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Creeping baselines and adaptive resistance to antibiotics

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ABSTRACT

The introduction of antimicrobial drugs in medicine gave hope for a future in which all infectious diseases could be controlled. Decades later it appears certain this will not be the case, because antibiotic resistance is growing relentlessly. Bacteria possess an extraordinary ability to adapt to environmental challenges like antimicrobials by both genetic and phenotypic means, which contributes to their evolutionary success. It is becoming increasingly appreciated that adaptation is a major mechanism behind the acquisition and evolution of antibiotic resistance. Adaptive resistance is a specific class of non-mutational resistance that is characterized by its transient nature. It occurs in response to certain environmental conditions or due to epigenetic phenomena like persistence. We propose that this type of resistance could be the key to understanding the failure of some antibiotic therapy programs, although adaptive resistance mechanisms are still somewhat unexplored. Similarly, hard wiring of some of the changes involved in adaptive resistance might explain the phenomenon of "baseline creep" whereby the average minimal inhibitory concentration (MIC) of a given medically important bacterial species increases steadily but inexorably over time, making the likelihood of breakthrough resistance greater. This review summarizes the available information on adaptive resistance.

1. Introduction

1.1. Antibiotics: tools for defense or communication?

The deliberate use of antibiotics in the context of human activities, including clinical and veterinary medicine as well as agriculture etc., is a very recent phenomenon. However, antibiotics have been widely present in nature throughout the evolution of microbes. At first glance, the most direct explanation for the production of antimicrobial compounds by microorganisms is to fight competitors. If this were so, it might be expected that the antibiotic concentrations found in the environment should be above inhibitory levels, which rarely seems to be the case. Another striking occurrence is that, in bacteria, antibiotics are commonly produced as secondary metabolites in the stationary phase of growth, even though it would appear that competition for nutrients should play a more significant role in the phase of logarithmic growth. Also, recent studies have shown that the presence of specific antibiotic concentrations can actually promote certain colonization- and virulence-related phenotypes such as biofilm formation, motility and even toxin production. Arguably, this would disagree with the exclusive function of antimicrobial agents as weapons to kill other bacteria, in which case it would be expected that such compounds would always have a deleterious effect on metabolism and pathogenic properties. Therefore, these responses that occur at sub-inhibitory concentrations make antibiotics good examples of hermetic compounds, since they exhibit opposite effects when present at low and high doses (Calabrese and Baldwin, 2002). With this information in mind, it has been proposed that antibiotics have a key environmental function as signaling molecules in the microbial world (Linares et al., 2006), perhaps even as a lingua franca that allows intra as well as interspecies communication. Indeed, there is evidence for the regulation of antibiotic production in *Streptomyces* spp. through a well known signaling device of microorganisms, mediated by so-called quorum sensing signals (Horinouchi, 2007). Quorum sensing involves the secretion by bacteria of specific chemicals that upon reaching a particular threshold concentration are taken up by other microbes and trigger adaptive changes appropriate to the community of organisms. Some of these quorum sensing molecules, such as the *Pseudomonas* quinolone signal (PQS) and the homoserine lactones from *Pseudomonas aeruginosa*, possess antimicrobial activity at very high concentrations (Dubern and Diggle, 2008; Kaufmann et al., 2005; Wells, 1952), indicating their dual nature. There is also abundant evidence that antibiotics can manipulate the immune system of higher organisms, which might be considered indicative of communication between bacteria and eukaryotes. For instance, macrolides are known to have anti-inflammatory properties (Tamaoki et al.,

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2004), most likely due to the downregulation of genes involved in the inflammatory cascade (Morimura et al., 2008). In contrast, the antibiotic rifampicin has a pro-inflammatory effect (Yuhas et al., 2009). These immunomodulatory activities need to be taken into account as they might pose beneficial or negative consequences in the treatment of specific infectious diseases; in cystic fibrosis (CF), for example, macrolides are often utilized more for their anti-inflammatory action than for any potency against the major CF pathogen *P. aeruginosa*.

Regardless of the role of antibiotics in nature, it is undeniable that from a human perspective they are excellent tools when used to combat infection. Traditionally, antibiotics have been classified on the basis of their chemical structure and the mechanism by which they kill bacteria or inhibit their growth. For example, some families of antibiotics act by inhibiting the synthesis of the cell wall like β-lactams and glycopeptides. Others, like aminoglycosides, macrolides, tetracyclines, ketolides, and oxazolidinones inhibit protein synthesis. The lipopeptide daptomycin as well as polymyxins and cationic antimicrobial peptides have been proposed to disrupt bacterial membranes, although it is almost certain that they are mechanistically far more complex. Fluoroquinolones and rifampicin inhibit DNA and RNA synthesis, respectively. Finally, sulphonamides and trimethoprim inhibit folic acid synthesis. These classifications generally consider that antimicrobials act by inhibiting a major single target. However, the recent development of genomic and proteomic approaches in microbiology has challenged this simplistic model. Indeed, it seems increasingly clear that antibiotics operate by complex mechanisms that generally involve multiple cellular components. Furthermore, these techniques have opened the door to an unprecedented look at the changes triggered by antimicrobial drugs in different bacteria. For example, microarray technology provides useful information about how the presence of certain antibiotic concentrations can modify the global gene expression pattern, or transcriptome, of a particular strain. A review by Brazas and Hancock (2005b) highlighted the usefulness of microarrays in terms of understanding the mechanisms of action of antibiotics, as well as in the development of new antimicrobials. In good agreement with a signaling role of antibiotics, the analysis of gene expression changes in bacteria in the presence of these compounds reveals the existence of complex transcriptional responses that can be referred to as gene expression signatures. Brazas and Hancock (2005b) distinguished four groups of gene expression responses to antibiotic treatment on the basis of their relation to the main antibiotic target. These groups are as follows: group 1, genes dysregulated as a direct effect of target inhibition by the drug; group 2, genes whose expression is altered as an indirect effect of the antibiotic action; group 3, genes dysregulated as a secondary effect of target inhibition; and group 4, genes not related to the target of the antibiotic but which are consistently dysregulated by a specific antibiotic in a particular species or strain. The latter are called bystander effects and an example would be the aforementioned dysregulation of virulence genes. Thus, the mechanism of action of a novel antimicrobial could potentially be predicted by comparing its signature transcriptome to those of well studied antibiotics. Additionally, microarray data can also provide information on possible resistance mechanisms. However, if the above classification is reconsidered in the light of the concept of antibiotics as signaling molecules, many of these signature responses might be a result of excessive interspecies communication with pathological consequences.

1.2. Bacterial resistance to antibiotics

1.2.1. Antibiotic resistance: cutting a long story short

The phenomenon of antibiotic resistance precedes the use of these compounds in a clinical context, which is not surprising, considering that they are ubiquitous in nature. For example, phylogenetic studies indicate that the β-lactamases, the principal mechanism of resistance to β-lactam antibiotics, originated more than 2 billion years ago (Hall and Barlow, 2004; Garau et al., 2005). It was, however, only after the introduction of antimicrobials as a therapy against infectious diseases that bacteria began to experience an accelerated evolution leading to the apparition and transfer of resistance mechanisms affecting most, if not all, antibiotics available today. This arms race between pathogenic bacteria and modern medicine started very early on in the antibiotic era, with bacteria achieving increasing success that has started to threaten medicine's most successful strategy. This clearly demonstrates that despite the simplicity of bacteria in an evolutionary context, their extraordinary feats of adaptability make them extremely successful organisms, able to colonize a wide range of ecological niches as well as endure and overcome major challenges.

There are a variety of mechanisms that lead to antibiotic resistance, including the production of enzymes that inactivate the drug, alteration of an antibacterial target, reduction in net permeability to that compound often through efflux, etc. The origin of these traits is diverse. For instance, genes encoding determinants of resistance can be horizontally transferred between different strains or even species via conjugation. This is a common mechanism for drug-inactivating enzymes that are often carried as cassettes on mobile elements. These mechanisms generally lead to high increases in resistance. Alternatively, mutations in certain genes can also result in decreased susceptibility. In this case, the result is usually not as dramatic, giving rise to low-level antibiotic resistance. This phenomenon has attracted particular attention lately, as it has become clear that this ability to modestly resist antibiotic inhibition is an ideal background for the development of high-level resistance, either from the accumulation of successive low-effect mutations or from the later acquisition of a high-level determinant (Baquero, 2001).

The emergence of antibiotic resistance to a new antimicrobial drug in hospitals and the community at large usually occurs quite soon after the novel agent starts being used in therapy and can often already be observed in clinical trials. At the beginning, the new agent is highly effective in killing most of the target microorganisms. However, relatively shortly afterwards, resistant strains occur. The proportion of resistant strains increases over time and, moreover, the average "baseline" MIC values of all strains also show a steady rise (Table 1). This observation has been made for all different antibiotic classes and ultimately leads to the appearance of multi-drug resistant strains. For example, the existence of clinical isolates of *P. aeruginosa* that exhibit multiple resistance mechanisms makes the strains untreatable by available antibiotics. These extremely resistant strains are also called Superbugs and are of major concern.

The Gram-positive pathogen *Staphylococcus aureus* provides a good example of how a microorganism can gradually become resistant to multiple antibiotics belonging to different classes. Penicillin, which was the first antibiotic widely deployed in the clinic, was identified by Alexander Fleming in 1928 by observing the ability of the fungus *Penicillum notatum* to produce a diffusible substance that killed *Staphylococci*. Penicillin was introduced for the treatment of *S. aureus* infections in 1940 and already in 1942 the first resistant strain was isolated (Kirby, 1944). In subsequent years, the proportion of resistant strains as well as the determined MIC values increased dramatically. Indeed, around 50% of *S. aureus* isolates were already resistant to penicillin by 1950. When penicillin was no longer successful in treating *S. aureus* infections, methicillin, a semisynthetic penicillin-related antibiotic, was introduced in 1959. Also in this case, methicillin resistant *S. aureus* (MRSA) were identified two years later, by which time already 80% of all isolates were penicillin resistant. The first MRSA strain

Table 1
Examples of the increase of antibiotic resistance to different antimicrobials over time.

Antibiotic class[a]	Antibiotic	Mechanism of action	MIC values			Species
			Introduction of antibiotic[b]	Intermediate point[c]	Last decade[d]	
β-Lactams	Penicillin	Disruption of cell wall synthesis	0.04 μg/ml in 1940 Kislak et al. (1965)	0.12–1 μg/ml in 1980 Anderson et al. (1980)	No longer used	*S. pneumoniae*
Glycopeptides	Vancomycin	Inhibition of peptidoglycan synthesis	0.25–0.5 μg/ml in 1986 Foster et al. (1986)	>7 μg/ml in 1997 Hiramatsu (1998)	>32 μg/ml in 2002 MMWR (2002)	*S. aureus*
Macrolides	Erythromycin	Protein synthesis inhibition by binding to 50S ribosomal subunit	1 μg/ml in 1994 Shortridge et al. (1999)	16 μg/ml in 1994–1999 Shortridge et al. (1999)	256 μg/ml in 2004 Farrell and Jenkins (2004)	*S. pneumoniae*
Quinolones	Ciprofloxacin	Inhibition of DNA replication and transcription by inhibiting bacterial DNA gyrase and topoisomerase IV	<0.5 μg/ml in 1990 Masecar et al. (1990)	8 μg/ml in 1998 Huczko et al. (2000)	32–64 μg/ml in 2008 Fujimura et al. (2009)	*P. aeruginosa*
Aminoglycosides	Tobramycin	Inhibition of protein synthesis by binding to 30S ribosomal subunit	<2 μg/ml in 1980 Dibb et al. (1983)	8 μg/ml in 1987 Stratton et al. (1987)	>16 μg/ml in 2000 MacLeod et al. (2000)	*P. aeruginosa*

[a] One drug of each class was selected to illustrate this phenomenon in a particular pathogen.
[b] MIC values obtained when the drug was introduced in the clinic.
[c] Example of an intermediate point, indicating an increase in resistance over time.
[d] MIC values show the MIC that we observe right now for most isolates of a particular species. However, other isolates with a higher MIC exist, though they are rare and not mentioned here.

was observed in 1961 in the United Kingdom (Jevons, 1961), followed by several other strains in the 1980s, and by 1990 MRSA strains were observed worldwide. Now, MRSA strains that are resistant to all β-lactams cause the majority of nosocomial infections worldwide (Grundmann et al., 2006). The same resistance problem occurred with vancomycin, a glycopeptide antibiotic, which is one of the few antimicrobials that have activity against MRSA. Usually, such strains are sensitive to vancomycin and have an MIC of 0.25–1 μg/ml. After vancomycin started to be intensively administered, vancomycin resistant *S. aureus* (VRSA) strains appeared. The first vancomycin resistant strain, with an MIC > 7 μg/ml, was isolated in Japan in 1997 (Hiramatsu, 1998). Although still rare, vancomycin resistance is currently increasing. In 2002, a VRSA with an MIC of 32 μg/ml was isolated (MMWR, 2002). In addition to the vancomycin resistant isolates, also susceptible clinical MRSA isolates have been showing a stepwise increase in MIC over the years (MIC creep) (Steinkraus et al., 2007). For instance, the geometric mean MIC shifted from 0.62 μg/ml to 0.94 μg/ml for the populations over the years 2001–2005 as shown in Fig. 1. Although small, these changes are of great concern as such gradual MIC increases over the years may eventually lead to resistant isolates. It must be noted, however, that the technique used to determine the MIC can also impact the specific values obtained. For example, the vancomycin MICs for *S. aureus* and coagulase-negative staphylococci (CoNS) as determined using Etest are generally higher than those obtained with broth microdilution. In fact, some authors explain the gradual increase attributed to MIC creep by differences in the methodology or even the type of statistical treatment employed to analyze the data (Sader et al., 2009). Taking this into account, it should be recommended to utilize homogeneous methods of MIC determination when trying to track slight changes in resistance over time.

Penicillin also used to be the first drug of choice against *Streptococcus pneumoniae* strains. Originally, this pathogen was susceptible to penicillin with an MIC of <0.04 μg/ml (Appelbaum, 1992; Kislak et al., 1965), but resistant strains (MIC > 2 μg/ml) began to appear, albeit at a slower rate than in *S. aureus*, and the baseline level of susceptibility started to creep up. This slow increase in baseline MIC was observed up to 1980. During this period, only sporadic cases of resistance occurred, such as the ones in Australia and New Guinea (Hansman and Bullen, 1967) and in the US (Cates

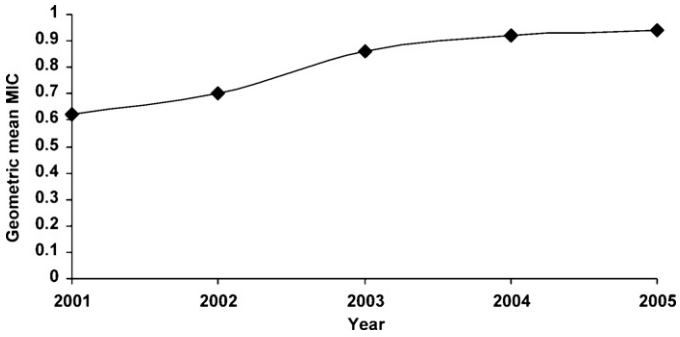

Fig. 1. Graphic representation of the step-wise increase in geometric mean MIC (MIC creep) to vancomycin of MRSA isolates during the years 2001–2005. The represented values were taken from Steinkraus et al. (2007).

et al., 1978). However, in addition to the resistant isolates, several intermediate isolates (MIC 0.12–1 μg/ml) were being identified (Anderson et al., 1980). More recently, penicillin resistance has increased at a faster rate and, by 1990, 40–50% of the *S. pneumoniae* strains were resistant to penicillin (Goldstein, 1999); even susceptible isolates now tend to have MICs of around 0.5 μg/ml, making intermediate or full resistance just a single mutation away. Other β-lactam antibiotics have also experienced resistance issues. Of great concern, most penicillin-resistant pneumococci now tend to have increased resistance to macrolides. Macrolide resistance itself has risen over time due to a major increase in administered prescriptions. Shortridge et al. (1999) showed that the MIC for erythromycin in 1994 ranged from 1 μg/ml to 16 μg/ml, but ten years later the range had increased and included some strains with MICs of up to 256 μg/ml (Farrell and Jenkins, 2004).

Another interesting case is that of carbapenems, a group of β-lactams that show the broadest spectra of effect within this family of antibiotics. Resistance is known to occur in some Gram-positive bacteria such as MRSA, *Enterococcus faecium* and some pneumococci; however, until recently, carbapenems remained very effective against Gram-negative microorganisms. Nevertheless, the greater use of carbapenems is leading to a steady increase in resistance (Meyer et al., 2010). Thus, resistance has now been observed at appreciable frequencies in *Pseudomonas*, *Acinetobacter*, *Klebsiella* and *Escherichia coli*. The development of this resistance over the last fifteen years was recently summarized by Pfeifer et al. (2010).

Several quinolone antibiotics were introduced into the market between 1963 and 1990 in order to treat infections by both Gram-negative and Gram-positive pathogens (Notari and Mittler, 1989; Modai, 1989; Gibson et al., 2003). Nalidixic acid was the first to be administered in clinical trials in 1963, but rapidly failed due to rapid resistance development. Subsequently the fluroquinolones norfloxacin and then ciprofloxacin were introduced and the latter has become a very important drug in human medicine. The more frequent use of ciprofloxacin in hospital settings has led to a rising level of resistance, and there is a strong correlation between fluoroquinolone prescription frequency and resistance (Hsu et al., 2010). Ciprofloxacin resistance was very uncommon in 1985 for *Enterococci*, when only 1.4% of strains were resistant, but this increased to 15.2% in 1990 (Schaberg et al., 1992). Furthermore, *Neisseria gonorrhoeae* and *Salmonella enterica* were almost completely susceptible to fluoroquinolones in 1999, with 0.4% and 0% of resistant strains, respectively, but became substantially more resistant within five years, with 4.1% and 80% of resistant strains (Wang et al., 2006, 2007). Clinical isolates of *P. aeruginosa* were originally susceptible to ciprofloxacin and their MICs were <0.5 μg/ml (Masecar et al., 1990), but over the years the isolates have become increasingly resistant. Mouneimne et al. (1999) concluded that the level of ciprofloxacin resistance varies according to the mechanisms involved. Thus, the identified resistance of clinical isolates correlates with the number and types of mutations in the target site topoisomerases, namely *gyrA*, *gyrB*, *parC* and *parE*. The specific mutations then determine the degree of resistance. Worryingly, clinical *Pseudomonas* isolates with MICs of 32–64 μg/ml have been recently isolated around the world (Fujimura et al., 2009; BSCA, 2008). Analysis of the ciprofloxacin resistome of *P. aeruginosa* PAO1 and PA14 revealed that mutations in many genes can lead to low-level resistance to fluoroquinolones (Brazas et al., 2007; Breidenstein et al., 2008). Therefore, it is plausible that an accumulation of these types of mutations in a particular strain have contributed to the observed MIC baseline creep.

Analogous *P. aeruginosa* resistome investigations for other antibiotics, including aminoglycosides (Schurek et al., 2008) and β-lactams (Alvarez-Ortega et al., 2010) have indicated that resistance genes determining low-level resistance are surprisingly common in this organism. Thus, transposon mutants in more than 150 genes showing modest changes in tobramycin MIC were identified. It was proposed that such mutants may occur in the bacteria affecting the lungs of CF patients where aminoglycoside resistance arises over time and thus could be important in clinical outcome. In addition to such low-level resistance, the major mechanisms of high-level resistance to specific subsets of aminoglycosides are a broad range of aminoglycoside modifying enzymes. So-called impermeability resistance, that we now understand to be likely due to increased efflux, affects all aminoglycosides. Depending on the specific mechanism, clinical strains of *P. aeruginosa* show varied MIC values. As was the case with fluoroquinolones, *Pseudomonas* was initially susceptible to tobramycin, with an MIC < 2 μg/ml, but soon after the administration of this antibiotic started, isolates with decreased susceptibility or clinically meaningful resistance were identified worldwide in increasing numbers. In Europe, around 30% of the strains were resistant to tobramycin in 1998 (Schmitz et al., 1999). Currently, the MICs have increased dramatically and values of 16 μg/ml frequently occur in isolates from CF patients, whereas an MIC of >128 μg/ml appears sporadically (MacLeod et al., 2000).

A first look at the numbers in Table 1 might give the impression that the level of antibiotic resistance periodically jumps, thus leading to major increases in the MICs of the isolated microorganisms. However, there is growing evidence that this is not the case. More thorough evaluation of the MIC values over time shows that there is a gradual increase of the average MICs of the isolates of a particular pathogen, which is marked by the observation of subtle changes that usually go unnoticed in clinical screenings, as they do not appear to directly influence clinical success or failure. However, as discussed above, this low-level resistance is known to facilitate, and might even be required for, the subsequent acquisition of high-level breakthrough resistance (leading to clinical failure of a drug). Therefore, the identification of new isolates with intermediate resistance should always be considered as potential indicators of a likely evolution towards a highly resistant phenotype.

1.2.2. Adaptive resistance

The best studied mechanisms of resistance thus far correspond to intrinsic and acquired resistance, both of which are characterized by an irreversible phenotype and are independent of the presence of the antibiotic or the environmental conditions surrounding the microorganism (Table 2). Not so well understood is the phenomenon of adaptive resistance, which can be defined as the induction of resistance to one or more antimicrobial agents in response to the presence of a specific signal (Table 2). This increase generally reverts upon removal of the triggering factor, although in many cases the original level of resistance cannot be restored (Mawer and Greenwood, 1978). The phenomenon of adaptive resistance has been known for decades. Initial observations reflected how in vitro incubation of a microorganism in a medium supplemented with a sub-inhibitory antibiotic concentration turned the cells more resistant to subsequent exposures to that antimicrobial and, in some occasions, also to other drugs of the same or even different classes (cross-resistance). We now know that, in addition to antibiotics, other signals can also trigger this adaptation response. These include environmental cues like pH, anaerobiosis, cation levels, etc., as well as social activities like biofilm formation and swarming motility. Because of its transient nature, this type of resistance is difficult to detect and has been disregarded in many cases; however, it is now clear that this phenomenon might play a significant role in the differences in antibiotic resistance observed when comparing in vitro with in vivo studies and, consequently, it could be involved in clinical failure of some antibiotic administration regimes. This is especially so because the antibiotic levels at some points during treatment are in the sub-inhibitory range. Moreover, the development of adaptive resistance under certain conditions appears to favor the subsequent acquisition of intrin-

Table 2
Comparison between the three major types of antibiotic resistance.

Type of resistance	Intrinsic	Acquired	Adaptive
Acquisition	Not acquired, part of the genetic make-up of the strain or species	Mutation	Changes in gene expression triggered by environmental factors or presence of antimicrobials
		Horizontal transfer	
Characteristics	Inheritable Stable Irreversible	Inheritable Stable Irreversible	Not inheritable Transient Generally reverts upon removal of inducing signal
	Independent of environment	Independent of environment	Dependent on environment

sic and/or acquired resistance traits (Driffield et al., 2008; Hausner and Wuertz, 1999; Molin and Tolker-Nielsen, 2003). The molecular mechanisms behind adaptive resistance have only recently started to be understood, with most studies corresponding to only a few microorganisms. The results obtained so far show that these mechanisms are more complex than initially thought, involving intricate regulatory responses. In that sense, the use of transcriptome analysis will be very useful to identify which genes are modulated by a specific antibiotic.

Here we summarize the information available to date on adaptive resistance to antibiotics (Fig. 2), as well as evaluate how these mechanisms can participate in the slow but relentless increase in baseline MIC values. Some of the examples provided here show how a better understanding of the mechanisms involved in adaptive resistance is invaluable in the design of more effective antibiotic treatment strategies. Furthermore, the data provided by numerous studies points towards a role of adaptive resistance in the differences obtained in laboratory susceptibility tests and the antibiotic failure observed in the clinic.

2. Factors that trigger adaptive resistance

2.1. Environmental cues

Bacteria respond to changes in the environment by modulating their gene expression. Taking this into account, it seems likely that one reason for the differences in antibiotic resistance between in vitro and in vivo conditions could be due to adaptations of bacteria to the host milieu. Thus, the adaptive resistance triggered by environmental factors such as anaerobiosis, concentration of ions, carbon source, polyamines and pH has been investigated over the years in an attempt to understand whether adaptation to these conditions helps pathogens to resist higher concentrations of antimicrobial agents. Here, we describe how such environmental factors can modulate gene expression and induce antibiotic resistance. Furthermore, we summarize the mechanisms of adaptive resistance induced by the above-mentioned cues (Fig. 2).

2.1.1. Anaerobiosis

To date, the importance of anaerobiosis (absence of oxygen) in the development of antibiotic adaptive resistance has not been studied in great depth. However, several studies indicate that the amount of oxygen available has a substantial influence on the increased resistance to antibiotics of some pathogens. The best known example is *P. aeruginosa* when infecting the lung of CF patients. CF is an autosomal-recessive genetic disorder that, in 70% of cases, is due to a Δ508 deletion in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. This mutation leads to impaired chloride transport at the apical surface of the epithelial cells and increased sodium absorption. This results in thickened and dehydrated mucus in the lungs, which impairs mucociliary clearance of bacterial pathogens. Subsequently, invading *P. aeruginosa* cells get caught in the mucous layer and migrate through the mucus and strongly adhere to epithelial surfaces. There is an oxygen gradient across the mucus and the pericellular region under the thickened mucus is essentially anaerobic (Grimwood, 1992; Starner and McCray, 2005; Govan and Deretic, 1996). Although the precise environmental factors involved are unknown, these adhered cells adapt to the new (anaerobic) environment by acquiring certain phenotypic changes, including a greater production of some virulence factors such as the production of exoproducts, e.g. exotoxin A, elastase, lipase, exoenzyme, etc. (Grimwood, 1992), as well as losing their LPS O-antigen (Hancock et al., 1983), and forming microcolonies/biofilms (Davies, 2002; Singh et al., 2000). These adaptive changes allow *Pseudomonas* to maintain a chronic infection and coincidently acquire increased antibiotic resistance. One phenotypic change that has been related to *P. aeruginosa* adaptation to the CF lung is the development of small colony variants (SCVs) (Haeussler et al., 2003). In addition to having a small colony size, SCVs show a low growth rate and atypical colony morphology, and their presence has been linked to chronic and persistent infections (Kahl et al., 2003). The isolation of SCVs from the CF lung shows that, presumably through adaptation to anaerobic conditions, *Pseudomonas* can become more persistent and resistant to a broad range of antipseudomonal agents (Haeussler et al., 1999). In vitro studies on the SCVs isolated from CF lungs showed a hyperpiliated, autoaggregative phenotype with an increase in twitching motility and biofilm formation. In addition, SCVs show increased fitness under stationary phase conditions, indicating that the nutrient limitation and anaerobic conditions present in the CF lung favor the growth of SCVs. The occurrence and subsequent selection of these variants in the CF lung makes it even more difficult to overcome resistance and persistence. This correlation between SCVs and oxygen limitation has also been found in other species. For instance, in a clinical case of chronic aortic valve endocarditis, which is caused by *Enterococcus faecalis*, it was demonstrated that microaerobic conditions favor the growth of SCVs. Thus, a higher optical density was observed when the SCVs were grown under microaerobic conditions, while their growth was significantly impaired in aerobic conditions (Wellinghausen et al., 2009).

Karlowsky et al. (1997) and others showed that anaerobiosis reduces bacterial killing by aminoglycosides. Generally, aminoglycosides have a high bactericidal activity; however, this activity is strongly reduced under anaerobic conditions. This is especially of concern in patients with CF as an anaerobic environment exists in the lung. For the same killing effect, a much higher dose of antibiotic is required under anaerobic compared to aerobic conditions. A lethal aerobic concentration for *P. aeruginosa* would

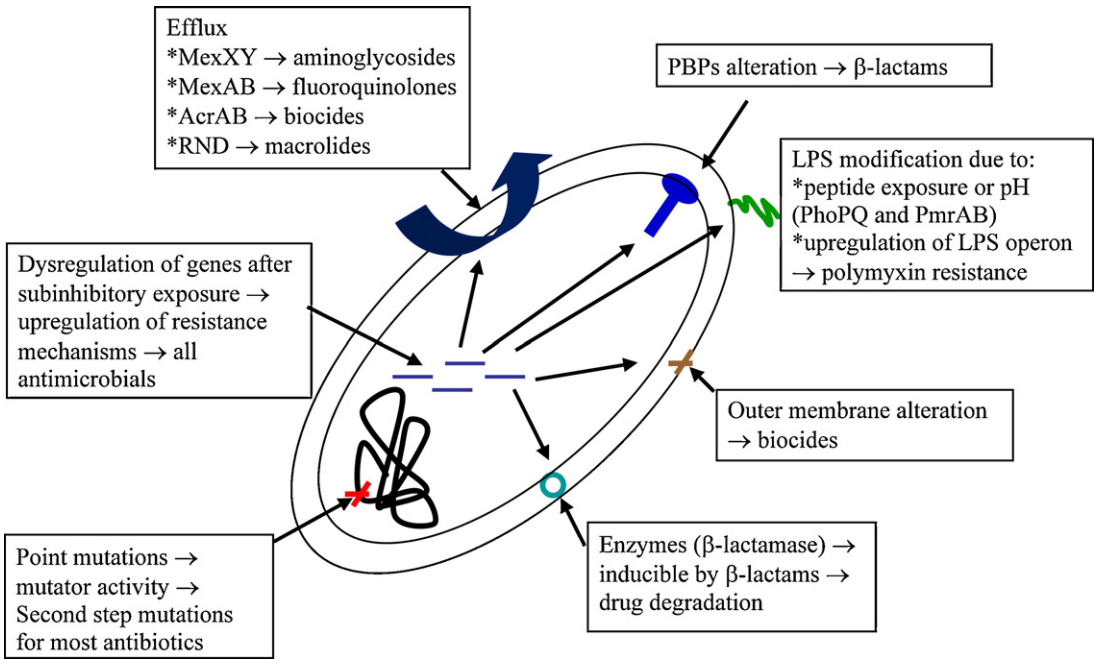

Fig. 2. Schematic representation of some of the major known adaptive resistance mechanisms. RND, PBP and LPS stand for resistance-nodulation-cell division type efflux, penicillin binding protein and lipopolysaccharide, respectively.

be 2 μg/ml, whereas the lethal concentration under anaerobiosis would be 20 μg/ml (Kindrachuk and Hancock, unpublished data). The mechanisms leading to the reduced susceptibility to aminoglycosides under anaerobic conditions are most likely due to limited cytoplasmic accumulation of the drug and differences in uptake between cells growing under aerobic and anaerobic conditions (Bryan et al., 1979; Bryan and Kwan, 1981, 1983; Mates et al., 1983). Although the mechanisms of adaptive aminoglycoside resistance will be discussed in another section of the review, it is worth emphasizing here that anaerobiosis contributes to aminoglycoside resistance. In fact, two genes that have been shown to be highly expressed in aminoglycoside-adapted *P. aeruginosa* cells, *denA* and *anr*, encode a nitrate reductase and a regulator controlling anaerobiosis (Karlowsky et al., 1997). This indicates that genes involved in the anaerobic respiration pathway are induced in response to aminoglycosides, and strengthens the overall picture that varying oxygen concentrations in the environment can have an impact on adaptive responses.

2.1.2. Ions and ionic binding

Ions, such as divalent cations, are commonly present in the environment. An early study investigating the influence of the divalent cations Mg^{2+} and Ca^{2+} on adaptive resistance to aminoglycosides and fluoroquinolones was performed on clinical isolates of *Enterobacteriaceae* and *P. aeruginosa* (Gould et al., 1991). This work studied the influence of ionic binding on aminoglycoside and fluoroquinolone killing using stationary phase cultures, as this growth state is more similar to in vivo conditions. The authors observed that there was a concentration-dependent killing in the case of the aminoglycoside netilmicin at 37 °C as well as at 4 °C. However, the killing effect at 4 °C could to some extent be inhibited by EDTA, but not by Ca^{2+}. This suggested that ionic binding to the cell wall is of great importance for netilmicin uptake. Moreover, this study showed that this was not the case for ciprofloxacin at 4 °C. Furthermore, adaptive resistance was observed for both antibiotics, although it was more obvious in the case of netilmicin. Divalent cations are known to have a role in stabilizing the outer membrane and, as a result, their presence can limit the self-promoted uptake of aminoglycosides and cationic peptides, thus contributing to resistance. Therefore, a higher concentration of divalent cations would be predicted to have a protective effect against these antimicrobial compounds and cells would be expected to be more sensitive at lower concentrations of divalent cations. Conversely it has been demonstrated that cells of some species, like *Salmonella* and *Pseudomonas*, actually acquire a higher degree of resistance to cationic antimicrobials when divalent cations are limiting (Nicas and Hancock, 1980, 1983; Gunn et al., 1998b; Heithoff et al., 1999; Soncini and Groisman, 1996).

Initially, the molecular mechanisms involved in this adaptive resistance were poorly understood. Now thanks to extensive research carried out in *Salmonella* and *Pseudomonas*, we have a quite clear picture of these mechanisms as well as the regulatory pathways involved. Two-component regulatory systems, consisting of a sensor histidine kinase and a transcriptional regulator, allow microorganisms to sense changes in the environment and adapt to the new conditions. Two different two-component systems, namely PhoPQ and PmrAB, were found to be necessary for *Salmonella* to become adaptively resistant in low Mg^{2+} concentrations. In *S. enterica* serovar Typhimurium, the sensor protein PhoQ can detect Mg^{2+} limitation and activate the transcriptional regulator PhoP, which will then induce the transcription of the *pmrAB* operon. The PmrAB system, identified by Roland et al. (1993), cannot sense magnesium levels and is only directly activated by high Fe^{3+}, low pH and vanadate. Cross-talk between the PhoPQ and PmrAB systems is mediated by the protein PmrD (Kato and Groisman, 2004; Kox et al., 2000), whose expression is induced by PhoP. PmrD interacts with and stabilizes the phosphorylated form of PmrA, which then leads to the upregulation of the lipopolysaccharide (LPS) modification operon (*pmr* operon). The *pmr* operon codes for genes involved in the addition of 4-aminoarabinose to lipid A. This modification reduces the net negative charge of LPS and, as a result, of the cell surface, thereby limiting the interaction with positively charged antimicrobials. Also, PhoP induces the expression of the genes *pagP*, *pagL* and *lpxO*, all of which are involved in other modifications of LPS (Gunn et al., 1998a). This all leads to adaptive polymyxin B resistance. In the case of *Salmonella*,

functional PhoP is required for activation under magnesium starvation. In vivo, PhoPQ and PmrAB are activated when *Salmonella* cells undergo phagocytosis by macrophages (Alpuche Aranda et al., 1992). Under these conditions, it is generally considered that the activating signal is acidic pH rather than a low concentration of Mg^{2+} and Ca^{2+} ions (Alpuche Aranda et al., 1992; Bearson et al., 1998; Prost et al., 2007).

The regulation of divalent cation sensing in *P. aeruginosa* is similar to that of *Salmonella*, although some notable differences can be observed, which might be related to the different environmental niches occupied by the two species. In *Pseudomonas*, both PhoPQ and PmrAB get activated independently from each other by low concentrations of Mg^{2+} (20 μM), which reflect starvation conditions. PhoPQ is involved in autoregulating the *oprH-phoPQ* operon (Macfarlane et al., 1999) and PmrAB also autoregulates its cognate operon (McPhee et al., 2003), as reviewed by Gooderham and Hancock (2009). Once activated, both PhoP and PmrA are able to independently upregulate the expression of the LPS modification (*arn*) operon. The *arn* operon is a homologue of the *Salmonella pmr* operon and, as in *Salmonella*, its induction makes the cells more resistant to polymyxins and other cationic antimicrobial peptides (Ernst et al., 1999; McPhee et al., 2006; Moskowitz et al., 2004). Like polymyxins, aminoglycoside resistance in *Pseudomonas* is also dependent on the Mg^{2+} concentration (Hancock et al., 1981). However, in contrast to *Salmonella*, no functional PhoP protein is required for this induction. Thus, a *phoP* mutant shows the same resistance to polymyxin B as the wild type, whereas a *phoQ* mutant is even more resistant to this drug (McPhee et al., 2003). This membrane remodeling is an important adaptive mechanism. In fact, the LPS lipid A modifications seen under low Mg^{2+} concentrations are highly similar to those observed in isolates from chronic CF patients (Ernst et al., 1999). This suggests the presence of an inducing signal within the CF lung. In *Pseudomonas*, all evidence obtained to date seems to point to peptides as the inducing signals, although this will be discussed in a later section of this review.

To date, *Salmonella* and *Klebsiella* are the only known organisms for which the PmrD protein actually activates PmrA. A recent study (Cheng et al., 2010) clearly showed how the expression of the *pmr* operon in *Klebsiella pneumoniae* CG43 is regulated by PhoP, PmrA and PmrD in a similar way to *Salmonella*. In *E. coli*, PmrD is present but it fails to activate PmrA, most likely due to the fact that it is highly divergent (Gibbons et al., 2005). As a result, *E. coli* does not modify its LPS in response to low Mg^{2+}. *Yersinia*, like *Pseudomonas*, does not have PmrD and both PhoPQ and PmrAB get activated independently by low magnesium (Winfield et al., 2005).

In summary, the cation concentration in the surrounding milieu can have a high impact on the adaptive resistance to certain drugs like aminoglycosides and, in particular, cationic antimicrobial peptides. Therefore, the possibility that the pathogen may find these conditions within the host could be related to a reduced efficacy of the antibiotic treatment.

2.1.3. Others

Several other factors like carbon sources, the presence of polyamines, and pH have been identified to have an influence on adaptive antibiotic resistance.

In *P. aeruginosa*, resistance to several antibiotics was shown to be influenced by the carbon source used in the growth medium (Conrad et al., 1978). The authors demonstrated that adaptive resistance to polymyxin B and colistin exists if either D-glucose or L-glutamate is used as a carbon source, whereas other carbon sources such as L-isoleucine, L-valine and isobutyrate have the opposite effect and lead to increased susceptibility. Interestingly, if carbon sources with opposite effects, such as D-glucose and L-isoleucine, are combined, then resistance occurs. This indicated that resistance to antibiotics will vary depending on the available carbon source in a specific milieu, which is relevant as this might change at different stages of an infection or in different host tissues.

That the presence of polyamines affects antibiotic resistance has been known for a while for *E. coli* (Samartzidou and Delcour, 1999). Polyamines are polycationic compounds, e.g. cadaverine, putrescine, spermidine and spermine that *Pseudomonas* can use as the sole carbon and/or nitrogen source (Lu et al., 2002). They can increase or decrease antibiotic susceptibility according to the particular organism and antibiotic (Tkachenko and Nesterova, 2003; Kwon and Lu, 2006), and there has been evidence that this relates in part to the induction of the *oprH-phoPQ* operon (Kwon and Lu, 2006; Chou et al., 2008).

Another factor that influences adaptive resistance is pH. It has been shown for the *Bacteroides fragilis* group that the susceptibility towards a wide variety of antibiotics (including ciprofloxacin, clindamycin, trovafloxin, imipenem, meropenem, piperacillin-tazobactam and ampicillin-sulbactam) decreased at an acidic pH (Falagas et al., 1997). Furthermore, for several other organisms including *E. coli*, *S. aureus*, *P. aeruginosa*, *K. pneumoniae*, *Serratia marcenscens* and *Proteus mirabilis* it was demonstrated that pH can affect adaptive resistance to aminoglycosides (Young and Hewitt, 1973; Damper and Epstein, 1981; Davies, 1991). A study on a clinical *Pseudomonas* isolate showed that the killing effect of amikacin diminished as the pH became more acidic. Thus, amikacin killing was lower at pH 6.5 than at pH 7.4. Furthermore, at an even more acidic pH (pH 5.5), amikacin did not demonstrate a killing effect at all. This seemed to indicate that the underlying mechanism is a failure of drug-uptake at a lower pH. The likely cause here is a compensatory decrease in the electrical potential gradient $\Delta\Psi$ across the cytoplasmic membrane that compensates downwards to keep the protonmotive force constant when pH is lowered, thus increasing the ΔpH (Xiong et al., 1996). The pH of the medium is also important with regards to polymyxin and peptide resistance. In fact, in the *Enterobacteriaceae* a lower pH (pH 6.1 vs. pH 7.5) acts as an inducing signal for the two-component systems PhoPQ and, as a result, PmrAB. Thus, mild acidification leads to the expression of the PhoP- and PmrA-regulated genes, including the previously described LPS modification operon. Foster and Hall (1990) described the induction of *psiD*, which is upregulated 50-fold, when *Salmonella* is grown at pH 6.1 instead of pH 7.5. Furthermore, other PmrA-regulated genes, such as *pbgP*, *pbgE*, *pmrC* and *ugd*, are induced at a mildly acidic pH (pH 5.8) and are dependent on a functional PmrA (Soncini and Groisman, 1996). Interestingly, Soncini and Groisman (1996) could also show that the induction of some genes under acidic conditions is independent of the PhoPQ system, as *phoP* and *phoQ* mutants still showed an induction of the above-mentioned genes. Overall, this shows that PmrA-regulated genes can either be induced by low Mg^{2+} or mildly acidic conditions. The major significance of this finding is that the macrophage intracellular environment has a slightly acidic pH. Therefore, it could well be the case that this is the in vivo inducing-signal of LPS modification and, as a consequence, of polymyxin resistance.

In conclusion, several environmental factors including carbon source, pH, anaerobicity and ionic strength can all modify antibiotic resistance in pathogenic bacteria. It has also been suggested that osmolarity has an impact on antibiotic resistance. An increase in osmolarity subsequently leads to a decreased antibiotic susceptibility (Rodriguez et al., 1990). As in most topics related to adaptive resistance, more studies need to be carried out in order to characterize the effect of these cues and evaluate their significance in the clinic.

2.2. Social behavior

Over the past two decades, there has been a growing interest in the study of bacterial communities and social activities, which has given rise to the field termed by one group as sociomicrobiology (Parsek and Greenberg, 2005). Under certain environmental conditions, microorganisms exhibit coordinated multicellular behaviors that require cell-to-cell communication and are often controlled in part by quorum sensing signals. Two well known examples are biofilm formation and swarming motility, both of which have been related to an increase in virulence as well as in resistance to antibiotics and host defense mechanisms (Verstraeten et al., 2008).

2.2.1. Biofilms

Biofilms are microbial communities in which the cells adhere to a surface and to other cells constituting dense aggregates of a single or multiple species. Although the existence of biofilms has been known for a long time, it was not until the 1980s that mechanistic research regarding this form of microbial growth began. Since then, scientists have recognized the great importance of biofilms in nature, medicine and industry, as well as their evolutionary interest as a community of microorganisms. When forming part of a biofilm, bacterial cells show notable phenotypic and transcriptional differences compared to their planktonic counterparts, and they mimic more the behavior of a multicellular organism. Even though most research on bacteria has been carried out using planktonic (free living) bacterial cultures, it is likely that biofilms are, in fact, a more natural form of bacterial growth in many environments, as well as in industrial and clinical settings (Davey and O'toole, 2000).

It has been suggested that biofilms are responsible for over 60% of all infections in man (NIH, 2002). Some of the clinically relevant types of biofilms include those formed on the teeth (Kolenbrander and Palmer, 2004), heart valves (endocarditis) (Høiby et al., 1986), the lungs of CF patients (Bjarnsholt et al., 2009), prosthetic joint infections (del Pozo and Patel, 2009), catheters and stents (Taconelli et al., 2009), and chronic wounds (Kirketerp-Møller et al., 2008). One of the major complications associated with biofilms in the clinical environment is their high resistance to antibiotics and detergents, as well as to the immune system, all of which make them very difficult to eradicate. Biofilm cells can be up to thousands fold more resistant to antibiotics than planktonic cells (Hoyle and Costerton, 1991). Two clear examples are provided by the pathogens *Staphylococcus epidermidis* and *P. aeruginosa*, both of which have been implicated in multidrug-resistant urinary tract infections associated with catheters. Initially observed in the clinical context, in vitro studies confirmed the suspicion that the reason behind the difficulty to treat infections related to prosthetic materials was the formation of biofilms on the surface of these devices (Richards et al., 1989; Taylor et al., 1988; Nickel et al., 1985). For instance, a *P. aeruginosa* isolate from a urinary infection patient showed a 1000-fold increase in resistance to tobramycin in the biofilm state (Nickel et al., 1985). The sensitive phenotype could be recovered by taking cells from the biofilm and growing them in a liquid culture, which corroborated the adaptive nature of this type of resistance. The same phenomenon has since been found in a number of other microorganisms such as the yeast *Candida albicans* (Hawser and Douglas, 1994), and the bacteria *Porphyromonas gingivalis* (Hansen et al., 2000), *E. coli* (Ito et al., 2009), *Corynebacterium urealyticum* (Soriano et al., 2009) and *K. pneumoniae* (Anderl et al., 2000). A general characteristic of biofilm resistance is its tendency to affect a wide range of drugs, which hinders even more the possibilities of finding an appropriate therapy.

Ever since the initial observation of this phenomenon, numerous studies have attempted to understand the mechanisms underlying this form of adaptive resistance (for review, see Drenkard, 2003; Høiby et al., 2010; Stewart, 2002). The basic mechanisms suggested include restricted penetration, antimicrobial destroying enzymes, limited or anaerobic growth at the base of biofilms, specific quorum sensing regulated resistance mechanisms for individual classes of antibiotics, the presence of persister cells and general stress responses.

Some of these mechanisms are directly derived from the way biofilms are structured, while others are part of the coordinated adaptation involved in the biofilm mode of growth. Cells in biofilms produce an extracellular matrix that consists of polysaccharides, proteins and DNA. This polymer matrix surrounds the cells conferring at the same time stability to the biofilm structure and protection from external agents. It is generally believed that this matrix can delay the penetration of antibiotics to some degree. For instance, the positively charged aminoglycosides exhibit a lower ability to penetrate into biofilms, which is probably due to interactions with the negatively charged polymers (Kumon et al., 1994). Additionally, bacteria produce enzymes, such as β-lactamases, which can accumulate in this matrix, resulting in the inactivation of a large fraction of the antibiotic molecules before reaching the cells. Thus, a β-lactamase-negative mutant of *K. pneumoniae* showed a higher diffusion rate of ampicillin into the biofilm than did the parent strain (Anderl et al., 2000). It must be noted, however, that most studies regarding penetration of antibiotics concluded that diffusion was not very significantly impeded (Dunne et al., 1993; Shigeta et al., 1997; Vrany et al., 1997), and that other mechanisms are probably more important with relation to biofilm-specific resistance. New evidence suggests that the participation of the extracellular matrix goes beyond merely hindering the penetration of drugs. Thus, a recent paper revealed that, in addition to providing support to the biofilm structure, DNA has the property of chelating cations, thereby destabilizing the outer membrane (OM) LPS (Mulcahy et al., 2008). As a result, DNA has antimicrobial properties at certain concentrations and, more importantly in the context of adaptive resistance, at sub-inhibitory concentrations it upregulates the expression of the PhoPQ and PmrAB two-component systems. As we have discussed above, both of these systems can induce the LPS modification operon. This induction increased the resistance of the biofilm to cationic peptides and aminoglycosides by 2560-fold and 640-fold, respectively (Mulcahy et al., 2008).

Some studies seem to indicate that the conditions of the biofilm production facilitate the acquisition of inheritable resistance traits. In fact, biofilm cells exhibit an increased mutation rate. For example, *P. aeruginosa* biofilm cells show a downregulation of genes related to mechanisms of protection against DNA damage, which could actually be the cause of the increased mutability in the CF lung (Driffield et al., 2008). Also, close contact between the cells is thought to facilitate plasmid transfer by conjugation (Hausner and Wuertz, 1999; Molin and Tolker-Nielsen, 2003). This enhanced development of acquired resistance in biofilm-forming cells would be particularly dangerous, as the already more resistant biofilm cells would then become intrinsically multidrug resistant.

Further proposals about biofilm resistance are related to the heterogeneity of these communities, even when they are formed by cells of a single species. Because of the special structure of biofilms, there are gradients of nutrients and oxygen that cause the cells to be in distinct growth states (Fig. 3). Thus, the cells at the biofilm surface will have greater access to nutrients and oxygen and will be metabolically active. In contrast, the cells at the center of the biofilm will be under nutrient-limited and almost anaerobic conditions and will adopt a slow growth mode as well as many adaptations to these nutrient and oxygen deprivation conditions. Consequently, cells in different layers of the biofilm will be affected differently by different types of antimicrobials depending on their mechanism of action. For example, those antibiotics, such as β-lactams, that require the cells to be metabolically active in order to be killed, will not eradicate the inner layers of the biofilm, which will then

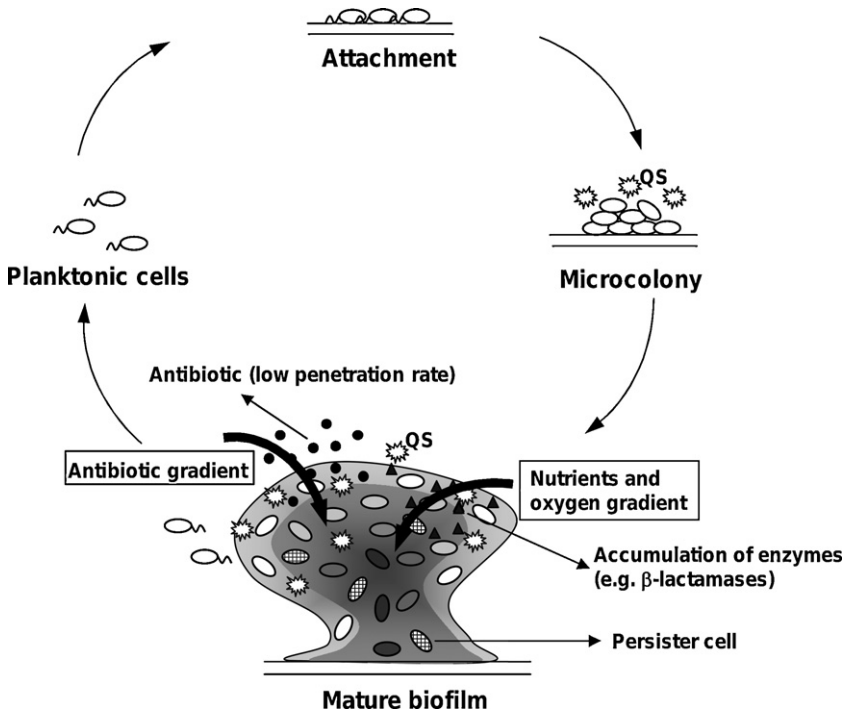

Fig. 3. Schematic representation of the major stages of biofilm formation and some of the principal resistance mechanisms observed in mature biofilms. The grey background represents the extracellular matrix that consists of exopolysaccharide and DNA, both of which contribute to antibiotic resistance. The intensity of the color varies according to the nutrients and oxygen gradient (represented with a thick arrow), i.e. the lighter the color the greater the concentration of oxygen and nutrients and vice versa. The cells embedded in the biofilm also show a different shade on the basis of their growth and metabolic rate, ranging from the metabolically active cells, in white, to the slow growing cells, in dark grey. QS is the quorum sensing signal.

start growing again right after the treatment stops. In fact, it is generally the less active cells in the biofilm that are responsible for the observed increased resistance (Brown et al., 1988; Gilbert et al., 2002). Nevertheless, tolerance has been associated with the more active bacteria in some cases. For example, the antimicrobial peptide colistin can effectively kill *P. aeruginosa* cells from the slow growth areas of the biofilm, whereas the active cells are able to develop tolerance to this antimicrobial by induction of the LPS-modification operon and the efflux pump MexAB-OprM (Pamp et al., 2008). It was also observed that ciprofloxacin and tetracycline had precisely the opposite effect, being able to kill only the active cells. As a result, the use of combination therapy was proposed to eradicate *P. aeruginosa* biofilms.

In addition, growth under anaerobic conditions has been demonstrated to contribute to antibiotic tolerance of *P. aeruginosa* biofilms against different antibiotics, including tobramycin, ciprofloxacin, carbenicillin, ceftazidime, chloramphenicol, and tetracycline (Borriello et al., 2004). The effect of the growth rate on biofilm antibiotic resistance has also been observed in *E. coli* (Evans et al., 1991; Ito et al., 2009). Ito et al. (2009) recently showed that the cells from the deeper layers of an *E. coli* biofilm remained sensitive to ofloxacin and kanamycin, whereas ampicillin was ineffective at killing these cells. Gene expression analysis of cells at different stages of biofilm formation revealed that *rpoS* plays an important role in regulating the transcriptional changes that mark the maturation of a biofilm, including the acquisition of increased resistance to certain antibiotics in later stages (Ito et al., 2009). Another characteristic of biofilms is the presence of a larger subpopulation of persister cells than in the planktonic state (Lewis, 2008). Persistence can be defined as the ability of some cells belonging to a genetically homogeneous population to withstand stress conditions, including antibiotic pressure, that are lethal to the rest of the cells in that population (Gefen and Balaban, 2009). In the case of *S. epidermidis*, persister cells could be found in both monolayer and multilayer biofilms but not in planktonic cultures at log phase in a strain- and antibiotic-dependent manner (Qu et al., 2010). The authors concluded that, together with high cell density growth, persisters are the most important determinants of biofilm antibiotic resistance in this pathogen.

Several screening studies have attempted to identify specific genes involved in biofilm adaptive resistance, with the aim of gaining a better understanding of the molecular basis for these adaptations. For example, some recent papers described the identification of gene mutations that led to the formation of supersusceptible biofilms, i.e. biofilms structured like those of the parent strain but which are more sensitive to the action of antibiotics. In the case of *P. aeruginosa*, Mah et al. (2003) found a gene, *ndvB*, necessary for the synthesis of periplasmic glucans. The authors proposed that such molecules might bind antibiotics in the periplasm, thereby preventing them from reaching their targets. Also, Zhang and Mah (2008) identified a novel efflux pump that was upregulated in *P. aeruginosa* biofilms that when mutated resulted in a decreased resistance to aminoglycosides and fluoroquinolones. In a similar manner, azithromycin resistance of *P. aeruginosa* biofilms depends on the presence of the MexCD-OprJ efflux pump (Gillis et al., 2005). The gene *rapA*, which encodes a homologue of helicase-like proteins, was demonstrated to determine biofilm-specific resistance in a uropathogenic *E. coli* strain. Mutants in this gene formed apparently normal biofilms that were more sensitive to penicillin G, norfloxacin, chloramphenicol and gentamicin, while resistance of planktonic cells remained unaltered (Lynch et al., 2007). This mutant also showed a different transcriptional profile from that of the parent. Thus, the gene *yhcQ*, encoding a putative multidrug efflux pump, was downregulated in the *rapA* mutant. Analysis of an *yhcQ* mutant revealed a greater sensitivity than the wild-type, but lower than the *rapA* mutant. This means that additional mechanisms are involved in the sensitivity phenotype. Also downregulated was the gene *yeeZ*, which is

thought to participate in cell wall-related processes. It seems likely that the product of this gene is related to the lower polysaccharide content of the *rapA* biofilms, which results in a higher penetration rate of antibiotics.

Overall, biofilms constitute a major threat in the clinical environment by acting as reservoirs of multidrug resistant pathogenic bacteria. It is now evident that the increased resistance of biofilms is due in part to the characteristics and adaptations of the biofilm state, including the dysregulation of antibiotic resistance determinants. Further studies will be very useful in determining the most effective treatment strategies in biofilm-related infections.

2.2.2. Swarming motility

When growing on a semisolid surface, certain bacterial species show a special type of motility, known as swarming, that is characterized by a coordinated and rapid movement of the cells (Fraser and Hughes, 1999). Swarming is generally considered a social phenomenon because it relies on intercellular communication. This type of motility has been observed in many different bacteria, both Gram-negative (genera *Proteus*, *Vibrio*, *Serratia*, *Salmonella*, *Escherichia*, *Azospirillum*, *Aeromonas*, *Yersinia* and *Pseudomonas*) and Gram-positive (*Bacillus* and *Chlostridium*), and it entails highly complex adaptations and cell differentiation (Fraser and Hughes, 1999; Overhage et al., 2008a; Verstraeten et al., 2008; Yeung et al., 2009). Thus, swarming can be regarded as a specific physiological state, clearly distinct from the vegetative state. Since, for example, mucosal surfaces in the body represent regions of higher viscosity, with conditions similar to those which encourage swarming in vitro, it is felt that swarming is relevant to microbial infections in man.

In some species, swarming cells have been demonstrated to show upregulation of certain virulence-related genes. For example, *P. aeruginosa* swarmers have an increased expression of the type three secretion system (TTSS), alkaline protease, pyoverdin and pyochelin (Overhage et al., 2008a). Likewise, bacterial cells are more resistant to the action of antibiotics under swarming conditions than their vegetative counterparts. The first observation of this characteristic was by Kim et al. (2003) in *S. enterica* serovar Typhimurium. This study demonstrated that *Salmonella* swarmer cells are more resistant to a wide range of antimicrobials, including β-lactams, polymyxins, aminoglycosides and quinolones. More recently, the same phenomenon has been observed in *P. aeruginosa* (Lai et al., 2008; Overhage et al., 2008a), *E. coli* (Lai et al., 2008), *S. marcescens* (Lai et al., 2008), *Burkholderia thailandensis* (Lai et al., 2008) and *Bacillus subtilis* (Lai et al., 2008). In the case of *B. subtilis*, *P. aeruginosa* and *E. coli*, Lai et al. (2008) found that swarm colonies were still sensitive to polymyxins, unlike those of *Salmonella*. Conversely, Overhage et al. (2008a) demonstrated an increased resistance to polymyxin B in *P. aeruginosa* swarm cells by using a different assay. Besides antibiotics, *P. aeruginosa*, *B. subtilis* and *E. coli* swarm cells develop increased resistance to arsenite and, in the last two species, they are also more resistant to the biocide triclosan (Lai et al., 2008). Resistance to all the antimicrobials tested reverted to planktonic levels in all three species when the cells were taken from the swarming colony and grown under non-swarming conditions; therefore, this is clearly a type of adaptive resistance and not the result of mutant selection (Kim et al., 2003; Lai et al., 2008; Overhage et al., 2008a). Nevertheless, the adaptive phenotype can persist as Kim and Surette (2003) showed that while polymyxin B resistance was reduced drastically after inoculating the *Salmonella* swarming cells into liquid medium, resistance to kanamycin decreased in a more gradual manner.

The molecular mechanisms underlying this resistance are still largely unknown, although it is to be expected that they will be dependent on the bacterial species and the antimicrobial agent. Also, the mechanisms might be distinct from those identified in biofilms due to the significant differences that characterize these two types of multicellular communities. In the case of *S. enterica*, it appears that the aminoarabinose modification of the LPS is involved in this phenotype. Kim et al. (2003) demonstrated that the LPS modification operon (*pmrHFIJKLM*) is upregulated during swarming and that *pmrK* mutants are swarming deficient. As previously mentioned, the products encoded by this operon participate in resistance to cationic antimicrobial peptides in this microorganism (Gunn et al., 1998a,b) and, as a result, it would explain the increased resistance of *Salmonella* swarmers to polymyxins, but not to all the affected antibiotic classes. A later work revealed that L-cysteine is essential for complete differentiation of swarm cells in *S. enterica*, including the increased resistance phenotype (Turnbull and Surette, 2008). This study described the identification of cysteine auxotrophs that can still swarm, albeit showing an altered morphology, but do not display induced antibiotic resistance. It also showed that the CysB regulon is upregulated under swarming conditions, independently of the presence of an inorganic sulphur source. These results link the specialized metabolic state of swarming cells and their reduced drug susceptibility. Conversely Butler et al. (2010) recently concluded that the increased antibiotic resistance during swarming in *Salmonella* is not due to the physiology of swarming cells, but to the high cell density found within the swarming colony as well as to mobility. Additionally, quorum sensing signals did not appear to play a role in adaptive resistance. These observations would agree with the limited differences in gene expression between swarming and non-swarming cells in this microorganism (Wang et al., 2004). The authors also tested this hypothesis in *Bacillus* and *Serratia* with similar results. Unlike the situation in *Salmonella*, comparison of the transcriptome between *P. aeruginosa* swarming and non-swarming cells revealed a very complex transcriptional response (Overhage et al., 2008a). In fact, screening of a transposon-mutant library demonstrated that hundreds of different genes corresponding to a wide range of functional classes are involved in swarming motility (Yeung et al., 2009). Therefore, it seems likely that many different genes might be involved in the adaptive resistance phenotype. Moreover, it does seem that the mechanisms are different from those of *Salmonella* swarm cells, since neither the *pmr* operon nor the *cys* regulon appear to be induced. Lai et al. (2008) tested a series of *P. aeruginosa* mutants for their resistance during swarming with the aim of identifying some of the genes involved. Thus, mutants in the efflux pumps MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM did not show any difference in swarming-specific resistance and neither did the aforementioned *ndvB* mutant, which is defective in biofilm-specific resistance to tobramycin, gentamicin, ciprofloxacin, chloramphenicol and ofloxacin (Mah et al., 2003).

Clearly, more research needs to be done to understand why swarming cells resist the action of antimicrobial drugs more efficiently than vegetative cells. This is particularly important because of the relevance of swarming motility in the context of pathogenesis. Not only do swarmers overexpress certain virulence determinants, but also swarming is the form of motility utilized by some bacteria at certain stages of the infectious process. For instance, the ability of *P. mirabilis* to invade uroepithelial cells requires differentiation into swarmer cells (Allison et al., 1992), and *P. aeruginosa* is thought to use this type of motility when moving across the epithelial surface of the CF lung, which is covered by a layer of thick mucus (Overhage et al., 2008a). Therefore, if bacterial cells are in the antibiotic-resistant swarming state inside the host, the infection would be more difficult to eliminate. Thus, like other types of adaptive resistance, swarming would contribute to antimicrobial therapy failure even if the strains seem to be sensitive in routine in vitro analysis.

2.3. Antimicrobial compounds

As discussed above, the presence of antimicrobial compounds at certain concentrations can modulate bacterial gene expression, thereby altering the transcription of genes involved in metabolism, virulence and antibiotic resistance (Brazas and Hancock, 2005b). A consequence of this is that, following the first exposure to the antibiotic, the bacterial cells are often able to withstand increasingly higher concentrations in successive exposures. Furthermore, cross-resistance to other classes of antimicrobials can also be observed in some cases. The level of this increase in resistance and its duration after removal of the antibiotic signal depends on the type of antibiotic, dose and time of exposure, as well as on the bacterial species. It is our supposition that decreased susceptibility due to one or more adaptive changes in the clinic can be stabilized by mutation and the same sorts of genetic events lead to both adaptation (through dysregulation of one or more resistance genes) or stepwise (creeping baseline) mutations. In this section we will describe how this phenomenon affects the major antibiotic families and the specific mechanisms for each one of them.

2.3.1. Aminoglycosides

The development of adaptive resistance to aminoglycosides in vitro has been well documented for numerous bacterial species, including *E. coli* (Mawer and Greenwood, 1978), *S. aureus* (Barber and Waterworth, 1966) and *P. aeruginosa* (Barber and Waterworth, 1966), with the latter being the best described. Early studies seemed to indicate that this type of resistance was unlikely to be relevant in clinical settings. Thus, strains adapted to high levels of the aminoglycoside gentamicin in the laboratory were significantly less virulent than their parent strain, which made them less likely to effectively cause an infection (Weinstein et al., 1971). However, an interesting paper by Mawer and Greenwood (1978) provided data that suggested this idea needed to be revised. These authors described the acquisition of an adaptive phenotype after exposure of *E. coli* cultures to sub-inhibitory concentrations of gentamicin or tobramycin that was characterized by a modest increase in resistance to both antibiotics. This adaptation was not as dramatic as the one described by Weinstein et al. (1971), but it did not affect the growth of the microorganism and was fairly stable. Indeed, complete reversion to the original susceptibility was not achieved even after 24 passages in antibiotic-free medium. Worryingly, the antibiotic levels utilized in this study were in a similar range to those encountered by bacteria at some points during treatment. Furthermore, the incubation of strains carrying the gentamicin-specific resistance factor (R factor) in gentamicin-supplemented medium induced the cells to become more resistant to other aminoglycosides like tobramycin. This could easily be the case during therapy. Thus, the use of gentamicin would not only be inefficient, due to the resistance of the strain, but it would render more difficult subsequent treatment with other aminoglycosides due to the induction of class-specific cross-resistance. In the case of *P. aeruginosa*, some clinical strains possess a so-called "impermeability type of resistance", which confers low-level resistance to aminoglycosides (Gerber and Craig, 1982). This mechanism was originally thought to be the result of the selection of resistant subpopulations, but Gilleland et al. (1989) demonstrated that the same phenotype could be reproduced in vitro by exposing the cells to sub-inhibitory levels of aminoglycosides. After the exposure, the cells were able to withstand concentrations 128-fold greater than the original MIC of the strain. Induction of adaptive resistance could also be achieved by using a dynamic in vitro model, in which the initial concentration of the antibiotic is diluted exponentially and, as a result, it reflects more accurately the conditions found in vivo (Barclay et al., 1992). The results obtained with this model confirmed that the use of a higher antibiotic dose produces a greater and longer response. The phenomenon of aminoglycoside adaptive resistance has also been demonstrated to occur in vivo in animal models like neutropenic mice (Daikos et al., 1991) and rabbit endocarditis (Xiong et al., 1997), as well as in CF patients (Barclay et al., 1996b). In the latter study, adaptation occurred 1–4 h after the administration of antibiotic and it did not disappear until 24–48 h had passed. For this reason, these and other authors suggested that higher and longer dose intervals would be more effective (Barclay et al., 1996b; Daikos et al., 1991). However, due to the known toxicities of aminoglycosides, a compromise needs to be reached for the selection of an effective concentration that does not present a high toxicity risk for the patient. The development of aminoglycoside adaptive resistance in *S. aureus* has also been suggested to be responsible for aminoglycoside resistance in clinical isolates lacking aminoglycoside modifying enzymes (AMEs) (Chandrakanth et al., 2008). Aminoglycosides are often administered in combination with other drugs. Therefore, the analysis of possible cross-resistance is very important. Fortunately, aminoglycoside-induced adaptive resistance did not seem to affect antibiotics from other classes, including the cationic drug polymyxin B (Gilleland et al., 1989), as well as ceftazidime, imipenem, aztreonam, ciprofloxacin and piperacillin (Barclay et al., 1996a). The effect of rifampicin was actually potentiated after incubation with aminoglycosides, but the basis for this is unknown (Barclay et al., 1996a).

The mechanisms involved in adaptive resistance to aminoglycosides are still not completely understood. Again the most studied organism is *P. aeruginosa*. Initial studies indicated that neither the outer membrane protein H1 (now OprH and part of the PhoPQ operon) nor phospholipid alterations were involved (Gilleland et al., 1989). It was originally believed that the reduced intracellular accumulation of aminoglycosides following pre-exposure was due to the downregulation of aminoglycoside uptake during the phase of accelerated energy dependent drug transport (EDP II) (Daikos et al., 1990). More recently, it has been demonstrated that the reason is actually an increased efflux through the MexXY-OprM pump. Thus, the genes encoding MexXY are induced by aminoglycosides, and mutants in these genes do not acquire adaptive resistance (Hocquet et al., 2003). The upregulation of the *mexXY* operon involves the participation of the product of PA5471, which is thought to eliminate the repression exerted by the negative regulator protein MexZ (Morita et al., 2006; Yamamoto et al., 2009). The exact mechanism behind this process is, however, still to be determined. Moreover, an upregulation of the *mexXY* operon was found in aminoglycoside-resistant clinical isolates (Westbrock-Wadman et al., 1999). In addition to this, exposure to aminoglycosides also appears to induce the expression of genes involved in the anaerobic respiratory pathway, such as *denA* and *anr* encoding a nitrite reductase and a regulatory protein, respectively (Karlowsky et al., 1997). Besides protonmotive force, cytoplasmic accumulation of aminoglycosides relies on the possession of a functional aerobic respiratory pathway (Taber et al., 1987). Thus, deviation of cellular energetics to the anaerobic route might confer on the cell a protective effect against antibiotic killing. Interestingly, a screening for *P. aeruginosa* mutants with reduced susceptibility to tobramycin identified numerous genes involved in energy metabolism (Schurek et al., 2008). Amongst these, certain mutants in genes encoding cytochromes displayed a small colony phenotype. As mentioned previously, the SCVs have been shown to be more resistant to antibiotics than their corresponding revertant strains and their observation in clinical isolates has been related to treatment with tobramycin or colistin (Haeussler et al., 1999). Tobramycin exposure also activates the two-component regulator AmgRS, which seems to be involved in adaptive responses to membrane stress. This, in turn, upregulates the expression of a number of genes including three transporter proteins (two membrane proteases and a protease-associated protein), but not the efflux pump

MexXY (Lee et al., 2009). This is, however, just the tip of the iceberg and many different genes can have an impact on aminoglycoside resistance (Schurek et al., 2008).

The study of adaptive resistance to aminoglycosides provides a good example of how understanding of this phenomenon might help design more efficient therapeutic programs, with more appropriate dosing levels and times.

2.3.2. Fluoroquinolones

Fluoroquinolones, such as nalidixic acid, ciprofloxacin, enoxacin, norfloxacin and gatifloxacin, are synthetic broad spectrum antibiotics that inhibit bacterial DNA gyrase and topoisomerase IV. As a result of this, the bacterial cells are killed by inhibition of DNA replication and transcription. Adaptive resistance to fluoroquinolones has not yet been studied as extensively as, for example, the adaptations triggered by aminoglycosides. However, some studies have investigated the mechanisms leading to adaptive resistance to this group of drugs as well as cross-resistance to other types of antibiotics.

Dudley (1991) described the pharmacodynamic and pharmacokinetic properties of fluoroquinolones and specifically highlighted the fact that ciprofloxacin has a 99% bactericidal killing effect when used at supra-MIC concentrations. Interestingly, at sub-inhibitory concentrations ciprofloxacin has a killing effect, but, since not all the cells get killed by the first exposure, regrowing subpopulations can be observed after 4–6 h, indicating the appearance of resistance in the population (Dudley et al., 1991). A second exposure to fluoroquinolones had no or very little bactericidal effect on these strains. Thus, those cells not killed by the first exposure would have become highly adaptively resistant to subsequent treatment (Dudley et al., 1991), a phenomenon that is often termed persistence and thought to be due to (reversible) transcriptional induction of resistance genes. Earlier, Blaser et al. (1987) had already shown this nicely in a population analysis of regrowing cultures of *P. aeruginosa* after treatment with enoxacin. The authors explained this phenomenon as a selection of resistant mutants during antibiotic therapy. However, they highlighted the fact that this resistance is unstable and compared it to the phenomenon of adaptive resistance in aminoglycosides. Thus, their observations suggest that this process might be actually due to an adaptive process. After these early studies, other authors have demonstrated the development of adaptive resistance by initial pre-exposure to a non-lethal dose of the fluoroquinolone (Brazas et al., 2007; Gould et al., 1990). In these cases, the adaptation led to an enhanced survival when the cells were later exposed to a lethal dose. Therefore, ciprofloxacin can give rise to adaptive resistance in *P. aeruginosa* and it has been suggested that a combination of several mechanisms leads to high level adaptive resistance.

Generally, the usefulness of antimicrobial therapy depends on the pharmacokinetics and pharmacodynamics of the antibiotic. Although Gould et al. (1991) considered that ciprofloxacin-induced resistance was not clinically significant, some authors have suggested that a higher concentration (often termed the "mutant prevention concentration") of fluoroquinolones is more useful, as the bactericidal properties of the drug lead to killing of the organism and decrease the likelihood of resistant mutant selection (Dudley, 1991). It is important to note here that the observed resistance is not due to a genetic mutation, but rather to a survival mechanism (Massey and Buckling, 2002). Cross-resistance to other fluoroquinolones can be observed; however, efflux differences can occur as different fluoroquinolones exhibit different hydrophobicity (Piddock and Zhu, 1991; Takenouchi et al., 1996).

Studies of the effects of sub-inhibitory concentrations of ciprofloxacin on gene expression in several microorganisms revealed dramatic changes in the transcriptome (summarized in Brazas and Hancock, 2005b). These alterations enable the organism to adapt to the new challenge and switch on mechanisms leading to survival. Furthermore, some factors play a role in enhancing the adaptation to suboptimal antibiotic concentrations, such as growth in a biofilm and poor absorption (Baquero, 2001). Microarray experiments demonstrated that, at sub-inhibitory concentrations, there was a significant dysregulation of genes encoding proteins from different functional classes including chaperones and heat shock proteins, phage-related, translation, modification and degradation, etc. Notably, some genes involved in intrinsic resistance to ciprofloxacin showed an upregulation, which would partly explain the increase in resistance (Brazas and Hancock, 2005a; Walsh et al., 2000). An example of this is the induced expression of the genes encoding the MexAB efflux pump (Brazas and Hancock, 2005a). Overexpression of MexAB due to sub-inhibitory ciprofloxacin treatment can contribute to adaptive resistance, as fluoroquinolones would be pumped out of the cell via active efflux (Poole, 2000). Normally, the operon encoding MexAB-OprM is repressed by a negative regulator, MexR, which binds to its promoter region (Poole et al., 1996). A recent study suggested that the presence of antibiotics, like ciprofloxacin, creates an oxidative stress in the cell that leads to a conformational change of MexR (Chen et al., 2008). In its oxidized form, this regulator dissociates from the promoter region of *mexAB-oprM* and, ultimately, results in increased transcription of this efflux pump. Several studies have also shown that fluoroquinolones can induce the SOS response (Brazas and Hancock, 2005a; Cirz et al., 2006; Hastings et al., 2004; Ysern et al., 1990), which is not surprising considering that these drugs exert a DNA-damaging effect. Thus, following DNA damage by the fluoroquinolone, the bacterial cells switch on the SOS response in order to enhance their chances of survival and, consequently, become more resistant to the antibiotic. Early studies demonstrated that nalidixic acid, ofloxacin, enoxacin and ciprofloxacin are able to induce genes involved in the SOS response including *recA*, *umuC* and *sulA* (Piddock and Wise, 1987). Ysern et al. (1990) showed that the *umuC* expression is the highest amongst the SOS response genes tested. It is known that *umuDC* is involved in SOS mutator activity, which leads to the introduction of mutations through the error-prone repair mechanism. Furthermore, McKenzie et al. (2001) highlighted that the SOS mutator DNA polymerase IV is important for causing resistant point mutations. This would suggest that fluoroquinolones are involved in mutagenesis. It is well studied that mutation or dysregulation of mutator and antimutator genes generally lead to a resistance phenotype towards multiple classes of antibiotics (Wiegand et al., 2008). Fluoroquinolones would then induce a transitory hypermutation state in the cells, which would lead to the acquisition of reduced sensitivity to fluoroquinolones, and perhaps other antibiotics. This phenomenon is known as adaptive mutation. Although the effects of these mutations would be permanent, they should be included in this review due to the fact that the transient increase in the mutation frequency would return to its normal levels after removal of the quinolone. Therefore, this is another example of a stable resistance mechanism induced as a result of the adaptation to the environmental conditions, in this case the presence of quinolones. Several studies showed that adaptive mutations can either occur after prolonged exposure to a fluoroquinolone or after second-step mutations (Riesenfeld et al., 1997; Hansen and Heisig, 2003). This increase in the mutation rate is dependent on two factors: antibiotic concentration and time (Fung-Tomc et al., 1993). Riesenfeld et al. (1997) observed that when *E. coli* cells were plated on LB supplemented with ciprofloxacin and incubated for 7 days, the number of colonies increased every day, indicating the appearance of resistant cells. High-level quinolone resistance has also been associated with adaptive mutations in *N. gonorrhoeae* (Bhuiyan et al., 1999). Another interesting perspective is that it is likely that this also happens in the clinic. Indeed, several studies showed that fluoroquinolone resistant strains occurred after antibiotic treatment in

the hospital (Follath et al., 1986; Desplaces et al., 1986). This has been generally associated with the selection of resistant strains within the population. However, we cannot dismiss the possibility of fluoroquinolones inducing mutations in originally susceptible cells. This phenomenon could be due to inappropriate dose regimes, which inevitably result in the exposure of the pathogen to sub-inhibitory levels of the drug and subsequent adaptation.

In addition to the analysis of the transcriptional responses to fluoroquinolones, some studies have investigated which open reading frames are important in response to ciprofloxacin by identifying mutants with an increased or decreased MIC compared to the parent strain (Brazas et al., 2007; Breidenstein et al., 2008). These screenings revealed the existence of a complex ciprofloxacin resistome, although further work will be required to understand the specific roles of the identified genes.

In summary, several adaptive resistance mechanisms to fluoroquinolones have been identified and need to be investigated in more detail to understand the best administration protocols for clinical use. This is very important, as it has been shown that often antibiotics fall to sub-inhibitory concentrations for extended periods during therapy, which can increase the likelihood of adaptive resistance as well as the appearance of adaptive mutations conferring stable increases in resistance.

2.3.3. β-Lactams

β-Lactams are a broad class of antibiotics, which include penicillins, penicillin-derivatives, cephalosporins, (carba)penems and monobactams. Their major killing mechanism is through inhibition of bacterial cell wall synthesis and events that require this process including cell division. Bacteria possess several resistance mechanisms that can overcome the deleterious effects of these compounds. Some of these mechanisms are known to be adaptively triggered by the presence of the β-lactams themselves and, therefore, constitute examples of adaptive resistance.

The best known mechanism of adaptive resistance to β-lactams is the one mediated by the inducible class C chromosomal β-lactamase, which has been identified in several bacterial species. β-Lactamases are enzymes produced by bacteria that can break open the β-lactam ring, thereby deactivating the antibacterial properties of the molecule and leading to resistance. The gene encoding the class C β-lactamase is *ampC*, which is under the control of the *ampR* regulator and other accessory proteins. Normark et al. (1986) described in detail how in some bacteria, like *E. coli* and *Shigella*, *ampC* is non-inducible and, therefore, not related to adaptive resistance, although in these and other species mutations leading to increased β-lactamase synthesis can still occur. However, in many Gram-negative bacteria such as *Enterobacter* spp., *Morganella morganii*, *S. marcescens*, *Citrobacter freundii* and *P. aeruginosa*, the chromosomally encoded β-lactamase is inducible by particular β-lactam antibiotics. Thus, the expression of the β-lactamase gene in the absence of β-lactams is generally low. However, in the presence of certain β-lactams, there is a high induction in the transcription of *ampC*, resulting in a greater β-lactamase production. Due to increased drug degradation, this leads to adaptive resistance (Lindberg and Normark, 1986; Livermore, 1987). The inducible β-lactamase is a major clinical problem, because resistance has been related to therapeutic failure. Thus, an initial upregulation of β-lactamase by the β-lactam antibiotic used in therapy is stabilized by mutation often in the AmpD gene that controls the levels of the cell wall byproduct that is the co-inducer of this β-lactamase (although other mutations can also occur affecting co-inducer levels). Several clinical studies identified patients that carried microorganisms with significantly increased resistance towards β-lactam antibiotics. The appearance of these resistant clinical isolates has been associated with the overexpression of *ampC* during antibiotic administration, which is in turn related to unsuccessful therapeutic outcome and occasionally to mortality (Chow et al., 1991; Choi et al., 2008; Bergstrom and Normark, 1979; Olsson et al., 1983). For example, a study by Choi et al. (2008) examined 732 patients suffering from infections caused by *Enterobacter* ssp., *S. marcescens*, *C. freundii* and *M. morganii*. After cephalosporin treatment, 5% of the treated isolates had become resistant. Similar trends have been observed in other independent studies (Chow et al., 1991; Kaye et al., 2001). β-Lactam resistance due to *ampC* overexpression was shown to be a particular problem in *Enterobacter aerogenes* and *Enterobacter cloacae* infections, where originally susceptible isolates can become resistant during the process of therapy. Moreover, resistant *Enterobacter* strains often occur in blood isolates (Kaye et al., 2001). Adaptive resistance to several β-lactam antibiotics (ceftazidime, penicillin, cefotaxime) is frequently related to a poor clinical outcome (Pai et al., 2004). Therefore, it is thought that drugs with a weak *ampC*-inducing effect, such as cefepime, might be a better choice (Sanders, 1993), although caution is still required. It must be taken into account the fact that even a small temporary increase in resistance can facilitate the acquisition of high resistance determinants to the antibiotic used and in some cases multidrug resistance. An example of such a mechanism would be the amplification of the gene copy number of the $bla_{\text{TEM-1}}$ gene encoding TEM-1-β-lactamase, which has been observed with increasing concentrations of cephalosporins (Sun et al., 2009). Several *Salmonella* strains developed resistance to increasing concentrations of cephalosporins and the importance of gene amplification, eventually allowing to stabilization of resistance through secondary point mutations, was shown.

However, adaptive resistance to β-lactams is not due solely to β-lactamase production. In fact, the exposure of a microorganism to sub-inhibitory concentrations of β-lactams induces dramatic changes in the transcriptome, some of which will allow the organism to adapt and eventually become resistant against killing by a follow-up exposure. This was clearly shown by Rogers et al. (2007) in a study carried out to investigate the global stress response of *S. pneumoniae* after pre-exposure to penicillin. Several of the genes identified as dysregulated in the gene expression profile had already been associated with penicillin resistance. The dysregulation of these genes highlights their importance in the defense against cell wall damage after lethal penicillin exposure. These genes include *luxS*, *ciaR-ciaH* and cell envelope genes that are upregulated upon penicillin exposure, whereas genes involved in competence, fatty acid and capsule biosynthesis are downregulated. The operon *ciaR-ciaH* encodes a two-component signal transduction system that negatively regulates competence (Guenzi et al., 1994). This regulation is one mechanism by which microorganisms can defend against the cell wall damage invoked by penicillin, resulting in a protective response due to cell envelope stress; intriguingly many other cell wall biosynthesis inhibiting antibiotics trigger the same regulon. Changes in the composition of the cell wall occur due to downregulation of genes involved in capsular polysaccharide and fatty acid biosynthesis and favor cell survival, as well as provide additional protection against β-lactam antibiotics. Also, alterations in the peptidoglycan composition of the cell wall, specifically an increase in disaccharide pentapeptide, confer resistance to β-lactams (Garcia-Bustos and Dougherty, 1987). The Gram-negative pathogen *P. aeruginosa* also experiences significant transcriptional changes after exposure to sub-inhibitory concentrations of ceftazidime, a penicillin binding protein 3 (PBP3) inhibitor. In this case, the dysregulated genes found were involved in the categories SOS response, adaptation, protection, multidrug efflux and antibiotic resistance (Blazquez et al., 2006). This is in agreement with observations in *E. coli*, where the SOS response genes were induced upon inhibition of the cell wall synthesis enzyme PBP3 (Miller et al., 2004; Perez-Capilla et al., 2005). Furthermore, alterations of the PBPs can occur, leading to a decreased

antibiotic affinity and a resistance phenotype (Smith et al., 2005).

Of note, exposure of bacteria to sub-inhibitory β-lactams has been associated with a reduction in virulence and growth inhibition. For instance, the production of autolytic enzymes is inhibited and exoenzyme expression is decreased (Grimwood et al., 1989). This has been shown under in vitro and in vivo conditions. *E. coli* cells exposed to sub-MIC β-lactams are less able to adhere to human urinary tract epithelial cells (Svanborg-Eden et al., 1978) and, in a mouse model, no *E. coli* cells were regrown in peritoneal fluid after the mouse was injected with sub-inhibitory ampicillin (Zak and Kradolfer, 1979). However, virulence might be regained once the antibiotic is removed because of the transient nature of this adaptation.

Together, adaptive β-lactam resistance is a major concern as, in the clinic, β-lactams can be reduced to sub-optimal concentrations leading to alterations of the PBPs, the cell wall and the gene expression profile.

2.3.4. Polymyxins and other peptides

It has been known for decades that the presence of sub-inhibitory concentrations of polymyxins can induce adaptations leading to increased resistance in several microorganisms such as *P. aeruginosa*, *E. coli* (Greenwood, 1975), and *Proteus* sp. (Shimizu et al., 1977). This phenomenon has been studied in detail in the Gram-negative bacteria *Salmonella* and *P. aeruginosa*. Thus, early articles reported the existence of alterations in the outer membrane structure that were linked to growth in a medium supplemented with polymyxin B (Gilleland and Murray, 1976; Gilleland and Conrad, 1982; Gilleland and Lyle, 1979). Upon polymyxin treatment none of the adapted strains showed the deleterious effects on the cell envelope and the cytoplasmic contents that are generally associated with polymyxin B damage (Gilleland and Murray, 1976). An early association between adaptive resistance and mutational resistance was made (Nicas and Hancock, 1980) but remained largely unresolved for decades.

The mechanisms of polymyxin-mediated adaptive resistance in *P. aeruginosa* have remained elusive until recently. In 2003, McPhee et al. observed that the presence of antimicrobial peptides upregulated the expression of the LPS modification (*arn*) operon. We discussed above how this mechanism is also responsible for the adaptive resistance to cationic antimicrobials in low Mg^{2+} concentrations via the two-component systems PhoPQ and PmrAB. However, mutants in these systems did not show any loss in induction of the LPS modification operon by peptides. A recent study reported the identification of a novel two-component system, ParRS, which is required for the acquisition of adaptive resistance to polymyxins and the bovine cathelicidin indolicidin (Fernández et al., 2010). Nevertheless, the results of this work also indicated that more regulators are likely to be involved. Thus, some peptides like CP28 and polyphemusin did not seem to be dependent on the ParRS system in order to induce resistance. Another interesting finding was that some peptides, like the human defense peptide LL-37, do not significantly induce the LPS modification operon, which agrees with previous microarray analysis (Overhage et al., 2008b). Intriguingly, a protein of unknown function encoded by the gene PA1797 also seems to participate in the adaptive resistance mediated by ParRS, although its specific function is still undetermined. The resistance induced via ParRS leads not only to resistance to polymyxins and other peptides but also to the aminoglycosides tobramycin and gentamicin, which also have a cationic nature. Since the polymyxin colistin and the aminoglycoside tobramycin are used in the treatment of CF patients, it is particularly important to understand the regulatory pathway initiated by different peptides. Moreover, some of the modifications typically associated with adaptive resistance to peptides, such as aminoarabinose in lipid A, have been observed in isolates from CF patients (Ernst et al., 2007). This could indicate that either the endogenous peptides or the polymyxins used to treat the infection are playing a role in the adaptation of *Pseudomonas* to the CF airways. Also, Schurek et al. (2009) described the analysis of clinical isolates with a high level of resistance to polymyxins and demonstrated the existence of an adaptive type of resistance termed the "skipped-well phenomenon" that appeared to be associated with alterations in the expression of genes involved in adaptive resistance, in particular the *arn* and *pmrAB* operons.

The regulatory network of peptide adaptive resistance in *Salmonella* is different from that of *Pseudomonas*. In this bacterium, sub-inhibitory concentrations of cationic antimicrobial peptides induce the expression of the PhoPQ and RpoS regulons. The result of this is an increase in virulence, as well as in resistance to cationic antimicrobial peptides from different classes like polymyxin B and protegrin-1 (Bader et al., 2003). This inducible resistance depends on the presence of a functional PhoP. PhoPQ regulates genes involved in cell envelope remodeling and proteolytic cleavage of peptides. For example, PagP adds palmitate to the LPS and is involved in resistance to α-helical peptides, and the *pmr* operon, homologous to the *P. aeruginosa arn* operon. The detection of antimicrobial peptides is achieved via interaction of these cationic molecules with an acidic patch in the sensor domain of PhoQ, which is also responsible for sensing cations (Bader et al., 2005). For this reason, Kindrachuk et al. (2007) proposed the use of PhoQ-activating ability as a parameter for optimization in the development of new antimicrobial peptides.

Adaptive resistance to peptides has also been demonstrated in the Gram-positive pathogen *S. pneumoniae* (Majchrzykiewicz et al., 2010). Although the presence of the peptides bacitracin, LL-37 and nisin did not produce a drastic change in the transcriptional profile, this study reports the dysregulation of several genes of interest, some of which encode putative transporters and regulators. In fact, analysis of strains with mutations in several of these genes displayed a greater sensitivity towards antimicrobial peptides than the parent. However, further work is still required in order to understand fully the mechanisms involved in peptide-induced adaptations in Gram-positive bacteria.

As mentioned previously, pathogenic bacteria can easily come into contact with antimicrobial peptides in the host, as host defense peptides are produced by the immune system either by induction or degranulation from neutrophils, as well as through administration as an antibiotic treatment in the form of polymyxins. Therefore, it is paramount to attain a better understanding of how these compounds are able to induce adaptive resistance to other antimicrobial peptides and, in the case of *P. aeruginosa*, to aminoglycosides. Furthermore, host defense peptides have also become the object of research for the development of promising new therapeutics because of their antimicrobial and immunomodulatory properties (Hancock and Sahl, 2006). In that sense, the capacity of a new peptide to induce adaptive resistance in a specific target species should be tested in order to troubleshoot the development process.

2.3.5. Biocides

Biocides are chemical substances with the ability to kill living organisms, including bacteria. There are several types of biocides according to their chemical structure, e.g. alcohols, aldehydes, phenols, quaternary ammonium compounds, etc. Biocides are effectively used as disinfectants, antiseptics and anti-fouling agents in the household as well as in different fields like medicine, agriculture and industry. However, the overuse of biocides as disinfectants and antiseptics, particularly in the clinic and the household, is to some extent responsible for the growing phenomenon of biocide and antibiotic resistance, as shown by several studies discussed in this section.

Biocide resistance in bacteria was first identified in the 1950s (Adair et al., 1969), shortly after these compounds started to be utilized. Over the last decades, in addition to intrinsic and acquired resistance, many examples of adaptive resistance induced by biocides have been observed. The first study describing the effect of a biocide on adaptive resistance was carried out on benzalkonium chloride, a quaternary ammonium compound, in *P. aeruginosa* (Adair et al., 1969). This study demonstrated that *Pseudomonas* could survive in increasing concentrations of benzalkonium and that the adapted cells exhibited cross-resistance to other quaternary ammonium compounds, but not to aminoglycosides, polymyxins or carbenicillin. This indicated that exposure to a biocide can induce resistance mechanisms; however, at that time, the nature of these mechanisms was not known. A more recent study by Loughlin et al. (2002) also showed that *P. aeruginosa* isolates can grow in increasing concentrations of benzalkonium chloride after serial passages in sub-inhibitory concentrations. Additionally, in this case, cross-resistance to other biocides and some antibiotics was observed. In fact, this cross-resistance phenomenon is commonly found in experiments of adaptation to biocides, which further complicates the problem of biocide overuse. For instance, the PAO1 laboratory strain exhibited cross-resistance to other quaternary ammonium compounds (cetylpyridinium chloride and cetrimidine) as well as to the antibiotics chloramphenicol and polymyxin B. However, no cross-resistance to other membrane-active disinfectants, such as dodecyl trimethyl-ammonium bromide and chlorhexadine, or to the antibiotics imipenem, ciprofloxacin and tobramycin could be found (Loughlin et al., 2002). Of note, Russell et al. (1998) had previously shown that the adaptation of *Pseudomonas stutzeri* cells to chlorhexadine, a biguanide type biocide, conferred cross-resistance to quaternary ammonium compounds. Another interesting observation of the study by Loughlin et al. (2002) was that the cross-resistance profiles of individual isolates differed. Indeed, numerous reports seem to indicate that the mechanisms leading to biocide adaptive resistance are very specific to the strain and the biocide used. For instance, Braoudaki and Hilton (2004) investigated the development of biocide adaptive resistance in *S. enterica* and *E. coli*, and observed that cross-resistance varied with the serotype. Thus, *S. enterica* serovar Enteritidis showed cross-resistance to chloramphenicol, whereas *S. enterica* serovar Typhimurium displayed cross-resistance to chlorhexadine. In the same study, benzalkonium chloride-adapted cells of the pathogenic *E. coli* O157 strain showed cross-resistance to amoxicillin, chloramphenicol, imipenem, tetracycline and trimethoprim. Cross-adaptive responses had already been described in *E. coli* (Nunoshiba et al., 1991). This paper described the acquisition of adaptive resistance in *E. coli* cells pre-treated with the biocide hydrogen peroxide to the biocides formaldehyde, glutaraldehyde, glyoxal, methyl glyoxal and chloroacetaldehyde. The authors suggested the involvement of the SOS response in this cross-adaptive response, as *recA*- and *lexA*-mutant strains did not show this response and lacked the induction of the expression of the *recA* promoter by H_2O_2. Hydrogen peroxide can also induce oxidative stress and damage DNA, lipids and proteins. Early studies discovered that if *E. coli* cells are exposed to low concentrations of hydrogen peroxide, they exhibit reduced susceptibility to a second higher dose (Demple and Halbrook, 1983; Christman et al., 1985; Nunoshiba et al., 1991). The usage of chloramphenicol inhibited this adaptive resistance, indicating that new protein biosynthesis is required.

It is important to note that there is controversy on whether biocide adaptive resistance can be considered a stable process. Several studies showed that adaptive resistance decreases once the cells are grown without the biocide, which seems to indicate that this phenomenon is unstable (Jones et al., 1989; Joynson et al., 2002). However, other studies, like the one by Braoudaki and Hilton (2004), showed that there is a certain degree of stability. Thus, the resistance levels seen once the cells were grown without the biocide never reached the pre-adaptation sensitivity values. Besides, adaptive resistance to benzalkonium chloride has been described not only for laboratory strains, but also for clinical isolates of *P. aeruginosa* (Joynson et al., 2002). Clinical isolates were sub-cultured into increasing concentrations of benzalkonium chloride and exhibited adaptive resistance behavior. It is thought that this is due to an impermeability mechanism and no cross-resistance to aminoglycosides was observed, indicating that the mechanisms leading to adaptive resistance are different between biocides and aminoglycosides.

Although diverse, the mechanisms leading to biocide-induced adaptive resistance in microorganisms have been described and include outer membrane alterations, fatty acid changes and active efflux. The study of Loughlin et al. (2002) showed that several changes occurred in biocide-adapted *P. aeruginosa* PAO1 cells compared to non-adapted cells. These differences included an alteration of the outer membrane, with an increased presence of a 25 kDa outer membrane protein (maybe OprG), as well as changes in the fatty acid composition of the cytoplasmic membrane. However, no changes in LPS or hydrophobicity could be observed. Conversely, the *P. aeruginosa* environmental isolate OO14 showed an increase in a 44 kDa outer membrane protein. These differences between isolates further indicated that the mechanisms are very specific, although the associations were never demonstrated genetically to be causally linked to resistance. Several other early studies revealed that an increase in the lipid content of the outer membrane corresponds to higher levels of benzalkonium chloride resistance (Chaplin, 1951; Hugo, 1967). Usually, if cells are exposed to benzalkonium chloride, blebbing of the outer membrane of *P. aeruginosa* cells occurs (Hoffmann et al., 1973; Jones et al., 1989). In contrast, no blebbing occurs in benzalkonium chloride adapted cells. All these results are consistent with the concept that alterations of the outer membrane are important mechanisms of adaptation to biocides, although the specific sites of action may vary. The fact that adaptive resistance to biocides is frequently linked to cross-resistance to antibiotics might be due to a common change in membrane structure which might be indicative of a common site of uptake. Generally, biocides exhibit more than one target site (Denyer, 1995), although it is possible that under sub-inhibitory concentrations they only interact with one cell target (Hugo, 1967). Therefore, adaptive resistance might occur when the target sites are blocked and this induces the observed resistance. Merianos (1991) described that the initial site of action for benzalkonium chloride is the adsorption to the outer membrane and then the inner membrane. As a result, damage of the outer membrane would leave the inner membrane unprotected. Therefore, possible resistance mechanisms include changes in the outer membrane structure (blebbing), changes in LPS (LPS as a quencher) (Jones et al., 1989) and increases in the lipid content of the membrane components (Chaplin, 1951). Blebbing not only leads to cell death after benzalkonium chloride treatment, but it is also a possible resistance mechanism, since it can act as a quencher by neutralizing the biocide action therefore leading to survival of the cell. Another suggested adaptive resistance mechanism is active efflux. The MIC of adapted cells changes dramatically from 4 μg/ml to 512 μg/ml for benzalkonium chloride in *E. coli* O157. The underlying mechanism for this change is not entirely clear yet; however, it was proposed to be due to active efflux. This dramatic change indicated that it is very dangerous to use biocides at sub-inhibitory concentrations in disinfection reagents, as this can give rise to the appearance of highly adapted resistant strains (Levy, 2001). Studies on triclosan (polychloro phenoxy phenol) adaptive resistance in *E. coli* investigated the putative role of the efflux pump AcrAB and a mutation in *fabI* (fatty acid biosynthesis) as possible resistance mechanisms

(McMurry et al., 1998). AcrAB is a stress-induced efflux system (Ma et al., 1995) with an unknown physiological role; however, it has been shown that deletion of this efflux pump leads to increased susceptibility to triclosan.

Biocides induce adaptive resistance primarily in Gram-negative bacteria which is thought to be related to their interaction with the outer membrane, although adaptive resistance has also been observed in some Gram-positive species (Russell et al., 1998). For example *Listeria monocytogenes*, a Gram-positive pathogen often associated with processed foods, shows adaptive responses to several biocides, quaternary ammonium compounds and tertiary alkylamine, as well as antibiotics. Several studies previously showed that eradication of *L. monocytogenes* is difficult and the occurrence of persisting strains is relatively frequent (Rorvik et al., 1995; Unnerstad et al., 1996; Autio et al., 1999; Miettinen et al., 1999). The adapted survivors from antimicrobial treatment can live on poultry products, raw meat and cheese products until they find a human host in which *Listeria* cells can multiply and cause an infection (Aase et al., 2000; Lemaitre et al., 1998). The already-mentioned quaternary ammonium compounds are used in the food processing industry and adaptive resistance in *Listeria*, as well as cross-resistance to other disinfectants, occurs when these products are used at sub-inhibitory concentrations (Aase et al., 2000; Lunden et al., 2003). After sub-lethal exposure for 2 h, high adaptation to several disinfectants, including one tertiary alkylamine and two quaternary ammonium compounds, was observed, but not to potassium persulphate and sodium hypochlorite (Lunden et al., 2003). The adapted cells had their highest increase in MIC, up to 15-fold, towards *n*-alkyldimethyl ethylbenzyl ammonium chloride. This further emphasizes the elevated resistance caused by some of the disinfectants routinely used in the food processing industry. Adaptive resistance can also occur due to cellular changes, such as modification of the cytoplasmic membrane, which can be seen even after short exposures to the disinfectant (McDonnell and Russell, 1999). Lemaitre et al. (1998) proposed that the problem of cross-resistance could be overcome by rotating the use of different disinfectants; however, it is not yet entirely clear how this should be done, and more studies regarding the interactions between the different classes of biocides are still required.

Overall, biocide adaptive resistance is an increasingly concerning problem as disinfectants are often used at sub-inhibitory concentrations in the household, clinic and food processing industry. This can eventually lead to increased resistance due to adaptations, which include changes in the outer membrane structure, efflux pumps and cytoplasmic membrane. Further work must be undertaken to fully understand all the mechanisms involved. It is also imperative to determine the extent of cross-resistance with antibiotics because of the implications that this might have in the clinic where such products are a mainstay of disinfection.

2.3.6. Others

The adaptive resistance triggered by other types of antimicrobials has not been studied in great depth, perhaps because it has not yet been associated with therapy failure. Nonetheless, there is evidence that antibiotics from the macrolide and tetracycline families can induce resistance.

In the case of the macrolides, a study on different serovars of *S. enterica* indicated that exposure to sub-inhibitory concentrations of erythromycin led to a considerable increase in resistance to subsequent antibiotic challenges (Braoudaki and Hilton, 2004). Furthermore, the authors noted that, in antibiotic-free medium, the level of resistance decreased gradually, not reaching the levels pre-exposure for at least 30 passages. This resistance seemed to involve the participation of an RND efflux pump, whereas the outer membrane and LPS profiles did not show significant differences between the parent and the adapted strains. In some *S. enterica* serovars, the adaptation to erythromycin also decreased the susceptibility to chloramphenicol and trimethoprim, as well as to the biocides triclosan and chlorhexadine, depending on the specific serovar (Braoudaki and Hilton, 2004). Adaptive resistance to erythromycin was also observed in *E. coli* O157 for which cross-resistance to ciprofloxacin, trimethoprim and tetracycline, as well as the biocide triclosan could be observed. As mentioned above, the development of adaptive resistance to the macrolide azithromycin has been reported in *P. aeruginosa* biofilm cells (Gillis et al., 2005). As observed for *Salmonella*, the induction of efflux pumps also seemed to play a significant role in this case, but no cross-resistance with other antibiotic classes could be found. In the Gram-positive bacterium *Streptococcus faecalis*, an inducible erythromycin resistance marker is carried on the transposable element Tn917 within the non-conjugative plasmid pAD2 (Tomich et al., 1980). In addition to inducing the expression of the resistance gene, exposure to sub-inhibitory erythromycin also promotes transposition of Tn917 to the mobilizable plasmid pAD1, thus increasing its possibilities of horizontal transfer to other strains or species. Inducible resistance to erythromycin is also carried in the plasmid pSCFS1 from *Staphylococcus sciuri*, which also harbors inducible resistance to chloramphenicol and florfenicol (Schwarz et al., 2000).

Adaptive resistance to tetracyclines has been demonstrated in *E. coli* strains carrying a resistance factor (Franklin, 1967). Pre-incubation of the cells in sub-inhibitory concentrations of tetracyclines for a short period of time resulted in a diminished uptake of this group of antibiotics. The authors proposed that the resistance marker had an inducible nature. It is now well understood that the plasmid-borne tetracycline resistance efflux pump on the transposon Tn10, for example, is inducible (McMurry et al., 1980) Thus, it would only result in a low-level resistance phenotype if cells are grown in a drug-free medium, but a very short incubation with low tetracycline doses would cause the cells to express a much more resistant phenotype. Other cases of inducible tetracycline markers are those encoded in the *E. faecalis* transposon Tn916 (Celli and Trieu-Cuot, 1998) and a plasmid of *B. fragilis* (Privitera et al., 1979). Interestingly, in these two species exposure to tetracycline also induces the mobilization of the respective genetic elements containing the resistance gene. In contrast, other species like *Pasteurella multocida* show a very low propensity for the development of adaptive resistance to tetracyclines (Champlin et al., 1988).

3. Concluding remarks: tackling the problem of adaptive resistance

After using antimicrobial agents for less than a century, widespread antibiotic resistance in pathogenic bacteria is making us reconsider the way these compounds have been utilized. Antibiotics are routinely used for treating human and animal infections, as well as in other fields like agriculture. Likewise, biocides are of common use in households, hospitals, food-processing plants, etc. with the intent of creating a bacteria-free environment. However, all evidence suggests that we could actually be getting closer to achieving the opposite effect, by promoting the development of antibiotic resistance. This review shows how easily this indiscriminate use of antimicrobial agents can lead to adaptations in the bacterial cells that turn them more resistant to drugs. It also illustrates how bacteria can be more resistant under specific environmental conditions and in certain physiological states, such as biofilms and swarming.

Due to its inducible nature, adaptive resistance was not considered to be relevant in the context of the overall increase in antibiotic resistance of pathogenic bacteria. This assumption, however, neglects several circumstances surrounding this type of antibiotic resistance. First, the increase in resistance induced by dif-

ferent environmental conditions might not completely revert upon removal of the signal, leading to a low-level resistance phenotype. Secondly, exposure to certain agents, such as the fluoroquinolone antibiotics, can transiently induce a hypermutator phenotype. This can result in adaptive mutations that lead to reduced susceptibility, potentially to several classes of antibiotics. Finally, the ability to adapt to and survive at high concentrations of antimicrobials facilitates the acquisition of high resistance markers, for example by horizontal transfer from other resistant species. Therefore, the baseline MICs are not creeping up solely due to selection, but also because of adaptation. Given the increasing significance of low-level resistance in pathogenic bacteria, it might be useful to evaluate this type of resistance more thoroughly and keep track of small increases in antibiotic resistance by utilizing quantitative assays in the clinic.

Within the clinical setting, the phenomenon of adaptive resistance can lead to failure, especially in blind therapy (target organism undetermined), but also in those cases where the dose is not adequate or when the patients do not follow the prescribed treatment adequately. In all these circumstances, the levels of the drug within the patient might be in the sub-inhibitory range. We show here, with the example of aminoglycosides, how the knowledge of the mechanisms involved in adaptive resistance can help to design the most effective antibiotic doses and times of administration. It is also important to determine which environmental conditions can trigger increased resistance to certain antibiotics. This will permit the evaluation of whether these conditions exist at some point during the infection, as this would inevitably lead to unsuccessful treatment. Another example is the treatment of biofilm-related infections, which require drastic eradication programs.

This type of resistance also needs to be taken into account for the development and troubleshooting of new antimicrobial compounds. Thus, in addition to bacteria-killing ability and toxicity, it has become apparent that the capacity to trigger adaptive resistance is another factor to consider in drug development. A clear example of this is the development of novel cationic peptides, in which the analysis of their capacity to induce adaptive resistance in the target bacterial species should be a crucial step.

In conclusion, a better understanding of the mechanisms of adaptive resistance to antimicrobials will help design more effective treatment programs, as well as to develop novel compounds with the same therapeutic effects but a lower risk of making pathogens more resistant to antibiotics. Additionally, more attention should be paid to the small increases in resistance observed in routine clinical analysis, as they can be indicative of adaptive resistance during the infection and, moreover, predictive of the later acquisition of high resistance markers.

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