Bacterial persistence: some new insights into an old phenomenon

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Bigger discovered more than 60 years ago, at the very beginning of the antibiotic era, that populations of antibiotic-sensitive bacteria contained a very small fraction (approximately $10^{-6}$) of antibiotic-tolerant cells (persisters). Persisters are different from antibiotic-resistant mutants in that their antibiotic tolerance is non-heritable and reversible. In spite of its importance as an interesting biological phenomenon and in the treatment of infectious diseases, persistence did not attract the attention of the scientific community for more than four decades since its discovery. The main reason for this lack of interest was the difficulty in isolating sufficient numbers of persister cells for experimentation, since the proportion of persisters in a population of wild-type cells is extremely small. However, with the discovery of high-persister (hip) mutants of *Escherichia coli* by Moyed and his group in the early 1980s, the phenomenon attracted the attention of many groups and significant progress has occurred since then. It is now believed that persistence is the end result of a stochastic switch in the expression of some toxin–antitoxin (TA) modules (of which the *hipA* and *hipB* genes could be examples), creating an imbalance in their intracellular levels. There are also models invoking the involvement of the alarmone (p)ppGpp in the generation of persisters. However, the precise mechanisms are still unknown. Bacterial persistence is part of a wider gamut of phenomena variously called as bistability, multistability, phenotypic heterogeneity, stochastic switching processes, etc. It has attracted the attention of not only microbiologists but also a diverse band of researchers such as biofilm researchers, evolutionary biologists, sociobiologists, etc. In this article, I attempt to present a broad overview of bacterial persistence to illustrate its significance and the need for further exploration.

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1. Prologue

The discovery of the progenitor antibiotics penicillin and streptomycin in the 1940s was an event of great significance in the history of medicine. It heralded the advent of the antibiotics era. Subsequently, several antibiotics of natural and synthetic origin became available for clinical use. Antibiotic therapy of bacterial (and fungal) infections in humans and animals came to be established as standard medical practice. There is no gainsaying that the use of antibiotics, called ‘magic bullets’ in popular parlance, mitigated a lot of suffering and saved millions of lives from an inevitable death that would have occurred otherwise, not to speak of the big spurt in the pharmaceutical and allied industries. On the other hand, two major problems have also arisen, raising questions about the continued and indiscriminate use of antibiotics. One is the emergence of heritable antibiotic resistance in susceptible microbial populations, their spread by horizontal gene transfer and lack of strategies to contain them (reviewed by Levy and

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Abbreviations used: c-di-GMP, (3′-5′) cyclic dimeric guanosine monophosphate; FMN, flavin mononucleotide; GFP, green fluorescent protein; *glnD*, glyceral-3-phosphate dehydrogenase; *hip*, high-persister; MIC, minimum inhibitory concentration; MRSA, methicillin-resistant *Staphylococcus aureus*; ORF, open reading frame; PCD, programmed cell death; *plsB*, glyceral-3-phosphate acyl transferase; (p)ppGpp; guanosine 3′-5′ bispyrophosphate; TA, toxin–antitoxin
Marshall 2004). Some pathogens have become resistant to most of the commonly used antibiotics. The notorious ‘superbug’ MRSA (methicillin-resistant \textit{Staphylococcus aureus}), which is resistant to all the $\beta$-lactam antibiotics and sensitive only to vancomycin (a glycopeptide antibiotic) is a case in point. This limits the choice of treatment for MRSA infections to vancomycin. Of late, vancomycin-resistant MRSA has also emerged, having acquired the trait from vancomycin-resistant enterococci by horizontal gene transfer (see Levy and Marshall 2004). The other problem that has emerged due to antibiotic therapy is the persistence of bacterial infections in which the pathogens linger in the host for long periods of time in spite of prolonged treatment. Some well-known examples are tuberculosis, syphilis, typhoid fever (the carrier state), gastric ulcer, lung infections in patients with cystic fibrosis and many more. In these diseases, the pathogens remain stably associated with the host, recalcitrant to therapeutic agents and host defence mechanisms. We need to gain a deeper understanding of the mechanism(s) of persistence in order to develop strategies to tackle problems associated with persistence. Some excellent reviews on this subject have appeared recently (Levin and Rozen 2006; Lewis 2007, 2008; Blaser and Kirscher 2007).

2. The persistent state

Bacterial persistence is the capacity of bacteria to tolerate exposure to lethal concentrations of bactericidal antibiotics. This property was first noticed by Bigger (1944), soon after penicillin was introduced for clinical use. Bigger noticed that treatment of cultures of \textit{Staphylococcus aureus} with high concentrations of penicillin did not kill all the cells; a small fraction, of the order of 10$^{-6}$ or less, survived. This fraction regrew after removal of the antibiotic and was, surprisingly, as sensitive to penicillin as the original population, again leaving behind a tiny fraction of survivors. Bigger coined the terms ‘persistence’ and ‘persisters’ to denote the phenomenon and the survivors of antibiotic exposure, respectively. He also suggested that persisters are in a state of dormancy, insusceptible to the action of penicillin, a view which has remained essentially unchanged even after 60-odd years. At that time, the only antibiotic known was penicillin whose mechanism of action was yet to be understood. In retrospect, Bigger’s ingenuity in testing persisters for antibiotic sensitivity upon regrowth seems remarkable. In all likelihood, a present-day microbiologist would have discarded them as being spontaneous antibiotic-resistant mutants! Unfortunately, the phenomenon of persistence did not attract the serious attention of microbiologists till the 1980s, although there were sporadic reports on the topic (McCune and Tompsett 1956; McDermot 1958).

3. Drug indifference versus persistence

Studies on the mode of action of penicillin and its descendents (the $\beta$-lactam group of antibiotics) as well as other antibiotics revealed a close relationship between antibiotic sensitivity and growth rate of bacteria (Lee et al 1944; Trumanen \textit{et al} 1986; Zeller and Voigt 1987; Aridesi \textit{et al} 2003). In general, non-growing or slow-growing bacteria are less sensitive to antibiotics. This property is called ‘drug indifference’. Drug indifference has been known for more than fifty years and has been demonstrated \textit{in vivo} using experimental animal infection models (for details, see Levin and Rozen 2006). Drug indifference is not the same as persistence. It is a reflection of the overall reduced sensitivity of dormant/slow-growing microbial populations, with no specific mechanistic basis. The property is elicited by the whole population. Persistence, on the other hand, is a special case of drug indifference, restricted to a small subpopulation and has a mechanistic basis of origin which is not fully understood (see below).

4. Persistent state and biofilms

The traditional view of bacteria is that they are free-floating and free-swimming organisms. However, in nature, many bacterial species exist in organized structures called ‘biofilms’. Biofilms consist of an exopolysachharide matrix in which several bacterial species (bacterial consortia) are embedded. They are highly organized, surface-adherent structures, found on a variety of biological and non-biological surfaces. Many clinical infections are ascribed to biofilms. Some examples are dental plaques, gingivitis, infections of the middle ear, lung infections in patients with cystic fibrosis, etc. Many informative reviews on biofilms are available (Hall-Stoodley \textit{et al} 2004; Kolter and Greenberg 2006; Jayaraman and Wood 2008).

The characteristic property of biofilm-associated bacteria relevant to the present discussion is their recalcitrance to eradication by antibiotics (Fux \textit{et al} 2005; Lewis 2001, 2005, 2007, 2008). Actually, biofilm-associated bacteria are not antibiotic resistant since the same cells are sensitive in the planktonic state. Moreover, Spoering and Lewis (2001) and Harrison \textit{et al} (2005 a, b) showed that biofilm cells are sensitive to fluoroquinolones (ciprofloxacin, ofloxacin, etc.) and metal oxyanions such as chromate, arsenate, tellurate, etc. which could kill growing as well as non-growing cells. Even at high doses of ciprofloxacin, a fraction of cells survives in biofilms suggesting that they could be persisters (Brooun \textit{et al} 2000). Based on these observations, Lewis suggested a model for the insensitivity of biofilm-associated cells to elimination by antimicrobials. According to his model (outlined in detail in Lewis 2008), treatment with ciprofloxacin eliminates the planktonic cells and the

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majority of biofilm-associated cells except the persisters, while the persister cells outside the biofilm matrix are killed by the host’s immune mechanisms. Once the antibiotic is withdrawn, the persisters inside the biofilm grow and repopulate the niche leading to secondary infection. Secondary infection, in turn, generates antibiotic-sensitive cells as well as antibiotic-tolerant persisters. The infection is perpetuated in this manner in spite of prolonged therapy.

5. Persistence and infectious diseases

Persisters pose formidable difficulties in the treatment of many infectious diseases. This problem has been discussed at length by Levin and Rozen (2006) and Lewis (2007, 2008) in general terms, and by Gomez and McKinney (2004) with special reference to tuberculosis. Although not appreciated very much, the problems posed by persisters are no less intractable than those posed by genetically antibiotic-resistant organisms. For example, biofilm-associated *Pseudomonas aeruginosa* infections in the lungs of patients with cystic fibrosis, a typical persister state, are incurable (see Lewis 2007, 2008). Using a mathematical model and computer simulation, Wiuff et al. (2005) showed that persistence (phenotypic tolerance, as they called it) could have profound effects on clearing of bacteria by antibiotics, and could actually prevent clearing resulting in treatment failure. Moreover, antibiotic-tolerant persisters pose another confounding problem in that they are often shielded from the host’s immune defence systems as well; apparently they ‘hide’ in various niches such as the central nervous system (*Treponema pallidum*), macrophages or granulomas (*Mycobacterium tuberculosis*), biofilms (*Pseudomonas aeruginosa*), stomach (*Helicobacter pylori*), gallbladder (*Salmonella typhi*), etc. As suggested by Levin and Rozen (2006), with the help of a mathematical model, a reservoir of such shielded persisters are a potential source for the emergence of heritable antibiotic resistance. Effective therapy of bacterial infections should aim not only at eradicating antibiotic-sensitive pathogens but also at eliminating persisters or preventing their emergence. At the same time, associated problems such as the development of heritable resistance, toxicity, undesirable side-effects, costs, etc. should be kept in focus. These issues have been exhaustively dealt with in the reviews of Levin and Rozen (2006) and Lewis (2007, 2008).

6. Nature and generation of persisters

As pointed out earlier, Bigger’s suggestion that persisters are in a state of dormancy, which enables them to tolerate antibiotic exposure, has not changed with time. Over the years, a large body of evidence has accumulated to substantiate the idea. However, there has not been much progress in our understanding of how persistence occurs. The main reason for this was the difficulty in obtaining persister cells in sufficient quantity for experimentation, since the frequency of their occurrence is extremely small. However, a breakthrough occurred in the 1980s with the work of Moyed and his group who isolated a high persister (*hip*) mutant of *E. coli* (Moyed and Bertrand 1983; Moyed and Broderick 1986; Black et al. 1991). The *hip* locus maps at 34.2–34.3 min on the chromosome and consists of two genes *hip A* and *hip B*. In the best studied *hip* allele, *hip A7*, the frequency of persisters after ampicillin exposure could reach as high as 1% of the population (a 10 000-fold increase over the wild-type). The *hip* genes form an operon, the *hip* BA operon, the expression being B → A (Black et al. 1991). We will return to *hip* BA later on. Another high-persister locus (*hip Q*) has been identified in *E. coli* by Wolfson et al. (1990). However, *hip* Q mutants have not been as well studied as *hip A* mutants in terms of physiology and genetics.

With the availability of the *hip* mutants of *E. coli*, it became possible to isolate persister cells in sufficient numbers for analysis. Two methods have been developed for the isolation of persister cells. The extremely simple method developed by Keren et al. (2004) is to expose log phase cultures of *E. coli* to ampicillin till lysis (about 3 h) and collect the surviving cells (persisters) by centrifugation. Another method developed by the same group (Shah et al. 2006) exploits the low transcriptional output in persisters due to their dormancy. By inserting a green fluorescent protein (GFP) reporter gene placed under the control of the *rrnB* promoter at the *att* site of the *E. coli* chromosome and sorting out the cells into bright green and dim green types, slow-growing subpopulations of cells could be isolated. This population showed a 20-fold higher tolerance to ofloxacin than the bright green (normally growing) cells. However, this method is time-consuming, expensive and not suitable for large-scale preparations. ‘A rapid method for isolating large numbers of persisters is yet to be developed,’ (Lewis 2007).

Making direct observations on single cells of wild-type, *hip A7* and *hip Q* mutants growing in a specially designed microfluidic chamber coupled with mathematical analysis, Balaban et al. (2004) have identified several features of persistence. In the *hip A7* mutant the persisters (which they call type I persisters) are a subpopulation of non-growing cells generated at the stationary phase of the previous growth cycle and are therefore proportional to the inoculum size. These cells take a very long lag (~14 h) to exit the stationary phase, compared to ~40 min by normal stationary-phase cells. The persisters in the *hip Q* mutant (called type II persisters) are generated during growth. They are also slow growers relative to non-persisters, but are not as growth arrested as type I persisters. Their proportion in
a growing population is dependent on the number of cells but independent of the inoculum size. Wild-type cells, on the other hand, show a more complex population dynamics than the hip mutants. They consist of three subpopulations, namely, normal (non-persister) cells, type I persisters carried over from the inoculum and type II persisters generated during growth. The significance of the work of Balaban et al (2004) lies in its beauty and elegance. It provided direct, visual evidence for the existence of two classes of cell types in cultures of hip A7 and hip Q mutants; one, a fast-growing and ampicillin-sensitive class (normal cells) and the other, a slow-growing and ampicillin-tolerant class (persisters). Apparently, both were present before antibiotic exposure. The work also showed that the slow-growing (ampicillin-tolerant) cells switched to the fast-growing (ampicillin-sensitive) type. Unfortunately, it is not possible to conduct such elegant experiments with wild-type cells since the frequency of persisters is ~1000-fold less. Subsequently, Kussell et al (2005) developed a theoretical model, using which they compared the population dynamics of wild-type and hip Q-mutant cells. Their results show that in wild-type cells, persistence is optimized to face very rare antibiotic challenges (one day or less in 100 years), while in the hip Q-mutant, it is optimized to frequent and intense antibiotic challenges. The fitness loss due to reduced growth rate in persisters pays off as a risk-reducing strategy. Kussell et al (2005) call this an ‘insurance’ strategy!

While mutations in hip A and hip Q increase the frequency of persisters, mutations in other genes such as glpD (glycerol-3-phosphate dehydrogenase) and plsB (glycerol-3-phosphate acyl transferase) reduce persister frequency in stationary-phase cultures by mechanisms not yet understood (Spoering et al 2006). A suggestion has been made that these genes might be required for maintenance of the persistent state rather than its generation (Lewis 2007, 2008). Perhaps pho U may also belong to this list (Li and Zhang 2007). Recently, more mutations that reduce persister frequency have been identified (Hanssen et al 2008; see below). The hip A7 mutant shows increased persistence not only to β-lactam antibiotics (penicillin, ampicillin, etc.) but also to fluoroquinolones, (ciprofloxacin), aminoglycosides (tobramycin), DNA damaging agents, heat, etc. (see Keren et al 2004 for references).

8. Toxin–antitoxin modules and persistence

Genomes of prokaryotes and plasmids have some genetic elements called TA modules (Gerdes et al 2005). Typically, they consist of two genes expressed as an operon. One of the genes encodes a stable toxin (which inhibits some important cellular functions) and the other an unstable antitoxin which neutralizes the toxin and also acts as an autoregulator of expression. While they constitute a maintenance function in plasmids (Hayes 2003), their role in bacteria is not precisely known. They have been suggested to trigger programmed cell death (PCD) in response to stress (starvation, antibiotic encounter, DNA damage, heat, etc.) (Aizenman et al 1996; Sat et al 2001; Hazen et al 2004). On the other hand, some workers believe that they do not cause cell death but act as inhibitors of macromolecular synthesis leading to reversible inhibition of growth (bacteriostasis) (Christensen et al 2001; Gerdes 2000). This view is supported by observations that the stasis brought about by the toxin could be reversed by expression of the cognate antitoxin gene or tmRNA (Pedersen et al 2002; Christensen et al 2003).

In their experiments on gene expression in persistent cells of E. coli, Keren et al (2004) observed that some well-characterized TA modules (relBE, mazEF, dinJ/yafQ) were among the overexpressed genes. Pursuing this observation further, they found that overexpression of plasmid-borne rel E, the toxin gene component of the rel BE operon, coding for a translation inhibitor (Christensen et al 2001) resulted in a 10–100 000-fold increase in persisters in response to ofloxacin (a fluoroquinolone), cefotaxime (a cephalosporin) and tobramycin (an aminoglycoside). In order to see whether the hip BA operon could also be a TA module they exploited the cold-sensitive phenotype of the hip A7 mutant as follows. The high persister hipA7 mutant is cold sensitive (no growth at 30°C) possibly due to the inability of the mutant Hip A protein to complex with the wild-type Hip B protein at 30°C. If true, this might lead to the failure of neutralization of the Hip A toxin by Hip B antitoxin and hence the dominance of the HipA toxin, resulting in growth
inhibition at 30°C. In agreement with this hypothesis, the hip A7 mutant yielded a lot more persisters at 30°C than 37°C (Karen et al. 2004). Falla and Chopra (1998) have reported earlier that overexpression of hip A increased the production of persisters tolerant to β-lactam and fluoroquinolone antibiotics. These observations imply that hip BA could be a TA module and suggest a general role for TA modules in generating persisters in response to antibiotic and other stresses. It is interesting to note that ygi U (which, along with ygi T, could be a TA module) is the most overexpressed gene in slow-growing cells and causes a dramatic increase in tolerance to ofloxacin, more than hip A7 or rel E (Shah et al. 2006). However, Vazquez-Laslap et al (2006) reported that very limited overexpression (causing only minimal inhibition of cell growth) of not only hip A and maz F (TA modules) but also unrelated (non-TA) genes such as dna J of E. coli and pmr C of Salmonella typhimurium resulted in persister cells tolerant to ampicillin and ciprofloxacin. Therefore, it seems that expression of many genes besides TA modules could lead to the production of dormant persister cells.

9. More on hip A alleles and persister formation

Korch et al (2003) showed that the hip A7 mutant of E. coli carries two point mutations in hip A – a G22S and a D291A. Both the mutations were shown to be necessary for its cold-sensitive phenotype while the D291A lesion alone, at low levels of expression, was sufficient for persistence. Interestingly, inactivation of the rel A–spo T gene pair, involved in the synthesis of the alarmone guanosine 3’ 5’ bispyrophosphate, (p)ppGpp, brought the persister levels of the hip A7 mutant to that of the hip A’ wild-type strain. This defect could be complemented in trans by the wild-type rel A’ gene. Based on these observations, they proposed a model of persister formation invoking a causative role for (p)ppGpp (see below). Overproduction of ppGpp by induced expression of the cloned rel A gene was earlier shown by Rodionov and Ishiguro (1995) to result in growth inhibition and ampicillin tolerance. However, as pointed out by Korch et al (2003), the experiments of Rodionov and Ishiguro used cultures in which ppGpp synthesis was induced prior to exposure to ampicillin. Therefore, the apparent ‘tolerance’ to the antibiotic could be due to populationwide drug indifference consequent upon growth inhibition brought about by high ppGpp levels rather than persistence, which is restricted to a small subpopulation. Korch and Hill (2006) reported in a recent publication that overexpression of plasmid-borne, wild-type hip A inhibited DNA, RNA and protein synthesis, and resulted in growth arrest in a majority (~90%) of the population. Cells in which growth was not arrested (colony formers post-induction of hip A) emerged as persisters after ampicillin exposure. Interestingly, the fraction of persisters arising after hip A overexpression in hip A’ cells was comparable to that obtained with the hip A7 mutant under normal growth conditions. An intriguing observation of Korch and Hill (2006) was that overexpression of plasmid-borne hip A7 caused neither marked growth arrest nor inhibition of macromolecular (protein) synthesis. Still, hip A7 is a high persister-yielding mutant. Moreover, inhibition of protein synthesis by tetracycline or chloramphenicol did not increase persister frequency. Therefore, it is difficult to strictly correlate persister formation with the adverse effects of hip A on macromolecular synthesis and growth inhibition alone. In addition, the loss of colony-forming ability after hip A overexpression was shown to be bacteriostatic rather than bactericidal and could be reversed by induced expression of the cognate antitoxin gene hip B (Korch and Hill 2006). Correia et al (2006) reported that hip A is a member of the phosphatidylinositol 3/4 kinase superfamily, the first bacterial member to be identified. They showed that replacement of the conserved aspartate residues at 309 (active site) or 322 (Mg2+ binding site) of the Hip A protein with glutamine (D309Q and D322Q, respectively) failed to arrest growth or yield persisters upon overexpression. The Hip A protein has a serine residue at 150 which is phosphorylated in about 50% of the molecules in vivo. Replacing the serine at 150 with alanine (S150A) had similar effects as the other mutants with altered aspartate residues at 309 and 322 (see above). However, the target(s) of Hip A kinase activity is/are still unknown; other than autophosphorylation of serine at 150, kinase activity has not been shown with any other substrate. Summing up, the recent reports discussed above have shown a close linkage between hip A (probably its protein/lipid kinase activity) and induction of dormancy and persister formation. Precisely how this is effected is still elusive.

The model of persister formation proposed by Keren et al (2004; reviewed by Lewis 2007, 2008) suggests that toxins such as Hip A, Rel E, etc. inactivate the targets of action of antibiotics (ribosomes, cell wall synthetic machinery, DNA gyrase/topoisomerase, etc.). When this happens, the antibiotics can no longer ‘corrupt’ their targets into malfunctioning. In the absence of toxins, the antibiotics would corrupt their targets to malfunction and lead ultimately to cell death. Therefore, whether or not the toxin-inactivated antibiotics bind to the target, the net result will be antibiotic tolerance (persistence). (This is like keeping a criminal behind bars to prevent him form committing crimes!) The model proposed by Korch and Hill (2003) is based on their observations that deletion of the rel A and spo T genes, responsible for (p)ppGpp synthesis, almost abolishes the high-persistence phenotype of the hip A7 mutant and this defect could be complemented by the rel A’ gene in trans. (The rel A’ gene encodes a truncated from of Rel A protein and could synthesise (p)ppGpp without ribosomal involvement (Schreiber et al 1991.) According
to this model, the primary event in persister formation is increase in intracellular levels of (p)ppGpp, which leads to dormancy and persister formation. According to Korch and Hill (2003), dormancy may not be a critical requirement since overexpression of hip A7 does not cause growth inhibition but still results in high persister generation (Korch and Hill 2006). Although a lot more is now known about persisters and their origin than earlier, the exact mechanism(s) which generate(s) them remains largely unknown. It is possible that there could be more than one mechanism of persister formation (see below). The two mechanisms proposed for persister formation are discussed in detail later.

10. Possible involvement of other loci in persister formation

In a recent report, Hansen et al (2008) presented data on the screening of an E. coli knockout library comprising 3985 open reading frames (ORFs) encoding non-essential functions for decrease/abolition of persister formation using an improvised screening procedure. Their search ultimately led to the identification of ten gene knockouts which were not affected in either growth rate or minimum inhibitory concentration (MIC) for oxacillin, but showed decreased persister formation. There were: dna J and dna K (chaperones), apa H (diadenosine tetraphosphatase), sur A (peptidyl-prolyl cis-trans isomerase), fis and hns (global regulators), hmr (response regulator of rpo S), dks A (transcriptional regulator of rRNA synthesis), ygf A (5-formyl tetrahydrofolate cycloligase) and yig B (flavin mononucleotide [FMN] phosphatase). The preponderance of global regulatory genes in this list suggests redundancy in the mechanisms of persister formation. Interestingly, none of them resulted in total abolition of persister formation, but only resulted in a ~10-fold decrease, strengthening the idea of operation of redundant pathways and mechanisms in the process. They have also discussed in detail the possible role(s) of these genes. Due to limitation of space, it is not possible to discuss all of them here; the reader is referred to the report of Hansen et al (2008) for detailed information. Deletion of dna K has been shown earlier to decrease persister formation in Staphylococcus aureus exposed to oxacillin (Singh et al 2007).

Hansen et al (2008) have emphasized the possible effects of knockout mutations in two loci, namely, ygf A and ygi B. The mammalian homologue of the former converts the stable, 5-formyl tetrahydrofolate into unstable (degradable) 5,10-methenyl tetrahydrofolate (Anguera et al 2003). They suggest that if this be true of E. coli also, ygf A knockouts will lead to depletion of the cellular folate pool and ultimately to inhibition of the metabolic pathways that require 5-formyl tetrahydrofolate as a co-factor. Similarly, knockout of ygi B, which is an FMN phosphatase (Kuznetsova et al 2006), will result in depletion of the cellular FMN pool. Therefore, both the knockouts will result in dormancy, which could be a prerequisite for persister formation. Intriguingly, knockouts of other FMN-specific phosphatase genes are neutral with respect to persister formation (Hansen et al 2008). Moderate overexpression of cloned ygf A and ygi B resulted in elevated persistence in cells exposed to oxacillin.

11. Persistence as a stochastic process

The traditional thinking in microbiology has been to consider clonal (genetically homogeneous) populations of bacteria as phenotypically homogeneous also. To some extent, this view was inevitable because the technology available in earlier times enabled only studies on bacterial populations rather than individual bacterial cells. However, in recent times, newer techniques have come into use such as flow cytometry, observations on single cells growing in microfluidic channels by single-cell time-lapse fluorescent microscopy along with other conventional techniques such as mathematical modelling, computer simulations, etc. The traditional view of phenotypic homogeneity in clonal populations is changing. It is now well established that a lot of phenotypic heterogeneity exists in genetically homogeneous populations. In other words, a clonal population may have two (bistability) or more (multistability) subpopulations with distinct phenotypic properties, arising due to ‘noise’ in gene expression patterns. Examples of such bistable/multistable states have been known for a long time and new insights are being gained. Some of the well known examples are the lysis/lysogeny decision in E. coli infected with phage λ; development of ‘competence’ (ability to take up extracellular DNA), sporulation, cannibalism, swimming and chaining, etc. in B. subtilis; chemotaxis, induction of the Lac operon, spontaneous SOS induction, etc. in E. coli, and many more. The underlying mechanism of phenotype variability has been attributed to ‘noise’ in the level of expression of a key regulatory factor. For instance, the decision to ‘choose’ either the lytic or the lysogenic mode of development in λ phage-infected cells is determined by the dominance of Cro versus CI proteins early in the infection process with the activity of the CII activator protein being a crucial determinant of the decision (Ptashne 2004; Dodd et al 2005). Likewise, competence development in B. subtilis is governed by the level of the ComK protein (van Sinderen et al 1995). Switching to generate alternate phenotypes can occur in response to environmental perturbations (responsive switching) or spontaneously (stochastic switching). The latter equips cells to face environmental perturbations: an ‘insurance’ against future catastrophe whether it occurs or not. Several excellent reviews and reports on phenotypic heterogeneity in bacteria have appeared recently (Balaban et al 2004; Kussell et al 2005; Kussell and Leibler 2005;
Many of the experiments discussed earlier in this review have shown that overexpression of hip A and other TA modules results in a high frequency of persisters. Moreover, upsetting the possible Hip A–Hip B balance at 30°C elevated persister frequency in the hip A7 mutant (Keren et al 2004).

The experiments of Balaban et al (2004) discussed earlier showed two types of persisters in E. coli. Type I persisters arise during the stationary phase of growth and are carried over during subculturing. Type II persisters, on the other hand, arise during growth. Both types were shown to be present in cultures before exposure to antibiotic stress. Apparently, both are generated by stochastic switching. This raises an interesting question. Since persisters are dormant, slow-growing/non-growing subpopulations, they obviously have a reduced fecundity. Why has evolution selected stochastic switching over response switching for the generation of persisters? Dhar and McKinney (2007) have raised and discussed this point in some depth. Under catastrophic situations when the very survival of the population is threatened and when response switching would be impossible to achieve, having an ‘insured’ subpopulation would ensure propagation of the genome after the crisis blows over (see also Lewis 2007, 2008). Using a mathematical model, Kussell and Leibler (2005) have suggested that stochastic switching would be evolutionarily favoured when environmental perturbations are infrequent, while response switching would be favoured in rapidly fluctuating environments. Moreover, as Dhar and McKinney (2007) have pointed out, use of E. coli strains maintained in the laboratory for long periods of time in the absence of environmental perturbations of any kind, especially antibiotic stress, would not be expected to maintain mechanisms of response switching to antibiotic tolerance (persistence). It would be more appropriate to use wild strains (as against laboratory-maintained strains) and antibiotic-refractory species such as Mycobacterium tuberculosis for examining persister mechanisms (Dhar and McKinney 2007).

12. Where do we stand today: an appraisal

From the preceding discussion, it is apparent that much effort has gone into the identification of genes responsible for the generation of persisters and in delineating the molecular mechanism(s) of persistence. From the available data, the case for involvement of TA modules in the generation of persisters seems strong in spite of some uncertainties. Not only are many TA modules overexpressed in isolated persisters, but also their effects on macromolecular synthesis leading to stasis or death have been well documented. Several studies have pointed to hip A as one of the genes crucial to persister formation; however, it is not among the overexpressed genes in the expression profile reported by Keren et al (2004). Although their report is silent on this aspect, it is possible that hip A might not have been overexpressed in the early log phase cells used by them for isolation of persisters. The characteristic phenotype of hip A7 is pronounced only in the stationary phase of growth while other TA modules such as rel E are expressed in mid-log cells also. Even among the 300-odd genes overexpressed in persisters, only a few (~2%; which include TA modules, SOS genes, heat and cold shock genes, etc.) are implicated in persister formation. It is conceivable that the rest of the overexpressed genes could be involved in other functions such as maintaining the structural integrity of the cells, viability, ability to resume growth, housekeeping functions, etc. Some of them could also overlap with genes expressed in other stress responses such as starvation, DNA damage, etc. It is also ironic that hip A as well as other TA modules do not figure in the screening of Hansen et al (2008). Since there are more than ten TA modules in E. coli (Pedersen et al 2002) it is possible that insertions in hip A or any other TA module could have escaped detection by the activity of other TA modules. However, it is also known that deletion of only hip BA, but not other TA modules, reduces persister frequency, an observation that runs counter to the above argument. This only shows how little we know of the genes responsible for persistence. Moreover, there are many inconsistencies between the reports of different workers such that data from one are different from another and cannot be strictly compared. For instance, in the gene expression profile reported by Keren et al (2004) din J, rel E and max F were among the overexpressed TA modules, whereas in the report of Shah et al (2006) the list included din J, yeo B and yef M; only din J was common between the two. Similarly, ygi U, which was reported by Shah et al (2006) as being highly overexpressed, does not figure in the list of Keren et al (2004). While the latter authors used the hip A7 mutant for their experiments, the former used a wild-type strain. Whether the differences are due to differences in the nature of persisters in the two strains, as suggested by Shah et al (2006) is a moot question. The downregulated genes might be responsible for the generation and maintenance of dormancy required for persister formation.

Among the models on the mechanism(s) of origin of persistence, the one proposed by Keren et al (2004; reviewed by Lewis 2007, 2008) has strong experimental support. Several cases in which overexpression of TA modules (and a few non-TA genes) resulted in persistence...
have been reviewed above. All these rely on the (untested) hypothesis that the toxins inactivate or inhibit their targets (components of cell wall synthesis, replication, translation, etc.) and prevent them from corrupting their activities and thereby rendering the cells antibiotic tolerant. Korch et al. (2003) reported a model invoking a causative role for the alarmonc (p)ppGpp in persistence. To the best of the author’s knowledge, this is the only report implicating (p)ppGpp in persistence (except that of Rodionov and Ishiguro 1995; see below). The hypothesis of Korch and Hill that elevated levels of (p)ppGpp pushes the cells into the persistent state has not yet been substantiated; nevertheless, it is an interesting possibility. However, according to Korch et al. (2003), antibiotic stress is needed to increase the levels of (p)ppGpp. If this were true, one would expect it to be not restricted to a subpopulation but occur in all cells, as is generally the case in stress responses. Balaban et al. (2004) have shown that persisters exist in cells before antibiotic stress is imposed. Therefore, even if high (p)ppGpp levels were to be responsible for the generation of persistence, it could be brought about by stochastic switching (see above) in a subpopulation, either by increased synthesis or decreased degradation or both. The dormancy of persisters has been documented very well. While persisters are dormant, all dormant cells are not persisters. Something else besides dormancy seems necessary. Dormancy requires shutting or slowing down of the metabolic processes, especially macromolecular synthesis. While this requirement poses no problems in hip A+ strains, it does in hip A7 mutants. Korch and Hill (2006) have shown that overexpression of hip A+ inhibits macromolecular synthesis, but overexpression of hip A7 has only marginal effects on the process. This paradox has not been resolved satisfactorily although some explanations have been offered (Korch and Hill 2006). Both the models discussed above have one piece of strong experimental support each. The report of Correia et al. (2006) that mutational alterations at the aspartate residues at positions 309 and 322 of the Hip A protein abolished its ability to arrest cell growth and afford protection against cefotaxime and ofloxacin exposure strongly supports the Karen–Lewis model. On the other hand, the observation of Korch et al. (2003) that deletion of rel A and spo T (based on which they proposed their model) abolished persistence in the hip A7 mutant and that the defect could be complemented by the rel A+ gene in trans supports the (p)ppGpp-mediated persistence model. This strengthens the notion that there may not be a single unique mechanism giving rise to persistence. Many, perhaps not mutually exclusive, mechanisms might be involved. It is tempting to speculate that the two models discussed above might be two aspects of the same process and could be combined to give a ‘unified’ model of persistence. According to this speculation, stochastic increase in (p)ppGpp levels in a subpopulation might ‘activate’ toxins (especially Hip A) to interact with its/their targets and lead to persistence or, alternatively, stochastic increase in toxin levels and binding to their respective targets might ‘activate’ rel A–spo T to overproduce (p)ppGpp leading to persistence. Intuitively, the former seems more appealing and plausible. Many experiments could be designed to test the above hypothesis. As pointed earlier, the (p)ppGpp-mediated ampicillin tolerance reported by Rodionov and Ishiguro (1995) seems to be a case of populationwide drug indifference rather than persistence, since the whole population seems to be protected from lysis by ampicillin. Although multidrug tolerance (synonymous with persistence) has been shown by Wüff et al. (2005), they have focused more on the clinical relevance of the phenomenon than on the molecular mechanism(s). Another mechanism of antibiotic (ampicillin) tolerance is that proposed by Miller et al. (2004) invoking the SOS response as the trigger. Levin (2004) has commented that this may not be persistence but an additional synergistic process producing the same end result, namely, antibiotic tolerance. SOS-triggered antibiotic tolerance has not been explored further.

Summing up, the identification of genes involved in persister formation and the mechanism of persistence are still in a nebulous state. We are not anywhere near to arriving at a comprehensive and satisfactory picture of how persisters arise and how persistence works. Many uncertainties remain. Obviously, more efforts are needed to understand and clarify the multitude of problems and issues.

13. Persistence as a form of social behaviour in bacteria

The phenomenon of bacterial persistence has recently attracted the attention of evolutionary biologists and sociobiologists. Contrary to the earlier traditional belief that bacteria are simple and independent creatures, living and minding ‘their own business’ of life, reproduction and death, it is now known that they indulge in an astonishing array of complex social behaviours. Some examples are: quorum sensing, biofilm formation, persistence (a recent addition), resource utilization, bacteriocin synthesis, etc. (reviewed by West et al. 2006, 2007a). Gardner et al. (2007) have developed a theoretical model to investigate the social aspects of bacterial persistence. Without going into the subtleties of modelling, their work is summarized below from a non-specialist’s perspective. As pointed out in the earlier sections of this review, persistence is conceived as an insurance policy to guarantee population survival when faced with catastrophic situations such as antibiotic exposure. Evolutionary biologists reject the idea of traits being favoured because they benefit populations (West et al. 2007 b). No doubt persistence primarily benefits the
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persister because it enables survival during adversity (selfish benefit). However, because of their reduced growth rate (dormancy), they make critical resources available to non-persisters and facilitate their growth (social benefit), more so if the latter happen to be relatives (kin selection). Thus, persistence confers cooperative, mutual benefits on the self and others. The model of Gardner et al (2007) also predicts that at high population densities (late log/stationary phase) when resource competition would be intense, the fraction of cells that becomes persisters would be high. This is what has been found experimentally and also in clinical settings by several workers. Many parallels can be drawn between persistence and another, well-studied microbial social behaviour, namely, biofilms. For one, both involve reversible lifestyle switches. In the case of persistence, it is a switch from active growth to slow growth or dormancy. In the case of biofilms, it is a switch from a nomadic (planktonic) to a sedentary lifestyle (biofilm). The mechanisms involved or presumed to be involved are also similar. The transition from a nomadic to a sedentary state in biofilms has been shown to be caused by an increase in the levels of a compound called (3′-5′) cyclic dimeric guanosine monophosphate (c-di-GMP; see Kolter and Greenberg 2006 for references). This is similar to the increase in the levels of toxins such as Hip A over those of antitoxins such as Hip B in the case of persistence. Persisters are believed to confer cooperative growth benefits on their kin by virtue of their dormancy. A parallel to this in biofilms is the exit of some cells from the matrix to reduce competition inside and thereby help the biofilm cells to grow. Such parallels can be drawn between many social behaviours of bacteria, but it is beyond the scope of this review to attempt such an exercise. The excellent review by West et al (2007 b) gives a detailed account of many social behaviours of bacteria and could be consulted for more information. Studies on phenomena such as persistence, quorum sensing, etc. in several species of bacteria have led to the emergence of an interdisciplinary offshoot of Microbiology which has been christened ‘Sociomicrobiology’ by Parsek and Greenberg (2005). Hopefully, the nascent discipline of Sociomicrobiology will make great strides in future.

14. Epilogue

In contrast to the enormous efforts to understand mechanisms of persister generation and action, the properties of persisters, especially their mutagenic potential, seem to have received very little attention. Many reports have suggested that persisters are a potential source for the emergence of heritable antibiotic resistance. However, this idea does not seem to have been put to experimental test. Persisters are not as dormant as spores but are slow or very slow growers. (Refer to type I and type II persisters described by Balaban et al 2004.) A vast body of literature is available on the mutagenic potential of cells in the slow-growing state or stationary phase. I have earlier suggested that ‘leaky’ growth of E. coli could be mutagenic (Jayaraman 2000). It is tempting to speculate that mechanisms of stationary phase mutagenesis may also operate in persisters. The mutagenic potential of persisters may not be restricted only to the emergence of antibiotic resistance but may be generalized. In other words, the ‘insurance’ that persisters enjoy may cover many risks. Evolutionarily also, it makes sense and it is more advantageous to be prepared to face threats of different kinds than just one. The overexpression of SOS genes like rec A, umu C, umu D etc. in persister cells (Keren et al 2004) and SOS induction by ampicillin (Miller et al 2004) lend some credence to the above notions. Although persisters are a clinical menace, they may have some evolutionary value in the generation of spontaneous genetic variability.

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Note added in proof: While this manuscript was in press, I became aware of a report (Smith P A and Romesberg F E 2007 Combating bacteria and drug resistance by inhibiting mechanisms of persistence and adaptation; Nature Chem. Biol. 3 549–555) in which the authors have presented a brief review of persistence and suggested some possibilities to exploit inhibition of persistence as a potential therapeutic strategy to counter drug resistance/tolerance.

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