5

IMPORTANCE OF ADAPTIVE AND STEPWISE CHANGES IN THE RISE AND SPREAD OF ANTIMICROBIAL RESISTANCE

Lucia Fernandez, Elena B. M. Breidenstein, and Robert E. W. Hancock

Department of Microbiology and Immunology, University of British Columbia, Vancouver, British Columbia, Canada

5.1 INTRODUCTION

One of the major problems we are facing today in the context of infectious diseases is the relentless increase and spread of antimicrobial resistance. However, the origins of this phenomenon can be traced to well before the clinical antibiotic era. Compounds with antimicrobial activity are known to be produced by practically all living organisms, including bacteria, fungi, plants, and animals. Consequently, microorganisms, especially antibiotic producers themselves, have evolved mechanisms to protect themselves against the toxicity of these compounds. Also, existing cellular components with a totally different function can, in many cases, confer resistance to a given antimicrobial, thus acquiring a new function. All these traits would often be associated with a reduction in fitness and even virulence when the antimicrobial agent is not present. For pathogens, this means that maintaining these determinants would only be beneficial under antibiotic pressure. The corresponding genetic markers would otherwise be lost as part of the natural selection process. Nevertheless, this changed drastically with the use of antibiotics as therapeutics. In the beginning, antibiotics were very effective in clearing the infections, but shortly afterward resistant organisms started to arise. The high selective pressure caused by intensive utilization of these compounds would inevitably lead to the selection of those

Antimicrobial Resistance in the Environment, First Edition.
Edited by Patricia L. Keen and Mark H.M.M. Montforts.

microbes able to withstand the concentrations administered to the patients. Indeed, there have been reports of resistance toward practically all antibiotics available today. Alarmingly, there is a group of microorganisms, named Superbugs, that are resistant to most antimicrobials currently prescribed, and these include some of society's most prevalent causes of hospital infections including methicillin-resistant *Staphylococcus aureus* (MRSA), multidrug-resistant *Pseudomonas aeruginosa*, *Enterococcus* sp., and the like. Overall, antibiotic resistance represents a serious threat to modern medicine as we might one day be left helpless to combat bacterial infections. It is therefore imperative to tackle this problem and minimize the rise of resistance as well as develop novel and more efficient therapeutics. A further problem is that antimicrobial agents are not only used in human medicine but also in veterinary medicine, agriculture, industry, and the like. As a result, the levels of antibiotics found in many different settings, including areas adjacent to farms and sometimes natural ecosystems, can be fairly high. These niches have now become reservoirs of resistance markers that could be later transferred to human pathogens. This occurrence also needs to be taken into account if we want to succeed in the battle against antibiotic resistance.

There are three principal types of antibiotic resistance, namely intrinsic, acquired, and adaptive. Intrinsic resistance comprises all the natural, underlying characteristics of a particular species or strain that make it immune to the action of one or more types of drugs. Resistance determinants can also be acquired via horizontal transfer or as a result of mutations. This acquired resistance may lead to a high-level decrease in susceptibility, although in many cases, in particular in the case of mutational resistance, only a low-level (stepwise) increase is observed. Finally, microorganisms can become adaptively resistant to antibiotics in response to certain changes in the environmental conditions. In contrast to intrinsic and acquired mechanisms of resistance, which are permanent and stable, adaptive resistance is characterized by its transient nature. For a long time, clinical research focused on resistance phenotypes with a high impact and of a permanent nature. For this reason, phenomena such as stepwise and adaptive resistance have been largely neglected and are, as a result, poorly understood. Nonetheless, a growing number of studies highlight the importance that these mechanisms might have in the rise and geographical expansion of antimicrobial resistance. In fact, the ability of microorganisms to survive at concentrations around the minimal inhibitory concentration (MIC) facilitates the subsequent acquisition of high-level resistance markers (Baquero, 2001). Furthermore, there is more and more evidence that the decrease in susceptibility of a particular species does not necessarily occur in a single large step but rather in a gradual and steady manner. Thus, it is now clear that the baseline MICs are creeping, that is, slowly rising over time. Stepwise and adaptive resistance are good candidates to play a role in this MIC creep. Interestingly, it has transpired that some of the mechanisms responsible for these two different types of resistance are overlapping or related to some extent.

One of the best studied microorganisms in terms of resistance mechanisms is the opportunistic human pathogen *P. aeruginosa*. This ubiquitous bacterium can live in numerous habitats including soil, water, plants, and animals. Moreover, it is one of the leading causative agents of hospital-acquired infections as well as morbidity and mortality in cystic fibrosis (CF) patients. One constant characteristic of *P. aeruginosa* infections is that they are very difficult to eradicate. This pathogen is not only

intrinsically resistant to antibiotics, but it too can easily become less susceptible through the acquisition of new traits or adaptation via complex regulatory pathways. All these reasons make *Pseudomonas* an excellent and intriguing model to describe what we currently know about adaptive and stepwise acquired resistance. Specifically with reference to this book, *Pseudomonas* is found throughout the environment as well as being a prominent opportunistic pathogen of humans and animals, causing more than 160,000 infections annually in the United States. Nonetheless, we will also include some examples corresponding to other microorganisms. This chapter intends to summarize the most recent findings on the adaptive and stepwise types of resistance, as well as illustrate how these may contribute to the overall decrease in susceptibility to antimicrobials in bacteria.

5.2 TRANSCRIPTIONAL RESPONSES TO ANTIMICROBIALS

The use of novel molecular techniques in microbiology has permitted the observation of changes experienced in the transcriptome or proteome of microbes under different environmental conditions. One such technique is microarray analysis, which reveals the differences in gene expression between two samples. Particular attention has been paid to the responses displayed by bacteria upon exposure to sublethal and lethal doses of antimicrobial compounds. The results obtained to date in these experiments have revealed interesting hints about the mechanisms involved in antibiotic activity and antibiotic resistance. Moreover, these data suggest that antibiotics actually have complex modes of action, thereby challenging the traditional view of antibiotics having a single target in the bacterial cell. Brazas and Hancock (2005a) described the existence of gene signatures specific to a given antimicrobial compound that should be distinguished when interpreting microarray data. These genes may, according to the authors, be classified in four groups: genes dysregulated as a direct or indirect effect of the mechanism of action of the drug are, respectively, groups 1 and 2; group 3 comprises those genes altered as a downstream effect of target inhibition but that do not participate in the antibiotic activity or in antibiotic resistance. Finally, the genes included in group 4 are specific to a particular species, strain, or antibiotic and are termed bystander effects. An example can be provided by analyzing the transcriptional profile of *P. aeruginosa* cells exposed to ciprofloxacin (Brazas and Hancock, 2005b), which is summarized in Table 5.1. Fluoroquinolones interfere with DNA (deoxyribonucleic acid) replication resulting in DNA damage. The direct effect of this is the induction of the SOS response genes, which could be classified into group 1. The *Pseudomonas* SOS response involves the upregulation of the genes in the R2/F2 pyocin region. Interestingly, mutation of these genes resulted in a considerable decrease in susceptibility to ciprofloxacin. This seems to indicate that the higher expression of pyocins in the presence of this antibiotic is contributing to its killing action. It is likely that these pyocins constitute a phage lytic system that leads to cell lysis upon its activation (Nakayama et al., 2000). Genes in group 2 include those involved in metabolic changes, general stress responses, and antibiotic resistance mechanisms, which are the indirect result of the target inhibition. For instance, exposure to ciprofloxacin induces the expression of the efflux pump MexAB-OprM and the sigma factor *algU*, which coordinates an environmental stress response. In turn, the upregulation of *algU* results in a higher expression of genes involved in

TABLE 5.1 Examples of Genes Included in the Four Groups of the Signature Response to Ciprofloxacin[a]

	Characteristics	Examples
Group 1	**Direct effects** of target inhibition	SOS response genes
Group 2	**Indirect effects** elicited by target inhibition aimed at compensating for the damage caused by the antibiotic	Efflux pump *mexAB-oprM; algU* (cell wall stress response)
Group 3	**Secondary effects** of target inhibition that are not related to antibiotic activity or contribute to antibiotic resistance	Alginate production genes (*algR*, *algB*, etc.)
Group 4	**Bystander effects**, unrelated to target inhibition but constant for a particular microorganism or antibiotic	Motility and attachment genes (*pilG*, *fimV*, etc.)

[a]These examples have been taken from the microarray data published by Brazas and Hancock (2005a).

alginate biosynthesis, such as *algB*, which can be considered an example of group 3. Finally, exposure to ciprofloxacin leads to a downregulation of genes involved in motility and attachment. These could be described as bystander effects and would belong to group 4.

These experiments have also revealed intriguing results that might be linked to the ecological role of antibiotics. It is of particular interest that subinhibitory concentrations of antibiotics can sometimes induce the expression of virulence-related factors. A good example of this phenomenon is provided by Linares et al. (2006) who studied the transcriptome of *P. aeruginosa* in the presence of sublethal doses of three antibiotics belonging to different classes, namely tobramycin, ciprofloxacin, and tetracycline. This study found, for instance, that tobramycin upregulates the expression of genes involved in pyoverdine synthesis, whereas tetracycline induces pyochelin biosynthesis and type III secretion (T3S) genes. Moreover, the effect of subinhibitory antibiotics on some virulence-related phenotypes was also determined. Thus, all three antibiotics induced biofilm formation, but only tobramycin and tetracycline promoted swimming and swarming motility. In contrast, ciprofloxacin seemed to inhibit motility phenotypes. A previous study by Marr et al. (2007) had previously shown that subinhibitory tobramycin induces the expression of motility-related genes. Also, the presence of subinhibitory tetracycline increased cytotoxicity in *P. aeruginosa*, which is in good agreement with the induction of T3S. The T3S genes were also upregulated in the presence of subinhibitory concentrations of the macrolide azithromycin (Nalca et al., 2006). However, this antibiotic appeared to downregulate the expression of other quorum sensing-regulated genes such as *lasA* and *rhlB*. The adaptability of *P. aeruginosa*, whereby it is able to live in many different niches including soil, water, as well as human and animal hosts, affords a good model to try to pinpoint the responses of bacteria to antibiotics in nature. As mentioned previously, antibiotics are widespread in the natural environment. However, the concentrations detected in this context are well below those used in therapy, although within antibiotic-intensive farming operations concentrations may be higher. For this reason, it is thought that the role of these compounds cannot be

the inhibition of the growth of competitor organisms and is unlikely to directly select for high-level acquired resistance. In fact, the results discussed above provide significant evidence that antibiotics might be signaling molecules in a similar way to quorum sensing signals (Linares et al., 2006). Indeed, in *P. aeruginosa*, quorum sensing regulators such as the *Pseudomonas* quinolone signal (PQS) and the homoserine lactones display antimicrobial activity at high doses (Dubern and Diggle, 2008; Kaufmann et al., 2005; Wells, 1952).

In the context of antibiotic resistance, microarray analyses give very helpful information about possible mechanisms activated in bacteria to fight against the toxic effects of antimicrobials. Overall, high-throughput molecular techniques prove that microorganisms can easily alter their expression profile and adapt to the presence of antibiotics, thereby becoming more resistant. Furthermore, these experiments show how small changes in gene expression and small changes in resistance can be determinants in increasing bacterial fitness in the presence of an antimicrobial insult. This tactic would allow certain cells in the population to survive, multiply, and, in many cases, have a greater probability of attaining stable determinants of high-level resistance. Without these novel techniques, we probably would not have even begun to comprehend the relevance or to understand the underlying mechanisms of the types of resistance described in this chapter. Characterization of the expression patterns of known antibiotics can also be very useful in the design and development of new antimicrobials. Thus, comparison of the gene signatures between a novel and a well-described antibiotic can give information about possible cell targets, induction of adaptive resistance mechanisms, and the like. This will allow for a more efficient selection of the best candidates for further development and subsequent introduction into the clinic.

5.3 STEPWISE INCREASE IN RESISTANCE (INTRINSIC RESISTOME AND MUTATIONAL RESISTOME)

In clinical settings it is well known that pathogens have, throughout the years, increasingly become resistant to the different antibiotics currently available. Thus, the initial susceptibility observed when an antibiotic was first introduced in the market gave way relatively soon to phenotypes with varying degrees of resistance. One example would be the rise of resistance to the aminoglycoside tobramycin in *P. aeruginosa*. Originally, *Pseudomonas* strains were susceptible with an MIC $< 2\,\mu g/mL$ (Dibb et al., 1983), but some time after administration became frequent, resistance increased constantly. Indeed, right now we are facing the problem that resistant isolates with an MIC of $16\,\mu g/mL$ are isolated on a frequent basis, and sometimes strains with an MIC $> 128\,\mu g/mL$ are identified (MacLeod et al., 2000). These highly resistant strains represent a serious threat, especially because they are often resistant to more than one antibiotic. Multidrug-resistant bacteria, including *Pseudomonas*, are extremely difficult to eradicate and have become a major problem in the treatment of infections. As mentioned previously, such organisms are also referred to as Superbugs.

It was originally thought that these increases in antibiotic resistance happened suddenly. However, recent studies demonstrate that the "baseline" MIC actually shows a stepwise rise over time. This phenomenon has been clearly shown by

Steinkraus et al. (2007) in the case of susceptible MRSA isolates. A stepwise increase in MIC in the years 2001–2005 was observed for originally susceptible clinical MRSA isolates. The geometric mean MIC for vancomycin shifted from 0.62 to 0.94 μg/mL. This change is small; however, it is of concern as it can lead to a higher level of antibiotic resistance over time. Little attention has been paid to these small changes, which are often unnoticed in clinical screenings. However, over the last years it has become clear that a subtle increase in the MIC could actually contribute to the high-level resistance that leads to clinical failure. The phenomenon of low-level antibiotic resistance and its impact on high-level antibiotic resistance has been extensively reviewed by Baquero (2001). This review describes clearly how several compounds can select for low-level resistance and explains how these phenotypes may lead to high-level resistance. For instance, even though a single mutation might lead to just a small increase in the MIC, the occurrence of additional mutations in the same strain would inevitably result in a more dramatic change (El'Garch et al., 2007). These authors showed that a mutation in *galU*, *nuoG*, *mexZ*, or *rplY* led to a twofold increase in resistance to aminoglycosides. For example, the MIC for tobramycin was 1 μg/mL for all the single mutants, whereas the MIC of the wild-type was 0.5 μg/mL. Interestingly, an increase in resistance could be observed once double, triple, and quadruple mutants were tested. A *galU*/*nuoG*/*mexZ*/*rplY* quadruple mutant exhibits a tobramycin MIC of 8 μg/mL. This clearly shows the increase in resistance for additional mutations.

Although the mechanisms involved in low-level resistance have just started to be understood, different authors already suggest that they are far more complex than originally anticipated (Alvarez-Ortega et al., 2010, Breidenstein et al., 2008, Dötsch et al., 2009; Schurek et al., 2008). Recently, several screening studies revealed that many different mutations can lead to low-level resistance to antibiotics in *P. aeruginosa*. Moreover, while some mutations lead only to low-level resistance to one antibiotic class, several others result in low-level resistance to different antibiotic classes at the same time. The antibiotics used for these screens belonged to different classes and included fluoroquinolones, aminoglycosides, β-lactams, tetracycline, and sulfonamide. In this section, we will discuss the results obtained in these screenings with particular focus on the mutations that lead to decreased susceptibility and, therefore, to low-level resistance (mutational resistance). Nonetheless, we will also briefly mention some of the identified genes that led to increased susceptibility when mutated (involved in intrinsic resistance). Most of the studies were carried out using the comprehensive Harvard library (Liberati et al., 2006), which has been created in a PA14 strain background and comprises an array of more than 4500 individual mutants in separate genes. However, some studies also used the PAO1 miniTn5- *luxCDABE* library (Lewenza et al., 2005), and due to strain specificity, certain differences were observed (Brazas et al., 2007; Fajardo et al., 2008). Also, one has to keep in mind that the screens were performed on libraries of nonredundant genes and all essential genes are missing. Therefore, the gene list is not comprehensive and it is possible that more genes have an impact on resistance. Interestingly, all of these studies confirm the view that nonclassical antibiotic resistance genes participate in resistance, an idea that has developed in recent years. A better knowledge of these genes may play an important role in identifying new antimicrobial targets.

The ciprofloxacin resistome was analyzed in two independent studies by Brazas et al. (2007) and Breidenstein et al. (2008), which used the PAO1 and PA14

backgrounds, respectively. These screens found many genes that, once inactivated, led to altered susceptibility. Most times, the fold changes observed were only twofold, and such small changes can be easily missed in the clinic or environment. However, the large number of different genes that can lead to a small increase or decrease in susceptibility is important as it reflects a very large reservoir of mechanisms by which bacteria can become resistant. The ciprofloxacin resistome is not only large but also diverse in that a variety of genes involved in different cellular functions has an impact on ciprofloxacin resistance. Cross resistance between ciprofloxacin and other fluoroquinolones, such as nalidixic acid and levofloxacin, could also be observed. Breidenstein et al. (2008) discovered that 114 out of the approximately 4500 transposon mutants screened showed an altered susceptibility to ciprofloxacin. Thus, 35 mutants had an increase and 79 mutants had a decrease in susceptibility. As mentioned above, most of these showed only a twofold change, and many of these genes are distinct from those traditionally found to be involved in antibiotic resistance. Indeed, many genes from different functional classes are involved including those involved in transport of small molecules, membrane proteins, energy metabolism, DNA replication and recombination, cell division, hypothetical proteins, phage proteins, and the like. Interestingly, prior microarray analysis revealed that 43 out of the 114 identified resistance genes were dysregulated after exposure to 0.3× or 1× MIC of ciprofloxacin in the wild-type strain (Brazas and Hancock, 2005b). This overlap between global gene expression and resistance determinants highlights the fact that *Pseudomonas* can activate certain defense mechanisms in order to combat the bactericidal activity of ciprofloxacin.

Of particular interest among the mutants that led to increased susceptibility to ciprofloxacin, reflecting intrinsic resistance mechanisms, were those with mutations involved in DNA replication and repair. These genes included the Holliday junction helicases *ruvA* and *ruvB*, the recombinase *xerD*, the site-specific recombinase *sss*, the adenosine-triphosphate-(ATP)-dependent helicase *recG*, the ATP-dependent protease *lon*, DNA topoisomerase I *topA*, and DNA binding protein *fis* (Breidenstein et al., 2008; Dötsch et al., 2009). For example, the inactivation of *lon* leads to a four to eight fold change in increased susceptibility, making *lon* very important for ciprofloxacin resistance. The cell division protein *ftsK*, when mutated, also showed an eightfold increase in susceptibility. Other studies showed that inactivated *ftsK* also had a supersusceptible phenotype to different classes of antimicrobials represented by levofloxacin, ceftazidime, imipenem, meropenem, ertapenem, and cefotaxime (Alvarez-Ortega et al., 2010; Dötsch et al. 2009). The major intrinsic efflux pump *mexAB-oprM*, which is known to be involved in antibiotic resistance, was also demonstrated to have an increased susceptibility to all tested antibiotic classes (fluoroquinolones, β-lactams, tetracycline, and sulfonamide) upon mutation. It is therefore clear that the antibiotic resistance mechanisms are not specific for one antibiotic class as significant overlap exists (Fig. 5.1). The intrinsic ciprofloxacin resistome was thus shown to involve a huge gene pool.

On the other hand, several genes that when mutated led to mutational resistance and MIC creep over time, were identified. These included phage-related genes, Hydrogenated nicotinamide adenine dinucleotide (NADH) dehydrogenases, DNA mismatch repair proteins *mut*, the *mexCD-oprJ* efflux regulator *nfxB* as well as genes involved in iron transport. Interestingly, Brazas and Hancock (2005b) previously observed, using the PAO1 mutant library, that mutants in the bacteriophage-like R2/F2 pyocins

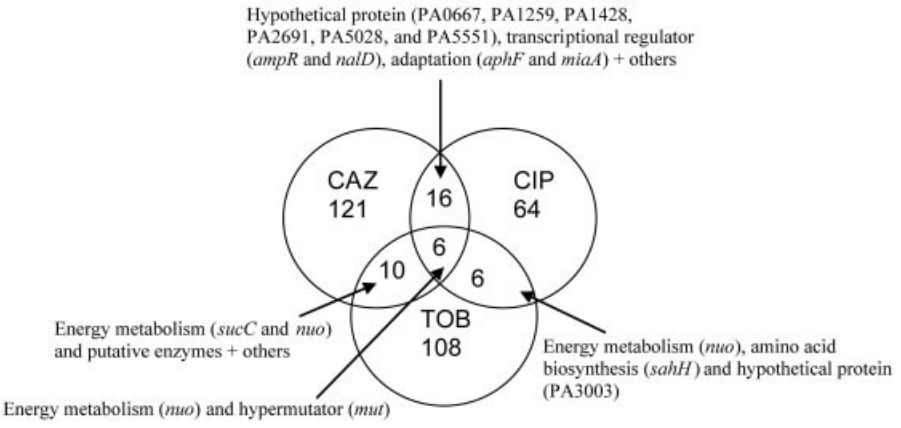

FIGURE 5.1 Analysis of the mutants that showed increased resistance to three antibiotics, representing different antibiotic classes, found in several screens (Alvarez-Ortega et al., 2010; Breidenstein et al., 2008; Dötsch et al., 2009; Schurek et al., 2008). The figure represents the number of resistant mutants to each antibiotic as well as the overlaps between the different antibiotics. Examples of the overlapping genes are given for each group. The abbreviations are as follows: CAZ, ceftazidime, CIP, ciprofloxacin, and TOB, tobramycin.

(PA0613-PA0648) are resistant to ciprofloxacin. These genes are also highly upregulated upon ciprofloxacin exposure, making them a susceptibility determinant.

Likewise, a study of the aminoglycoside resistome indicated that free radicals may impact on antibiotic killing. Thus, mutation of genes involved in aerobic respiration, such as cytochrome and NADH reduction genes, showed a decrease in tobramycin susceptibility (Schurek et al., 2008). Kindrachuk et al. (2011) demonstrated that these genes are downregulated in wild-type *P. aeruginosa* upon lethal exposure to tobramycin under anaerobic and aerobic conditions. In contrast, studies in *Escherichia coli* carried out by Kohanski et al. (2007, 2008) showed that the components of the tricarboxylic acid cycle (TCA) and electron-transport chain are upregulated upon exposure to aminoglycosides (gentamicin and kanamycin), suggesting possible mechanistic differences in these two species. The study by Schurek et al. (2008) also identified many other determinants of low-level tobramycin resistance in *Pseudomonas*. In fact, 135 genes leading to a twofold change upon mutation were identified. These genes are thought to contribute to the gradual low-level increase in tobramycin resistance. These small changes were also validated for selected genes in the PAO1 background. Mutated genes leading to low-level tobramycin resistance are primarily involved in energy metabolism (cytochrome, *nuo*, *nos*, and *nqr*), DNA replication and repair (*mut*, *mic*, *uvr*, and *radA*), and lipopolysaccharide (LPS) biosynthesis (*wbp*). Generally, aminoglycosides cross the cytoplasmic membrane via an energy-dependent process involving the electron-transport chain (Hancock, 1981). Therefore, it is not surprising that energy metabolism mutants exhibit an increase in resistance. This resistance can be easily explained by an alteration of the membrane potential that causes a decrease in aminoglycoside uptake (Mates et al., 1982). In contrast, a different study by Dötsch et al. (2009) did not observe the acquisition of resistance to tobramycin after inactivation of the *nuo* operon (NADH oxidoreductase). These mutants did,

however, display resistance to piperacillin, piperacillin-tazobactam, cefepime, cefotaxime, ceftazidime, and ciprofloxacin. Schurek et al. (2008) further observed that mutants in genes involved in the assembly of the A-band of LPS exhibit increased resistance to tobramycin (*wbpZ*, *wbpY*, *wzt*, *wzm,* and *wbpW*). By using the 1-*N*-phenylnaphthylamine (NPN) assay (Loh et al., 1984), the interaction between tobramycin and the outer membrane could be determined for the wild-type and the resistant LPS mutants. The authors could clearly demonstrate that tobramycin is less able to permeabilize the outer membrane of the LPS mutants, therefore enabling them to become more resistant. The tobramycin resistome analysis demonstrated for the first time that aminoglycoside resistance is affected by a wide range of different factors. The variety of identified genes that, when mutated, lead to minor changes in the MIC indicates that it is highly possible that if more mutations occur they would have an additive effect. This accumulation phenomenon had previously been suggested by Baquero (2001) and later demonstrated by El'Garch et al. (2007). Therefore, the observation that hundreds of mutations can lead to tobramycin resistance is of great importance, especially in the context of clinical settings. CF patients often undergo aminoglycoside treatment in order to treat *Pseudomonas* infections in their lungs; however, it is known that increasingly resistant strains are selected in the lungs. Thus, antibiotic therapy failure can be partly explained by the occurrence of tobramycin resistance mutations, such as those investigated in the study by Schurek et al. (2008).

Another important antibiotic class for the treatment of *Pseudomonas* infections is the β-lactams, which includes cephalosporins, carbapenems, and penicillins. β-lactams bind to penicillin-binding proteins (PBP) leading to peptidoglycan synthesis blockage (Walsh, 2003). Resistant mutants are known to involve mutations in membrane proteins, efflux, and the enzyme β-lactamase (Poole, 2004). However, the independent low-level β-lactam resistance studies by Dötsch et al. (2009) and Alvarez-Ortega et al. (2010) revealed that far more genes lead to an increase in β-lactam resistance if mutated. The study of Alvarez-Ortega et al. (2010) focused on the cephalosporin ceftazidime and 2 carbapenems, namely imipenem and meropenem, while Dötsch et al. (2009) included the penicillins. Although the overall structure of the β-lactams is similar, distinct differences between the tested antibiotics could be observed. In total, Alvarez-Ortega et al. (2010) found 78 mutants with an alteration in susceptibility. Of these, 41 mutants showed reduced susceptibility and 37 showed an increase in susceptibility toward at least one antibiotic. Most mutants leading to a low-level increase in resistance were observed for ceftazidime (37 ceftazidime/9 imipenem/14 meropenem resistant mutants). Only one hypothetical mutant, PA0908, exhibited a 2- to 3-fold increase in MIC toward all 3 antibiotics tested, while 14 mutants showed reduced susceptibility to 2 antibiotics. This small overlap indicates somewhat specific resistance mechanisms depending on the particular compound. The fact that the largest number of resistant mutants appeared in the ceftazidime screen highlights the easier development of ceftazidime-resistant mutants through mutation. This is very important for clinical treatment as all three antibiotics are currently used for antipseudomonal therapy. However, one has to keep in mind that some cross resistance to other antibiotic classes was observed through the different screens and, therefore, overlap between the resistant mechanisms can exist. Based on these observations, it would be important to reconsider the administration regimes of certain β-lactams in the light of their potential for

resistance. Albeit the changes are modest, they correlate with the low-level resistance phenotype described by Baquero (2001) and in combination could lead to significant antibiotic resistance and play a part in MIC creep over time. Regarding ceftazidime, the isolated mutants were involved in efflux as well as cell wall and LPS biosynthesis; some exhibited increased β-lactamase production. In the aminoglycoside resistome analysis described above, mutants involved in LPS biosynthesis were also identified. However, these were not identical to the ones found in the β-lactam screen. LPS biosynthesis mutants in *wapR*, *wpmM*, *wbpL*, *wspE*, *galU*, and the operon PA5001-5005 exhibited an increase in resistance to cephalosporins, penicillins, and sometimes to carbapenems (Alvarez-Ortega et al., 2010; Dötsch et al., 2009). The basis for this phenomenon might be a decrease in antibiotic penetration, although any such effect would be indirect as, unlike the aminoglycosides, β-lactams pass through the channels of outer membrane porins. β-lactam resistance also occurs due to an overexpression of efflux systems, such as MexAB-OprM, which occurs when the negative regulator, *nalC*, is mutated. Several other mutations leading to ceftazidime resistance could be identified including mutants in the following genes: *ampR*, *ampD*, *dacB* and *mpl*. These strains exhibited different degrees of *ampC* β-lactamase overproduction (6.4- to 55-fold increase) which would make these mutants more resistant to ceftazidime. Interestingly, *ampR* mutants were also shown to be more resistant to almost all other tested antibiotics, except tobramycin and carbapenems. In the case of the carbapenems (meropenem and imipenem), the most important mechanism of attaining increased resistance via mutation is the inactivation of the OprD porin through which carbapenems are known to enter the cell.

A comparison of the results obtained in a variety of different screens revealed that some genes are involved in resistance to more than one antibiotic class (Fig. 5.1). These include *mutS* and *mutL*, also known as hypermutators. If either one of these two genes is mutated, the spontaneous mutation rate increases dramatically (100- to 1000-fold) (Oliver et al., 2000). The result of this is that they exhibit an accumulation of secondary mutations, and this makes these strains rapidly acquire high resistance to several antibiotics. These same hypermutators are commonly found in CF patients (Ciofu et al., 2005) and help reduce the effectiveness of the antibiotic therapy administered for chronic lung infections. At an early stage of the infection, weak hypermutators are isolated from patients with CF (Kenna et al., 2007). Some of these weak hypermutators in *P. aeruginosa* were identified by Wiegand et al. (2008). A mutator phenotype is generally related to a disruption in DNA repair genes and, therefore, it was not surprising to find a modest increase in mutation frequency for mutants in *mutT*, *mutY*, and *mutL*. Two other gene deletions led to an increase in mutation frequency, namely *radA* (PA4609) and PA3959. The DNA repair protein RadA, once mutated, enhances the mutation frequency by 15-fold, whereas PA3958, belonging to the endonuclease/exonuclease/phosphatase family, increases the mutational frequency by only 3.4-fold. Thus it is evident that there are a variety of hypermutator mutants that could play a role in the early infection stages of CF patients. The investigation of these new hypermutator strains emphasizes the antibiotic treatment problem in the clinic as well as the need to find new strategies for overcoming these resistance problems.

Overall, the identification of hundreds of genes that when mutated contribute to antibiotic resistance provides a new perspective on resistance. The large number of mutations leading to low-level antibiotic resistance might provide an explanation for

the clinical phenomenon of MIC creep, and ultimately lead to high-level antibiotic resistance. Indeed, such mutations might occur in environmental situations and, while significant, be easily missed.

5.4 MECHANISMS OF ADAPTIVE RESISTANCE

The induction of resistance determinants by certain environmental cues is known as adaptive resistance. This type of resistance is transient and, in general, the initial levels of susceptibility can be restored after removing the inducing signal. In some cases, however, several passages under noninducing conditions are required (Mawer and Greenwood, 1978). Some of these triggering signals correspond to environmental factors in the milieu surrounding the microorganism such as anaerobiosis, altered pH, and decreased levels of nutrients including ions or particular carbon sources. This means that when the pathogen is in certain ecological niches or mediating an infection, it might exhibit greater resistance to antimicrobial agents than predicted when grown on typical laboratory nutrient-rich medium. This is further complicated by the fact that the presence of antibiotics, in particular at subinhibitory concentrations, can also make the bacteria able to withstand a subsequent challenge with an otherwise lethal dose. In addition, when bacterial cells form multicellular structures such as biofilms or swarming colonies, they are also less susceptible to antibiotics. As biofilms are currently regarded as one of the most common forms that bacteria use to live in natural environments and within the host, this is of particular significance. Furthermore, all of these factors can potentiate one another. For instance, exposure of biofilms to antibiotics can increase their level of resistance even more (Bagge et al., 2004; Pamp et al., 2008).

Microbiologists have known for decades that the exposure of bacterial cells to sublethal concentrations of an antimicrobial drug in the laboratory increases the microbe's ability to resist a subsequent antibiotic insult. For example, the existence of adaptive resistance to aminoglycosides in *P. aeruginosa* was described by Barber and Waterworth (1966). Interestingly, strains adapted to high levels of gentamicin in vitro showed a loss in virulence compared to their parent strain (Weinstein et al., 1971). This would make them less effective when infecting the host. Therefore, it was considered at the time that adaptive resistance would not be important in the clinical context. However, as early as 1978, an intriguing study demonstrated that after inducing adaptation with gentamicin or tobramycin at lower concentrations, *E. coli* exhibited a modest adaptive resistance phenotype that did not significantly reduce virulence or growth (Mawer and Greenwood, 1978). This led to decreased susceptibility toward several aminoglycosides and not just the one used in the initial exposure. A consequence of this is that if bacteria carrying a gentamicin-specific high-level resistance marker get exposed to gentamicin, they acquire increased resistance to other aminoglycosides, for example, tobramycin, preventing it from being used later in the treatment. In spite of these data, the phenomenon of adaptive resistance was still deemed irrelevant by many. It was not until the 1990s that the development of adaptive resistance to aminoglycosides was taken into account in order to improve the effectiveness of administration regimes. At that time several studies recommended the use of higher doses and longer intervals between doses within the range allowed by the toxicity associated with this class of antibiotics (Barclay et al., 1996; Daikos et al., 1991).

As mentioned above, we now know that adaptive resistance is induced in the presence of many different environmental cues, including subinhibitory (sub-MIC) concentrations of antimicrobials. However, its impact and the specific underlying mechanisms are just starting to be understood. One of the main problems in evaluating the clinical impact of adaptive resistance is that it is not a stable phenotype, and, as a result, it cannot be easily detected with traditional antimicrobial resistance screening methods. Nevertheless, it seems very likely that adaptation to host conditions or to antibiotic exposure during treatment is one of the reasons for clinical failure of antibiotic therapy. One implication of this type of resistance is that the susceptibility profile of a pathogen might be completely different under in vitro and in vivo conditions. Furthermore, such adaptations sometimes confer protection from, that is, cross resistance to, several antimicrobial agents, including those from different classes. Another cause of concern is that, like stepwise resistance, the development of adaptive resistance can be associated with a greater probability of evolution toward a high-level resistance phenotype (Driffield et al., 2008; Hausner and Wuertz, 1999; Molin and Tolker-Nielsen, 2003).

Albeit insufficient, the knowledge we currently have seems to indicate that adaptive resistance is a complex and tightly regulated phenomenon. In fact, many advances in this field were made through understanding transcriptional regulation. In the following sections we will summarize the different tactics employed by bacteria in acquiring adaptive resistance and provide examples for each of them.

5.4.1 Efflux and Influx

One of the most common mechanisms used by bacteria to become resistant to an antimicrobial compound is by limiting its accumulation inside the cell. There are two different ways of achieving that, namely limiting the entrance of the drug into the cell or actively pumping it out of the cell. In many cases, these mechanisms are intrinsic and are characteristic of a particular species. For example, *P. aeruginosa* possesses a low permeability outer membrane and as a result antibiotics cannot penetrate easily into the bacterium. Indeed, it is believed that it is the synergy between this reduced permeability and active efflux (as well as the above-mentioned enzymes like β-lactamase) that makes *Pseudomonas* an intrinsically highly resistant species. The genes encoding efflux pumps, porins, and the like are sometimes only expressed at a very low level under normal growth conditions and require the presence of a specific environmental cue to upregulate their expression. In these situations, one can observe clear examples of adaptive resistance.

Active extrusion of toxic compounds, including antibiotics, out of the bacterial cell is mediated by transport proteins called efflux pumps. Five families of efflux pumps have been identified in bacteria to date (Schweizer, 2003). Among these, four obtain energy from the proton motive force. These are the RND (resistance–nodulation–division), the MF (major facilitator), the SMR (small multidrug resistance), and the MATE (multidrug and toxic compound extrusion) families. In contrast, the energy source for the ABC (ATP-binding cassette) family is ATP hydrolysis. Some of these transporters are specific and can only export one compound, whereas others have a broad range of substrates, thus participating in the development of multidrug resistance. Constitutive expression of efflux pumps may have deleterious effects on bacterial fitness and even virulence. This was

described for the *P. aeruginosa nalB* and *nfxB* multidrug-resistant mutants, which overexpress the RND efflux pumps MexAB-OprM and MexCD-OprJ, respectively (Sanchez et al., 2002). These mutants showed impairments in different virulence-related phenotypes such as phenazine and protease production or virulence in a *Caenorhabditis elegans* model, but not in biofilm formation. They also showed reduced fitness in water or on dry surfaces, environments that reflect potential ecological reservoirs of this pathogen. As a result of these potential disadvantages, there is a tight control of the transcription of the genes encoding pumps so that they are only upregulated when they provide an advantage to the cell. Some well-described cases have related this induction with the presence of antimicrobial compounds. *P. aeruginosa* possesses multiple efflux pumps, including 12 putative candidates belonging to the RND family, although not all of them have yet been characterized (Stover et al., 2000). Each pump has a different range of exported compounds. Likewise, specific types of antimicrobials tend to induce only certain pumps. For example, aminoglycosides are known to induce the MexXY efflux pump, which accounts for a significant part of adaptive resistance to this class of antibiotics (Hocquet et al., 2003). In fact, mutants in *mexXY* do not acquire as great an increase in resistance upon exposure to aminoglycosides as does the parent strain (Hocquet et al., 2003). In a similar manner, part of the adaptive resistance triggered by subinhibitory ciprofloxacin is due to the upregulation of the efflux pump MexAB-OprM (Brazas and Hancock, 2005b). Due to the ability of some of these pumps to extrude multiple drugs, a worrying consequence of this is that exposure to one antibiotic would induce the expression of the corresponding efflux pump. This would then make the cells less susceptible to several classes of antibiotics. Figure 5.2 provides an example of this phenomenon based on the upregulation of *mexAB-oprM* by ciprofloxacin. Also of concern is the existence of cross resistance between biocides and antibiotics. Biocides are chemical substances generally used as disinfectants, antiseptics, and antifouling agents in diverse human activities. These include hospitals, households, industry, and agriculture. As a result, the possibility that exposure to these compounds is inducing resistance not only to biocides themselves but also to clinical drugs is alarming indeed. One of the predicted mechanisms for this phenomenon is the induction by biocides of efflux pumps (Chuanchuen et al., 2001; Hegstad et al., 2010; McMurry et al., 1998).

The upregulation of efflux pumps has also been observed in biofilms. For instance, Zhang and Mah (2008) identified an MF efflux pump in *P. aeruginosa* that participates in increased resistance to tobramycin and ciprofloxacin in the biofilm state but not in a planktonic culture. Transcriptional analysis showed that this pump, encoded by the operon PA1874-77, was upregulated during biofilm formation. Also, the resistance of *Pseudomonas* biofilms to azithromycin was found to require the presence of MexCD-OprJ (Gillis et al., 2005). Another interesting occurrence is the induction of the genes *mexAB-oprM* upon exposure of the cells in the upper layers of *Pseudomonas* biofilms to colistin (Pamp et al., 2008). In contrast to biofilms, the increased resistance of *P. aeruginosa* swarming cells does not seem to involve the participation of any of the well-characterized efflux pumps (Lai et al., 2008).

Of note, *P. aeruginosa* mutants showing an overexpression of efflux pumps are commonly isolated from patients after antibiotic therapy. These isolates show a reduced susceptibility toward multiple antibiotics. For example, Westbrock-Wadman et al. (1999) reported the overexpression of *mexXY* in aminoglycoside-resistant

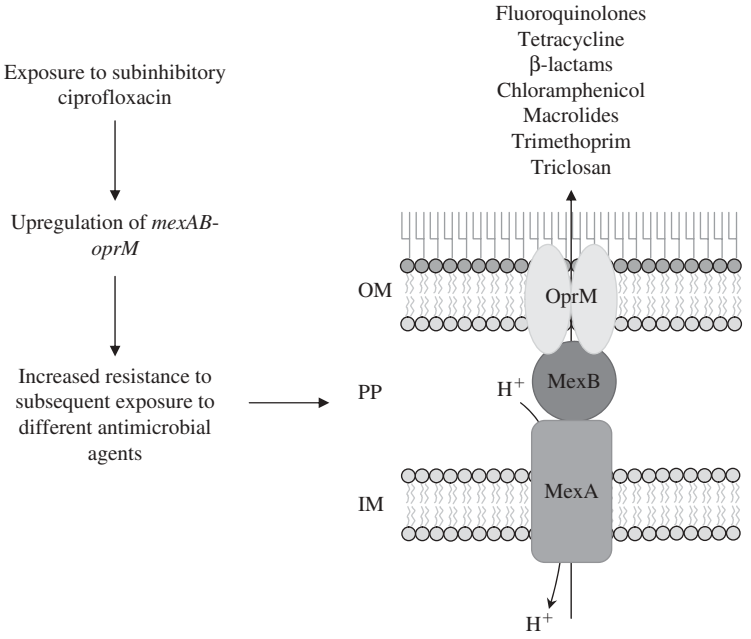

FIGURE 5.2 Example of the acquisition of adaptive resistance via the upregulation of efflux pumps. The presence of subinhibitory concentrations of ciprofloxacin induces the transcription of the operon encoding MexAB-OprM. As a result of this, the bacterium becomes more resistant to a wide range of antimicrobials from different classes because the cells will pump the drugs out more efficiently. Some examples of the known substrates of MexAB-OprM are indicated (Poole, 2001). The following abbreviations are used: OM, outer membrane; PP, periplasm, IM, inner membrane.

isolates, and another multiresistant clinical isolate displayed simultaneous overexpression of MexAB-OprM and MexEF-OprN (Pumbwe and Piddock, 2000). Additionally, a study reported that fluoroquinolone resistance in CF isolates was due to overexpression of MexCD-OprJ and MexEF-OprN in the majority of cases (Jalal et al., 2000), whereas in strains isolated from wounds and urine samples the principal mechanism is the mutation of *gyrA* and *parC* (Jalal and Wretlind, 1998).

Besides increased efflux, another mechanism to achieve a greater level of resistance is by reducing the uptake and, as a result, the accumulation of the antibiotic in the cytoplasm. In 1982, Gerber and Craig described a low-level aminoglycoside resistance phenotype in *P. aeruginosa* that they designated "impermeability type of resistance." Some years later, Gilleland et al. (1989) observed that this phenotype could be reproduced by in vitro exposure to increasing concentrations of aminoglycosides, starting with a dose equaling the MIC for each individual antibiotic. This suggested that it was actually adaptive resistance and not mutant selection that led to the decreased susceptibility trait. Around the same time, another study demonstrated that cytoplasmic accumulation of aminoglycosides requires the proton motive force as well as a functional aerobic respiration pathway (Taber et al., 1987). For this reason, the fact that subinhibitory aminoglycosides upregulate genes involved in the anaerobic pathway could well contribute to the development of adaptive resistance to

these compounds. Examples of this are *anr* and *denA*, which code for a regulatory protein and a nitrite reductase, respectively, and whose expression is induced by tobramycin (Karlowsky et al., 1997). In fact, *P. aeruginosa* cells are considerably more resistant to aminoglycosides when grown under anaerobic conditions Kindrachuk et al. (2011). Thus, the concentration required to produce similar killing rates is at least 10 times higher under anaerobic conditions.

The limited capacity of the antibiotic to access cells is also a characteristic of biofilms. In this case, it is the extracellular matrix rather than the cell envelope that hinders the access of the antibiotic molecules to their cell targets. This polymeric matrix is constituted of polysaccharides, proteins, and DNA, and it is thought to retard the penetration of the antimicrobial agents. One example is the aminoglycosides that, because of their cationic nature, would interact with the negatively charged polymers (Kumon et al., 1994) and perhaps with anionic DNA molecules present in the extracellular matrix (Mulcahy et al., 2008). However, it is not very clear if this delayed penetration of antibiotics is sufficient to result in a significant level of resistance. Furthermore, this reduced penetration into the biofilm has been observed only for β-lactams and aminoglycosides and not all reports agree on that (Hoyle et al., 1992; Shigeta et al., 1997; Yasuda et al., 1993). In contrast, studies regarding other antibiotic classes seem to indicate that the antimicrobial drugs can diffuse sufficiently into the biofilm, thereby suggesting that other mechanisms must account for the increased resistance of these communities (Shigetta et al., 1997; Suci et al., 1994; Vrany et al., 1997).

5.4.2 Modifications of the Cell Envelope

One of the mechanisms of bacterial resistance to antibiotics is the alteration of the cell envelope such that interactions with the antimicrobial molecules are limited. A well-studied example of this is the modification of the lipopolysaccharide (LPS), which leads to increased resistance to polymyxins and other cationic antimicrobial peptides. The most important among these modifications is the addition of 4-aminoarabinose to the lipid A portion of LPS. This inhibits the interaction with LPS and consequent self-promoted uptake of cationic molecules like the aforementioned peptides. Aminoarabinose addition in *P. aeruginosa* is carried out by the products of the LPS modification (*arn*) operon, which is a homolog of the equivalent *Salmonella* operon. The upregulation of this operon is under tight control, and it generally entails the participation of two-component regulatory systems (TCS). TCS regulators generally consist of two proteins, a sensor histidine kinase, which spans the cytoplasmic membrane, and a cytoplasmic DNA-binding response regulator (Stock et al., 2000). The kinase senses changes in the surrounding milieu via a periplasmic loop. Then, it autophosporylates and transduces the stimulus to the cytoplasmic response regulator, also by phosphorylation. The final result of this process is the binding of the phosphorylated response regulator to a recognition sequence in the promoter of multiple genes and the consequent up- or down-regulation of the target genes involved in responding to the environmental stress. *P. aeruginosa* possesses one of the largest collections of TCSs known so far, which comprises 64 sensor kinases and 63 response regulators (Gooderham and Hancock, 2009; Stover et al., 2000). As in *Salmonella*, the *Pseudomonas arn* operon is induced at low concentrations (in the μM range) of divalent cations (Mg^{2+}, Ca^{2+}). Under these conditions, two TCS regulators are activated independently, namely PmrAB and

PhoPQ, and their response regulators independently increase the transcription of the *arn* operon (Macfarlane et al., 1999; McPhee et al., 2003). The overall result is a greater resistance to antimicrobial peptides under suboptimal Mg^{2+}or Ca^{2+} growth conditions. However, the level of divalent cations in the human body is not limiting (about 1–2 mM), and therefore it seems unlikely that this pathway plays a significant role in the clinical context. Nevertheless, modifications akin to those induced in vitro in a medium with a low content of divalent cations have been observed in *P. aeruginosa* isolated from CF patients (Ernst et al., 1999, 2007). In their article, McPhee et al. (2003) revealed a finding that hinted at the possible explanation behind this intriguing dilemma. This study demonstrated that the induction of the LPS modification operon could be achieved by exposing the cells to subinhibitory concentrations of the antimicrobial peptide CP11CN, a derivative of the bovine cathelicidin indolicidin. Although the authors observed that the operon containing the *pmrAB* genes was upregulated under these conditions, neither PmrAB nor PhoPQ was essential for peptide-dependent adaptation. Thus, strains with mutations in these two two-component regulators showed induction levels similar to those obtained for the parent strain. Recently, Fernández et al. (2010) identified the system ParRS, which is necessary for the upregulation of the *arn* operon elicited by indolicidin as well as the bacterial peptides polymyxin B and colistin. In contrast, other peptides like CP28 or polyphemusin induced similar levels of adaptive resistance in the *parRS* mutants and the parent strain. Therefore, it is very likely that additional two-component systems participate in sensing the presence of antimicrobial peptides. Of note, the human host defense peptide LL37 does not significantly induce the transcription of the *arn* operon (Fernández et al., 2010; Overhage et al., 2008a).

Altogether these studies seem to indicate that cationic peptides are good candidates for the induction of aminoarabinose modification of the lipid A in the CF airways. Indeed, the polymyxin colistin is a common therapeutic agent in CF, and the bacterial cells might also come in contact with host defense peptides during the infection. Alarmingly, Fernández et al. (2010) showed that the LPS modification observed in the presence of indolicidin and polymyxins increased resistance to tobramycin and gentamicin, as well as to antimicrobial peptides, probably due to the positive charge of these molecules. Like colistin, tobramycin is very frequently used in CF treatment. This makes it imperative to understand how adaptations leading to resistance to both antibiotics can be induced throughout the course of the infection. Moreover, study of clinical polymyxin-resistant isolates revealed the acquisition of altered expression patterns of the genes involved in adaptive resistance to peptides (Schurek et al., 2009).

5.4.3 Stress Responses

The ability to adapt rapidly to changes in their surrounding milieu is paramount for bacteria, both in their ecological niches and inside the host, as they usually live in dynamic environments. Thus, chemical and physical parameters such as pH, temperature, nutrients, and oxygen concentration are constantly varying. This is especially important when these changes are drastic and potentially compromise the survival of the cells. As a result, bacteria have evolved stress responses that allow them to rapidly modify their transcriptional and proteomic profiles until the conditions return to their normal range. In addition to the conditions listed above, antibiotics are also a source of stress, in particular when they approach lethal levels.

Fluoroquinolones are synthetic antibiotics that interfere with DNA synthesis by inhibiting the activity of DNA gyrase and topoisomerase IV. Therefore, fluoroquinolones can be classified as DNA-damaging agents. In bacteria, DNA damage triggers the so-called SOS response. The SOS network detects and repairs the DNA damage, but, if this is unsuccessful, it will lead to error-prone repair resulting in mutagenesis and/or death of the bacterium. The topoisomerase inhibitor ciprofloxacin is known to upregulate the expression of the genes involved in the SOS response in several microorganisms, including *P. aeruginosa* (Brazas and Hancock, 2005b; Cirz et al., 2006; Hastings et al., 2004). This fluoroquinolone can induce double-strand breaks in the DNA. Therefore, exposure to this antibiotic, even at subinhibitory concentrations, switches on the SOS network in order to repair the damage and increase the survival chances of the cells. In that sense, the fluoroquinolone-mediated induction of this response makes the cells less susceptible to antibiotic challenge. Another consequence of this upregulation is the appearance of adaptive point mutations that may confer protection not only from fluoroquinolones but also from other antibiotic classes. This phenomenon is the consequence of the aforementioned error-prone repair systems, such as the SOS mutator DNA polymerase IV (McKenzie et al., 2001). Wiegand et al. (2008) showed how the mutation of genes with mutator or antimutator activity may lead to resistance to several antibiotic classes. Several studies on *E. coli* have also demonstrated that the SOS response can be triggered by the presence of β-lactams, thereby contributing to enhanced survival to lethal antibiotic doses (Miller et al., 2004). In this case, it is thought that the activating signal is the inactivation of the penicillin binding protein 3, encoded by *ftsI*, which is sensed by the two-component system DpiBA. This would interrupt cell division and result in an increased tolerance to antibiotic exposure.

Another vital stress response is the one triggered by heat shock, which has been mostly characterized in *E. coli* (Guisbert et al., 2008). This regulatory network is generally initiated by a temperature shift. The temperature rise results in protein misfolding, which once detected will increase the cytoplasmic levels of active $\sigma^{32}(\sigma^{H})$ transcription factor. This, in turn, will upregulate a regulon mainly constituted of genes encoding proteases and chaperones that will either degrade or refold the damaged proteins. This system is fairly conserved among bacteria. In addition to high temperature, there are other signals that can trigger this response. One of them is the exposure to aminoglycosides. These compounds interfere with protein synthesis and cause errors during translation, which increases the level of misfolded proteins in the cytoplasm and activates the heat shock response. This has been observed in bacteria such as *E. coli* (Shaw et al., 2003) and *Bacillus subtilis* (Lin et al., 2005). In *P. aeruginosa*, the heat shock response has not yet been studied in great depth. However, we do know that there are homologs of the proteins described in *E. coli*, including the heat shock sigma factor, which in *Pseudomonas* is called RpoH (Potvin et al., 2008). Furthermore, recent evidence indicates that exposure to lethal tobramycin induces the heat shock network in *P. aeruginosa* Kindrachuk et al. (2011). Interestingly, Schurr and Deretic (1997) found that the regulation of mucoidy conversion and the heat shock response is coordinated in *P. aeruginosa*.

Also induced by tobramycin is the two-component system AmgRS, which regulates an envelope stress response in *P. aeruginosa* (Lee et al., 2009). This response contributes to increased resistance to the antibiotic challenge as mutants in the *amgRS* operon are significantly more susceptible to aminoglycosides. This system is

a homolog of *E. coli* CpxRA, which detects the stress caused by the presence of misfolded proteins in the membrane (Shimohata et al., 2002). The authors suggested the possible utilization of this two-component regulator as a target of new drugs. This could potentiate the action of aminoglycosides, thus permitting the use of smaller doses of these toxic antimicrobials.

5.4.4 Production of Inducible Enzymes

The production of enzymes able to degrade or inactivate antibiotic compounds is very widespread among bacteria. One of the best known examples is the group of the β-lactamases, which are the major resistance mechanism to β-lactams. The mechanism of action is by hydrolyzing the β-lactam ring, which results in deactivation of the antibiotic. Because β-lactams can be used to treat a wide range of infections and do not represent a high toxicity risk for the patient, resistance to this type of antibiotics is of great concern (Livermore, 1996). β-lactamases are ancient proteins that existed well before the clinical antibiotic era. In fact, evolutionary studies on these enzymes appear to indicate that they probably existed more than 2 billion years ago (Hall and Barlow, 2004; Garau et al., 2005). β-lactamases can be plasmid or chromosomally encoded. The chromosomal β-lactamase gene, *ampC*, is present in numerous species. In some bacteria, this gene is constitutively expressed, whereas in others its expression is inducible by β-lactams (Normark et al., 1986). *P. aeruginosa* is an example of the latter. Thus, in *Pseudomonas*, exposure to subinhibitory levels of many β-lactams significantly upregulates the transcription of *ampC* through the activation of the LysR-type transcriptional regulator AmpR (Hanson and Sanders, 1999). The consequence of this would be the acquisition of adaptive resistance to different β-lactams (Lindberg and Normark, 1986; Livermore, 1987). However, not all β-lactams induce *ampC* expression to the same degree and not all β-lactams are deactivated by this β-lactamase. In that sense, the utilization of drugs, like cefepime, that do not upregulate *ampC* is recommended (Sanders, 1993), especially since the development of adaptive resistance to certain β-lactams, such as ceftazidime, penicillin, and cefotaxime, during the course of an infection has been associated with clinical failure (Pai et al., 2004).

Although not described in *P. aeruginosa*, it is worth noting the fact that, in some cases, the exposure to subinhibitory concentrations of antibiotics induces not only a drug-inactivating enzyme but also the mobilization of the genetic element carrying it. This is the case of tetracycline resistance genes from *Enterococcus faecalis* (Celli and Trieu-Cuot, 1998) and *Bacteroides fragilis* (Privitera et al., 1979), which are carried by transposon Tn916 and a plasmid, respectively.

Accumulation of antibiotic-degrading or modifying enzymes has been observed in the extracellular matrix of biofilms. This would significantly reduce the amount of antibiotic able to penetrate the biofilm and reach the bacterial cells. This phenomenon has been observed for a β-lactamase of *Klebsiella pneumoniae* (Anderl et al., 2000).

5.4.5 Multicellular Behaviors

Although we tend to consider microorganisms as unicellular entities, it is clear that under specific conditions they can associate and exhibit social behaviors. These

activities are under tight control of cell-to-cell signals such as the quorum sensing molecules. Such bacterial communities have been attracting growing interest among microbiologists. In particular, the most well-known examples, namely biofilms and swarming motility, are known to be involved in antibiotic resistance, virulence, and even evasion of the host defense mechanisms (Overhage et al., 2008b; Verstraeten et al., 2008). The study of microbial multicellular behavior is also interesting from an evolutionary perspective, as it might provide hints about the evolution to a true pluricellular state. Finally, recent research indicates that biofilms and swarming colonies are probably very common growth forms in natural, clinical, and industrial environments, as well as during the course of bacterial infections (Davey and O'Toole, 2000; Overhage et al., 2008b).

The formation of dense microbial aggregates attached to surfaces has been known for centuries, but it was only about three decades ago that studies on biofilms began. Communities known as biofilms can be constituted by one or more species of microorganisms. Biofilm cells display considerable differences in the transcriptome and proteome compared to planktonic cells. Indeed, it could be said that they show similarities with the behavior of cells from a multicellular organism, including the differentiation of cells in the community. In the clinical setting, biofilms are a serious cause of concern due to their high level of resistance to antimicrobial agents. Indeed, some authors have demonstrated that biofilm cells can be 100- or 1000-fold more resistant than planktonic cells (Hoyle and Costerton, 1991). Furthermore, biofilms usually show increased resistance to multiple drugs, which further complicates the implementation of an adequate treatment regime. In the case of *P. aeruginosa*, biofilms have been observed in prosthetic materials (Donlan, 2001) as well as in the lungs of CF patients (Bjarnsholt et al., 2009) and in chronic wounds (Kirketerp-Møller et al., 2008). Already in 1985, a *P. aeruginosa* isolate from a urinary tract infection displayed a 1000-fold decrease in susceptibility to the aminoglycoside tobramycin when the cells were forming a biofilm (Nickel et al., 1985). Once the cells were taken out of the biofilm and grown in a liquid culture, they reverted to their original degree of resistance. This confirmed the adaptive nature of biofilm-associated resistance to antibiotics.

Biofilms show increased resistance by upregulation or accumulation of known mechanisms of antibiotic resistance, such as enzymes or efflux pumps, as well as by delayed penetration of the antibiotic through the extracellular matrix. However, there are other characteristics specific to this multicellular state that also contribute to the reduction in susceptibility. One major trait of biofilm communities is that they are very heterogeneous. That is, cells forming part of the biofilm, even if they are of the same species, may be in totally different growth states. The explanation for this cell differentiation is that not all cells have equal access to oxygen and nutrients, and this will shape their metabolic profile (Fig. 5.3). Thus, the cells in the deep layers of the biofilm must adapt to a nutrient-deprived and nearly anaerobic environment, whereas the cells in the upper layer will have greater oxygen and nutrient availability. As a result, some cells will be in a slow growth mode while others will show a high metabolic activity. Heterogeneity in itself is an advantage as cells throughout the biofilm will show different responses to certain classes of antibiotics. For instance, if a given antimicrobial kills metabolically active cells, as is the case of β-lactams, ciprofloxacin and tetracycline, then the center of the biofilm will not be eradicated and will regrow once therapy is interrupted

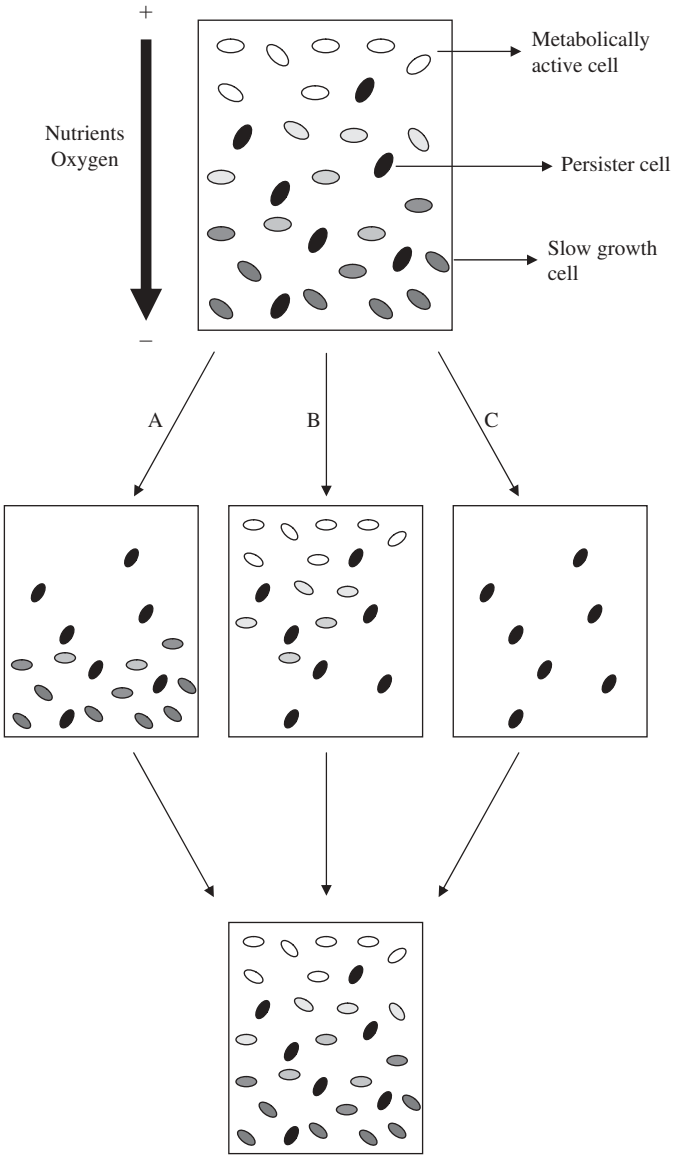

FIGURE 5.3 Schematic representation of cases in which biofilms show resistance to three different antibiotic treatments. A, exposure to an antimicrobial that kills metabolically active cells; B, elimination of slow growth cells from the deeper layers of the biofilm while the more active ones acquire adaptive resistance and survive; and C, treatment that eradicates both the active and the inactive cells but allows for the survival of persisters. The result of all three scenarios is the regeneration of a mature biofilm with identical structure and resistance profile as the original one.

(Pamp et al., 2008). In contrast, other antibiotics such as colistin kill the cells in a deficient metabolic state but are not as effective against more active cells that are able to acquire tolerance to the antibiotic, for example, through upregulation of

the expression of the LPS modification operon and the MexAB-OprM efflux pump, as mentioned above (Pamp et al., 2008). However, in most cases, increased resistance in biofilms is due to the less active cells (Brown et al., 1988; Gilbert et al., 2002). In fact, anaerobic growth conditions favor the development of tolerance toward diverse antibiotics, including ciprofloxacin, carbenicillin, tobramycin, chloramphenicol, ceftazidime, and tetracycline in *P. aeruginosa* biofilms (Borriello et al., 2004). Finally, it is important to mention that biofilms have a greater fraction of persister cells than planktonic cultures (Lewis, 2008). Persistence is a dormant state during which cells show greater tolerance to the action of antimicrobial agents. Upon removal of the toxic compound, these cells can start growing again and give rise to a new population with identical levels of antibiotic resistance to those of the original one (Fig. 5.3). Alarmingly, a high rate of persisters has been observed in samples from CF patients (Mulcahy et al., 2010; Smith et al., 2006).

Swarming is a social type of motility, which requires complex intercellular communication and involves the fast and coordinate movement of the cells over a semisolid surface (Fraser and Hughes, 1999). Overhage et al. (2008b) proposed that swarming could be the state adopted by some *P. aeruginosa* cells when moving across the thickened mucus covering the lung epithelial surface of CF patients. This makes it important to understand the characteristics of this distinct physiological state. Recent studies have demonstrated that swarmer cells show a higher level of expression of virulence determinants. In *Pseudomonas*, Overhage et al. (2008b) observed the upregulation of the genes involved in the type III secretion system, alkaline protease, pyochelin, and pyoverdin, all of which have been implicated in pathogenicity. Furthermore, like biofilms, swarming colonies have a greater resistance to antimicrobial compounds (Lai et al., 2008; Overhage et al., 2008b). This has also been demonstrated for other species such as *Salmonella*, *Serratia marcescens*, *E. coli*, and the like. In all cases, the vegetative levels of resistance could be restored by incubating the cells from a swarming colony under nonswarming conditions, such as a liquid culture (Kim et al., 2003; Lai et al., 2008; Overhage et al., 2008b). Despite being a clear adaptive response, the recovery of the initial susceptibility depends on the antibiotic and the bacterium. Thus, *Salmonella* swarming cells reverted to planktonic levels of resistance to polymyxin B after one pass in a liquid medium, while resistance to kanamycin only decreased gradually (Kim and Surette, 2003). The mechanisms of adaptive resistance during swarming are yet to be identified. Studies in *Salmonella* have reported the possible participation of the LPS modification (*pmr*/*arn*) operon (Kim et al., 2003) and the CysB regulon (Turnbull and Surette, 2008). However, a more recent article argued that it was not the physiological adaptations during swarming but the elevated cell density and the mobility of swarming cells that led to increased resistance (Butler et al., 2010). However, it is possible that this is not so in *Pseudomonas*. In fact, swarming in *Pseudomonas* is accompanied by a highly complex transcriptional response (Overhage et al., 2008b), whereas in *Salmonella* there were not many significant differences in the expression pattern (Wang et al., 2004). The precise molecular mechanisms of swarming adaptive resistance in *Pseudomonas* remain, however, elusive, and more studies need to be done to understand this interesting multicellular state.

5.5 CONCLUSION

The generalized misuse and overuse of antimicrobial compounds over the last decades have resulted in an accelerated evolutionary process leading to bacterial resistance. Microorganisms are being exposed to relatively high doses of these compounds due to human activity not only in the clinic, households, and industrial settings but also in natural environments.

Throughout this chapter, we have shown how bacteria such as *P. aeruginosa* can easily acquire a low-level increase in resistance, usually via mutation, or transiently become less susceptible to an otherwise lethal antibiotic challenge. Moreover, some of the molecular mechanisms responsible for these two types of antibiotic resistance are shared or at least related to some degree. For instance, the same gene may be downregulated under specific environmental conditions or carry a point mutation in a particular strain, resulting in both cases in reduced susceptibility to an antimicrobial agent. These phenomena are worrying enough because they might hinder the efforts to treat an infection or to eradicate the bacteria from a specific environment. In fact, it is now clear that antibiotic resistance during an infection is quite different from what is predicted on the basis of laboratory tests. Moreover, the dangers related to the acquisition of stepwise or adaptive resistance do not end there. Indeed what is really alarming is that, over time, these apparently irrelevant types of resistance might lead to a permanent high-level resistance phenotype. For example, several low-impact mutations can accumulate in the same strain, and/or the cells will be more likely to horizontally acquire high-level resistance determinants from other bacteria due to their ability to resist lethal antibiotic concentrations, even if only in a temporary manner.

A better understanding of the possible triggers and mechanisms of stepwise and adaptive resistance is essential in order to plan new treatment strategies, specifically conceived to circumvent these issues. One such example is the design of novel therapeutics. For instance, new drugs could be developed that do not significantly induce adaptive resistance or that target specific molecules involved in adaptive responses (e.g., prevent quorum sensing or disaggregate biofilms). Also important are the use of combination therapy and rational planning of more adequate programs in terms of dose and timing. In that sense, it is very important to avoid the exposure of the pathogens to subinhibitory concentrations of antimicrobials during treatment.

In conclusion, the application of novel molecular technologies has opened the door for us to comprehend in greater depth the gradual process of antibiotic resistance acquisition. This gives hope for the development of new more effective antimicrobials and administration regimes, which might slow down the relentless increase in resistance of bacterial pathogens.

ACKNOWLEDGMENTS

Our work on adaptive and stepwise resistance was funded by a grant from Cystic Fibrosis Canada (CFC). L.F. received a postdoctoral fellowship from the Fundacion Alfonso Martin Escudero (Spain), and E.B.M.B. was supported by a scholarship from the CFC. R.E.W.H. holds a Canada Research Chair in Microbiology.

REFERENCES

Alvarez-Ortega C, Wiegand I, Olivares J, Hancock REW, Martinez JL (2010). Genetic determinats involved in the susceptibility of *Pseudomonas aeruginosa* to beta-lactam antibiotics. *Antimicrob Agents Chemother* 54(10):4159–4167.

Anderl JN, Franklin MJ, Stewart PS (2000). Role of antibiotic penetration limitation in *Klebsiella pneumoniae* biofilm resistance to ampicillin and ciprofloxacin. *Antimicrob Agents Chemother* 44(7):1818–1824.

Bagge N, Schuster M, Hentzer M, Ciofu O, Givskov M, Greenberg EP, Høiby N (2004). *Pseudomonas aeruginosa* biofilms exposed to imipenem exhibit changes in global gene expression and beta-lactamase and alginate production. *Antimicrob Agents Chemother* 48(4):1175–1187.

Barber M, Waterworth PM (1966). Activity of gentamicin against *Pseudomonas* and hospital staphylococci. *Br Med J* 1(5481):203–205.

Barclay ML, Begg EJ, Chambers ST, Thornley PE, Pattemore PK, Grimwood K (1996). Adaptive resistance to tobramycin in *Pseudomonas aeruginosa* lung infection in cystic fibrosis. *Antimicrob Chemother* 37(6):1155–1164.

Baquero F (2001). Low-level antibacterial resistance: A gateway to clinical resistance. *Drug Resist Updat* 4(2):93–105.

Bjarnsholt T, Jensen PØ, Fiandaca MJ, Pedersen J, Hansen CR, Andersen CB, Pressler T, Givskov M, Høiby N (2009). *Pseudomonas aeruginosa* biofilms in the respiratory tract of cystic fibrosis patients. *Pediatr Pulmonol* 44(6):547–558.

Borriello G, Werner E, Roe F, Kim AM, Ehrlich GD, Stewart PS (2004). Oxygen limitation contributes to antibiotic tolerance of *Pseudomonas aeruginosa* in biofilms. *Antimicrob Agents Chemother* 48(7):2659–2664.

Brazas MD, Hancock REW (2005a). Using microarray gene signatures to elucidate mechanisms of antibiotic action and resistance. *Drug Discov Today* 10(18):1245–1252.

Brazas MD, Hancock REW (2005b). Ciprofloxacin induction of a susceptibility determinant in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 49(8):3222–3227.

Brazas MD, Breidenstein EBM, Overhage J, Hancock REW (2007). Role of lon, an ATP-dependent protease homolog, in resistance of *Pseudomonas aeruginosa* to ciprofloxacin. *Antimicrob Agents Chemother* 51(12):4276–4283.

Breidenstein EBM, Khaira BK, Wiegand I, Overhage J, Hancock REW (2008). Complex ciprofloxacin resistome revealed by screening a *Pseudomonas aeruginosa* mutant library for altered susceptibility. *Antimicrob Agents Chemother* 52(12):4486–4491.

Brown MR, Allison DG, Gilbert P (1988). Resistance of bacterial biofilms to antibiotics: A growth-rate related effect? *J Antimicrob Chemother* 22(6):777–780.

Butler MT, Wang Q, Harshey RM (2010). Cell density and mobility protect swarming bacteria against antibiotics. *Proc Natl Acad Sci USA* 107(8):3776–3781.

Celli J, Trieu-Cuot P (1998). Circularization of Tn916 is required for expression of the transposon-encoded transfer functions: Characterization of long tetracycline-inducible transcripts reading through the attachment site. *Mol Microbiol* 28(1):103–117.

Chuanchuen R, Beinlich K, Hoang TT, Becher A, Karkhoff-Schweizer RR, Schweizer HP (2001). Cross-resistance between triclosan and antibiotics in *Pseudomonas aeruginosa* is mediated by multidrug efflux pumps: Exposure of a susceptible mutant strain to triclosan selects *nfxB* mutants overexpressing MexCD-OprJ. *Antimicrob Agents Chemother* 45(2):428–432.

Ciofu O, Riis B, Pressler T, Poulsen H, Hoiby N (2005). Occurrence of hypermutable *Pseudomonas aeruginosa* in cystic fibrosis patients is associated with the oxidative stress caused by chronic lung inflammation. *Antimicrob Agents Chemother* 49(6):2276–2282.

Cirz RT, O'Neill BM, Hammond JA, Head SR, Romesberg FE (2006). Defining the *Pseudomonas aeruginosa* SOS response and its role in the global response to the antibiotic ciprofloxacin. *J Bacteriol* 188(20):7101–7110.

Daikos GL, Lolans VT, Jackson GG (1991). First-exposure adaptive resistance to aminoglycoside antibiotics in vivo with meaning for optimal clinical use. *Antimicrobial Agents Chemother* 35(1):117–123.

Davey ME, O'Toole GA (2000). Microbial biofilms: From ecology to molecular genetics. *Microbiol Mol Biol Rev* 64(4):847–867.

Dibb WL, Asphaug Kjellevold V, Digranes A (1983). *Pseudomonas aeruginosa* and *Acinetobacter calcoaceticus*: In vitro susceptibility of 150 clinical isolates to five beta-lactam antibiotics and tobramycin. *Chemotherapy* 29(5):332–326.

Donlan RM (2001). Biofilms and device-associated infections. *Emerg Infect Dis* 7(2):277–281.

Dötsch A, Becker T, Pommerenke C, Magnowska Z, Jänsch L, Häussler S (2009). Genome-wide identification of genetic determinants of antimicrobial drug resistance in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 53(6):2522–2531.

Driffield K, Miller K, Bostock JM, O'Neill AJ, Chopra I (2008). Increased mutability of *Pseudomonas aeruginosa* in biofilms. *J Antimicrob Chemother* 61(5):1053–1056.

Dubern JF, Diggle SP (2008). Quorum sensing by 2-alkyl-4-quinolones in *Pseudomonas aeruginosa* and other bacterial species. *Mol Biosyst* 4(9):882–888.

El'Garch F, Jeannot K, Hocquet D, Llanes-Barakat, Plesiat P (2007). Cumulative effects of several nonenzymatic mechanisms on the resistance of *Pseudomonas aeruginosa* to aminoglycosides. *Antimicrob Agents Chemother* 51(3):1016–1021.

Ernst RL, Yi EC, Guo L, Lim KB, Burns JL, Hackett M, Miller SI (1999). Specific lipopolysaccharide found in cystic fibrosis airway *Pseudomonas aeruginosa*. *Science* 286(5444):1561–1565.

Ernst RK, Moskowitz SM, Emerson JC, Kraig GM, Adams KN, Harvey MD, Ramsey B, Speert DP, Burns JL, Miller SI (2007). Unique lipid A modifications in *Pseudomonas aeruginosa* isolated from the airways of patients with cystic fibrosis. *J Infecti Dis* 196(7):1088–1092.

Fajardo A, Martinez-Martin N, Mercadillo M, Galan JC, Ghysels B, Matthijs S, Cornelis P, Wiehlmann L, Baquero F, Martinez JL (2008). The neglected intrinsic resistome of bacterial pathogens. *PloS ONE* 3(2):e1619.

Fernández L, Gooderham WJ, Bains M, McPhee JB, Wiegand I, Hancock REW (2010). Adaptive resistance to the "last hope" antibiotics polymyxin B and colistin in *Pseudomonas aeruginosa* is mediated by the novel two-component regulatory system ParR-ParS. *Antimicrob Agents Chemother* 54(8):3372–3382.

Fraser GM, Hughes C (1999). Swarming motility. *Curr Opin Microbiol* 2(6):630–635.

Garau G, Di Guilmi AM, Hall BG (2005). Structure-based phylogeny of the metallo-β-lactamases. *Antimicrob Agents Chemother* 49(7):2778–2784.

Gerber AU, Craig WA (1982). Aminoglycoside-selected subpopulations of *Pseudomonas aeruginosa*: Characterization and virulence in normal and leukopenic mice. *J Lab Clin Med* 100(5):671–681.

Gilbert P, Maira-Litran T, McBain AJ, Rickard AH, Whyte FW (2002). The physiology and collective recalcitrance of microbial biofilm communities. *Adv Microb Physiol* 46:202–256.

Gilleland LB, Gilleland HE, Gibson JA, Champlin FR (1989). Adaptive resistance to aminoglycoside antibiotics in *Pseudomonas aeruginosa*. *J Med Microbiol* 29(1):41–50.

Gillis RJ, White KG, Choi KH, Wagner VE, Schweizer HP, Iglewski BH (2005). Molecular basis of azithromycin-resistant *Pseudomonas aeruginosa* biofilms. *Antimicrob Agents Chemother* 49(9):3858–3867.

Gooderham WJ, Hancock REW (2009). Regulation of virulence and antibiotic resistance by two-component regulatory systems in *Pseudomonas aeruginosa*. *FEMS Microbiol Rev* 33 (2):279–294.

Guisbert E, Yura T, Rhodius VA, Gross CA (2008). Convergence of molecular, modeling, and systems approaches for an understanding of the *Escherichia coli* heat shock response. *Microbiol Mol Biol Rev* 72(3):545–554.

Hall BG, Barlow M (2004). Evolution of the serine β-lactamases: Past, present and future. *Drug Resist Updat* 7(2):111–123.

Hancock REW (1981). Aminoglycoside uptake and mode of action—with special reference to streptomycin and gentamicin. I. Antagonists and mutants. *J Antimicrob Chemother* 8(4):429–445.

Hanson ND, Sanders CC (1999). Regulation of inducible AmpC beta-lactamase expression among Enterobacteriaceae. *Curr Pharm Des* 5(11):881–894.

Hastings PJ, Rosenberg SM, Slack A (2004). Antibiotic-induced lateral transfer of antibiotic resistance. *Trends Microbiol* 12(9):401–404.

Hausner M, Wuertz S (1999). High rates of conjugation in bacterial biofilms as determined by quantitative in situ analysis. *Appl Environ Microbiol* 65(8):3710–3713.

Hegstad K, Langsrud S, Lunestad BT, Scheie AA, Sunde M, Yazdankhah SP (2010). Does the wide use of quaternary ammonium compounds enhance the selection and spread of antimicrobial resistance and thus threaten our health? *Microb Drug Resist* 16(2):91–104.

Hocquet D, Vogne C, El Garch F, Vejux A, Gotoh N, Lee A, Lomovskaya O, Plésiat P (2003). MexXY-OprM efflux pump is necessary for adaptive resistance of *Pseudomonas aeruginosa* to aminoglycosides. *Antimicrob Agents Chemother* 47(4):1371–1375.

Hoyle BD, Costerton JW (1991). Bacterial resistance to antibiotics: The role of biofilms. *Prog Drug Res* 37:91–105.

Hoyle BD, Alcantara J, Costerton JW (1992). *Pseudomonas aeruginosa* biofilm as a diffusion barrier to piperacillin. *Antimicrob Agents Chemother* 36(9):2054–2056.

Jalal S, Wretlind B (1998). Mechanisms of quinolone resistance in clinical strains of *Pseudomonas aeruginosa*. *Microb Drug Resist* 4(4):257–261.

Jalal S, Ciofu O, Hoiby N, Gotoh N, Wretlind B (2000). Molecular mechanisms of fluoroquinolone resistance in *Pseudomonas aeruginosa* isolates from cystic fibrosis patients. *Antimicrob Agents Chemother* 44(3):710–712.

Karlowsky JA, Hoban DJ, Zelenitsky SA, Zhanel GG (1997). Altered *denA* and *anr* gene expression in aminoglycoside adaptive resistance in *Pseudomonas aeruginosa*. *J Antimicrob Chemother* 40(3):371–376.

Kaufmann GF, Sartorio R, Lee SH, Rogers CJ, Meijler MM, Moss JA, Clapham B, Brogan AP, Dickerson TJ, Janda KD (2005). Revisiting quorum sensing: Discovery of additional chemical and biological functions for 3-oxo-*N*-acylhomoserine lactones. *Proc Natl Acad Sci USA* 102(2):309–314.

Kenna DT, Doherty CJ, Foweraker J, Macaskill L, Barcus VA, Govan JR (2007). Hypermutability in environmental *Pseudomonas aeruginosa* and in populations causing pulmonary infection in individuals with cystic fibrosis. *Microbiology* 153(Pt6):1852–1859.

Kim W, Surette MG (2003). Swarming populations of *Salmonella* represent a unique physiological state coupled to multiple mechanisms of antibiotic resistance. *Biol Proced Online* 5:189–196.

Kim W, Killam T, Sood V, Surette MG (2003). Swarm-cell differentiation in *Salmonella enterica* serovar Typhimurium results in elevated resistance to multiple antibiotics. *J Bacteriol* 185(10):3111–3117.

Kindrachuk KN, Fernández L, Bains M, Hancock REW (2011). Involvement of an ATP-dependent protease, PA0779/AsrA, in inducing heat shock in response to tobramycin in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 55(5):1874–1882.

Kirketerp-Møller K, Jensen PØ, Fazli M, Madsen KG, Pedersen J, Moser C, Tolker-Nielsen T, Høiby N, Givskov M, Bjarnsholt T (2008). Distribution, and ecology of bacteria in chronic wounds. *J Clin Microbiol* 46(8):2717–2722.

Kohanski MA, Dwyer DJ, Hayete B, Lawrence CA, Collins JJ (2007). A common mechanism of cellular death induced by bactericidal antibiotics. *Cell* 130(5):797–810.

Kohanski MA, Dwyer DJ, Wierzbowski J, Cottarel G, Collins JJ (2008). Mistranslation of membrane proteins and two-component system activation trigger antibiotic-mediated cell death. *Cell* 135(4):679–690.

Kumon H, Tomochika K, Matunaga T, Ogawa M, Ohmori H (1994). A sandwich cup method for the penetration assay of antimicrobial agents through *Pseudomonas* exopolysaccharides. *Microbiol Immunol* 38(8):615–619.

Lai S, Tremblay J, Déziel E (2008). Swarming motility: A multicellular behaviour conferring antimicrobial resistance. *Environ Microbiol* 11(1):126–136.

Lee S, Hinz A, Bauerle E, Angermeyer A, Juhaszova K, Kaneko Y, Singh PK, Manoil C (2009). Targeting a bacterial stress response to enhance antibiotic action. *Proc Natl Acad Sci USA* 106(34):14570–14575.

Lewenza S, Falsafi RK, Winsor G, Gooderham WJ, McPhee JB, Brinkman FS, Hancock REW (2005). Construction of a mini-Tn5-luxCDABE mutant library in *Pseudomonas aeruginosa* PAO1: A tool for identifying differentially regulated genes. *Genome Res* 15(4):583–589.

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

Liberati NT, Urbach JM, Miyata S, Lee DG, Drenkard E, Wu G, Villanueva J, Wei T, Ausubel FM (2006). An ordered, nonredundant library of *Pseudomonas aeruginosa* strain PA14 transposon insertion mutants. *Proc Natl Acad Sci USA* 103(8):2833–2838.

Lin JT, Connelly MB, Amolo C, Otani S, Yaver DS (2005). Global transcriptional response of *Bacillus subtilis* to treatment with subinhibitory concentrations of antibiotics that inhibit protein synthesis. *Antimicrob Agents Chemother* 49(5):1915–1926.

Linares JF, Gustafsson I, Baquero F, Martinez JL (2006). Antibiotics as intermicrobial signaling agents instead of weapons. *Proc Natl Acad Sci USA* 103(51):19484–19489.

Lindberg F, Normark S (1986). Contribution of chromosomal β-lactamases to β-lactam resistance in *enterobacteria*. *Rev Infect Dis* 8(Suppl. 3):S292–304.

Livermore DM (1987). Clinical significance of β-lactamase induction and stable derepression in gram-negative rods. *Eur J Clin Microbiol* 6(4):439–445.

Livermore DM (1996). Are all beta-lactams created equal? *Scand J Infect Dis Suppl* 101: 33–43.

Loh B, Grant C, Hancock REW (1984). Use of the fluorescent probe 1-*N*-phenylnaphthylamine to study the interactions of aminoglycoside antibiotics with the outer membrane of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 26(4):546–551.

Macfarlane EL, Kwasnicka A, Ochs MM, Hancock REW (1999). PhoPQ homologues in *Pseudomonas aeruginosa* regulate expression of the outer-membrane protein OprH and polymyxin B resistance. *Mol Microbiol* 34(2):305–316.

MacLeod DL, Nelson LE, Shawar RM, Lin BB, Lockwood LG, Dirks JE, Miller GH, Burns JL, Garber RL (2000). Aminoglycoside-resistance mechanisms for cystic fibrosis *Pseudomonas aeruginosa* isolates are unchanged by long-term, intermittent, inhaled tobramycin treatment. *J Infect Dis* 181(3):1180–1184.

Marr AK, Overhage J, Bains M, Hancock REW (2007). The Lon protease of *Pseudomonas aeruginosa* is induced by aminoglycosides and is involved in biofilm formation and motility. *Microbiology* 153(2):474–482.

Mates SM, Eisenberg ES, Mandel LJ, Patel L, Kaback HR, Miller MH (1982). Membrane potential and gentamicin uptake in *Staphylococcus aureus*. *Proc Natl Acad Sci USA* 79(21):6693–6697.

Mawer SL, Greenwood D (1978). Specific and non-specific resistance to aminoglycosides in *Escherichia coli*. *J Clin Pathol* 31(1):12–15.

McKenzie GJ, Lee PL, Lombardo M-J, Hastings PJ, Rosenberg SM (2001). SOS mutator DNA polymerase IV functions in adaptive mutation and not adaptive amplification. *Mol Cell* 7(3):571–579.

McMurry LM, Oethinger M, Levy SB (1998). Overexpression of *marA*, *soxS* or *acrAB* produces resistance to triclosan in *Escherichia coli*. *FEMS Microbiol Lett* 166(2):305–309.

McPhee JB, Lewenza S, Hancock REW (2003). Cationic antimicrobial peptides activate a two-component regulatory system, PmrAB. That regulates resistance to polymyxin B and cationic antimicrobial peptides in *Pseudomonas aeruginosa*. *Mol Microbiol* 50(1):205–217.

Miller C, Thomsen LE, Gaggero C, Mosseri R, Ingmer H, Cohen SN (2004). SOS response induction by β-lactams and bacterial defense against antibiotic lethality. *Science* 305(5690):1629–1631.

Molin S, Tolker-Nielsen T (2003). Gene transfer occurs with enhanced efficiency in biofilms and induces enhanced stabilisation of the biofilm structure. *Curr Opin Biotechnol* 14(3): 255–261.

Mulcahy H, Charron-Mazenod L, Lewenza S (2008). Extracellular DNA chelates cations and induces antibiotic resistance in *Pseudomonas aeruginosa* biofilms. *PLoS Pathogens* 4(11): e1000213.

Mulcahy LR, Burns JL, Lory S, Lewis K (2010). Emergence of *Pseudomonas aeruginosa* strains producing high levels of persister cells in patients with cystic fibrosis. *J Bacteriol* 192(23):6191–6199.

Nakayama K, Takashima K, Ishihara H, Shinomiya T, Kageyama M, Kanaya S, Ohnishi M, Murata T, Mori H, Hayashi T (2000). The R-type pyocin of *Pseudomonas aeruginosa* is related to P2 phage, and the F-type is related to lambda phage. *Mol Microbiol* 38(2): 213–231.

Nalca Y, Jänsch L, Bredenbruch F, Geffers R, Buer J, Häussler S (2006). Quorum-sensing antagonistic activities of azithromycin in *Pseudomonas aeruginosa* PAO1: A global approach. *Antimicrob Agents Chemother* 50(5):1680–1688.

Nickel JC, Wright JB, Ruseska I, Marrie TJ, Whitfield C, Costerton JW (1985). Antibiotic resistance of *Pseudomonas aeruginosa* colonizing a urinary catheter in vitro. *Eur J Clin Microbiol* 4(2):213–218.

Normark S, Lindquist S, Lindberg F (1986). Chromosomal beta-lactam resistance in enterobacteria. *Scand J Infect Dis Suppl* 49:38–45.

Oliver A, Canton R, Campo P, Baquero F, Blazquez J (2000). High frequency of hypermutable *Pseudomonas aeruginosa* in cystic fibrosis lung infection. *Science* 288(5469):1251–1254.

Overhage J, Campisano A, Bains M, Torfs EC, Rehm BH, Hancock REW (2008a). Human host defense peptide LL-37 prevents bacterial biofilm formation. *Infect Immun* 76(9):4176–4182.

Overhage J, Bains M, Brazas MD, Hancock REW (2008b). Swarming of *Pseudomonas aeruginosa* is a complex adaptation leading to increased production of virulence factors and antibiotic resistance. *J Bacteriol* 190(8):2671–2679.

Pai H, Kang CI, Byeon JH, Lee KD, Park WB, Kim HB, Kim EC, Oh MD, Choe KW (2004). Epidemiology and clinical features of bloodstream infections caused by AmpC-

type-β-lactamases-producing *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* 48(10): 3720–3728.

Pamp SJ, Gjermansen M, Johansen HK, Tolker-Nielsen T (2008). Tolerance to the antimicrobial peptide colistin in *Pseudomonas aeruginosa* biofilms is linked to metabolically active cells, and depends on the *pmr* and *mexAB-oprM* genes. *Mol Microbiol* 68(1): 223–240.

Poole K (2001). Multidrug efflux pumps and antimicrobial resistance in *Pseudomonas aeruginosa* and related organisms. *J Mol Microbiol Biotechnol* 3(2):255–264.

Poole K (2004). Resistance to β-lactam antibiotics. *Cell Mol Life Sci* 61(17):2200–2223.

Potvin E, Sanschagrin F, Levesque RC (2008). Sigma factors in *Pseudomonas aeruginosa*. *FEMS Microbiol Rev* 32(1):38–55.

Privitera G, Sebald M, Fayolle F (1979). Common regulatory mechanism of expression and conjugative ability of a tetracycline resistance plasmid in *Bacteroides fragilis*. *Nature* 278(5705):657–659.

Pumbwe L, Piddock LJV (2000). Two efflux systems expressed simultaneously in multidrug-resistant *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 44(10):2861–2864.

Sánchez P, Linares JF, Ruiz-Díez B, Campanario E, Navas A, Baquero F, Martínez JL (2002). Fitness of in vitro selected *Pseudomonas aeruginosa nalB* and n*f*xB multidrug resistant mutants. *J Antimicrob Chemother* 50(5):657–664.

Sanders CC (1993). Cefepime: The next generation? *Clin Infect Dis* 17(3):369–379.

Schurek KN, Marr AK, Taylor PK, Wiegand I, Semenec L, Khaira BK, Hancock REW (2008). Novel genetic determinants of low-level aminoglycoside resistance in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 52(12):4213–4219.

Schurek KN, Sampaio JL, Kiffer CR, Sinto S, Mendes CM, Hancock REW (2009). Involvement of *pmrAB* and *phoPQ* in polymyxin B adaptation and inducible resistance in non-cystic fibrosis clinical isolates of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 53(10):4345–4351.

Schurr MJ, Deretic V (1997). Microbial pathogenesis in cystic fibrosis: Co-ordinate regulation of heat-shock response and conversion to mucoidy in *Pseudomonas aeruginosa*. *Mol Microbiol* 24(2):411–420.

Schweizer HP (2003). Efflux as a mechanism of resistance to antimicrobials in *Pseudomonas aeruginosa* and related bacteria: Unanswered questions. *Genet Mol Res* 31;2(1):48–62.

Shaw KJ, Miller N, Liu X, Lerner D, Wan J, Bittner A, Morrow BJ (2003). Comparison of the changes in global gene expression of *Escherichia coli* induced by four bactericidal agents. *J Mol Microbiol Biotechnol* 5(2):105–122.

Shigeta M, Tanaka G, Komatsuzawa H, Sugai M, Suginaka H, Usui T (1997). Permeation of antimicrobial agents through *Pseudomonas aeruginosa* biofilms: A simple method. *Chemotherapy* 43(5):340–345.

Shimohata N, Chiba S, Saikawa N, Ito K, Akiyama Y (2002). The Cpx stress response system of *Escherichia coli* senses plasma membrane proteins and controls HtpX, a membrane protease with a cytosolic active site. *Genes to Cells* 7(7):653–662.

Smith EE, Buckley DG, Wu Z, Saenphimmachak C, Hoffman LR, D'Argenio DA, Miller SI, Ramsey BW, Speert DP, Moskowitz SM, Burns JL, Kaul R, Olson MV (2006). Genetic adaptation by *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients. *Proc Natl Acad Sci USA* 30;103(22):8487–8492.

Steinkraus G, White R, Friedrich L (2007). Vancomycin MIC creep in non-vancomycin-intermediate *Staphylococcus aureus* (VISA), vancomycin-susceptible clinical methicillin-resistant *S. aureus* (MRSA) blood isolates from 2001–2005. *J Antimicrob Chemother* 60(4): 788–794.

Stock AM, Robinson VL, Goudreau PN (2000). Two-component signal transduction. *Annu Rev Biochem* 69:183–215.

Stover CK, Pham XQ, Erwin AL, Mizoguchi SD, Warrener P, Hickey MJ, Brinkman FS, Hufnagle WO, Kowalik DJ, Lagrou M, Garber RL, Goltry L, Tolentino E, Westbrock-Wadman S, Yuan Y, Brody LL, Coulter SN, Folger KR, Kas A, Larbig K, Lim R, Smith K, Spencer D, Wong GK, Wu Z, Paulsen IT, Reizer J, Saier MH, Hancock REW, Lory S, Olson MV (2000). Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature* 406(6799):959–964.

Suci PA, Mittelman MW, Yu FP, Geesey GG (1994). Investigation of ciprofloxacin penetration into *Pseudomonas aeruginosa* biofilms. *Antimicrob Agents Chemother* 38(9):2125–2133.

Taber HW, Mueller JP, Miller PF, Arrow AS (1987). Bacterial uptake of aminoglycoside antibiotics. *Microbiol Rev* 51(4):439–457.

Turnbull AL, Surette MG (2008). L-Cysteine is required for induced antibiotic resistance in actively swarming *Salmonella enterica* serovar Typhimurium. *Microbiology* 154(11): 3410–3419.

Verstraeten N, Braeken K, Debkumari B, Fauvart M, Fransaer J, Vermant J, Michiels J (2008). Living on a surface: Swarming and biofilm formation. *Trends Microbiol* 16(10): 496–506.

Vrany JD, Stewart PS, Suci PA (1997). Comparison of recalcitrance to ciprofloxacin and levofloxacin exhibited by *Pseudomonas aeruginosa* biofilms displaying rapid-transport characteristics. *Antimicrob Agents Chemother* 41(6):1352–1358.

Walsh C (2003). Antibiotics that act on cell wall biosynthesis. In C Walsh (Ed.), *Antibiotics: Actions, Origins, Resistance*. ASM Press, Washington, DC, pp. 23–49.

Wang Q, Frye JG, McClelland M, Harshey RM (2004). Gene expression patterns during swarming in *Salmonella typhimurium*: Genes specific to surface growth and putative new motility and pathogenicity genes. *Mol Microbiol* 52(1):169–187.

Weinstein MJ, Drube CG, Moss EL Jr, Waitz JA (1971). Microbiologic studies related to bacterial resistance to gentamicin. *J Infect Dis* 124:S11–17.

Wells IC (1952). Antibiotic substances produced by *Pseudomonas aeruginosa*; syntheses of Pyo Ib, Pyo Ic, and Pyo III. *J Biol Chem* 196(1):331–340.

Westbrock-Wadman S, Sherman DR, Hickey MJ, Coulter SN, Zhu YQ, Warrener P, Nguyen LY, Shawar RM, Folger KR, Stover CK (1999). Characterization of a *Pseudomonas aeruginosa* efflux pump contributing to aminoglycoside impermeability. *Antimicrob Agents Chemother* 43(12):2975–2983.

Wiegand I, Marr AK, Breidenstein EBM, Schurek KN, Taylor P, Hancock REW (2008). Mutator genes giving rise to decreased antibiotic susceptibility in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 52(10):3810–3813.

Yasuda H, Ajiki Y, Koga T, Kawada H, Yokota T (1993). Interaction between biofilms formed by *Pseudomonas aeruginosa* and clarithromycin. *Antimicrob Agents Chemother* 37(9):1749–1755.

Zhang L, Mah TF (2008). Involvement of a novel efflux system in biofilm-specific resistance to antibiotics. *J Bacteriol* 190(13):4447–4452.