Antibiotic resistance: an overview of mechanisms and a paradigm shift

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Antibiotic resistance is the biggest challenge to the medical profession in the treatment of infectious diseases. Resistance has been documented not only against antibiotics of natural and semi-synthetic origin, but also against purely synthetic compounds (such as the fluoroquinolones) or those which do not even enter the cells (such as vancomycin). The wide range of occurrence of antibiotic resistance suggests that, in principle, any organism could develop resistance to any antibiotic. The phenomenon of horizontal gene transfer compounds the problem by facilitating rapid spread of antibiotic resistance. Unfortunately, the discovery and development of newer antibiotics have not kept pace with the emergence of antibiotic resistance. In this article a broad overview of the various mechanisms of antibiotic resistance will be presented mainly to illustrate their variety, rather than to catalogue everything that is known. Of late, a paradigm shift in the traditional perception of antibiotics and antibiotic resistance is emerging. Antibiotics are beginning to be viewed as intermicrobial signalling agents and even as sources of nutrition to microorganisms, rather than as weapons in the hands of antibiotic producer organisms to fight against competitors which might cohabit with them. Likewise, mechanisms of antibiotic resistance are being believed to have evolved not as defence strategies which microbes use to thwart the action of antibiotics, but as integral components of processes involved in basic bacterial physiology. However, when these mechanisms get placed out of their natural context their resistance property alone gets highlighted. Many leading workers in the field call this emerging trend of thought as a Copernican turning point. Some of these trends will be discussed towards the end.

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THE quest for antibacterial agents for the treatment of infectious diseases began towards the end of the 19th century and early 20th century. One of the early successes was the discovery of Salvarsan (an arsenical, arsphenamine, discovered by Paul Ehrlich in 1910). Salvarsan was used as an anti-syphilitic drug during the World War I. Another remarkable early antibacterial drug was Prontosil (a conjugate of sulphanilamide with an aromatic dye), discovered by Gerhard Domagk. Prontosil is the progenitor of the family of sulpha drugs which are in use even today, either alone or in combination with other antibacterials. It is documented that one of the early patients to be treated with Prontosil was Domagk's own daughter, who was critically ill with a streptococcal infection and made a miraculous recovery after receiving the drug. While Ehrlich was already a Nobel laureate when he discovered Salvarsan, Domagk was awarded the Nobel Prize in 1939 for the discovery of Prontosil. (However, he could not receive the Prize because of the political climate of Nazi Germany at that time.) The discoveries of Salvarsan and Prontosil mark the beginnings of the age of chemotherapy of bacterial infectious diseases. The real breakthrough occurred in the late 1930s and early 1940s when penicillin and streptomycin were discovered by Alexander Fleming and Selman Waksman respectively.

The discovery of the two progenitor antibiotics is an event of greatest significance in the history of medicine and a blessing to humanity. The clinical use of antibiotics has mitigated lot of suffering and has saved (and continues to do so) millions of lives from certain death. There was also an unprecedented development of the pharmaceutical and health-care industries. However, there was also an unanticipated and undesirable consequence, namely the emergence and spread of antimicrobial resistance which impedes the efficacy of treatment, escalates the costs and often results in treatment failure. As more and more antibiotics were discovered, the emergence of drug resistance also kept mounting. Another confounding problem was/is the phenomenon of multidrug resistance (MDR). When a pathogen acquires resistance to a given antibiotic and gets selected after prolonged use of that antibiotic, it becomes a favoured host to acquire resistance to several others. Pulmonary tuberculosis which was once considered to have been vanquished by the use of streptomycin, rifampin, isoniazid, etc. emerged in the MDR form in the 1980s. When synthetic antimicrobials such as the fluoroquinolones were introduced, it was hoped that the bacteria would not develop resistance to synthetic compounds; however, such hopes were short-lived since bacteria did develop resistance soon. Another unexpected case is the development of resistance to vancomycin (a glycopeptide) which binds noncovalently to the cell walls of

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Gram-positive bacteria and blocks an essential step in cell-wall synthesis which gives rigidity to the cell walls (see below). Since the antibiotic acts on the exterior of the cell and no proteins or other intracellular factors are involved. It was hoped that vancomycin resistance would not occur, but it did (see below). Worse still, vancomycin resistance from enterococci got transferred to another notorious 'superbug' methicillin-resistant *Staphylococcus aureus* (MRSA), to give rise to vancomycin-resistant *Staphylococcus aureus* (VRSA), which is even more deadly. Recent reports of the emergence of MDR in some opportunistic pathogens such as *Acinitobacter baumanni* are alarming.

The history of antibiotic resistance coincides with the history of antibiotics themselves. Ironically, penicillin resistance was discovered even before penicillin was put to clinical use[1]. Reports of resistance to penicillin and streptomycin started appearing soon after they were introduced into clinical medicine[2,3]. Watanabe[4] discovered MDR in enteric bacteria brought about by the resistance transfer factors (RTFs) which are autonomous, extra-chromosomal and often self-transmissible plasmids. The number of antibiotics belonging to various families, their varied modes of action and the number of bacteria in which antibiotic resistance has been documented suggest that, in principle, any microbe could develop resistance to any antibiotic. The phenomenon of antibiotic resistance has been covered in several reviews[5–9]. A monograph dedicated to Stuart Levy has also appeared[10]. Some modern approaches to the prediction of antibiotic resistance have been suggested[11,12]. Smith and Romesberg[13] have suggested some possibilities that could be explored to tackle the problem of drug resistance. In addition, there are several reports dealing with particular antibiotics and/or particular mechanisms of resistance. Some of these will be discussed below.

An antibiotic has to go through a number of steps in order to exert its antibacterial action. First of all it has to enter the cells (influx). Once inside, it has to remain stable and accumulate to inhibitory concentrations. In some cases it has to be activated to an active form. Finally it has to locate and interact with its target(s) to exert its action. Alterations in any one or more of these processes can render the cells resistant to the antibiotic. All possible alterations have been realized in clinical as well as laboratory isolates of resistant bacteria. In the following pages a broad overview of the mechanisms of antibiotic resistance will be presented. Towards the end, some current notions on antibiotics and antibiotic resistance from a biological perspective will be presented and discussed.

Antibiotic resistance by influx–efflux systems

Bacterial cells have an intrinsic capacity to restrict the entry of small molecules. This property is more pronounced in Gram-negative bacteria, whose outer membrane provides an effective barrier and constitutes a first-line defence against antimicrobial challenge. Gram-positive organisms lack the outer membrane and hence lack this front-line defence. This is perhaps one of the reasons for their high sensitivity to many antibiotics. However, Gram-positive organisms also restrict antibiotic influx by physiological means[9]. It has been estimated that *Escherichia coli* has a large number of genes (~600) encoding small molecule transport proteins[14]. Intuitively, it is obvious that restriction of influx might not be specific to any one or any given family of antibiotics. It might be a generalized mechanism to protect cells from many toxic chemicals, including antibiotics. Moreover, restriction of influx could only delay the onset of toxicity, but is not enough to afford resistance. On the other hand, activation of efflux pumps has been observed to be a major means of antibiotic resistance in many bacteria and with many antibiotics. The efflux systems pump out the antibiotics that have managed to enter into the cells, thereby preventing their intracellular accumulation[15–19]. The most well-studied efflux system in *E. coli* is the AcrAB/TolC system. This system comprises of an inner membrane protein, Acr B, and an outer membrane protein, Tol C, linked by a periplasmic protein, Acr A. When activated, the linker protein is believed to fold on itself, bringing the Acr B and Tol C proteins in close contact, thus providing an exit path from the inside to the outside of the cell. Antibiotics are pumped out through this channel (see Normark and Normark[6], for details). In antibiotic-sensitive cells, the AcrAB/TolC system is under repression by the product of *acrR* gene. A mutation in *acrR*, causing an arg45cys change, activates expression of the system and consequent drug efflux[20]. Detailed information on antibiotic efflux systems and mechanisms are available in the reviews cited above. A few illustrative examples are described below. Resistance to fluoroquinolones and tetracyclines occurs by efflux mechanisms, although target mutations are also commonly encountered in fluoroquinolone resistance[21]. In the case of the transposon Tn10 which confers tetracycline resistance, the gene for the tetracycline efflux protein, *tetA*, is kept under repression by the tetracycline-sensitive repressor protein encoded by the *tetR* gene. Exposure to tetracycline inactivates the repressor and leads to *tetA* expression and efflux of the antibiotic.

Nine proton-dependent efflux pumps have been identified in *E. coli* so far[19]. These cause the efflux of many (two or more) antibiotics leading to MDR. They are classified into three major families: major facilitator super family (MFS), resistance nodulation cell division family (RND) and small multidrug resistance family (SMR). Of these, the major efflux pumps belong to the RND family. The AcrAB–TolC system is the most extensively studied member. The efflux pump systems are also regulated by the *marAB* global regulon[22,23]. Inactivation of the *marR*-

encoded repressor triggers the expression of *marA*, which, in turn, upregulates many efflux pumps and other genes and down regulates porin synthesis. Little is known about the Mar B protein.

Antibiotic resistance by chemical alteration of antibiotics *in vivo*

Some drugs need to be activated *in vivo* (usually by reduction) in order to elicit their biological activity. The cytotoxic antitumour compound, mitomycin C, is a well-known example. Among the antibiotics, members of the nitrofuran family (nitrofurantion, nitrofurazone, nitrofurazolidone, etc.), used in the treatment of urinary tract infections, are reduced by cellular reductases encoded by *nfsA* and *nfsB* genes. Mutations in these genes are associated with nitrofuran resistance[24–27]. In contrast to resistance developing due to inhibition of activation described above, in many cases chemical alterations of antibiotics inactivate their biological activity and lead to resistance. The classical example of this mode of resistance is the action of β-lactamase enzymes which cleave the β-lactam ring of penicillin, cephalosporin, etc. The number of β-lactamases identified so far runs into hundreds. They have been classified into a number of groups and subgroups based on structure and function[28]. The enzymes discovered early (the TEM-1, TEM-2 and SHV-1 β-lactamases) were capable of inactivating penicillin but not cephalosporin. But subsequent variants with a variety of amino acid substitutions in and around their active sites were identified in many resistant organisms. These have been collectively called 'extended spectrum β-lactamases (ESBLs)' and act on later generation β-lactam antibiotics also (reviewed by Bradford[29]). The early β-lactamases were sensitive to inhibitors such as clavulanic acid and sublactam. These compounds were incorporated in therapeutic formulations to inhibit β-lactamase activity and restore penicillin sensitivity (as a precaution in case the infection happens due to a resistant organism). However, some of the ESBLs are insensitive to these inhibitors. However, they are sensitive to another inhibitor called tazobactam. While most of the ESBLs are derivatives of the early enzymes, some are new[29]. Newer families of ESBLs have been discovered recently and are causing much concern. Notable among these are cefotaximases (CTM-X enzymes)[30–32]. The most potent among the members of the β-lactam family are the carbapenems (imipenem, meropenem, panipenem, ertapenem, etc.), which have a broad antibacterial spectrum, including ESBL-producing pathogens, and are used in the therapy of infections that are not controlled by other members of the family (reviewed by Shah[33]). Recently, carbapenemases, contributing to carbapenem resistance, have been discovered[34–36]. The CTM-X genes are believed to have descended from progenitor genes present in *Klyuvera* spp.[37–40]. β-Lactam resistance contiues to be a problem which has not yet been conquered. In a recent report, Lloyd *et al.*[41] have described differences in the cell walls of penicillin-sensitive and resistant strains of *Streptococcus pneumoniae*, which could be exploited in future to tackle penicillin resistance. Another important source of resistance to β-lactam antibiotics is the family of penicillin-binding proteins (PBPs). A discussion of their contribution is deferred to a later section of this review.

Like the β-lactam antibiotics, chloramphenicol is also inactivated by an enzymatic mechanism, namely acetylation (reviewed by Schwarz *et al.*[42]). This is the most common mechanism by which pathogens acquire resistance to chloramphenicol. Mosher *et al.*[43] have shown that O-phosphorylation of chlorophenicol affords resistance in *Streptomyces venezuelae* ISP 5230, which is a chloramphenicol-producing organism. Mechanisms of resistance to aminoglycosides (streptomycin, neomycin, amikacin, tobramycin, etc.) have been well studied and documented. A common mode of resistance in *Pseudomonas aeruginosa* and many other Gram-negative organisms are inhibition of drug uptake. This is a chromosomal mechanism and gives cross resistance to many aminoglycosides. Having many NH_2 and OH groups in the molecule, aminoglycosides offer scope for inactivation involving these moieties. Several aminoglycoside inactivating enzymes have been detected in many bacteria and plasmids[44,45]. Inactivation occurs through acylation of NH_2 groups and either phosphorylation or adenylation of the OH groups. Interestingly, the amino and hydroxyl groups in aminoglycosides have also been exploited by appropriate modifications not only to protect them from enzymatic inactivation, but also to expand their antibacterial activity. For example, chemical modification of kanamycin A by acylation of the C-1 amino group of the deoxy-streptamine moiety with γ-amino α-hydroxy butyric acid gives rise to amikacin, which is a better antibiotic than its parent in terms of its antibacterial spectrum as well as activity against aminoglycoside-resistant organisms[46]. Recently, a plasmid-mediated mechanism of aminoglycoside resistance involving methylation of 16S ribosomal RNA has come to light (reviewed by Doi and Arakawa[47]). The literature on amino glycoside resistance is vast. A few selected references are: Shaw *et al.*[48], Davies and Wright[49], Mingeot-LeClerc *et al.*[50], Magnet and Blanchard[51], Davies[52], Sakhya and Wright[53], Shakil *et al.*[54], and Courvalin[55].

Fluoroquinolones (ciprofloxacin, norfloxacin, ofloxacin, etc.) are a group of synthetic antimicrobials which target the DNA gyrase and topoisomerase IV enzymes leading to inhibition of DNA replication. As pointed out earlier, it was (vainly) hoped that resistance to synthetic compounds might not occur, but resistance to fluoroquinolones is common now. By and large, fluoroquinolone resistance occurs through mutations in the genes coding for the subunits of DNA gyrase (*gyrA* and *gyrB*) and topoisomeraseIV (*parC* and *parE*). Drug efflux is also a common mechanism. Quite often a combination of both

the mechanisms is seen[15–17,21,56]. Two recent reports[57,58] showed that a gene encoding an aminoglycoside-specific acetylase could mutate further to give an enzyme which will inactivate fluoroquinolones also. This is an example to show that genes encoding minor and perhaps unrecognized activities, besides the major activity, could mutate further to gain extended activity and could be selected by appropriate selection pressures. While the early reports on quinolone resistance were shown to be associated with mutations in the gyrase–topoisomerase genes, plasmid-mediated quinolone resistance has come to light recently. Two proteins, QnrA and QnrB, have been shown to bind to gyrase and protect it from inactivation by quinolones[54,59,60].

Type A and type B streptogramins which are cyclic polyketide–amino acid hybrids and cyclic depsipeptides respectively, inhibit translation by synergistic binding to the 50S ribosomal subunit[9]. Resistance to type A streptogramin has been found to be mediated by an enzyme called VatD (virginiamycin acetyl transferase), which acetylates the antibiotic[61,62]. Resistance to type B streptogramin is brought about by the product of the *vgb* gene, a C–O lyase[63]. Homologues and orthologues of the genes encoding both the enzymes have been detected in a variety of non-pathogenic bacteria, environmental bacteria and plasmids[9]. Few other streptogramins, such as quinupristin and dalfopristin, are used to treat vancomycin-resistant *Enterococcal* infections. Resistance to these compounds has been reported to occur in *Enterococcus faecalis* by efflux mechanisms[64].

Antibiotic resistance due to target alterations

Antibiotic resistance stemming from alterations in the target(s) of the drugs in such a way as to counter their toxic effects is common in pathogens and non-pathogens. The involvement of PBPs in penicillin resistance has been briefly mentioned earlier. The PBPs are trans-peptidases which catalyse the crosslinking reaction between two stem peptides, each linked to adjacent *N*-acetyl-muramic acid residues of the peptidoglycan backbone. This reaction which crosslinks the penultimate D-alanine residue of one peptide (the donor) with the third L-lysine residue of the next peptide (the acceptor) and elimination of the ultimate D-alanine of the donor, is responsible for conferring rigidity to the cell wall. Penicillin and other related antibiotics which are structurally similar to the D-ala–D-ala dipeptide form fairly stable covalent complexes with PBPs and thereby inhibit the crosslinking reaction, resulting in the weakening of the cell wall and ultimate lysis of the cell. Many mutational changes in PBPs have been shown to result in penicillin resistance. Some of them are: reduction in the affinity of PBPs to penicillin, over expression of endogenous, low-affinity PBPs encoding genes, etc. These have been reported and reviewed extensively[65].

Fluoroquinolone resistance resulting from *gyrA*, *gyrB*, *parC* and *parE* mutations has been mentioned earlier. As many as eight amino acid substitutions in *gyrA* and two in *gyrB* have been shown to cause fluoroquinolone resistance. Likewise three sites in *parC* and one in *parE* have been identified. The *gyrA* mutations are located predominantly in the quinolone resistance determining region (QRDR) of the protein. Ruiz[21] has reviewed quinolone resistance in detail (see also Courvalin[55]). Other examples of antibiotic resistance due to target alterations are the resistance to rifamycins, streptomycin, vancomycin and linezolid. These are briefly described below. Rifampin (also called rifampicin, a semi-synthetic rifamycin) is a frontline drug in the combination chemotherapy of tuberculosis. It is also extensively used in the treatment of many other bacterial infections, including those caused by MRSA. Rifampicin resistance in *Mycobacterium tuberculosis* and other pathogens and non-pathogens has been well documented. Rifampicin-resistant strains are mutated in *rpoB*, the gene encoding the β-subunit of RNA polymerase. Such mutations are also common in laboratory strains of *E. coli*[66–69]. Streptomycin has been and continues to be a frontline drug in the treatment of tuberculosis. In addition to the enzymatic mechanisms of aminoglycoside resistance discussed earlier, mutations in genes encoding the proteins of the 30 S ribosomal subunit, especially the S12 protein encoded by *rps*L, are common in *E. coli* and other organisms (for references see the earlier section on aminoglycoside resistance).

Enterococci as well as *Staphylococcus aureus* have been shown to acquire resistance to the glycopeptide antibiotic vancomycin[70–72] by a strategy which is known as antibiotic evasion. As mentioned at the outset, vancomycin binds non-covalently to the cell-wall precursors of Gram-positive bacteria. Specifically, the binding occurs through a set of five hydrogen bonds between the antibiotic and the *N*-acyl-D-ala–D-ala dipeptide portion of the stem pentapeptides linked to the *N*-acetyl muramic acid backbone. This binding blocks the crosslinking transpeptidase reaction catalysed by the PBPs (see above). Consequently the cell walls are rendered less rigid and more susceptible to lysis. In vancomycin-resistant organisms, the stem peptides terminate in D-lactate as against D-alanine in the sensitive strains. (Essentially an NH is replaced by an O.) This eliminates the formation of a crucial hydrogen bond and results in a 1000-fold decrease in the affinity for vancomycin and consequent resistance to the same. This process is regulated by a two-component regulatory system involving a set of five genes (*vanR*, *vanS*, *vanH*, *vanA* and *vanX*; for details on the mechanism of vancomycin resistance, see the reviews cited earlier and also Wright[9]). Interestingly, the same mechanism is used by vancomycin-producing bacteria also[73] (see Wright[9] for more information). Thickening of the cell wall has also been shown to confer vancomycin resistance[74].

Table 1. Some representative antibiotics, their modes of action and mechanisms of resistance

Category	Some members	Mode of action	Major mechanisms of resistance
β-Lactams	Penicillins, Cephalosporins, Cefotaximes, Carbapenems	Inhibition of cell-wall synthesis	Cleavage by β-lactamases, ESBLs, CTX-mases, Carbapenemases, altered PBPs
Aminoglycosides	Streptomycin, Gentamycin, Tobramycin, Amikacin	Inhibition of protein synthesis	Enzymatic modification, efflux, ribisomal mutations, 16S rRNA methylation
Quinolones	Ciprofloxacin, Ofloxacin, Norfloxacin	Inhibition of DNA replication	Efflux, modification, target mutations
Glycopeptides	Vancomycin	Inhibition of cell-wall synthesis	Altered cell walls, efflux
Tetracyclines	Tetracycline	Inhibition of translation	Mainly efflux
Rifamycins	Rifampin (Rifampicin)	Inhibition of transcription	Altered β-subunit of RNA polymerase
Streptogramins	Virginiamycins, Quinupristin, Dalfopristin	Inhibition of cell-wall synthesis	Enzymatic cleavage, modification, efflux
Oxazolidinones	Linezolid	Inhibition of formation of 70S ribosomal complex	Mutations in 23S rRNA genes followed by gene conversion

Linezolid, which is widely used against many Gram-positive pathogens, including MRSA, is a member of the oxazolidinone family. It inhibits protein synthesis by preventing the formation of the initiation complex[75]. Resistance to linezolid involves any one of the genes encoding the 23 S ribosomal RNA. Since there are multiple copies of them, mutation in any one gene is not enough to confer resistance. Intrachromosomal recombination (gene conversion) is necessary for the full manifestation of a resistant phenotype[8].

The list of antibiotics and resistance mechanisms is long. This review is not intended to present a comprehensive catalogue of everything that is known. Only a brief overview, illustrating the variety of mechanisms of antibiotic resistance and giving a flavour of the subject, has been presented above. For more information, the original papers and reviews cited above and the cross references therefrom have to be consulted. Secondly, the mechanism of spread of antibiotic resistance, namely horizontal gene transfer, is beyond the scope of this review and will not be discussed here. Table 1 presents a brief summary of some of the classes of antibiotics, their modes of action and some of the common resistance mechanisms.

Non-heritable antibiotic resistance

Some physiological states render bacteria insensitive to antibiotics. In general, slow growing or non-growing bacteria are less sensitive to antibiotics than actively growing cells. This property has been called drug indifference. It is a property shown by the whole population and so far no specific mechanism has been attributed to it. Nevertheless, drug indifference has been shown *in vivo* using animal infection models[76]. However, there are some specific physiological states in which bacteria show high antibiotic resistance. Strictly speaking, it is not correct to describe such states as resistant because they are transient, reversible and non-heritable; instead, it would be more appropriate to call them antibiotic-tolerant states. Three such antibiotic-tolerant states have been described, namely persistence, biofilms and swarming. These are briefly described below.

Persistence

The phenomenon of persistence has been known for more than six decades, but the underlying molecular mechanisms are still obscure. Antibiotic-sensitive bacterial populations have a small fraction ($\sim 10^{-6}$) of slow or non-growing, antibiotic-tolerant cells called persisters. How antibiotic-tolerant persisters arise only in a small fraction of the population while the majority of cells remain antibiotic-sensitive has been the subject of intense investigation for the past 30 years. It is currently believed that persistence is the end result of a stochastic switch in the expression of some toxin–antitoxin (TA) genes, occurring in a small fraction of the population of cells, resulting in an imbalance in their intracellular levels and also the involvement of the alarmone (p)ppGpp in an unknown manner in the process. Detailed information on persistence is available in Jayaraman[77] and other references cited therein.

Biofilms

In contrast to the traditional notion that bacteria are free-living and free-swimming organisms (planktonic), many

bacterial species in nature exist as organized structures called biofilms, which consist of a self-produced exopolysaccharide matrix in which bacterial cells (often cells of several species or consortia) are embedded. They are highly organized, surface-adherent structures and permit the transport of nutients and metabolic waste in and out. Many clinical infections such as gingivitis, otitis media, lung infections (in cystic fibrosis patients), etc. are attributed to biofilms[78]. Several reviews on biofilms are available[79–81]. One of the characteristic properties of biofilms is their tolerance to very high concentrations of antibiotics[82–87]. The apparent antibiotic resistance of biofilm-aassociated cells is not due to mutations, since sensitivity reappears when the biofilms are disrupted and the cells are returned to the planktonic state. Moreover, it has been shown that biofilm-associated cells are sensitive to several antibacterial agents (fluoroquinolones, metal oxyanions, etc.)[88–90]. Brooun *et al.*[91] showed that even at very high concentrations of ofloxacin (a fluoroquinolone), a tiny fraction of cells in the biofilm survived. Based on these observations, Lewis[85] suggested that antibiotic tolerance of biofilm-associated cells could be due to the presence of antibiotic-insensitive persisters (see above) in the biofilms. According to the model of Lewis, antibiotic treatment of biofilms eliminates most of the embedded cells, except the persisters which repopulate the matrix after the antibiotic is withdrawn, yielding a mixture of antibiotic-sensitive cells (majority) and antibiotic-tolerant persisters (minority). This process repeats itself after successive antibiotic exposures. This way the infection persists in spite of antibiotic therapy. Persisters may not be the sole determinants of antibiotic tolerance in biofilms; other factors such as reduced antibiotic influx due to the matrix, and lower metabolic and growth rates of cells in the biofims could also be involved[92,93].

Swarming

This is a form of multicellularity in many bacterial species characterized by the migration of highly differentiated cells (swarm cells) on semi-solid surfaces. Planktonic cells first differentiate into long, multi-flagellated swarm cells, remain in close contact with one another and migrate as a raft[94,95]. When swarm cells are subcultured in liquid media, they revert to the planktonic state. Multiple antibiotic resistance has been shown in swarm cells of *Salmonella typhimurium*[96]. In a recent report, Lai *et al.*[97] have shown that swarm cells of *E. coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Burkholdia thailandensis* and *Serratia marcescens* tolerate exposure to very high concentrations of antibiotics of many classes. As is the case with biofilms, the antibiotic tolerance of swarm cells is completely reversible when the swarm cells return to the planktonic state. The authors have hyothesized that antibiotic tolerance might be a general feature of multicellularity.

Antibiotics and antibiotic resistance genes: a paradigm shift

It is customary to think of antibiotics as 'good' for humans and animals, since they are useful as therapeutic agents and 'bad' for bacteria because they either kill them or inhibit their multiplication. Therefore, it is generally believed that bacteria try to get rid of antibiotics by all means at their disposal in order to survive and gain an upper hand during the infection process. This view is undergoing drastic changes in recent times. Antibiotics have been around for hardly 60 odd years. However, antibiotic biosynthetic pathways have evolved millions of years ago[98] and have been maintained till date. Enzymes such as β-lactamases are believed to be billions of years old[99]. Therefore, antibiotics and their resistance mechanisms must have some biological use other than what humans have put them to. The multiplicity of efflux pumps, most of them non-specific, in many organisms, the demonstration of antibiotic resistance in non-clinical settings with no prior antibiotic exposure (see below), the wide prevalence of resistance genes and enzymes in non-pathogens and antibiotic producers, etc. have raised several interesting biological questions, which many workers have begun to address lately. A brief overview of this trend is presented below.

Studies on antibiotic resistance have focused, by and large, on pathogenic bacteria because of their clinical importance. According to Wright[9], this is a narrow viewpoint; a broader view should include the pan-microbial genome which comprises of resistance genes in pathogens, non-pathogens, antibiotic producers, cryptic genes, precursor genes, etc. D'Costa *et al.*[100] and Wright[9] have coined the term 'antibiotic resistome' to denote the pan-microbial genome (with respect to antibiotic resistance). In an elegant study, D'Costa *et al.*[100] examined a library of ~500 actinomycetes, isolated from a variety of soils (urban, agricultural and forest: the soil resistome) for resistance to 21 antibiotics of natural, synthetic and semi-synthetic origin. Their remarkable observation was that every strain, without exception, exhibited MDR to at least two antibiotics; some were resistant to even 15 and on the average 7–8 antibiotics. A good fraction (5–80%) of the isolates that were screened inactivated many antibiotics tested after 48 h exposure. The importance of this report lies in the fact that soil microbes have proved to be an under-appreciated source of antibiotic resistance genes. Since the majority of microbes in the soil or other environments are unculturable, the resistance potential of the environments should be even greater. Horizontal transfer of resistance genes from environmental organisms to pathogens is a frightening possibility.

Orthologous and homologous genes for many antibiotic inactivating enzymes occur in many bacterial genera and species, covering pathogens, non-pathogens and antibiotic producers[9]. Some examples are the genes for vacomycin resistance, virginiamycin resistance, aminoglycoside acetylase, etc. Even non-antibiotic producers harbour genes for resistance. Sequence and structural similarities between genes/proteins for known/unknown functions and antibiotic resistance entities have been observed[9], suggesting that the former could evolve into the latter. The case of a gene for an aminoglycoside acetyltransferase mutating to gain the ability for fluoroquinolone inactivation[57] has been described earlier. All the above reinforce the notion that we have a great deal more to learn about antibiotic resistance from studying non-pathogenic and environmental organisms rather than focusing only on clinically relevant ones.

Many of the soil microbes, especially the actinomycetes, are antibiotic producers. As early as 1940, Selman Waksman, the discoverer of streptomycin, suggested that the ecological role of antibiotics might be to prevent the growth of competitors which might share the niche along with antibiotic producers. The producers, in turn, will have to have self-protective mechanisms in the form resistance to the antibiotics that they produce. In agreement with this idea, Benveniste and Davies[101] showed similarities between antibiotic resistance genes in pathogens and producer organisms. The neighbours which coexist with the producers have to evolve protective mechanisms of their own (or receive them by horizontal gene transfer) in order to ward-off the danger posed by antibiotics secreted into the environment by the producers. No doubt the secreted antibiotics will inhibit the growth of competitors, but whether the secretion would be sufficient to reach inhibitory levels in natural environments is uncertain. Since antibiotics were sought, discovered and developed either as bacteriostatic or bactericidal compounds for therapeutic use, it has been customary to use them at or above the MIC against target organisms in experimental work. However, Linares *et al.*[102] have shown that at sub-inhibitory concentrations, antibiotics could have very different effects, such as up- or down-regulation of the expression of several genes, and these effects could be relevant to many bacterial properties. Many workers now believe that at low concentrations (sub-MIC levels), feasible in real-life situations, antibiotics have biological activities other than being mere antibacterial compounds (see refs 103–105 for review). They display what has been called hormesis, that is, contrasting activities at low concentrations compared to high concentrations. In general terms, many studies have shown that there is a concentration-dependent modulation of transcription by antibiotics (see the reviews cited above). The effects are specific for the antibiotic and the organism. Although at inhibitory concentrations the effects might be the same or similar, at low concentrations there is no overlap in the pattern of transcriptional modulation[106]. These observations lead to the conclusion that the utility of antibiotics as antibacterial compounds might not be their real biological function[103,104].

Some interesting ideas that are gaining popularity of late concern the role of efflux pumps in many organisms. It seems that so many of them are neither required nor do all of them function in antibiotic efflux alone[107]. Their role could be the general detoxification and efflux of toxic metabolic intermediates, wastes, biocides, detergents, dyes, etc. as well as to serve as determinants of virulence, signal trafficking, etc. (see refs 108, 109 for review). Similarly, the antibiotic modifying enzymes may even enable organisms to use antibiotics as a source of food[110]. The above authors isolated organisms belonging to many phyla, some closely related to human pathogens, from 11 kinds of soils and screened them for growth on antibiotics. A surprising finding was that a majority of them were able to grow on antibiotics as the sole source of carbon! All the isolates could grow on penicillin, carbenecillin, ciprofloxacin and vancomycin. Many isolates could utilize at least 10–12 of the 18 antibiotics tested for growth. Therefore, it seems that the enzymatic modification of the antibiotics is not a mere resistance mechanism, but a step in some, perhaps unidentified, metabolic pathway which could serve basic physiological needs/functions. The lurking danger is that picking up the genes for antibiotic modifying enzymes from the soil microbes onto plasmids or integrons would place them out of context, such that their natural functions would be masked and the resistance property alone would be apparent. Moreover, the controls which regulate their expression in their natural hosts would be lost when they exist out of context. This suggests that in their natural hosts and in the genetic set up in which they evolved, their function may not be antibiotic resistance. They elicit this property only when placed out of context. In evolutionary terms, this is an example of what is known as co-option or exaptation in which a character shaped by natural selection for a given function is utilized (co-opted) for a different one[110].

Many microbes have an intrinsic (innate) resistance, that is, low sensitivity to antibiotics. A well-studied organism in this respect is *Pseudomonas aeruginosa*, a free-living, opportunistic pathogen thriving in many kinds of environments. Intrinsic resistance is usually ascribed as being due to poor influx and/or increased efflux of antibiotics and is a non-specific property. In an interesting publication Fajardo *et al.*[111] screened a library of 5952 transposon insertions in *P. aeruginosa* for increased innate resistance (MIC > control) or decreased innate resistance (MIC < control) to several antibiotics. This screening revealed that ~2% of the genome of this organism is involved in antibiotic sensitivity/resistance. Most of the insertions increased intrinsic resistance while a few decreased the same, that is, led to a hypersensitive pheno-

type. Setting apart the finer details, the study showed that intrinsic antibiotic resistance in *P. aeruginosa* and perhaps in other organisms also, is determined by more number of genes than known so far. The property seems to be under the control of a complex network of elements involving many loci which govern normal physiological functions such as amino acid biosynthesis, transcriptional regulation, chemotaxis, non-coding RNAs, etc. just to mention a few. Therefore, it appears that antibiotics and resistance mechanisms might not have evolved merely as weapons to fight-off antibiotic onslaught, as was believed earlier, but could be functions which are integral parts of bacterial physiology. Linares *et al.*[102] and Fajardo *et al.*[111] call this a Copernican turning point in looking at the phenomenon. We can look forward to many interesting developments in basic microbiology with this new approach.

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