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BIOFILM SUSCEPTIBILITY TO ANTIMICROBIALS

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Abstract—Microbial biofilms, where organisms are intimately associated with each other and a solid substratum through binding and inclusion within an exopolymer matrix, are widely distributed in nature and disease. In the mouth, multispecies biofilms are associated not only with dental plaque and tooth decay but also with soft tissues of the buccal cavity and with most forms of periodontal disease. Organization of micro-organisms within biofilms confers, on the component species, properties which are not evident with the individual species grown independently or as planktonic populations in liquid media. While many of these properties relate to the establishment of functional, mixed-species consortia within the exopolymeric matrices, others relate to the establishment of physico-chemical gradients, within the biofilm, that modify the metabolism of the component cells. A consequence of biofilm growth that has profound implications for their control in the environment and in medicine is a markedly enhanced resistance to chemical antimicrobial agents and antibiotics. Mechanisms associated with such resistance in biofilms will form the substance of the present review. While some aspects of biofilm resistance are yet only poorly understood, the dominant mechanisms are thought to be related to: (i) modified nutrient environments and suppression of growth rate within the biofilm; (ii) direct interactions between the exopolymer matrices, and their constituents, and antimicrobials, affecting diffusion and availability; and (iii) the development of biofilm/attachment-specific phenotypes.

Key words: Biofilm, antimicrobials, growth rate, glycocalyx, attachment to surfaces.

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The development and application, post-1940, of a wide variety of antibiotics were generally heralded as the advent of an 'Antibiotic Era'. Common infectious diseases, such as tuberculosis and pneumonia, became treatable and were essentially eradicated from the developed world. Since then, major developments of chemical antimicrobial agents such as bisbiguanides, isothiazolones, and peroxygens, for use in antisepsis, disinfection, and preservation, have also been made. In recent years, however, we have been forced to re-evaluate such antimicrobial strategies. Widespread, indiscriminate use of antibiotics has led to the development and emergence of antibiotic-resistant strains. Similarly, the widespread use, and dissemination within the environment, of chemical antimicrobials is leading to reductions in their effectiveness. This is coupled to increasing demand being placed by man on the need to control the presence and metabolism of bacteria in an ever-widening sphere of applications.

Thus, the development and use of a broad range of medical devices have led to the emergence and recognition of a variety of infections caused by organisms that were regarded previously as 'harmless'. In this respect, infections relate not only to biofilms associated with the surfaces of implanted medical devices such as prostheses, endocardial pacemakers, and catheters (Marrie and Costerton, 1983; Holmes and Evans, 1986), but also to our failure to disinfect adequately the organisms attached to medical equipment such as fiber-optic endoscopes. Biofilm infections, associated with indwelling medical devices, are often chronic and act as sources for bacteremia. While the latter respond readily to antibiotic treatment dictated by the results of conventional sensitivity testing (Thomson *et al.*, 1995), the biofilms from which they derive display a greatly enhanced resistance and often fail to respond to even the most aggressive antibiotic prescribing (Kunim and Steel, 1985; Nickel *et al.*, 1985; Gristina *et al.*, 1987; Costerton *et al.*, 1993). If the device is not surgically removed prior to antibiotic treatment, then the infection will generally recur. Resistance of biofilms is not restricted to antibiotics but is also shown with respect to a wide range of chemical biocides. These include isothiazolones (Costerton and Lashen, 1984), quaternary ammonium compounds (Costerton and Lashen, 1984; Evans *et al.*, 1990b), and halogens and halogen-release agents (Favero *et al.*, 1983). Failure of available antimicrobials to contend adequately with microbial biofilms, together with an increasing dependence of modern medicine upon the implantation of devices, has stimulated the search for antimicrobials which have activity directed primarily toward the biofilm phenotype (Gilbert and Brown, 1995). This process includes not only the development of our knowledge of biofilm physiology, in the search for novel antimicrobial targets, but also an examination of the various mechanisms associated with resistance of biofilms toward current antibiotic agents.

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Failure of micro-organisms to succumb to antimicrobial treatment may arise through: (i) an inherent insusceptibility to the agents used; (ii) the acquisition of resistance, by previously susceptible strains, either by genetic mutation or by transfer of genetic material from another species or genus; and (iii) the emergence of pre-existing but unexpressed resistance phenotypes. While it is unequivocal that biofilms resist conventional treatments, the extent and mechanisms through which adaptation toward a less-susceptible phenotype is influenced by growth as a biofilm, on soft tissue or hard surfaces, will remain a matter for debate until common *in vitro* susceptibility-testing methodologies are adopted that adequately replicate the *in vivo* complexities (Anwar and Costerton, 1990; Gilbert and Brown, 1995).

The present article considers those mechanisms of resistance that are considered likely to be associated with attachment of micro-organisms to surfaces and growth into microcolonies/communities entrapped within extracellular polymers. We will consider (i) the extent to which resistance is a reflection of the nutrient environment generated within the biofilm, (ii) direct modification of antibiotic action through the presence of extracellular polymers and antibiotic-modifying enzymes, and (iii) the development of attachment/biofilm-specific phenotypes.

ANTIBIOTIC RESISTANCE, NUTRIENT ENVIRONMENT, AND BIOFILMS

Often critical to the long-term survival of micro-organisms is their ability to attach to surfaces and form adherent biofilms. Biofilms are functional consortia of microbial cells enveloped within sometimes-extensive matrices of extracellular polymers (glycocalyx) and concentrated products of their own metabolism, together with ions and nutrients sequestered from the environment. Soft-tissue infection or infections associated with indwelling medical devices are often the result of the growth of mono-species biofilms. In the majority of situations in the human body (*i.e.*, gastro-intestinal tract and oral cavity), and in the general environment (*i.e.*, freshwater and marine ecosystems), biofilm consortia are composed of a variety of species and genera.

The structural organization of the glycocalyx, which forms the intercellular matrix, varies according to the prevailing physico-chemical environment. Thus, in situations of high shear (*i.e.*, tooth surface during mastication, gastro-intestinal tract, *etc.*), the biofilm population is organized within stratified compacts of exopolymeric material (*i.e.*, dental plaque; Newman and Barber, 1995). Under low to moderate shear, with nutrients accessed from the bathing fluids, the biofilms then appear as attached floccules. These anchor the micro-colony to the substratum yet maximize diffusive interactions with a nutrient-bearing environment (Costerton *et al.*, 1994a). If nutrients are derived from the substratum, rather than the bathing fluids, then diffuse layers of biofilm cells which completely coat the available surface are favored, such as in the colonization of the nasopharynx. With the exception of those cells that are located at the periphery of the biofilm, access and availability of nutrients and the elimination of metabolic by-products are restricted to a greater extent than it would be for the same cells growing individually in liquid culture. Thus, cells deep within the biofilm matrix have available to them only those materials from the bathing fluids that have failed to be sequestered by more outlying cells. Conversely, these micro-organisms have greater access to the secreted metabolic products of the neighboring cells. This may lead to spatial organization of species within mixed-species biofilm communities, with associated cross-feeding, and the development of a functional inter-species dependence. In both mono- and multi-species biofilms, nutrient and gaseous gradients, generated by metabolism, will cause nutrient availability and growth rates of the enveloped cells to vary with location relative to the substratum and biofilm/liquid phase interface.

Nutrient limitation and growth rate

The plasticity in structure and physiology of bacterial cells allows them to make rapid phenotypic responses not only to changes in their nutrient status and growth rate, but also to changes in temperature and pH, and following exposure to subeffective concentrations of antibiotics (Brown and Williams, 1985; Williams, 1988; Brown *et al.*, 1990; Gilbert *et al.*, 1990). Such responses may include changes in a wide variety of cellular components—including proteins, fatty acids, and phospholipids associated with the cell envelope—and production of extracellular enzymes and polysaccharides (Brown *et al.*, 1990; Gilbert *et al.*, 1990). Since all antimicrobial substances must interact with the cell envelope either as the primary target or as a means of accessing this target, then such changes in phenotype inevitably affect susceptibility toward a wide range of antibiotics, disinfectants, antiseptics, and preservatives (Brown *et al.*, 1990; Gilbert *et al.*, 1990).

In well-mixed suspension cultures, all members of the community experience a common environment at any particular time. In batch culture, these environmental conditions, and hence the phenotype, change with time, while in open environments, such as chemostat culture, steady-state conditions prevail. Single phenotypes dominate such cultures which, as a consequence, tend to demonstrate singular responses toward antimicrobial treatments. At any given time within biofilm communities, however, a plethora of phenotypes is represented for each component species. The breadth of phenotypes represented reflects the extent of the chemical heterogeneity within the biofilm and the presence of various concentration gradients. Thus, the outcome of any attempt to eliminate a biofilm community by antimicrobial treatment will often reflect only the susceptibility of the most resistant phenotype represented.

A distinction can be made between those effects related to the nature of the least available nutrient (nutrient limitation/depletion) and the cellular growth rate. Within the depths of a biofilm, growth rates will generally be suppressed relative to planktonic cells growing in the same environment. In this respect, Ashby *et al.* (1994) used biofilm:planktonic ratios of isoeffective concentration (growth inhibition and bactericidal activity), determined for a wide range of

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TABLE 1

ACTIVITY INDICES FOR VARIOUS ANTIBIOTICS AGAINST *Escherichia coli* (from Ashby *et al.*, 1994)

Antibiotic	Minimum Effective Concentration Activity Ratio*, Non-growing/ Growing Cells	Sessile Planktonic Index Activity Ratio#, Biofilm/Planktonic Cells
Cephamycins		
cefminox	1.0	1.8
cefoxitin	2.0	1.9
cefotetan	32	2.5
cefmetazole	1.0	1.5
Cephalosporins		
cefotaxime	16	5.4
ceftazidime	16	3.2
cefoperazone	8.0	1.7
cefpirome	> 32	3.2
Carbapenems		
imipenem	0.5	1.2
meropenem	8.3	1.6
Miscellaneous		
gentamicin	1.0	1.1
ciprofloxacin	33.3	1.2

* MIC (mg/L) *vs.* growing cells/concentration causing 50% reduction in OD_{600} nm for non-growing cells.

\# Ratio of concentrations causing a 50% inhibition of the incorporation of [3H]-leucine in biofilm and planktonic populations, respectively.

antibiotics against cells grown either in broth or on urinary catheter discs, to indicate the extent of biofilm resistance. They noted that such ratios (Table 1) closely followed those generated between non-growing and actively growing cultures. With the exception of ciprofloxacin, antibiotic agents that were most effective against non-growing cultures (*i.e.*, imipenem, meropenem) were also the most active against these biofilms. Other workers have used perfused biofilm fermenters (Gilbert *et al.*, 1989) directly to control and study the effects of growth rate within biofilms. Planktonic controls grown in chemostats can be used for the evaluation of the separate contributions of growth rate and association within a biofilm. Decreased susceptibility of *Staphylococcus epidermidis* to tobramycin (Duguid *et al.*, 1992a) and of *Escherichia coli* to tobramycin (Evans *et al.*, 1990a) and cetrimide (Evans *et al.*, 1990b) could be explained largely in terms of growth rate. Cells re-suspended from growth-rate-controlled biofilms and planktonic cells of the same growth rate possessed virtually identical susceptibilities to these agents. When intact biofilms were treated, however, susceptibility was decreased somewhat from that of planktonic and re-suspended biofilm cells, indicating some benefit to the cells of organization within a glycocalyx (see below).

Stewart (1994) developed a mathematical model which incorporated the concepts of metabolism-driven oxygen gradients and growth-rate-dependent killing to examine the susceptibility of *S. epidermidis* biofilms to various antibiotics. The model accurately predicted that susceptibility would be reduced in thicker biofilms due to oxygen limitation. Oxygen gradients within the biofilm may also directly influence the activity of some antibacterials (Shepherd *et al.*, 1988; Zabinski *et al.*, 1995). Since nutrient and gaseous gradients will increase within maturing biofilms, then growth-rate effects on susceptibility, such as these, will become particularly marked in aged biofilms (Anwar *et al.*, 1989, 1990) and might also lead to an onset of dormancy and the triggering of stringent-response genes (Zambrano and Kolter, 1995). Such changes probably contribute to reports that aged biofilms are more recalcitrant to antibiotic and biocide treatment than are younger ones (Anwar *et al.*, 1989).

The presence of sub-inhibitory levels of antibiotic agents within the depths of the biofilm will provide selective pressures for the development of more resistant phenotypes and for the selection and expression of resistance plasmids. Sub-inhibitory concentrations may be either generated through the failure of antibiotics to penetrate the glycocalyx adequately or caused through decreases in the susceptibility of the enveloped cells.

This view of biofilm resistance being related predominantly to the low growth rates within them (Prosser *et al.*, 1987; Brown *et al.*, 1988; Gilbert *et al.*, 1990) does not offer much hope to those searching for novel anti-biofilm/anti-plaque agents (Gilbert and Brown, 1995). In the study by Ashby *et al.* (1994), described above and presented in Table 1, ciprofloxacin, an agent that does not distinguish itself particularly against non-growing cells, nevertheless has good anti-biofilm activity. Similarly, experiments with this quinolone, utilizing the perfused biofilm fermenter (Gilbert et al., 1989), suggested that while growth rate played a crucial role in the ciprofloxacin susceptibility of *S. epidermidis* and *E. coli*, slow-growing ($\mu < 0.15\ h^{-1}$) biofilms were especially sensitive (Evans *et al.*, 1991; Duguid *et al.*, 1992b). These observations suggest that there might be some physiological properties, with potential to act as antimicrobial targets, that are unique to biofilm-grown cells.

MATRIX POLYMERS, GLYCOCALYX, AND EXTRACELLULAR ENZYMES

Electron microscopic examinations of antibody-stabilized biofilm preparations reveal ordered arrays of fine fibers that provide a relatively thick, hydrated, polyanionic matrix around the cells (Glycocalyx; Costerton *et al.*, 1981). While the exopolymers that compose the glycocalyx are predominantly highly hydrated, gelled polysaccharides, other polymers, particularly globular glycoproteins (Sutherland, 1985), are also represented. Whether the 'footprint polymers', which cement the primary colonizers to the substratum, differ from the matrix polymers, which bind cells to other cells, and whether these differ from the exopolymers

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found associated with planktonically grown cells is unknown (Sutherland, 1995). Nevertheless, in mixed-species biofilms, each component species will produce a different set of polymers, and these will merge to give heterogenous regions of polymers within the matrix (Cooksey, 1992). The physicochemical properties of the blended exopolymers will differ significantly from those of the purified components and will also be substantially affected by the ionic strength of the surrounding medium and the nature of the cationic species (Allison and Matthews, 1992).

Regulation of exopolymer synthesis

The synthesis of matrix polymers appears to be regulated by a variety of factors, of which surface attachment appears to be of particular importance. Thus, Davies *et al.* (1993) showed EPS (alginate) production to be de-repressed in biofilm cells compared with planktonics, and Evans *et al.* (1994) showed exopolysaccharide production to be increased at low growth rates and substantially higher for biofilm than planktonic cells. The latter effect would provide for increased exopolymer production within the slow-growing heart of a thick micro-colony/biofilm. This would alter the distribution and density of cells throughout the matrix, and once again confer some structural organization upon the community to provide customized micro-niches at various points within the biofilm (Costerton *et al.*, 1994a). Recently, it has also been suggested that in some Gram-negative organisms the production of exopolysaccharides, such as alginate, may be under the control of signal substances such as homoserine lactone (HSL). These are global regulators of transcriptional activation in bacteria (Williams *et al.*, 1992; Gambello *et al.*, 1993; Cooper *et al.*, 1995), responsible for cell-cell signaling, and implicated in cell-density-mediated events. In biofilms, signal substances such as HSL would become concentrated within the geometric center of the micro-colonies/biofilm, where cell physiology would be altered accordingly. The extent and nature of exopolymer production are also dependent upon physiological factors such as the relative availability of carbon and nitrogen (Sutherland, 1985).

Exopolymers and antimicrobial susceptibility

The exopolymers serve two major functions within biofilm communities. First, while not adhesins in their own right, exopolysaccharides are overproduced following the initial attachment of cells to surfaces. As such, they have been suggested to act as cements, which may reinforce the primary adhesion mechanisms (Allison and Sutherland, 1987). Second, the glycocalyx protects the cells contained within it against desiccation and against predatory phagocytic entities, such as white blood cells and protozoa, and may restrict the diffusion of agents from the surrounding medium through a combination of ionic-interaction and molecular-sieving events. Thus, in analogy to the penetration of agents through peptidoglycan (Marquis, 1968), the polymers of the glycocalyx may act as an ion-exchange resin where strongly charged molecules are actively removed from solution as they pass through. Incoming molecules would have to saturate all available binding sites, just as gentamicin must saturate all of the binding sites on the polysaccharide fibers before it can pass through a cellulose filter (Wagman *et al.*, 1975). While it has been suggested that the glycocalyx reduces access of antibiotics to the biofilm population in this manner (Gordon *et al.*, 1988; Nichols *et al.*, 1989), such effects are most pronounced for the activity of chemically, highly reactive biocides such as iodine and iodinepolyvinylpyrollidone complexes (Favero *et al.*, 1983). Access of such agents is substantially reduced by the presence of exopolymers which, in addition to acting as adsorption sites, will react chemically with, and neutralize, biocides.

The presence of high cell numbers within an exopolymer matrix will undoubtedly profoundly influence their access to molecules and ions, including protons (Costerton *et al.*, 1981). It is not surprising, therefore, that many groups of workers have suggested that the glycocalyx physically prevents access of antimicrobials to the cell surface, and that the recalcitrance of biofilms is purely and simply a matter of exclusion (Costerton *et al.*, 1987; Slack and Nichols, 1981; Suci *et al.*, 1994). Such universal explanations have been refuted (Gordon *et al.*, 1988; Nichols *et al.*, 1988, 1989), since reductions in the diffusion coefficients of antibiotics such as tobramycin and cefsulodin, within biofilms or microcolonies, are insufficient to account for the observed change in susceptibility. In this light, Gristina *et al.* (1989) compared the susceptibility of biofilms formed by slime-producing and non-slime-producing strains of *S. epidermidis*. Lack of any change suggested that the slime was not of great significance in antibiotic penetration. While a variety of antibiotic agents has been shown readily to perfuse biofilms (Dunne *et al.*, 1993) and to attain concentrations within the matrix that exceed the MIC/MBC observed for planktonic organisms (Darouchie *et al.*, 1994), there are also reports that sub-inhibitory concentrations of ß-lactams are selective of mucoid phenotypes (Govan, 1976). A solution to these apparent contradictions might be a reinforcement of the barrier properties of the glycocalyx through a local concentration, within the glycocalyx, of drug-inactivating enzymes such as beta-lactamases (Giwercman *et al.*, 1991). This will create marked concentration gradients of the antibiotic across them and protect, to some extent, the underlying cells.

Clearly, whether or not the glycocalyx constitutes a physical barrier to antibiotic penetration depends greatly upon the nature of the antibiotic, the binding capacity of the glycocalyx toward it, the levels of agent used therapeutically, and the rate of growth of the microcolony relative to the antibiotic diffusion rate (Kumon *et al.*, 1994). For antibiotics such as tobramycin and cefsoludin, therefore, such effects are suggested to be minimal (Nichols *et al.*, 1988, 1989), but for positively charged antibiotics such as the aminoglycosides, binding to the polyanionic matrix polymers is high and their access to cells impeded (Nichols *et al.*, 1988). Curiously, macrolide antibiotics, which are also positively charged but also very hydrophobic, are relatively unaffected by the presence of the exopolymers (Ichimiya *et al.*, 1994). Poor penetration through anionic matrices might therefore be a

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TABLE 2

RATIOS OF THE MINIMUM INHIBITORY CONCENTRATIONS (MIC) OF ANTIMICROBIAL AGENTS TOWARD BIOFILM AND PLANKTONIC POPULATIONS OF *Staphylococcus epidermidis* AND *Escherichia coli* AT VARIOUS TIMES AFTER INOCULATION OF THE PLANKTONIC PHASE (from Das *et al.*, 1995)

Micro-organism	Time (hrs)[1]	Biofilm:Planktonic MIC[2] Ratios			
		Phenoxy-ethanol	Cetrimide	PHMB[3]	Chloro-xylenol
S. epidermidis	0	1.00	1.14	2.86	1.00
	6	1.96	1.14	8.33	1.00
	14	1.92	1.14	7.14	0.97
	20	1.96	1.47	10.00	1.00
E. coli	0	1.00	0.94	2.03	1.00
	6	2.04	0.94	5.26	1.00
	14	2.00	0.94	6.25	1.00
	20	2.04	0.94	6.25	1.00

[1] Time between inoculation of the planktonic phase and addition of biocide.

[2] A ratio of greater than 1 indicates a higher MIC toward attached organisms and biofilms than toward planktonic cells.

[3] PHMB, polyhexamethylene biguanide (Vantocil).

phenomenon restricted to the more hydrophilic, positively charged agents.

Modification of exopolymer properties

The presence of adsorbed ions within the biofilm matrix polymers will affect its net charge and thereby its antibiotic-exclusion properties. In this respect, Hoyle *et al.* (1992) have shown the tobramycin-binding capacity of bacterial exopolysaccharides to be less important, in terms of reduced susceptibility, than is the reduction in diffusivity of the matrix imposed by Ca^{2+} condensation of the polymer. Such effects are dependent upon the nature of the adsorbed species and are not seen, for example, with Mg^{2+}. This might have profound effects upon the susceptibility of biofilms growing at different sites within the body or in patients with some predisposing clinical conditions (*i.e.*, cystic fibrosis, chronic renal failure, hypercalcemia) which create abnormal body chemistries which include markedly elevated calcium ion levels.

Novel treatment strategies

A potential application of these observations might be the use of adjuvants to alter the charge characteristics of the polymer matrix (Lee *et al.*, 1995), or the use of novel agents with non-lethal effects on exopolymer synthesis in combination with conventional antibiotics (Gagnon *et al.*, 1994; Pascual *et al.*, 1994).

Observation of the effects of charge on the functionality of the biofilm matrix in excluding antibiotic agents has led to various investigations of possible synergy between bioelectric treatments and antibiotics or biocides. Significant enhancements in killing action have been reported when low, direct-current fields (± 12 V/cm), at a low current density (± 2.1 mA/cm^2), were applied to biofilms together with various biocides. In this instance, several of these biocides were bactericidal against biofilms at concentrations that were less than the planktonic MIC (Blenkinsopp *et al.*, 1992; Costerton *et al.*, 1994b). Similar results have also been reported for the activity of antibiotics in combination with the bioelectric effect (Costerton *et al.*, 1994b). Modifications of implanted devices to facilitate bioelectric treatment are now in the developmental stages. Similar approaches to reducing the recalcitrance of biofilms through modification of the polymer matrix have involved the use of 67-kHz ultrasound (Pitt *et al.*, 1994) to produce synergy with gentamicin.

ANTIMICROBIAL SUSCEPTIBILITY AND ATTACHMENT-SPECIFIC PHYSIOLOGY

The possibility remains that bacteria are able to sense the presence of a surface to which they become attached, and, as a consequence, transcriptionally activate genes/operons to confer an attachment-specific phenotype which has a modified susceptibility toward antimicrobials. Variations of the 'bottle effect', whereby the metabolic activity of micro-organisms is stimulated by their attachment to surfaces, have been reported in the literature since the early 1940s (Zobell, 1943; Fletcher, 1984, 1986). Only recently, however, have attempts been made to define a genetic and physiological basis for such phenomena. Dagostino *et al.* (1991) utilized transposon mutagenesis to insert randomly into the chromosome of *E. coli* a marker gene which lacked its own promoter element. They then went on to isolate mutant cell lines which expressed the gene when attached to a polystyrene surface but not when grown on agar or in liquid media. The isolation of mutant cells such as these, with reporter genes that respond to attachment onto surfaces, has not diminished the level of debate as to the cause of surface-induced metabolic stimulation. This may reflect de-repression or induction of specific operons/genes, or it may be a physico-chemical manifestation (*i.e.*, localized concentration of nutrients, viscosity changes, pH effects, *etc.*) of the proximity of the surface (Van Loosdrecht *et al.*, 1990).

Evidence in favor of physico-cemical effects includes work on the regulation of lateral flagella gene transcription in *Vibrio parahaemolyticus*. This organism has been shown to produce a single polar flagellum in liquid, and numerous lateral, unsheathed flagella on solid culture media (Belas *et al.*, 1984, 1986; McCarter *et al.*, 1988). Changes in flagellation, in this instance, reflect an increased viscosity at the surface which restricts the movement of the polar flagellum and switches the *laf* genes. In a similar vein, Lee

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and Falkow (1990) recognized that reduced oxygen tension, as experienced by cells enveloped within a biofilm or in association with a surface, causes the triggering of expression of *Salmonella* invasins.

Other examples of surface-induced behavior defy such physico-chemical explanation. Thus, nitrilotriacetate does not adsorb to surfaces, but its breakdown is enhanced when the degradative organisms are attached to inert surfaces (McFeters *et al.*, 1990). This suggests increased expression of the degradative enzymes by attached cells. In a similar fashion, gliding bacteria do not synthesize extracellular polymers when they are grown in suspension culture (Humphrey *et al.*, 1979; Abbanat *et al.*, 1988), but do so rapidly after they become irreversibly bound to a surface.

On balance, it now seems probable that bacteria can sense the proximity of surfaces and, through cell density transcriptional activation (Cooper *et al.*, 1995), the proximity of other cells. Das *et al.* (1995) developed spectrophotometric methods which allowed for simultaneous monitoring of the growth of planktonic and biofilm bacteria within the wells of a microtiter plate. Bacterial cultures were exposed to a variety of agents at various times (from 0 to 20 hrs) following inoculation of the wells and the initiation of biofilm formation (Table 2). Minimum growth inhibitory concentrations were determined against the planktonic and attached subpopulations and expressed as activity ratios. The activities of both cetrimide and polyhexamethylene biguanide were affected immediately following bacterial attachment. In this respect, some bacterial cells were able to attach to the polystyrene surface and grow as a biofilm, while growth of the suspensions was inhibited (biofilm:planktonic MIC ratio > 1). Since there had been insufficient time for the formation of biofilms, then such data relate either to a decreased disposition of these agents at the colonized surface or to the expression of an attachment-specific phenotype. If biofilms were allowed to form for 6-20 hrs before exposure to biocide, then the MIC's were further reduced. Again, these incubation times are insufficient for an extensive biofilm to develop and might indicate the protective effects of microcolony formation. While it is likely that similar physiological responses, invoked as attachment-specific phenotypes, will affect antibiotic susceptibility, these have not been demonstrated.

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