with different SCC*mec* elements were reported subsequently. The epidemic hospital-acquired MRSA (HA-MRSA) clones reported so far mainly fall into three types (type I, II and III) based on the multilocus sequence typing (MLTS) method. Subsequent to the acquisition of SCC*mec*, MRSA further evolved to resist other classes of antibiotics such as aminoglycosides, tetracycline, sulphonamides and quinolones as a result of selective pressure on exposure to antibiotics and acquisition of various resistance genes through HGT. In the 1990s the enigmatic emergence of community-acquired MRSA (CA-MRSA) has been reported with different epidemiological and molecular profile than that of HA-MRSA. Initially CA-MRSA clones carried the single trait of *mecA* mainly in two SCC*mec* element types (type IV and V) and were susceptible to non-β-lactam antibiotics. But few typical CA-MRSA have been reported now to evolve as multidrug-resistant strains (e.g. USA 300 and ST80 clone) (Pantosti and Venditti 2009; de Lencastre et al. 2007).

Vancomycin-Resistant *Staphylococcus aureus* (VRSA) and Vancomycin-Resistant Enterococci (VRE)

Vancomycin served as a possible alternate therapy for the infections caused by the MRSA. Vancomycin inhibits cell wall synthesis by blocking the transglycosylation and transpeptidation reactions as it binds to the C-terminal peptide of D-Ala-D-Ala of pentapeptide precursor for the formation of bacterial peptidoglycan. The *van*

gene mediating vancomycin resistance was first observed in enterococci only. These genes are of seven types (*vanA*, *vanB*, *vanC*, *vanD*, *vanE*, *vanG*, *vanL*) which are known to synthesise a new target (peptidoglycan precursor) which replaces the normal D-Ala-D-Ala precursor, and hence, the antibiotic cannot find its target. The *vanA*-, *vanB*- and *vanD*-type genes produce the D-Ala-D-*Lac* target, whereas *vanC*, *vanE*, *vanG* and *vanL* gene types synthesise the D-Ala-D-Ser target. The acquisition of plasmid-borne *vanA* gene through conjugation from *Enterococcus* to *S. aureus* resulted in the development of VRSA. The evolution of *S. aureus* which were already resistant to multiple drugs into VRSA further complicated the treatment of infections caused by such bacteria (Perichon and Courvalin 2009).

Extended Spectrum β-Lactamase (ESBL)-Producing Bacteria

The emergence of β-lactamases served as a common mechanism of resistance for β-lactam antibiotics in Gram-negative bacteria. In the 1970s to 1980s, β-lactamases such as TEM-1, TEM-2 and SHV-1 that hydrolysed penicillin, ampicillin and early generation cephalosporins were detected. TEM-1 and TEM-2 were predominant in *E. coli* and SHV-1 was prevalent in *K. pneumoniae* (Chong et al. 2011). During the early 1980s, the emergence of modified β-lactamases carrying amino acid mutations in TEM-1, TEM-2 and SHV-1 enzymes was detected. As they were able to hydrolyse the third-generation cephalosporins such as cefotaxime, ceftriaxone, ceftazidime, cefuroxime and cefepime, apart from penicillin and ampicillin, they were termed as ESBLs. The TEM and SHV ESBLs were genetically evolved by amino acid substitutions from their non-ESBL progenitors TEM-1, TEM-2 and SHV-1, whereas another ESBL called CTX-M evolved independently of this lineage. Some other ESBLs different from TEM, SHV and CTX-M are OXA, BEL-1, BES-1, GES/IBC, SFO-1, TLA-1, TLA-2, PER and VEB enzyme families. ESBLs soon became pervasive and were reported all across the globe within two decades. So far more than 300 ESBLs have been described (Lynch et al. 2013). The increased incidents of dissemination of ESBL genes among bacteria through various MGEs which carry other antibiotic resistance genes have reduced the therapeutic options and caused an emerging threat to public health.

Quinolone-Resistant Bacteria

The increased drug resistance among bacteria towards various natural and semisynthetic antibiotics led to the introduction of synthetic drugs like quinolones and fluoroquinolones due to their broad spectrum of activity and possibilities of the absence of resistance mechanisms in bacteria to these synthetic drugs. Quinolones inhibit nucleic acid synthesis in bacteria by targeting DNA gyrase and topoisomerase IV enzymes which are involved in the essential activities of bacterial cell such as replication, transcription, recombination and repair. The mutations at the quinolone resistance determining regions (QRDRs) of subunits of DNA gyrase (GyrA, GyrB) and topoisomerase IV (ParC, ParE) enzymes cause their reduced affinity towards quinolones which lead to quinolone resistance phenotypes. Mutations that occur at the target sites of the antibiotics prior to the exposure of antibiotics help in selection and subsequent evolution of resistant bacteria. The accumulation of multiple mutations in the drug target facilitates the development of high-level resistance to quinolones in bacteria. The quinolone resistance can also be mediated by efflux pumps encoded by chromosome-borne genes such as *norM*, *norA*, *vcmA* and *bmrA* (Bhardwaj and Mohanty 2012). When quinolones were introduced, it was imprudently predicted that there would not be any quinolone resistance genes as these antibiotics were not naturally produced by any bacteria (Hernandez et al. 2011). Hence, the dissemination of resistance among bacterial communities through HGT was not expected. But the emergence of factors like target-protecting quinolone resistance proteins (Qnr), drug-modifying enzyme and plasmid-borne efflux pump genes falsified the latter belief.

Qnr proteins are pentapeptide repeat proteins which are believed to be evolved from the proteins like McbG that protect topoisomerases from the naturally occurring toxins like microcin B17, a topoisomerase poison. These pentapeptide repeat proteins occupy the DNA-binding region of the enzyme and protect them from the drug action. Though the Qnr proteins are of chromosomal origin, they are more often found in plasmids through which they tend to disseminate among different bacterial species. Similarly, as shown in Fig. 1b, a variant aminoglycoside acetyl transferase (AAC(6′)-Ib-cr) borne on plasmid was found to inactivate (by acetylation) ciprofloxacin and norfloxacin apart from aminoglycosides due to two amino acid changes (Trp102Arg and Asp179Tyr) in the active site of the enzyme (Robicsek et al. 2006). Two plasmid-mediated quinolone transporters (OqxAB and QepA) have been described to effectively efflux out quinolone antibiotics. All the above-mentioned resistance mechanisms may work alone or in synergy to combat the quinolone drugs. Apart from the mutation in the target sites, other genetic factors of quinolone resistance such as *qnr* genes, *aac(6′)Ib-cr* gene and *oqxAB* and *qepA* genes are often harboured by plasmids and cause plasmid-mediated quinolone resistance (PMQR). Though higher-level resistance through PMQR has not been reported, they could help the isolates to attain clinical breakpoint of resistance in combination with other mechanisms. Hence, the great plasticity of the bacterial systems allows them to educe their armaments to battle against these drugs.

Conclusions

Evolution of MDR is a very natural process, and from the discussions above, it can be concluded that the resistance determinants for antibiotics were always present in nature even before the miracle drugs were introduced for human use. These determinants just made their public appearances with the escalating use of antibiotics in many applications. With the selective pressures rising due to antibiotic use, the resistance genes kept appearing in their new avatars. Evolution of antibiotic resistance mechanisms paralleled evolution of antibiotics. Therefore, new strategies need to be used for keeping one-step ahead of the MDR pathogens. The advent of technologies based on microbial genomics, proteomics, combinatorial chemistry and high-throughput screening could lead to success stories in the development of new anti-infectives inspite of the large funds required for them. There are many innovative strategies being used to curb the problem of MDR (Breithaupt 1999; Tegos and Hamblin 2013). In pathogens *Neisseria gonorrhoeae* and *N. meningitides*, lipooligosaccharides on the bacterial surface have been shown to be crucial for their virulence-associated functions such as colonisation and immune evasion. The glycosyltransferases and hydrolases involved in the synthesis of these lipooligosaccharides have been used as targets for synthesis of small inhibitors as antibiotics. Companies such as TerraGen Diversity Inc. (Canada), ChromaXome Corp.(California) and GLYCODesign (Toronto) have been actively involved in the innovations pertaining to antibiotic research and development. Strategies of quorum-sensing inhibition and efflux pump inhibition also provide attractive alternatives to solve the problems of MDR (Bhardwaj et al. 2013; Kalia 2013; Bhardwaj and Mohanty 2012) though the possibility of evolution of resistance to these new molecules cannot be ruled out (Bhardwaj et al. 2013; Kalia et al. 2014). The governments should realise the seriousness of impending disaster due to MDR bacteria and urgency of the situation (Finch and Hunter 2006). Accordingly, new policies should be made to deal with this problem. In India, a national policy has been made for containment of antimicrobial resistance by the Directorate General of Health Services (2011), and a task force was constituted to work on various aspects related to antimicrobial resistance and its monitoring.

Opinion

From the concepts and facts presented in this chapter and reiterated throughout this book, it would be amply clear that the problem of