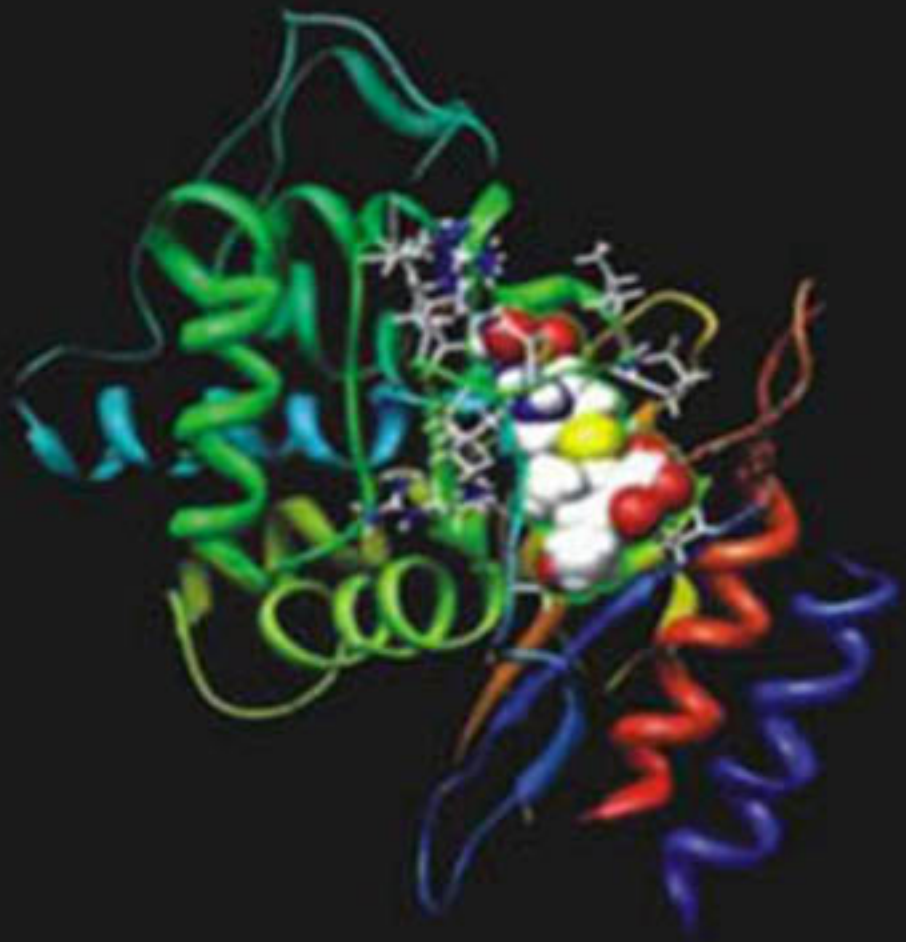

Current Trends in Antibiotic Resistance in Infectious Diseases

Editor
Asad U. Khan

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I.K. International Publishing House Pvt. Ltd.

NEW DELHI • BANGALORE

Published by

I.K. International Publishing House Pvt. Ltd.
S-25, Green Park Extension
Uphaar Cinema Market
New Delhi – 110 016 (India)
E-mail: info@ikinternational.com

ISBN 978-93-80026-70-1

10 9 8 7 6 5 4 3 2 1

Published by Krishan Makhijani for I.K. International Publishing House Pvt. Ltd., S-25, Green Park Extension, Uphaar Cinema Market, New Delhi – 110 016 and Printed by Rekha Printers Pvt. Ltd., Okhla Industrial Area, Phase-II, New Delhi – 110 020.

PREFACE

Infectious diseases are the major cause of premature deaths, killing almost 50,000 peoples everyday worldwide. An increase in the emergence of multidrug-resistant bacteria is threatening the world population. The presence of antibiotic-resistance genes on bacterial plasmids has further helped in the transmission and spread of drug resistance among pathogenic bacteria.

Multiple drug resistance is becoming a big challenge for the physicians and clinicians to treat various infections. It also affects the economy of the country especially the pharmaceutical industry where a huge amount of drugs goes into garbage. This challenge can be taken up by researchers working in the area of drug resistance in infectious diseases.

The β-lactam antibiotics are among the most widely prescribed antibiotics and are important components of empirical therapy. Because of extensive use, resistance to these drugs has become a major problem especially after the introduction of newer broad-spectrum cephalosporins, β-lactamase inhibitor/β-lactam antibiotics, monobactams and carbapenems. With the spread of ESBL producing strains in a hospital so as to formulate a policy of empirical therapy in high-risk units where infections due to resistant organism are much higher. The focus of this book is to compile the wide information about the present scenario of antibiotic resistance and its molecular mechanisms. This information would help to design new antibiotic as well as β-lactamase inhibitors to combat with infections caused by resistant strains of bacteria.

This book comprises ten chapters with special focus on beta lactamases of different kinds. One of the chapters covers notion of quorum-sensing in drug resistance. Moreover, ciprofloxacin resistance was also discussed in uropathoges. Antibiotic resistance among marine bacteria and *Vibrio cholerae* as special pathogen for deadly disease, cholera, were also discussed in detail.

I am indebted to members of my research group, over a period of 10 years especially for scientific discussions, exchange of ideas and of course experimental data on drug resistance. I express my deep sense of gratitude to Dr Raffaele Zarrilli to whom I used to discuss several problems on this topic. My special thanks to Prof M Saleemuddin for inspiring me to take up this task of compiling the information in form of a book. I also thank all the authors who have contributed their chapters in this book.

Asad U Khan

CONTENTS

Preface *v*

1. Quorum-Sensing and Multiple Antibiotic Resistance 1
Chritopher Skilbeck, Lilian Pumbwe, and Hannah M. Wexler

2. Genes Encoding Production of Metallo-β-Lactamases in Most Important Gram-Negative Pathogens 25
Pawel-Tomasz Sacha, Piotr Wieczorek and Elżbieta Tryniszewska

3. Ciprofloxacin Resistance Among Uropathogen 111
Zakaria Yehia El Astal

4. Metallo-β-Lactamase-Producing *Klebsiella pneumoniae* 136
Nilton Lincopan

5. Drug Resistance in Marine Bacteria 166
Rengathavasi Thavasi and Ibrahim M. Banat

6. An Overview of the Carbapenems with a Focus on Doripenem—The Latest Member of the Carbapenem Class 179
Shazi Shakil and Asad U. Khan

7. Increasing Importance of Newer Beta Lactamases in Indian Setting 193
Varsha Gupta

8. Vancomycin Resistant *Staphylococcus aureus*: A Global Threat 219
Hare Krishna Tiwari

9. Molecular Epidemiology of AmpC beta-Lactamases 234
Vikas Manchanda

10. Cholera and the Acquisition of Antimicrobial Resistance 253
M. Jain, P. Kumar, A.K. Goel, D.V. Kamboj and L. Singh

Index 269

Chapter 1

Quorum-Sensing and Multiple Antibiotic Resistance

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ABSTRACT

This review chapter examines the links between quorum-sensing and antibiotic resistance in bacteria, with particular attention to multidrug resistance. Biofilms have been shown to account for many, if not most, bacterial diseases, at least in the more economically developed world, and these biofilms typically exhibit massively increased drug resistance. The principle aim is to examine the simple hypothesis that quorum-sensing triggers increased antibiotic resistance, principally by initiating or assisting biofilm formation and by increasing horizontal gene transfer and multidrug efflux pump expression. It will be shown that the effects of quorum-sensing on drug resistance are multifaceted and dependent on the strain. Mechanisms whereby biofilm formation may contribute to multiple drug resistance will be considered. Finally, the strategy of targeting quorum-sensing in drug therapy as a means to circumvent the problems of multidrug resistance will then be discussed.

1. INTRODUCTION

Quorum-sensing (QS) is the predominant mechanism of cell-cell communication in bacteria. During the process of QS, bacteria release signal molecules called autoinducers (AIs). These molecules are called autoinducers because they induce a response in the

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same type of bacterium that is emitting the signal. For example, *Pseudomonas aeruginosa* cells communicate with one another via QS and the signal will induce a response in these cells. As the bacterial population grows the concentration of these secreted extracellular signalling molecules increases until it reaches a critical threshold, corresponding to a critical cell density or 'quorum', at which point the signal triggers dramatic changes in gene expression within the population. This process is called quorum-sensing since the bacteria sense the presence of other bacteria nearby and thus monitor the density of their own population and react once their population density exceeds the quorum threshold. The QS process is a cell density-dependent gene regulation mechanism. Bacteria can also listen in to the communications of other species within the same locale if they are able to decode their signals (to sense and decode the signals they need the correct receptor). For a review on quorum-sensing see Waters and Bassler (2005). As way of introduction, QS in *P. aeruginosa* will now be examined in more detail.

In *P. aeruginosa*, there are two QS systems – the *las* (LasIR) and *rhl* (RhlIR) systems. LasI and RhlI are the autoinducer synthases. LasI synthesises the *las* AI *N*-(3-oxododecanoyl) homoserine lactone (3O-C12-HSL or PAI-1) and RhlI synthesises *N*-butyryl homoserine lactone (C4-HSL or PAI-2). These AIs are the signalling molecules that are exported from the cell. When the AI reaches the threshold concentration in the extracellular medium it binds to a transcriptional activator, which is LasR in the *las* system and RhlR in the *rhl* system, to form a LasR-AHL or RhlR-AHL active complex, respectively (De Kievit *et al*., 2001). These transcriptional activators activate key genes involved in biofilm formation and antibiotic resistance.

The LuxIR QS system is found in *Vibrio fischeri*, the bacterial mutual symbiont that gives squid photophores their luminescence. LuxI is an analogue of LasI and RhlI, which synthesises the 3O-C6-HSL AI, an acyl-homoserine lactone (AHL) with a six-carbon acyl side-chain, whilst LuxR is the receptor for 3O-C6-HSL. The LuxR-3O-C6-HSL complex activates transcription of the luciferase operon (*luxICDABE*) which generates light with the enzyme luciferase. Luciferase operon activation also produces more LuxI, the AI synthase, creating a positive-feedback circuit. Most bacteria possess more than one QS system (see the review by Waters and Bassler, 2005). Gram-negative bacteria possess homologues of the LuxI and/or LuxR system. The LASIR and RhlIR systems in *P. aeruginosa* are LuxIR type systems. Each such system consists of a sensor-autoinducer (R-I) pair. Gram-positive bacteria possess a slightly different QS system in which short peptides act as AI signal molecules which bind to and activate membrane-bound histidine kinase receptors.

These various LuxIR-type QS systems differ in the nature of the autoinducer they produce. In each case, the AI is an acyl-homoserine lactone (AHL) made by the addition of the acyl chain from acyl-acyl carrier protein to S-adenosyl-L-methionine, the process of acylation, and lactonisation of the methionine moiety to give acyl-homoserine lactone (Watson, Minoque, Val, von Bodman and Churchill, 2002). (Lactonisation is the formation of the lactone ring; a lactone ring is a hetero ring containing –C(=X)-O- where X is a chalcogen, i.e. oxygen, sulphur, selenium or tellurium – oxygen in this case). The length and chemical nature of the acyl chain

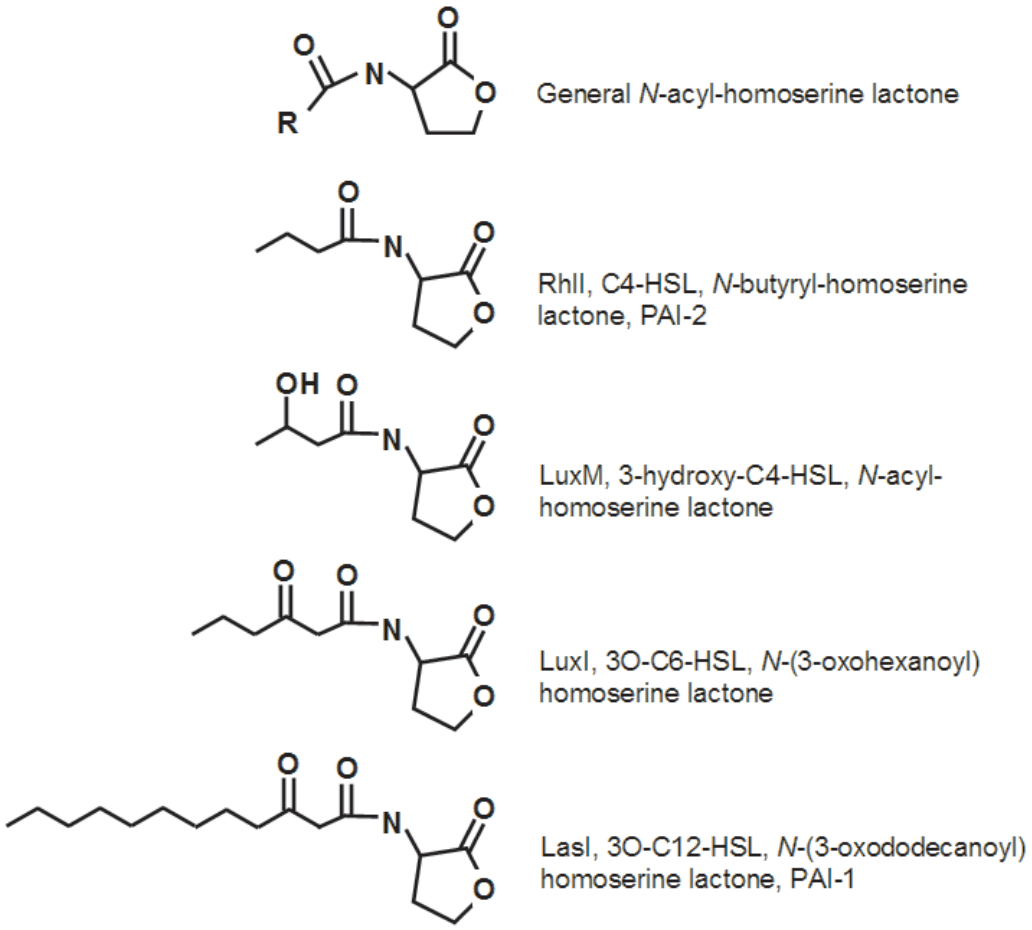


Fig. 1. The structures of some *N*-acyl-homoserine lactones (AHLs). These molecules all consist of a lactone ring, that is a carbon-ring containing a chalcogen atom, in this case oxygen, and which is derived from serine by closure of the ring (specifically L-serine, hence AHLs are also called *N*-acyl-homoserine-*L*-lactones). An acyl side-chain, that is a carbon chain of general formula R-C(=O)- is attached to the nitrogen atom of the homoserine lactone to form an *N*-acyl side-chain. The length of this chain and any additional chemical groups it contains determines the name of the molecule. RhlI and LasI occur in *P. aeruginosa*, LuxI in *V. fischeri* and LuxM in *V. harveyi*. Some alternative names are listed for each AHL.

determines the signal specificity – each species listens in to its own particular AI as each receptor is specific to its paired AI whose acyl chain fits its matching acyl-binding pocket in the receptor presumably in a lock-and-key or induced-fit manner. Figure 1 illustrates the molecular structures of some of the AHLs that shall be mentioned in this chapter, along with an explanation of their nomenclature. The structures of additional signalling molecules, including those of Gram-positive bacteria can be found in the reviews by Lyon and Muir (2003) and Waters and Bassler (2005).

Gram-positive bacteria have similar systems, but instead of AHL AIs they secrete alternative signals, such as small autoinducing peptides (AIPs) (Waters and Bassler,

2005) which bind to extracellular histidine kinase receptors, whereas in Gram-negative bacteria the AHL binds to an intracellular receptor. Like Gram-negative bacteria, Gram-positives may use QS to help regulate biofilm development (Yarwood, Bartels, Volper and Greenberg, 2004). This picture is an oversimplification and other autoinducer molecule types are known to be produced by certain species (see the review by Diggle, Crusz and Camara, 2007 for examples). Some Gram-positive bacteria also produce AHLs through a *luxS* analogue (Xu *et al*., 2000) and there is evidence of cross-talk between Gram-positive and Gram-negative bacteria as many Gram-positive bacteria possess a *luxS* homologue (e.g. *luxS* in *Streptococcus pyogenes*, Marouni and Sela, 2003). In *Staphylococcus aureus* the AIP activates the *agr* operon and synthesis of a small regulatory RNA, RNAIII. RNAIII positively regulates secreted toxins and enzymes but represses certain surface proteins (Tegmark, Morfeldt and Arvidson, 1998).

Multidrug resistance (MDR) can be attributed to several factors, for example, in *P. aeruginosa* it is attributed to the presence of broadly specific drug efflux pumps and to low outer membrane (OM) permeability. However, the efflux pumps are widely regarded as the main factor, with the low OM permeability as an auxiliary factor. In *P. aeruginosa* the *mexAB-OprM* operon encodes a three-component efflux pump that expels a wide range of antibiotic substrates, including quinolones, tetracycline, β-lactams, trimethoprim, chloramphenicol, novobiocin and macrolides. MexB is the translocase protein (belonging to the resistance-nodulation-division or RND family), OprM is an OM porin and MexA is the membrane fusion protein that links MexB to OprM. Other MDR systems in Gram-negative bacteria (MFS, MATE and SMR) export to the periplasm (see the review by Poole, 2005). In the next section, we shall examine the links between efflux pumps and QS.

2. QUORUM-SENSING AND EFFLUX PUMP EXPRESSION

Efflux pumps work by rapidly and actively exporting antibiotics from the bacterial cell. Typically, these pumps have very broad substrate specificities, meaning that they export a wide variety of structurally diverse antibiotics and organic toxins. There is a vast amount of literature on these pumps and they have been shown to confer multiple antibiotic resistance (or more generally, multidrug resistance, MDR) in a wide variety of bacteria during the planktonic stage. Examples from the authors' own work include MDR efflux pumps in *Bacteroides fragilis* (Pumbwe, Chang, Smith and Wexler, 2007), *Campylobacter jejuni* (Pumbwe and Piddock, 2002) and *Pseudomonas aeruginosa* (Pumbwe and Piddock, 2000).

Quorum-sensing has been shown by several studies to increase the expression of multidrug efflux pumps. For example, in *Escherichia coli*, SdiA (a LuxR homologue) is the QS transcription factor. Overproduction of SdiA leads to increased expression of the *acrAB* operon, which encodes the AcrAB efflux system, and increased quinolone resistance (Yang, Lopez and Zechiedrich, 2005); whilst an absence of SdiA leads to a reduction in AcrAB and a reduction in drug resistance.

Pumbwe, Skilbeck and Wexler (2008) demonstrated that quorum signals decreased cell density, increased expression of four putative *luxR* genes, increased expression of BmeB efflux pumps, increased antibiotic resistance and also increased biofilm formation in *Bacteroides fragilis* (strain ATCC25285). Resistance was at least doubled to the following antibiotics: ampicillin, cefoxitin, cefoperazone, metronidazole, tetracycline and ethidium bromide, indicating that QS can increase MDR. The efflux pump inhibitor Carbonyl cynaide-*m*-chlorophenyl hydrazone (CCCP) significantly reduced the MICs (minimum inhibitory concentrations) by at least twofold, indicating that the observed increase in resistance could be entirely accounted for by efflux pumps. The quorum signals comprised synthetic *N*-hexanoyl homoserine lactone (C6-HSL) added to the medium. Interestingly, the *Bacteroides fragilis* genome contains no *luxI* homologues and nine *luxR* homologues, suggesting that *B. fragilis* cannot produce its own *lux* autoinducers but can listen in to such signals coming from other sources (passive sensing). Supernatants from *V. cholerae*, *P. aeruginosa* and *Yersinia enterocolitica* also reduced cell density and increased biofilm formation, whilst that from *E. coli* and *V. parahaemolytica* had no significant effect. In high concentrations C6-HSL had a growth inhibitory effect (measured as a MIC) and this susceptibility doubled when CCCP was added, suggesting that the C6-HSL is a substrate for the efflux pumps. This adds evidence to the notion that AIs may be a substrate for the efflux pumps and that this may be a prime natural function of these pumps.

However, this switching on of efflux by QS is not a simple one-way relationship. The study by Yang *et al*. (2005) used *acrAB/tolC* and *mdfA* efflux system deletion mutants which had the same growth rate as wild-type (WT) cells in exponential phase, but reached a higher cell density in stationary phase. Mutants overexpressing the pumps reached a lower maximum cell density. Conditioned medium (CM) from cells overexpressing *acrAB* decreased WT cell growth more than CM from wild type cells (CM from *acrAB* deletants also decreased cell growth, but less so than CM from WT cells). Overexpression and deletion of *mdfA* had no such effect on growth rate or final cell density. The authors conclude that AcrAB/TolC and NorE, but not MdfA, possibly have a natural function in exporting the QS signalling molecules (autoinducers) and so are required for optimal cell-cell communication. Mutants lacking the AcrAB efflux system would then reach a higher cell density since they release less AI and so underestimate their population. Mutants overexpressing AcrAB pump out more AI and overestimate their population and so regulate to a lower population density. The CM from the latter contains excess AI and so reduces WT population growth more strongly. If AIs are a natural substrate for efflux pumps, then it would seem natural that when cells activate quorum sensing they increase or switch on efflux pump expression.

A similar process occurs in *P. aeruginosa* which possesses the MexAB system (a homologue of AcrAB). *P. aeruginosa nalB* mutants hyperexpress the *mexAB-oprM* multidrug efflux operon and also exhibit a reduction in secretion of extracellular virulence factors regulated by quorum sensing and a reduction in secretion of the *las*-system autoinducer PAI-1 (Evans *et al*., 1998). These data suggest that PAI-1 may be a substrate for MexAB-OprM and excessive expulsion of 3O-C12-HSL (PAI-1) from the cells reduces PAI-1-dependent activation of *lasI* and so leads to a reduction in PAI-1

and a reduction in *las*-induced virulence factors. For example, increased expression of *mexAB-oprM* correlates with a reduction in the pyocyanin virulence factor. Two other exoproducts that function as extracellular virulence factors and were also reduced are elastase and casein protease (aggressins).

A study by Maseda *et al*. (2004) on *P. aeruginosa* found that expression of *mexEF-oprN* during mid-stationary phase induced resistance to fluoroquinolones and chloramphenicol and that expression of this efflux pump could be induced by addition of the autoinducer C4-HSL (the autoinducer 3O-C12-HSL had little effect). Wild-type cells do not express this efflux system under exponential growth conditions due to a mutation in the positive regulator gene *mexT*. However, Pearson, Van Delden and Iglewski (1999) demonstrated that tritium-labelled C4-HSL equilibrates either side of the *P. aeruginosa* cell envelope after 30 seconds and that at this equilibrium the intracellular concentration equals the extracellular concentration. This demonstrates that C4-HSL is freely permeable across the cell membrane and so does not require active efflux. However, these authors showed that tritium-labelled 3O-C12-HSL equilibrated after about five minutes with the intracellular concentration threefold higher than the extracellular concentration. Furthermore, azide, a proton gradient inhibitor, increased the intracellular concentration, thus demonstrating that 3O-C12-HSL is actively secreted. A mutant strain lacking the *mexAB-oprM* operon accumulated a high intracellular concentration of 3O-C12-HSL, similar to that in WT cells with azide. These results together suggest that the autoinducer 3O-C12-HSL is actively secreted by the MexAB-OprM RND efflux pump, whereas C4-HSL passively diffuses out of the cell.

A study by Chen, Yuan and Livermore (1995) demonstrated that MexAB-OprM had a small effect on increasing antibiotic resistance in biofilms but a large effect in planktonic cells (in stationary phase and non-growing cells). This is interesting, since biofilms generally have a far higher antibiotic resistance and this raises questions regarding the mechanisms. Quorum-sensing also has a role to play in biofilm formation, and we shall examine this role in the next section before returning to the question of resistance mechanisms in biofilms.

3. QUORUM-SENSING AND BIOFILM FORMATION

Most bacteria alternate between a multicellular 'slime city' stage, in which they form a slimy film over solid surfaces, and a dispersal, swarming or planktonic stage in which cells float, swim or glide away from the slime city in order to find new suitable habitats. Bacterial cells may continue to multiply in both stages and they always maintain some degree of individuality.

In fact for many decades microbiologists thought that only the minority of bacterial species possessed a multicellular stage, but now the majority appear to do so. Hence, there is an increasing tendency to view bacteria as multicellular organisms (Shapiro, 1998; see Stoodley, Sauer, Davies and Costerton, 2002 for a good review of bacterial biofilms) and biofilm formation as a developmental process (O'Toole, Kaplan and Kolter, 2000).

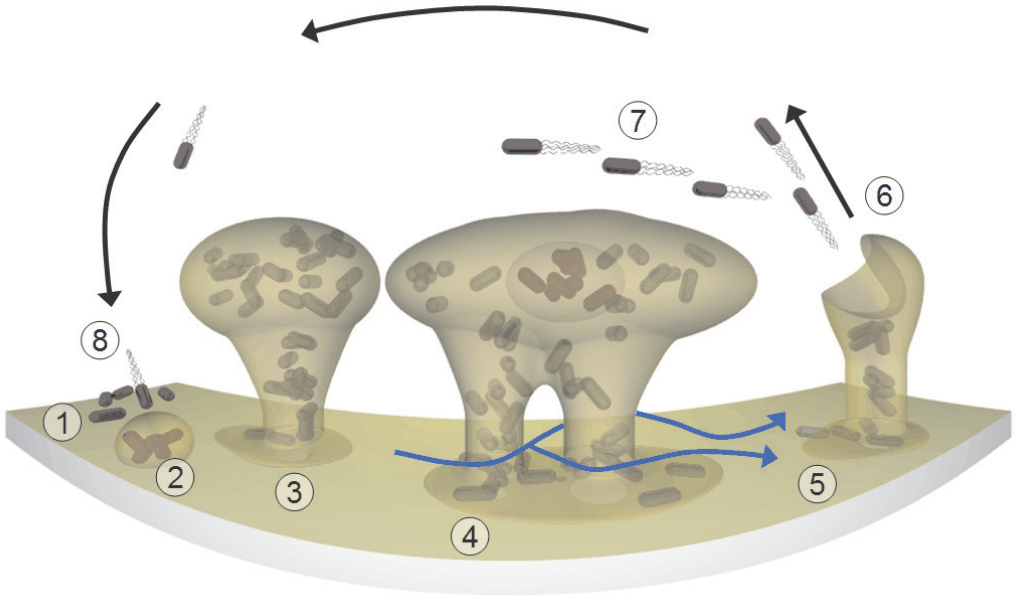


Fig. 2. The biofilm life cycle. Isolated cells adhere to the substrate (1) and move across the substrate until they aggregate into groups. Once aggregated they secrete slime to form a small microcolony (2) and undergo changes in gene expression, which suppresses flagellin synthesis and causes other changes to produce a phenotype more suited to biofilm 'city' life. These microcolonies eventually grow upwards from the substrate as mushroom-shaped or cylindrical columns (3). These larger microcolonies are exposed to higher fluid flows above the lower boundary layer and this flow may be conducted through water channels in the base of the biofilm (blue arrow). (4) Some cells begin to differentiate into flagellated swarmer cells which are released high above the substrate when the columns rupture (5). These swarmer cells disperse to new colonization site to repeat the cycle. Columns may undergo several such cycles of rupture, swarmer cells release and regrowth. The flagellated swarmer or planktonic cells (6) are dispersed both passively by fluid flow and actively, and can even swim upstream (7) as discussed in the text. Swarmer cells may undergo several stages of cell division in the planktonic stage, but eventually adhere to the substrate to complete the cycle (8). Additional mechanisms of cell dispersal from biofilms are discussed in the text.

Figure 2 illustrates the cycle of development in a typical biofilm. This process has been extensively reviewed by Watnick and Kolter (2000) who liken biofilms to a human city. Biofilm initiation begins when planktonic cells, which are often flagellated swarmer cells, adhere to the substrate. This adhesion step is widely regarded as random (Jenkinson and Lappin-Scott, 2001). However, recent research has shown that in flow conditions *E. coli* swarmer cells will preferentially swim to their left until they encounter a sheltered crevice or sidewall and then align to flow and swim upstream (Hill, Kalkanci, McMurry and Koser, 2007). This would surely bias their motion in favour of finding an upstream surface on which to set-up a biofilm that will allow dispersal downstream. Once they contact the surface, it is not clear how bacteria sense contact, but it leads to changes in gene transcription (Jenkinson and Lappin-Scott, 2001). This adhesion and the subsequent initiation of biofilm development are accelerated by force-generating appendages such as type IV pili (involved in twitching motility) and flagella, at least in static no-flow conditions (De Kievit *et al.*, 2001). In high flow it is probable that different adhesins

operate, using a catch-bond rather than a slip-bond. FimH is one such adhesin in *E. coli* which binds more strongly as the shearing force increases (Thomas, Forero, Sokurenko and Vogel, 2003). Once adherent, these appendages can be used for locomotion in two dimensions across the surface until cells contact one another and aggregate. Note that cells moving across the confines of a 2D surface are perhaps more likely to encounter one another, purely by random chance, than cells moving in a 3D space. However, if the flagella remain active then they may prevent the formation of a mature biofilm by reducing cell aggregation. In a study by Landry, An, Hupp, Singh and Parsek (2006) *P. aeruginosa* was found to form thick, mature biofilms with complex 3D structure, on a mucin substrate, whereas on glass they formed only thin, flat biofilms. The flagella cap protein, FliD acts as a mucin adhesin, such that the flagella become immobilised on adhesion to mucin, but the bacteria are still apparently able to use type IV pili in twitching locomotion. In contrast, on glass the bacteria are free to crawl rapidly across the surface by flagellar motion and so tend not to aggregate and form thin biofilm sheets lacking large cell aggregates (microcolonies).

In contrast to flagella, type IV pili have been shown to enhance microcolony formation (De Kievit *et al*., 2001). Quorum-sensing has been implicated in both adhesion and twitching motility (De Kievit *et al*., 2001). These aggregations become microcolonies as the cells switch off flagellin synthesis and secrete exopolysaccharide (EPS, extracellular carbohydrates which hydrate to form slime). In *P. aeruginosa* alginate is a principle slime component as is colanic acid in *E. coli*. These pioneering microcolonies must be distinguished from later structures, also called microcolonies. These later structures are cylindrical or mushroom-shaped towers that rise up from the slime sheet that covers the substrate. Their development requires *lasI* in *P. aeruginosa* (Davies *et al*. 1998). Bacterial cells occupy both the slime sheet covering the substrate and all regions of the towers. In addition motile cells may associate with the biofilm for a significant length of time before attaching or departing and these can be seen to swim between the towers (see the videos at: *http://gasp.med.harvard.edu/biofilms/jbmini/movie.html* cited in Watnick and Kolter (2000)). Water channels may penetrate the base of the biofilm (typically the lower 10 micrometres). These water channels are thought to be formed passively by hydrodynamic forces, but the biofilm community must synthesise enough EPS to allow the channels to form (and EPS synthesis is regulated by QS). The channels may assist the deliverance of oxygen and nutrients to the basal layers and waste removal from these layers (Stoodley *et al*., 2002).

The structural complexity of biofilms raises the question: what is the function of biofilms? The answer is that biofilms have multiple functions. First of all, colonization of a surface allows nutrients on or in that surface to be exploited, for example minerals, vegetation or chitin (see Keyhani and Rosman, 1996 for the utilisation of chitin by marine *Vibrio*). Second, the erection of tall microcolonies enables bacteria to clear the most stagnant part of the boundary layer for dispersal. When fluid flows over a surface, friction with the surface creates a region of retarded or slowed fluid movement just above the surface (the layer of fluid in contact with the surface is actually stationary) called the boundary layer. The speed of flow gradually increases asymptotically away from the surface, until the mainstream velocity is reached. One way of estimating the

thickness of this boundary layer (which has no exactly defined thickness as the speed increases asymptotically) is to use the displacement thickness (δ), given by the following numerical solution (see, for example, Acheson, 1990 or Batchelor, 2000):

$$\delta = 1.72 \left(\frac{\upsilon X}{U} \right)^{\frac{1}{2}}$$

Where υ is the kinematic viscosity (viscosity/density), x is distance along an unbroken surface and U is the mainstream speed. For a bacterium sitting one centimetre along on a flat, smooth pebble exposed to a mainstream flow of 1 m/s, the displacement thickness is about 170 μm at 20°C. Note that since the kinematic viscosity for water is about 15 times less than that for air (the viscosity of water is higher than that for air, but the kinematic viscosity of water is less due to the greater density of water) the displacement thickness is about 4 times less in water than air and is of the order of 100 μm. Thus, biofilm microcolonies, which measure 20-400 μm in height are well sized to reach the faster flowing water above the surface. It has been observed that cells at the base of the biofilm (especially in mono-species biofilms) become dormant and may fail to disperse – they have sacrificed their lineage to assist dispersal of their colleagues, a case of kin selection that is made more complex by the multi-species nature of natural biofilms. This situation is comparable to that in cellular slime moulds, such as *Dictyostelium*, in which cells forming the attachment disc and stalk produce no progeny and so sacrifice themselves to elevate the sporocarps above the boundary layer for efficient spore dispersal. Myxobacteria and streptomycetes also produce more integrated fungus-like multicellular structures bearing fruiting bodies, which are perhaps the next stage up from an aggregation of cells in a biofilm towards true multicellularity (see Reichenbach, 1984 for a good review of myxobacteria). In all these cases, one of the principle aims is to reach past the boundary layer, at least in part, to the fast-flowing fluid (water or air) for dispersal purposes and possibly to access nutrients and oxygen carried in the bulk flow of the fluid. In all cases only a fraction of the cell population is destined to produce progeny and this has raised interest in the evolution of social cheaters (Sandoz, Mitzimberg and Schuster, 2007).

In addition to dispersal of isolated swarmer cells, cell clusters and streamers may detach, especially from the tops of the towers, where flow is greatest, and on the leeward side fragments may detach and be carried downstream (Stoodley *et al*., 2002). Shearing forces may also cause slime to ripple downstream (see the video at *http://www.erc.montana.edu/MultiCellStrat/09-Going_Mass-Migration/Rippling.htm)* and cell clumps that are unstably bound to roll downstream along the surface. All these processes aid dispersal. Recirculating flow forming around the towers may facilitate this process by creating leeward drag and lift forces (Towler, Cunningham, Stoodley and McKittric, 2007). Interestingly, whole microcolonies have also been reported to migrate, but in any direction regardless of prevailing flow (Venugopalan *et al*., 2005).

Quorum-sensing regulates cell density-dependent gene expression including virulence factors and genes controlling biofilm differentiation. Autoinducers have been isolated from biofilms in nature, including those covering rocks in streams (McLean, Whiteley, Stickler and Fuqua, 1997) and catheters (Stickler, Morris, McLean and Fuqua, 1998). Biofilm formation is regulated by QS and mediated by the RpoS σ-factor (Brown and Smith, 2001). Miller and Bassler (2001) found that *rpoS* is induced earlier in cells overexpressing *acrAB* and later in *ΔacrAB* mutants. This adds more evidence to the notion that efflux pumps are an integral part of QS. The evidence supports the idea of a positive feedback loop in which QS induces an increase in efflux pump expression and this in turn exports the quorum signal more efficiently, enhancing quorum communication.

Recall that in *P. aeruginosa*, there are two QS systems – the *las* and *rhl* systems. LasI synthesises the autoinducer (AI) *N*-butanoyl-L-homoserine lactone (C4-HSL) whilst RhlI synthesises N-(3-oxododecanoyl)-L-homoserine lactone (3O-C12-HSL). A series of studies have refined our knowledge of the role QS plays in biofilm formation and antibiotic resistance in *P. aeruginosa*. Mutations in both the *lasI* and *rhlI* genes or in *lasI* only result in immature biofilm development, in which the biofilm remained as a thin sheet and failed to produce thick biofilms with microcolony towers (Davies *et al*., 1998). These differences appeared to result from a difference in the nature of the EPS matrix, though not in the quantity of EPS produced. A study by Shih and Huang (2002) also found that mutants of *P. aeruginosa* lacking quorum-sensing ability had a reduced ability to form biofilms. Deletant *ΔlasI* mutants formed tightly packed biofilms with little extracellular polysaccharide, whilst WT cells produced thicker biofilms with about ten times the cell density and with most cells situated near the surface of the biofilm. The *ΔlasI* mutants also formed biofilms that were easily removed by the surfactant SDS (sodium dodecyl sulphate), to which the WT biofilms are more resistant. Mutants also took longer to establish biofilms, with a 36 to 48 hour lag, whilst wild type cells increased rapidly over the first 24 hours and reached peak cell density by 72 hours. Double *ΔlasIΔrhlI* deletant mutants behaved similarly to the *ΔlasI* mutants, but *ΔrhlI* only mutants exhibited normal EPS production and exhibited a lag in biofilm formation similar to that of the *ΔlasI* mutants. However, all three QS deletants exhibited much less resistance to kanamycin than the wild type, with *ΔrhlI* mutants being twice as susceptible and *ΔlasI* mutants being ten times more susceptible. The susceptibility of the *ΔlasI* mutants was very similar to that of wild type planktonic cells. These results illustrate that different quorum-sensing subsystems have different roles to play in biofilm formation, but that a deficiency in either results in impaired biofilm formation and increased susceptibility to antibiotics.

De Kievit *et al*. (2001) have studied the role of QS in biofilm formation and gene expression in *P. aeruginosa*. They compared biofilm formation in WT cells, *lasI* mutants and *rhlI* mutants. The *rhlI* mutants produced biofilms physically similar to those of the WT, but *lasI* mutants (3O-C12-HSL deficient strains) produced thin and unstructured (essentially two-dimensional) biofilms which were easily dispersed by SDS, whereas the WT cells produced three-dimensional structured biofilms (with 'mushrooms', pillars and water channels) which were resistant to SDS. This confirms the results of Davies

et al. (1998) and Shih and Huang (2002). In WT, the expression of *lasI* was maximal on day four and declined on days 6 to 8 of biofilm establishment. This peak in LasI corresponded to the transition from thin two-dimensional undifferentiated biofilms to structured, thick and three-dimensional biofilms. In contrast, the expression of *rhlI* was confined to fewer cells and was maintained at a more or less constant level. The expression of both *rhlI* and *lasI* was highest nearer to the substrate at the base of the biofilm and declined with height in the biofilm, which the authors suggest may correlate with gradients of AI in the biofilm.

The study by De Kievit *et al*. (2001) also highlighted the important differences between biofilms grown in static conditions and those grown under fluid flow. In the static system they found that flagellar motility was important for initial adhesion and that type IV pili, responsible for twitching motility in *P. aeruginosa*, enhanced microcolony formation (for general reviews on twitching motility see, for example: Henrichsen, 1983, and Mattick, 2002). However, under flow conditions these appendages had no significant effect on biofilm formation. The choices of medium and carbon source also appear to be critical. In static cultures with a glucose carbon source in M9 medium, QS mutants exhibited reduced adhesion to glass, whilst in FAB medium with a citrate carbon source there was no difference in adhesion between WT and mutant strains and adhesion was reduced in all strains tested. In the M9/glucose medium twitching motility was reduced in the QS mutants, whilst in the FAB/citrate medium all strains exhibited poor twitching motility. These authors point out that the *pilA* gene encoding pilin protein is regulated by the Crc global regulator of carbon metabolism and that citrate possibly induced a reduction in PilA synthesis compared with glucose, via the Crc regulator and thus reduced twitching motility. Flagellar motility was also reduced when citrate was the carbon source. However, this difference only manifested in static conditions and under flow the behaviour of cells was similar in each medium. They suggest that flow may be inhibiting twitching motility (a reasonable assumption since twitching motility operates on a substrate where flow is low).

It is not at all intuitive that QS should activate biofilm formation, for although a certain critical cell density is required to form a biofilm, there comes a time when cells must begin swarming or shedding from a biofilm in order to colonise new habitats. In this instance, it seems reasonable to suppose that a higher cell density should trigger biofilm dispersal. Indeed, this is known to be the case in *Vibrio cholerae*, in which studies by Zhu and Mekalanos (2003) have shown that QS activates biofilm dispersal once a critical quorum is reached. These studies have shown that QS-deficient mutants produce thicker biofilms than WT. The autoinducer CAI-1 (synthesised by CqsA) represses LuxO and removes LuxO-induced repression of *hapR* which is repressed at low cell density. Biofilm formation is enhanced in *hapR* mutants, which overexpress the *Vibrio* polysaccharide synthesis *vps* operon. Thus, *hapR* causes a reduction in EPS and biofilm synthesis, by inhibiting *vps*, and QS activates (de-represses) *hapR*. A constitutively active *luxO* allele is known to repress *hapR* expression. The authors argue that biofilms allow this species to survive in the environment and to resist passage through the acidic stomach, but that once in the intestine QS triggers biofilm dispersal in order to colonise the intestine. In some other bacteria, e.g. *Staphylococcus epidermidis*, QS has also been

shown to repress biofilm formation (Xu *et al.*, 2006) so there is no simple relationship between QS and biofilm formation – the relationship is species dependent and QS may stimulate or inhibit biofilm development depending upon species. It remains a possibility that, although a critical quorum is required to activate the QS response that a higher concentration still has a different effect. In contrast, a study on *Aeromonas hydrophila* (Lynch *et al.*, 2002) which possesses an AhyIR LuxIR-homologous QS system, demonstrated that a mutant strain deficient in *ahyI* failed to produce a mature biofilm and viable cell counts in the biofilm were reduced. Addition of C4-HSL partially restored biofilm differentiation. This lends weight to the importance of QS in producing mature biofilms that are properly differentiated, at least in certain species.

Quorum-sensing in the Gram-positive *Staphylococcus aureus* has been demonstrated to be linked to spatial and temporal heterogeneity in the biofilm. After establishment, the biofilm undergoes several cycles of cell detachment, leaving voids which are refilled by cell division, followed by one or more further cycles of shedding. The cells that are shed are apparently the most metabolically active cells, indeed most of the remaining cells appear dormant, and it has been suggested that those cells that are shed are those involved most strongly in QS (Yarwood *et al.*, 2004). Eventually, after several cycles of shedding and re-growth, the biofilms studied became quiescent and QS declined.

In conclusion, the importance of QS in biofilm formation depends upon species. Quorum-sensing does not appear essential to the development of all biofilms and may even inhibit biofilm formation in some species, but QS does play a key role in those species in which biofilm formation is a well-programmed sequence of genetically controlled developmental events, but its role in the biofilm life cycle is variable and dependent on the individual species life strategy. Quorum-sensing may affect various stages of biofilm development, including adhesion, surface motility, microcolony and tower formation and dispersal. It is not sufficient to argue the simple hypothesis that quorum-sensing leads inevitably to biofilm formation.

4. QUORUM-SENSING, BIOFILMS AND GENE TRANSFER

One established process whereby bacteria acquire resistance to antibiotics is horizontal gene transfer. Three principle mechanisms of horizontal gene transfer, conjugation, transduction and transformation, have all been implicated in passing antibiotic resistance from one bacterial strain to another. The literature on this is vast and only examples shall be given here. Conjugation is the process whereby a bacterium possessing conjugation pili (F pili) adheres to a receptive cell, the pilus retracts and thus draws the cells together, enabling a bridge for DNA transfer to form between the cells. Plasmid DNA may be transferred in whole or in part as chromosomal DNA in the form of jumping-genes (transposons) and jumping gene-cassettes (integrons). For example, the transfer of vancomycin resistance from *Clostridium symbiosum* to *Enterococcus* species has been observed in the gut of mice (Launay, Ballard, Johnson, Grayson and Lambert, 2006). This transfer occurred by conjugation and the resistance gene was carried by a conjugative transposon. Conjugative plasmids, possibly carrying transposons and/or

integrons mediate transfer of multiple antibiotic resistance in *Acinetobacter* (see, for example, Towner, 2006, Lambert, Gerbaud and Courvalin, 1988, Devaud, Kayser and Bäch, 1982).

Transduction is the process whereby a phage virus infecting one bacterial strain picks up host DNA and incorporates it into its capsid and transfers this DNA to another host strain which then incorporates it into its genome. Bacteriophage 80 has been observed to transfer methicillin resistance between strains of *Staphylococcus aureus, in vitro* (Cohen and Sweeney, 1970). Transformation is the process whereby a bacterial cell takes up DNA fragments from its environment and incorporates these into its own genome. This process occurs naturally in some bacteria, for example in soil-dwelling *Acinetobacter* (Nielsen, Bones and van Elsas, 1997) but can also be induced by cell damage that perforates the membrane. This process has been implicated in transfer of antibiotic resistance, for example when induced in *E. coli* (Cohen, Chang and Hsu, 1972).

Conjugation, transformation and transduction have been shown to occur more readily in biofilms; furthermore conjugation and transformation have been implicated in enhancing biofilm formation – suggestive of another positive feedback loop. Molin and Tolker-Nielsen (2003) showed that released DNA stabilises biofilms by transformation and also suggest that conjugation may increase biofilm formation. Sumio *et al*. (2006) cocultured *E. coli* cells possessing non-conjugative plasmids (F+) with those lacking plasmid (F–) and observed that transformed cells were produced within 24-48 hours of biofilm formation. Ghigo (2001) has shown that natural conjugative plasmids induce bacterial biofilm development and act as factors that cause a switch from a planktonic to a colonial mode of life. Biofilms provide stable environments in which bacteria can come together for conjugation, and dense cell populations facilitate the spread of bacteriophage and the biofilm matrix helps retain dead and decomposing cells whose materials, including DNA, can be taken up by neighbouring cells during transformation.

Reisner, Höller, Molin and Zechner (2006) co-cultured 403 strains of *E. coli* with the *E. coli* reference strain K-12, which normally produces flat two-dimensional biofilms, and demonstrated that biofilm growth is dramatically enhanced in these co-cultures and that conjugative transfer apparently drives this biofilm expansion. They also demonstrated that the ability to promote this biofilm formation was transferable to the K-12 strain and was linked to conjugative gene transfer, indicating that conjugation is the driving process for biofilm development. Further, this process could be inhibited by a related plasmid inserted into K-12, which presumably inhibited conjugation. These data all point to conjugative gene transfer as the main factor stimulating this enhanced biofilm growth in 70% of cases studied. Thus, although not essential, conjugation appears to enhance biofilm formation. It is also possible that other factors may cause a similar enhancement. This study also demonstrates the need to advance the study of biofilms to multi-strain (and ultimately multi-species) biofilms which are more the norm in nature.

Together these empirical data suggest that conjugation may enhance antibiotic resistance in two ways – by transmitting genes for resistance, and by enhancing or triggering biofilm development. This makes sense, since conjugation is likely to be much more efficient in biofilms where the proximity of neighbours and the relative stability of the environment facilitate conjugation. Conjugation is more likely to be incomplete

in planktonic cells in which the shear forces of fluid flow may more frequently break the contact between cells.

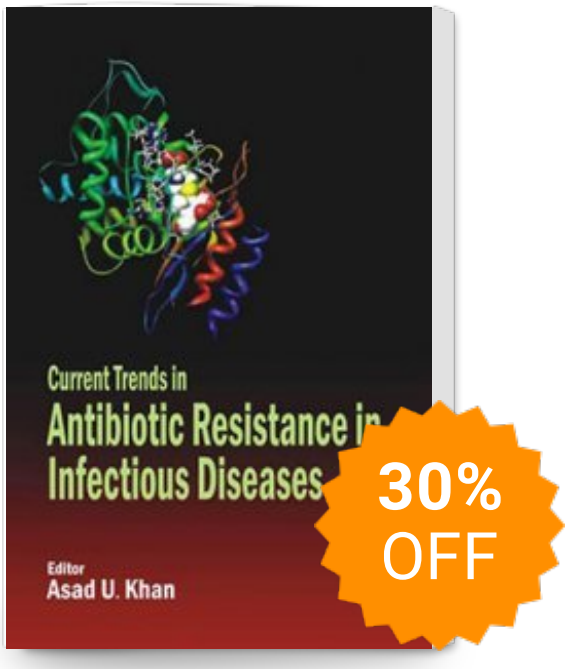
Since there is a direct link between horizontal transfer and biofilm formation, there is also an indirect link between QS and horizontal transfer because QS can affect biofilm development. However, direct links between QS and horizontal transfer have also been established. Quorum-sensing has been shown to directly induce natural competence (for transformation) in *Streptococcus pneumoniae* and *Bacillus subtilis* (see review by Lyon and Muir, 2003). In *S. pneumoniae* QS also induces synchronised DNA release from donor cells, which constitute a fraction of the total population (Steinmoen, Knutsen and Håvarstein, 2002), and competence has been shown to increase in *Streptococcus* biofilms in dental plaque (Li, Lau, Lee, Ellen and Cvitkovitch, 2001). Quorum-sensing regulates conjugation in *Enterococcus faecalis* (e.g. see Dunny 2007; see also the minireview by De Kievit and Iglewski, 2000 for further examples of the links between QS and horizontal gene transfer). Thus, QS may have a direct effect on the acquisition of antibiotic resistance by increasing the rate of horizontal gene transfer.

Evidence shows that QS can also dramatically enhance resistance of biofilms to antibiotics via mechanisms not associated with horizontal transfer. A study by Tomlin *et al*. (2005) investigated QS mutants in a cystic fibrosis isolate of *Burkholderia cenocepacia.* This organism has two QS systems: CepIR and CciIR. Mutants in either or both of these systems produced biofilms with a biomass less than half that of WT biofilms. Furthermore, planktonic cells of the mutants exhibited no change in antibiotic resistance. However, the biofilms of a *cepIcciI* double-mutant were more sensitive to ciprofloxacin and ceftazidime. Mutants defective in only one or other QS system were as resistant as WT. However, mutants lacking either system did produce structurally deficient biofilms that detached easily when treated with SDS. This demonstrates that both QS systems were involved in biofilm formation, and that an absence of both leads to hyper-susceptible biofilms. Next we shall examine the possible mechanisms for such direct QS-induced increases in antibiotic resistance in biofilms and whether these mechanisms confer selective or multiple antibiotic resistance.

5. DO BIOFILMS CONFER SPECIFIC OR MULTIPLE ANTIBIOTIC RESISTANCE?

Folkesson, Haagensen, Zampaloni, Strnberg and Molin (2008) studied biofilms of *E. coli* strains plus or minus an altered form of the IncF plasmid with altered forms of transfer pili. IncF plasmids are horizontal transfer-constitutive and induce their *E. coli* host to produce thick, three-dimensional *Pseudomonas*-type biofilms with elaborate towers or mushroom structures. This study looked at biofilms grown under flowing glucose medium and demonstrated that structured biofilms exhibited increased resistance to colistin, a peptide that alters cytomembrane permeability and is bactericidal in Gram-negative bacteria. These authors discovered that the structured biofilms were much more resistant to colistin and that this resistance was due to a genetically regulated tolerant sub-population. Interestingly resistance to the fluoroquinolone ciprofloxacin was no greater in the biofilms than in planktonic cells. The resistant sub-population did not

Current Trends In Antibiotic Resistance In Infectious Diseases



Publisher : IK International ISBN : 9789380026701 Author : Asad U. Khan