COMMENTARY

Novel mechanisms of emergence of multidrug resistance/tolerance

R. Jayaraman

Antibiotic resistance is a persistent healthcare problem worldwide. In spite of a large body of information already available on the phenomenon, newer insights are also coming to light. In this commentary, a few reports on novel mechanisms of emergence of antibiotic resistance, as well as their potential impact are discussed. Some emerging ideas on antibiotics and resistance mechanisms are also highlighted.

The indisputable success of antibiotics in the treatment of bacterial infections began to be clouded with the emergence of antibiotic resistance. As more and more new antibiotics and therapeutic strategies developed, the number of reports on antibiotic resistance also kept mounting. Presently, antibiotic resistance, especially multidrug resistance, has emerged as the major affront to health care, worldwide. The recent excitement over the New Delhi metallo-beta-lactamase (NDM-1) 'super bug' in our country is an example of the problem. *Current Science* also carried an editorial by Balaram[1] on this problem. The medical and economic impact of not only NDM-1 harbouring pathogens, but also others of its ilk such as the methicillin resistant *Staphylococcus aureus* (MRSA) are not difficult to appreciate. It is claimed that more mortality in the US could be attributed to MRSA than HIV. We can hope that the awareness NDM-1 generated at all levels will ultimately lead to the implementation of adequate control measures to tackle not only the present problem, but also future episodes of this kind. Although multidrug resistance has been known for a long time, we know precious little at the molecular level about how it occurs and how it could be controlled or prevented.

Antibiotic exposure is a survival stress to bacteria and antibiotic resistance can be viewed as a response of the bacteria to counter stress. Bacteria respond to stress in many ways. They have a reportoire of evolved stress-response mechanisms which could handle a limited number of stresses (heat, starvation, oxidative damages, radiation exposure, etc.). However, the stresses encountered by bacteria in nature are too many, too varied, unpredictable and unprovoked. It is inconceivable that bacteria could evolve and maintain response mechanisms for each and every possible stress. Genetic strategies (mutation and horizontal gene transfer) are best suited for adaptation to unforeseen stresses. The rates of spontaneous mutagenesis being low (10^{-10} to 10^{-6}/cell/generation), the chances of acquiring a useful (to the bacterium) mutation to resist an antibiotic are rather dismal, if not impossible. However, if the rates could be increased 100–1000 fold, it will be feasible. Such cells with spontaneous mutation rates several folds higher than normal are called mutators or hypermutators. A detailed discussion on the merits and demerits of hypermutability will be out of place here; however, hypermutability has been implicated in the emergence of antibiotic resistance (for references see Jayaraman[2] and Oliver and Mena[3]). In fact, clinical isolates of many pathogens have been reported to contain high proportions of mutators[2,3]. There are many ways by which bacteria could develop antibiotic resistance[4,5]. Resistance has been shown to occur against all kinds of antibiotics (natural, synthetic and semisynthetic). Interestingly, resistance has been observed even against antibiotics such as vancomycin which does not even enter the cells. It is not necessary that bacteria should be mutators in order to develop antibiotic resistance; non-mutators (normo-mutators) can also develop antibiotic resistance, but less frequently. Moreover, some of the phenotypic stress responses result in concomitant mutagenesis which has been called stress-induced mutagenesis, also called adaptive mutagenesis, stationary phase mutagenesis, etc.[6–8]. With so many mechanisms available to cells to develop antibiotic resistance, little wonder that it is widely prevalent and has grown into a formidable problem to deal with. In this context, reports from Collins and co-workers assume special significance not only for their scientific novelty, but also because of their possible impact in real-life situations. Although it is somewhat late in the day to write about these papers, I have ventured to do so because of the enormous interest generated by the NDM-1 story in the minds of scientists and general public.

In their first report, Kohanski *et al.*[9] showed that bactericidal antibiotics of different classes, regardless of their chemistry, targets or modes of action, generated reactive oxygen species (ROS), especially hydroxyl radicals, which could be correlated with their killing activities. They showed that the key event which is responsible for this is a rapid but transient depletion in the levels of NADH (more than five-fold increase in the NAD^+/NADH ratio; for detailed biochemical information see the original article). Two other significant observations they reported are: (i) only the bactericidal antibiotics tested (ampicillin, norfloxacin and kanamycin), but not a bacteriostatic antibiotic (spectinomycin) brought about this change, and (ii) inactivation of *recA* greatly potentiated the killing activity of the bactericidal antibiotics at concentrations which were non-lethal in the $recA^+$ genetic background. Pursuing these observations, Dwyer *et al.*[10] postulated that binding of the antibiotics to their targets altered cellular metabolism and through a series of biochemical reactions generated hydroxyl radicals which are highly reactive, toxic and cannot be enzymatically detoxified unlike the superoxides. The hydroxyl radicals could kill cells and also lead to mutagenesis. Their earlier observation[9] that $recA^0$ mutants were more sensitive to bactericidal antibiotics implies the involvement of the SOS response in ROS-mediated lethality. Subsequently, Kohanski *et al.*[11] provided direct experimental evidence in support of their contention. They showed that overnight exposure of *Escherichia coli* to sub-lethal concentrations of ampicillin (1 μg/ml) or norfloxacin (a fluoroquinolone: 50 ng/ml) or H_2O_2 (1 mM) increased the frequency of mutation to rifampicin resistance about 5–8-fold. Interestingly, inclusion of 100 mM thiourea (a scavenger of hydroxyl

radicals) during antibiotic or H_2O_2 exposure almost prevented the increase. Similarly, exposure under anaerobic conditions also prevented the increase. A correlation between the levels of hydroxyl radicals (measured flow cytometrically using a fluorescent dye probe) and increase in mutation frequencies was also observed. From cultures treated with a low concentration of ampicillin (as above) for five days, they picked up mutants resistant to either ampicillin, norfloxacin, kanamycin, chloramphenicol or tetracycline (primary selection). Single colonies of a given mutant class from primary selection, say ampicillin-resistant mutants, were screened for cross resistance to the other four antibiotics. Many of them showed significant cross resistance. For instance, among the ampicillin-resistant mutants, 89% were cross resistant to norfloxacin, 20% to kanamycin, 54% to tetracycline and 21% to chloramphenicol. This pattern of effects was reproduced in *S. aureus* also. Another significant observation which they reported was, if an ampicillin-treated culture was used to select mutants resistant to any other antibiotic, say norfloxacin, a (variable) fraction of them were sensitive to ampicillin itself. This means that the mutagenesis triggered by a given antibiotic does not always include mutants resistant to the triggering antibiotic. By DNA sequencing a few of the cross-resistant mutants, Kohanski *et al.*[11] detected sequence lesions in *gyrA*, *gyrB*, promoter of *acrAB* (multidrug effux system), etc.

Overall, the three reports of Collins and his group discussed earlier showed that exposure of cells to sub-lethal doses of antibiotics mimics treatment with mutagens. Collins and co-workers suggested that ROS, especially hydroxyl radicals, generated by antibiotic–cellular interactions, cause DNA damage and lead to mutagenesis directly and/or in conjunction with the SOS response whose mutagenic effects, in turn, are mediated by the upregulation of the error-prone DNA polymerases of the Y family, viz. Pol IV (product of *dinB*) and Pol V (product of *umuC* and *umuD*). Classically, the role of antibiotics in resistance development has been that they are only passive selective agents, having no role in the generation of mutants which they select out. This is a legacy of the Luria–Delbruck–Lederberg era of microbial genetics. A corollary to the idea of antibiotics as selectors of resistant bacteria has been that in doing so they may also select for mutators. The work of Collins and co-workers has shown that antibiotics could also act as mutagens via ROS and SOS. What is more interesting, even frightening, is that low concentrations of many antibiotics could induce multiple drug resistance. Usually, multidrug resistance is believed to occur through successive single mutational events, activation of multidrug efflux pump systems, or acquisition of resistance genes *en bloc* through plasmids, phages, integrons, etc. (horizontal gene transfer). The work of Collins and co-workers has shown that multidrug resistance can also be brought about by the mutagenic action of antibiotics, even at low concentrations. Low antibiotic levels can occur in many ways in clinical situations. Sub-standard and/or time-barred antibiotic formulations, patient negligence, time interval between successive doses, periods of recovery after withdrawl of the antibiotics, etc. can result in low levels of the drug which could trigger multidrug resistance. Moreover, if such resistance happens to occur in commensal organisms, there will be the danger of their getting passed onto pathogens at a later date. Kohanski *et al.*[11] observed that clonal isolates after exposure to low concentrations of ampicillin were heterogeneous with respect to their resistance against ampicillin or norfloxacin, ranging from total sensitivity (zero resistance) to high-level resistance. The low-level resistant mutants could use the trait as a spring board to achieve higher levels of resistance. These are some potentially serious issues which scientists should address. The paper by Collins and coworkers[11] was highly commended[12,13].

In a recent publication from the Collins' group, Lee *et al.*[14] reported that when wild-type *E. coli* was subjected to increasing concentrations of norfloxacin in a bioreactor over a period of 12 days and assayed at 24 h intervals for minimum inhibitory concentration (MIC) (defined here as the antibiotic concentration that would inhibit growth by 60%, but not more) for norfloxacin, an interesting result was obtained. At any given sampling time the population as a whole had a certain MIC; however, randomly selected members of the population either had MICs lower than that of the whole population (more abundant class) or higher than the population MIC (less abundant class). The former were called LRIs (less resistant isolates) and the latter were called HRIs (high resistant isolates). The MIC of LRIs was lower than the drug concentration in the bioreactor at which they were obtained, whereas that of the HRIs was higher than the bioreactor drug concentration. Lee *et al.*[14] speculated that HRIs produced some signalling molecule(s), which could be secreted into the medium and render the LRIs more drug tolerant so that both could survive. Cutting the long story short, the signalling molecule was identified as indole, produced by the action of tryptophanase, the enzyme which degrades tryptophan to indole, pyruvic acid and ammonia. They showed that addition of indole (0.3 mM) increased the norfloxacin MBC (minimum bactericidal concentration that would kill 99.9% of the cells) of a representative LRI (C10,6) from 800 to 1400 ng/ml. This would explain, at least in part, how C10,6 could have survived in the bioreactor till day-10 (the time of its isolation), when the concentration of norfloxacin in the reactor was 1500 ng/ml. Obviously, the few HRIs (C10,12, for example) could have secreted indole, which in turn could have induced higher tolerance to the antibiotic in the LRIs. Moreover, co-culturing a LRI and a HRI at a ratio 0.99 :: 0.01, the mixture grew better than either of them at a norfloxacin concentration of 1500 ng/ml. Both indole production in a HRI and the higher drug tolerance it conferred on a LRI were abolished when *tnaA*, the gene encoding tryptophanase, was deleted. Interestingly, the Δ*tnaA* HRI mutant grew better than the *tnaA*$^+$ HRI in the presence of 1500 ng/ml norfloxacin. This indicated that indole production in the HRIs is associated with a fitness cost. In spite of this, HRIs exert an altruistic effect on LRIs by increasing the latter's drug tolerance levels. Lee *et al.*[14] showed that indole induces two modes of antibiotic detoxification: drug efflux via multidrug efflux pump systems, and activation of oxidative stress protection mechanisms. These results were also reproduced with another antibiotic, viz. gentamycin. Based on their observations Lee *et al.*[14] proposed a population-based antibiotic resistance mechanism (see figure 4 of their paper). In the absence of antibiotic stress, wild-type cells produce and secrete indole. In the presence of the antibiotic, indole production ceases and most of the cells are killed. If drug-

resistant mutants emerge, they resist the antibiotic, survive and produce indole (at a fitness cost) to the same level as the unstressed wild-type cells do. The indole present in the medium induces drug tolerance in the antibiotic-sensitive cells and ensures their survival, a case of bacterial altruism. Since the resistant mutants under antibiotic stress secrete TnaA (tryptophanase) and a few other proteins into the medium, there is some uncertainty as to whether indole synthesis in such cells is intracellular followed by secretion or extracellular, possibly by the action of TnaA (on media components; J. J. Collins, pers. commun.). Interestingly, addition of indole to wild-type cells induces tolerance to subsequent antibiotic challenge (J. J. Collins, pers. commun.). Apart from being academically interesting, the findings of Lee *et al.*[14] could have some practical implications. Extrapolation of their findings to a clinical setting permits the speculation that a minority of drug-resistant cells will not only resist the antibiotic by themselves, but also altruistically induce higher drug tolerance in the majority drug-sensitive cells. This would impede clearance of the pathogens by antibiotic treatment and would also enhance the chances of emergence of more number of resistant mutants.

Perhaps a slight digression at this point may be worthwhile. There has been a paradigm shift in recent years in our perception of antibiotics and antibiotic resistance mechanisms[4,5,15]. In contrast to traditional thinking, antibiotics are now viewed by some workers as intermicrobial signalling agents, regulators of gene expression or even as sources of food to microbes, rather than as weapons used by producer organisms to fight against cohabiting competitors. Similarly, the various antibiotic resistance mechanisms are now believed by some workers to have evolved not as defence strategies deployed by microbes to conquer the antibiotics, but as integral parts of their physiology. They become problematic only when placed out of their natural context. Allen *et al.*[5] cite several instances of retrospective detection of antibiotic resistance determinants and conjugative plasmids in preserved bacterial cultures dating back to the pre-antibiotic era (ca. 1900–1950). Such elements seem to have existed in nature and probably indulged in horizontal gene transfer even before antibiotics appeared on the scene and exerted selection pressure. It is believed that β-lactamases existed billions of years ago[4,5]. Obviously, they must be important enough to have been conserved for so long. Antibiotics display a behaviour called 'hormesis', which denotes different, even contrasting effects at low and high concentrations. At sub-lethal levels, their binding to the targets leads to alteration in transcription patterns, which are implicated in metabolic adjustments. Hormetic changes could be advantageous to microbes in their struggle for survival and evolution. Couce and Blazquez[16] have reviewed many hormetic changes in bacteria at sub-lethal antibiotic concentrations and their possible consequences from a human perspective. Perhaps it is time we realize that antibiotics are not just passive selectors of resistant bacteria. They may do that, but they could also be triggers of multiple antibiotic resistance. Hopefully, the all-round awareness generated by the NDM-1 super bug will lead to a restrained and conscientious use of antibiotics.

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R. Jayaraman lives at R.H. 35, Palaami Enclave, New Natham Road, Madurai 625 014, India.
e-mail: aarjay007@rediffmail.com