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Modification by surface association of antimicrobial susceptibility of bacterial populations

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In the majority of natural situations in which bacteria are found, they are associated with and attached to surfaces. In the presence of moisture and nutrients, they grow to form extensive bacterial films which are often enveloped within copius exopolymeric matrices. Biofilms are ubiquitous to many different situations in industry, the environment and medicine. Their presence can be either beneficial or more commonly detrimental to such systems. In this respect, biofilm populations possess physiological properties distinct from those of unattached, planktonic bacteria. Moreover, it is generally accepted that bacteria growing within a biofilm are more resistant to antimicrobial agents than their planktonic counterparts. However, although the consequences of attachment to antimicrobial resistance have been known for many years, the mechanistic bases for such effects have still to be fully elucidated. In this article the nature of different resistance mechanisms, including those of the exopolymeric matrix, environmental modulation, attachment-specific physiologies and quorum sensing are reviewed.

Keywords: bacterial biofilms; antimicrobial resistance; exopolymeric matrix; environmental modulation; attachment-specific physiology; quorum sensing

Introduction

It is now generally accepted that the majority of bacteria in nature have a marked tendency to interact and grow in close association with surfaces [20]. They do so as biofilms, covered by and interspersed within matrices of extracellular polymers [17]. The physiological properties of the attached organisms, including susceptibility towards antibiotics and biocides, are markedly affected by attachment and are very different to those of free living, planktonic cells. Surface associated growth is therefore of considerable importance when selecting *in vitro* growing conditions for culture. In this respect, biofilms have been cultured, and studied, in association with a wide range of surface types, and by a variety of scientific disciplines, including medicine, immunology, biotechnology, biocorrosion, biofouling, biodeterioration and process engineering [55]. The extent to which attachment, *per se*, rather than biofilm formation affects antimicrobial susceptibility is unknown, but a number of studies have identified attachment-moderated phenotypes which affect susceptibility.

Biofilm growth, within tissues, is often associated with indwelling medical devices and commonly involves monocultures. Such growth on exposed tissue surfaces such as the nasopharynx and gut are more often comprised of mixed species [18]. In the general environment also, mixed species biofilms are found where bacteria are often joined by fungi, algae and protozoa to produce truly heterogeneous populations. In these, the community has a net metabolic capability which is unrepresented by any single member species. Moreover, the inter-species relationships represented are often synergistic. Biofilms may therefore be described as functional consortia of microorganisms organised within extensive exopolymer matrices [18]. From man's perspective the development of biofilms can sometimes be desirable, for example, in the removal of heavy-metal pollutants from sewage treatment processes, but is more often problematic. In an industrial context, biofilms can grow to be extensive and sufficient to cause biofouling and physical blockage of pipework and heat exchangers. They may also increase the frictional resistance to fluid flow on ship hulls and in water conduits and promote the corrosion of metallic substrata [41]. To these effects can be added a whole panoply of disease processes such as prosthetic device colonization [23], dental plaque formation [16], infections of the cystic fibrotic lung [50] and contamination and spoilage problems in the food industry [42]. Control and eradication of biofilms in those situations is difficult and costly since their resistance towards most antibiotics and biocides is substantially increased over that of planktonic populations. Resistance of biofilms to antimicrobial strategies is not a problem which is unique to the biomedical field [40,60] but is widely experienced in the environmental industries where all but the most vigorous antifouling strategies fail to eliminate or mollify the problem [19]. Despite the established ubiquity of bacterial biofilms and their recalcitrance towards antibiotic and biocide treatment regimens, the nature of such resistance mechanisms are not, as yet, fully understood.

Resistance mechanisms

Organisation of microorganisms into a biofilm is thought to confer many survival advantages upon the component organisms [4]. These include not only protection, *in vivo* from host defence mechanisms and *in vitro* from predatory protozoa, but also provide and maintain an appropriate physico-chemical environment for growth and survival.

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Regardless of their site of occurrence, bacterial biofilms are notably recalcitrant to antimicrobial treatments [13]. In this respect treatment with antimicrobial agents which have proven to be effective against suspension cultures in the laboratory, often fail to have any effect *in vivo/in situ*. Such resistance has been attributed to a number of properties associated with biofilms. These include reduced diffusion of materials through the extracellular matrix relative to liquids, overproduction of hydrolytic enzymes and concentration of these within the exopolymer, physiological changes due to reduced growth rates and/or the induction of attachment-specific, drug-resistant physiologies. It is unlikely that any single mechanism will account for the general observation of resistance; rather, that these mechanisms are compounded in biofilms to create extreme recalcitrance.

The extracellular polymeric matrix

A common feature of all bacterial biofilms is the presence of an extensive exopolymer matrix [17]. Following attachment, cells initiate production and accumulation of extracellular polymers which eventually surround and envelop the developing microcolony [6]. Accumulation of these extracellular matrices can occur within hours of initial adhesion. Although such matrices are often referred to as the 'glycocalyx', they do not provide a common defined structure. Whilst the predominant component of the exopolymer matrix is polysaccharide, other macromolecules such as nucleic acids, proteins and glycoproteins may also be present [70]. These matrix polymers determine the physical properties of the biofilm. Bacterial polysaccharides are chemically heterogeneous and contain non-carbohydrate substituents, many of which are species specific and are usually negatively charged [71]. They may also be neutral, or more rarely positively charged depending upon the component of the repeat units. Furthermore, whilst polysaccharides are generally hydrophilic, in nature they may also possess some hydrophobic properties [56]. The presence of such a matrix around individual cells or microcolonies will therefore influence the access of molecules and ions. It is therefore not surprising that many of the early workers in the field of biofilm resistance attributed much to the diffusion properties of the glycocalyx [68,73]. Recent work has shown, however, that the diffusion properties of the extracellular polymers are insufficient to account for much of the observed resistance [9,39,57,59]. Indeed, whilst the extracellular matrix can function as an ion-exchange column and exclude large, highly charged molecules, most solutes will equilibrate across it and access the resident population. In this respect, the reductions in diffusion coefficients for antibiotics such as tobramycin and cefsulodin within biofilms relative to suspensions are insufficient to account for the observed changes in susceptibility. Such studies, however, have generally focused on antibiotics rather than biocides and upon medically-relevant biofilm populations rather than biofouling situations. The dimensions of biofilms *in vivo* are in the order of tens of micrometres, whilst for industrial biofilms they may be in the order of centimetres thick [59]. Whilst thickness will not affect diffusion properties *per se*, it will affect the concentration of charged antimicrobial agents accessing the depths of industrial scale biofilms [57]. This is particularly the case when the extracellular matrix binds directly with and reduces availability of the drug. In this respect, if the antimicrobial substances are either strongly charged (ie tobramycin) or chemically highly reactive (ie halogens/peroxygens), then they will be bound to and quenched by the matrix as they attempt to diffuse through it. In this manner, the glycocalyx will function as an ion exchange column and will protect cells remote from the treated surface from the action of the agents.

Clearly, whether or not the extracellular matrix constitutes a physical barrier to the penetration of antimicrobial agents depends greatly upon the nature of the antimicrobial, the binding capacity of the matix towards it, the levels of agent used and the rate of growth of the microcolony relative to the diffusion rate of the antimicrobial agent. For antibiotics such as tobramycin and cefsulodin such effects have been suggested to be minimal [58,59]. On this basis, Gristina and colleagues [40] found no difference in the susceptibility towards antibiotics, of biofilms of slime-producing and non-slime producing strains of *Staphylococcus epidermidis*. By contrast, however, Evans *et al* [29] in a similar study assessed the susceptibility to the quinolone antibiotic ciprofloxacin of mucoid and non-mucoid strains of *Pseudomonas aeruginosa* grown as biofilms. The results from this study demonstrated that possession of a mucoid phenotype was clearly associated with decreased susceptibility. Similarly, activities of chemically highly reactive biocides such as iodine and iodine-polyvinylpyrollidone complexes are substantially reduced by the presence of protective exopolymers [32]. In such instances not only will the polymers act as adsorption sites but they will also react chemically, with, and neutralise, biocides.

Attached populations form glycocalices which are composed of exopolymer from each of the constituent species. The rheological interaction between these different polymers may lead to the formation of a heterodisperse polysaccharide solution that possesses increased gelation and viscosity characteristics. Preliminary studies demonstrated that diffusion of both charged and uncharged antibiotics may be reduced to a greater extent by heterodisperse gels than by the individual component polysaccharides [5].

Metabolically active cells within a biofilm will contribute to reduction in penetration of antibacterial agents by acting as metabolic 'sinks' [57]. This will be particularly the case when the cells produce extracellular and periplasmic hydrolases which denature the antibiotic as it diffuses across the biofilm. In so doing, the free concentration of the compound, which would be driving diffusion, would be reduced across the breadth of the biofilm, reducing the rapidity of penetration [59]. In these respects, extracellular products would be localised rather than released into the bulk phase. Many of these hydrolytic enzymes are induced/derepressed in adherent populations [38,51] and become trapped and concentrated within the biofilm matrix, thereby enhancing their protective properties. Such induction might be promoted via quorum sensing, transcriptional activation (see later).

In summary, reductions in the diffusion coefficient, across polymeric matrices relative to liquid media, are insufficient to account for the recalcitrance of biofilms since

at equilibrium concentrations at the cell surface and in the bathing medium will be the same. With losses of biocide occurring either through chemical or enzymic inactivation of the agent within the biofilm during diffusion, then the reduction in diffusion coefficient might tip the balance towards chemical/antibiotic recalcitrance.

Modulation of the growth environment

Adherent microcolonies are functional consortia which influence their micro-environment through localised concentration of enzymes and metabolic products and the relative depletion of gases such as oxygen [18]. As a consequence, cells deep within the biofilm are exposed to concentrations of substrates, hydrogen-ions, and also oxidation-potentials which are significantly altered from those experienced by planktonic cultures growing in the same medium. The nature of the growth-limiting nutrient might differ at different points in the biofilm and growth rates will be reduced through the imposition of nutrient deficiencies which might or might not reflect the composition of the bathing medium. Growth rate *per se* and also the response of cells to specific nutrient deprivations are known not only to be important factors in bacterial pathogenesis, but also are primary moderators of antimicrobial susceptibility [12,15].

Rate of cell growth is a major difference between growth of microorganisms in enriched, laboratory media and the real world. While many organisms have the potential to divide frequently *in vitro* (doubling times <30 min), *in situ/in vivo* division times are more likely to be measured in hours than minutes [10,12]. Growth rate control, in continuous culture systems, and the application of particular nutrient deprivations have been used extensively to model natural open growth habitats, such as infections. In these, the availability of critical nutrients regulates the rate of cell division. Imposition of different nutrient limitations gives rise not only to populations which divide slowly but also to cells with physiologies and cell envelopes which are peculiar to each limitation [15,27,43] and radically different from those of cells grown under nutrient-rich conditions. Changes in the cell envelope such as these have been widely reported to influence greatly susceptibility to a variety of antimicrobial agents [14,78] both for Gram-positive and Gram-negative microorganisms. Since mature biofilms are composed of multilayers of bacteria embedded in an exopolysaccharide matrix, diffusion and transport of nutrients through the biofilm becomes an important consideration. In nutrient-rich environments, oxygen and nutrients are rapidly utilised by aerobic bacteria at the biofilm:liquid interface, thereby diminishing the availability of such nutrients in the depths of the biofilm. This leads to the formation of anaerobic and anoxic zones [52]. In this manner, nutrient gradients are likely to be established within thick biofilms and will provide populations of cells which are very heterogeneous with respect to growth rate. Consequently, growth rates are likely to decrease with depth of location of the cells within the biofilm. Some workers have used chemostats to separate the effects of growth rate from those of nutrient limitation upon susceptibility for a range of antimicrobial agents. A general conclusion has been drawn that, for cells grown in suspension, slowly growing cells are particularly recalcitrant [33,37,74]. The recalcitrance has been commonly associated with changes to the bacterial cell envelope [12,27], specifically with respect to fatty acids and phospholipids [37], metal cations [49], envelope proteins [14,15] and extracellular enzymes and polysaccharides [61,71]. These, in turn, influence the access and susceptibility of the cells towards antimicrobial agents such as biguanides [46], gentamicin [64] and polymyxin [78], the initial binding and action of which is mediated through membrane phospholipids.

Since within most *in vitro* biofilm models growth rates will be significantly less than for the component organisms growing planktonically, biofilms will be reported as less susceptible to antimicrobial agents than their free-living counterparts [11]. The extent to which reductions in growth rate within biofilms can explain the observed differences in susceptibility of adherent populations has only recently received direct study. In these studies, a number of important observations have been made. Firstly, for the species studied, with cetrimide, ciprofloxacin and tobramycin, susceptibility increased with growth rate not only for the planktonic chemostat cultures but also for the biofilms [24,25,28–30]. With the exception of ciprofloxacin the susceptibilities of the resuspended biofilms and planktonic cells were similar at any given growth rate. Had slow-growing biofilms been compared with fast growing planktonic cells, as has been the case in some other studies [11], then erroneous statements about the resistance of biofilms would have been made. A major contributor to the recalcitrance of biofilms towards antimicrobials must therefore be their reduced growth rate. Secondly, when intact biofilms were exposed and their susceptibilities related to those of planktonic cells and resuspended biofilms then, at any given growth rate, organisation as a consortium increased resistance to some extent, thereby indicating resistance to be more than a growth-rate related event. For mucoid pseudomonads and ciprofloxacin this was significant, but for *E. coli* and tobramycin less so. Organisation of the cells within a polymeric matrix does, therefore, contribute towards their recalcitrance, but the extent of such protection depends upon the amount of exopolymer and the nature of the agent. Finally, perfusion of the intact biofilms *in situ* with antibiotics has enabled the effectiveness of chemotherapeutic regimens to be evaluated [24,25].

Attachment-specific physiologies

It is well established that bacteria often respond to environmental stimuli through changes in physiological process or morphology. In some cases responses to changes in the growth environment are different for sessile and planktonic bacteria. Very often bacteria require prolonged exposure in order to attach firmly to surfaces, indicating some physiological response. This time-dependent process has been termed active adhesion [34] and can result in a sequence of phenotypic changes in bacterial cells that determine the characteristics of the resulting biofilm. Such changes include the expression of specific cell wall proteins in *E. coli* and *P. aeruginosa* [21] and the conversion in *Vibrio parahaemolyticus* from a single polar flagellum in liquid media to numerous lateral flagella on solid culture media [8]. This latter response was subsequently found to be due

to a switching on of the *laf* genes through contact with a surface [53]. Similarly, gliding bacteria lack extracellular polymer biosynthesis when grown in suspension culture [1,45], but rapidly initiate/increase such synthesis following irreversible adhesion to a surface.

There is still, however, much debate as to whether surface-induced physiologies reflect derepression/induction of specific operons/genes or are purely manifestations of the physico-chemical presence of the surface on the surroundings of the cell [75]. Indirect effects of surfaces might include the accumulation of many substrates at surfaces which will therefore be available in increased abundance for attached organisms [66]. In this manner, the growth rate of attached cells, and associated physiologies, will be different to those of planktonic cells placed in the same medium.

Studies conducted using growth-rate controlled sessile and planktonic populations of *E. coli, P. aeruginosa* and *S. epidermidis* showed that all three organisms produced greater quantities of EPS when cultivated at slow rates of growth as a biofilm compared to their unattached equivalents [3,31]. Significantly, these differences in exopolymer synthesis became smaller with increasing growth rate. Clearly exopolymer production is enhanced not only by attachment but also through reduction in cellular growth rate. Moreover, the exopolysaccharide produced by *P. aeruginosa* when grown as a biofilm contained an additional, chemically distinct low-molecular weight polymer, and lipopolysaccharide profiles derived from *P. aeruginosa* and *E. coli* biofilm cells in all instances were 4–7 saccharide units longer than for the same cells grown planktonically. Interestingly, marked differences in cell surface hydrophobicity and electrokinetic potential were observed between biofilm cells and those cells spontaneously eluted from the biofilm during growth. This may indicate specific, controlled dispersal mechanisms. Thus, whilst some of the physiological responses at surfaces are probably nutritional, others may appear to be associated exclusively with surface growth *per se*.

At a genomic level, recent work has noted the presence of 'touch-promoters' which respond to the proximity of the substratum [22]. Studies by Dagostino and colleagues [22,52] demonstrated a distinct transition from reversible to irreversible adhesion with time in a marine pseudomonad, whereby reversible-adhesion phase cells appeared to be primed for firm attachment to the polystyrene surface. Candidate physiologies for touch-induction are now appearing regularly in the literature, but, with the exception of cell-density responsive transcriptional activation (below), molecular genetic bases for the phenomena have not been proposed.

Quorum sensing transcriptional activation

Whilst it has long been appreciated that the properties of some groups of microorganisms exhibit co-operative behavioural patterns [2,48], the complex molecular basis of bacterial sensing and its coordination at a multicellular level has only become apparent recently. The properties of individual cells within a multicellular system and genetic responses of populations have now been identified that are responsive to population density [47,76]. Initial attention focused on a signalling system in actinomycetes mediated by small molecules known as butyrolactones which were recognised to be involved in the control of gene expression [44]. A second type of common sensory system has been identified in bacteria, known as the two-component sensory system. In this system, a sensory protein component regulates the phosphorylation of a response regulator protein [62]. Recently, a new subclass of response regulators has been discovered that are adapted for intercellular communication and that use *N*-acyl homoserine lactones rather than phosphorylation as the signal [7,35,76]. The first such observations concerned bioluminescence in the marine bacteria *Vibrio fischerii* and *V. harveyi*. These organisms do not luminesce when grown in dilute planktonic cultures, but do so either in their symbiotic forms in the light organs of squid or fish-gut microflora respectively, or when, in batch culture, cell density exceeds a critical level. In this respect, both organisms show acyl-homoserine lactone mediated autoinduction of their bioluminescence at high cell densities. Moreover, bioluminescence will continue for as long as favourable conditions are maintained. Whilst the molecules involved in each of the two organisms are similar in structure, their activities are species-specific [72].

Clearly, concentrations of the autoinducer will become greater not only as cell density is increased through growth, but also if organisation of the cells restricts dilution of the autoinducer by diffusion. For an individual cell, conditions must be such that production of exoproducts is favourable. Concentration of an autoinducer such as homoserine lactone signals the potential benefit of exoproduct synthesis to the cells. Hence, growth of cells in association with surfaces and within biofilms offers the prospect of autoinducer accumulation with a concomitant switching-on of a new phenotype. In the case of *V. fischerii* two genes have been isolated and cloned, *luxR* and *luxI*, which control and regulate luminescence. The *luxI* gene codes for an autoinducer synthetase whereas *luxR* codes for a membrane-associated transcriptional activator that is switched by the detection of the autoinducer. Switching of the *luxR* gene can be both positive or negative according to the autoinducer concentrations [26,67]. Other genes in the *Lux* operon code for the machinery associated with luminescence. The *V. fischerii* autoinducer is *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL) [7,72]. LuxR and LuxI mediate cell density dependent control of *lux* gene transcription. At low cell densities, *luxI* is transcribed at a basal level and OHHL accumulates slowly in the medium. At sufficiently high OHHL concentrations a LuxR-OHHL complex is formed which stimulates transcription of the *luxI* operon leading to luminescence and the positive autoregulation of *luxI*[69]. Because OHHL is freely diffusible, the induction of one cell leads directly to the induction of others, creating a positive feedback loop that can generate a large and rapid response to a small stimulus. This provides a co-ordinated response from a population of cells [69]. The control and operation of these regulatory elements have recently been reviewed [54,72].

If cell-density responsive transcriptional activation was not restricted solely to luminescence then its implications in biofilms is enormous. Although the genetic bases for attachment-phenotypes have not been fully elucidated,

within the past two years regulators analogous to the *luxI/luxR* family have been identified in a wide range of bacterial species [69]. In addition to controlling luminescence in *V. fischerii*, the *luxI/luxR* superfamily have been associated with conjugal transfer in *Agrobacterium tumefaciens* Ti plasmids [65,79] and extracellular virulence factor production in *P. aeruginosa* [36,47,63]. There is also evidence for homologous systems in *Erwinia, Rhizobia* and *Escherichia* [35].

Substantial evidence now exists for a super-family of response-regulator proteins that recognise a number of chemically related autoinducers. Their role in the properties of attached bacterial populations remains to be determined. It is unlikely, however, that cell densities, and thereby concentration of autoinducer, will reach such critical levels in the natural world, other than in association with microcolonies and biofilms. As such, small signalling molecules produced by one microorganism might function as an attractant for a second microorganism, leading to the development of mixed cultures functioning co-operatively within a particular ecological niche [77]. As well as information about energy, nutrient levels and oxygen availability, the individual cell will sense the local population density and maturity to synchronise growth and developmental processes within the community. This will be of fundamental importance for the bacteria to adapt dynamically in response to prevailing environmental conditions. In addition, the ability of a microorganism to use such small molecules to monitor its own population density may postively switch cells to an attachment-phenotype through the expression of adhesive polymers and appendages or alternatively, facilitate induction of other genes essential for the maintenance of the biofilm mode of growth. Quorum sensing transcriptional activation might give rise to the recalcitrant physiology associated with biofilm populations and therefore offers the prospect for the design of novel antibiofilm agents.

Concluding remarks

Whilst there have been relatively few controlled studies of the antibiotic susceptibility of biofilms, it has recently been demonstrated that sensitivity may be profoundly affected when growth occurs as an adherent biofilm, rather than as planktonic cells, and that such resistance might contribute towards the recalcitrance of particular infections. There is now direct evidence that growth rate, nutrient limitation, exclusion by exopolymer matrix together with expression of adherence phenotypes each play a role in this recalcitrance. Workers in the area should therefore be aware of these contributory factors and select appropriate *in vitro* models and control populations when developing and testing novel antimicrobial regimens.

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