Medical Implications of Biofilms

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Contents

CHAPTER ONE

Biofilm-Dependent Regulation of Gene Expression

Philippe Lejeune

1 INTRODUCTION

Microbial development and biofilm formation on implanted biomaterials and hospital equipment are important sources of nosocomial infections, mainly because surface-associated contaminants express biofilm-specific properties such as increased resistance to biocides, antibiotics, and immunological defences. Although it has long been recognised that the presence of a solid phase can influence many bacterial functions (ZoBell, 1943; Costerton et al., 1987; Van Loosdrecht et al., 1990), we are just beginning to understand the regulatory processes at the molecular level. There is no doubt that the identification of the structures involved in the sensing of the particular microenvironments encountered at interfaces and in developing biofilms and the description of the regulatory networks allowing the appropriate genetic responses will lead to the development of surface coatings and preventive or curative drugs able to deal with these life-threatening infections.

2 BIOFILM FORMATION IS A DEVELOPMENTAL PROCESS

An invidual bacterium present on, or introduced into, the human body can reach the surface of an indwelling medical device by three different mechanisms (Van Loosdrecht et al., 1990): passive transport due to air or liquid flow, diffusive transport resulting from Brownian motion, and active movement involving flagella. Although contact is, therefore, frequently a question of chance, chemotactic processes can direct motile bacteria in response to any concentration gradient that may exist in the interfacial region. Following contact, the next stage may be initial adhesion. This is mainly a physicochemical

process based on weak interactions between molecules of the solid phase (or ions and polymers adsorbed on the surface) and appropriate cell surface structures, such as fimbriae and adhesins.

Numerous studies with model bacteria have identified genes and functions required for adhesion, and a picture of the early stages of colonisation has begun to emerge. First, an individual bacterium that approaches a solid surface has to overcome possible repulsive forces and then interact with the solid phase. Depending on the strength of the bonds that the bacterium is able to form with the substrate, motility and gliding properties are often of crucial importance to initiate efficient attachment. Transposon mutations leading to the suppression of the adherent phenotype of *Pseudomonas aeruginosa* (O'Toole and Kolter, 1998a), *P. fluorescens* (O'Toole and Kolter, 1998b), *Vibrio cholerae* (Watnick and Kolter, 1999), *Salmonella enterica* Serovar Typhimurium (Mireles, Togashi, and Harshey, 2001), and the W3110 strain of *Escherichia coli* (Pratt and Kolter, 1998) have been found in genes involved in flagellar motility. Accordingly, a non-adherent phenotype could be detected after transposon inactivation of two types of bacterial gliding movement: twitching motility relying on type IV pilus extension and retraction in *P. aeruginosa* (O'Toole and Kolter, 1998a), and swarming due to overflagellation in *S. enterica* (Mireles et al., 2001). Time-lapse microscopic observations of *P. aeruginosa* adhesion confirmed that the organisms move along the surface before attachment, almost as if they are scanning for an appropriate location for initial contact (O'Toole, Kaplan, and Kolter, 2000).

The next step in early attachment events is an interaction between the surfaces of the bacterium and the material sufficiently strong to prevent disruption by convective forces or Brownian motion. It has been recognised for some time that the introduction of a clean substratum in a natural fluid is immediately followed by fast and efficient adsorption of organic molecules to the surface (ZoBell, 1943), forming a so-called conditioning film. Two types of interactions are then possible: weak chemical bonding between the bacterial envelope and the solid surface or the conditioning film, and bridging mediated by specialised bacterial attachment structures. The first link between the bacterium and the material is presumably a combination of weak chemical bonds, dipole interactions, and hydrophobic interactions (Marshall, 1992).

Different genetic strategies have been used to identify the structural components of the bacterial envelope involved in these interactions. In various species, many natural isolates are not able to adhere to abiotic surfaces under laboratory conditions. But the reservoir of cryptic functions is so large that cyclic flow experiments (which exercise a strong selective pressure in favour of

adherent mutant cells) can easily reveal potential adhesion structures (Le Thi et al., 2001). Such an approach was followed to isolate a point mutation in a regulatory gene of *E. coli* K-12, which resulted in the overproduction of curli, a particular type of thin and flexible fimbrium, and allowed the overproducing strains to adhere to any type of material (Vidal et al., 1998). As *E. coli* is the most common bacterium found in biofilms that have developed on catheters introduced into the urinary tract, immunological and genetic studies were undertaken to investigate the role of curli in clinical strains isolated from patients with catheter-related infections. Immunogold labelling with curlin antibodies revealed the constitutive production of these fimbriae at the surface of the bacteria, and transduction of knock-out mutations in the curli-encoding genes demonstrated their essential role in adhesion to biomaterials (Vidal et al., 1998; Prigent-Combaret et al., 2000). Therefore, curli synthesis by other pathogenic strains, such as O157:H7 (Uhlich et al., 2002), has to be regarded as a potential biofilm-forming character.

Identification of bacterial structures of attachment has also been performed by transposon mutagenesis followed by screening for non-adherent clones. In the Gram-positive species *Staphylococcus epidermidis* (Heilmann et al., 1997), *Streptococcus gordonii* (Loo, Corliss, and Ganeshkumar, 2000), and *Staphylococcus aureus* (Cucarella et al., 2001), this approach allowed the characterisation of new envelope proteins involved in surface colonisation. Using a similar type of screening, Vallet and co-workers (2001) detected a new fimbrial adhesin of *P. aeruginosa* and demonstrated the requirement of a periplasmic chaperone involved in pilus synthesis via the so-called chaperone/usher pathway. Similar studies in *E. coli* revealed the importance of accurate lipopolysaccharide synthesis for bacterial attachment (Genevaux et al., 1999a) and identified type I fimbriae as another structure able to promote adhesion (Pratt and Kolter, 1998). Interestingly, the physicochemical processes of *E. coli* adhesion mediated by curli and type I fimbriae are clearly different, since type I pilus-associated attachment requires flagellar motility (Pratt and Kolter, 1998), whereas adhesion mediated by curli is independent of strain motility (Prigent-Combaret et al., 2000).

Recently, Ghigo (2001) demonstrated that the conjugative pili encoded by transferable plasmids (including F) of several incompatibility groups could by themselves act as adhesion factors and promote biofilm development. Furthermore, plasmid transfers by conjugation seemed to be favoured in biofilms (Hausner and Wuertz, 1999; Ghigo, 2001). These observations are of great medical and evolutionary significance because they raise the question of the role of biofilms as a place for the evolution of structures for bacterial

interactions and for the horizontal spreading of genes, such as those encoding antibiotic resistance mechanisms.

When an individual bacterium has reached an abiotic surface and is immobilised by relatively firm links, a cascade of physiological changes is initiated. More than 10 years ago, Van Loosdrecht and co-workers (1990) published an exhaustive review of the early literature on the influence of surfaces on microbial behaviour. Changes in global functions, such as growth rate, respiration, and assimilation, could be correlated with substratum-attached growth. More recently, the use of microscopy and reporter gene techniques to quantify gene expression in biofilms clearly established that precise changes in gene expression are triggered during the transition between the free-living and attached states (for a review of these methods, see Prigent-Combaret and Lejeune, 1999).

One of the first features of this transition is of pivotal importance for biofilm development. As fimbriae- and adhesin-mediated interactions are relatively weak, the early stages of the adhesion process are generally reversible. For a bacterium immobilized at the solid–liquid interface, the 'choice' of further surface-associated growth involves multiplication and production of extracellular polymers, resulting in the formation of a first slimy layer on the substratum. In *P. aeruginosa* biofilms, the bacteria are embedded in a polymer matrix that is composed mainly of alginate. Davies and co-workers (1993, 1995) examined the expression of *algC*, a gene required for alginate synthesis, within individual colonising cells. As early as 15 minutes after the initial attachment, they observed an activation of *algC* expression.

By using a library of *lacZ* fusions and a colourimetric assay based on biofilm development in the wells of microtitre plates, Prigent-Combaret and co-workers (1999) showed that the expression of about 40 per cent of the genes of an *E. coli* biofilm-forming strain was modified during the colonisation process. As in *P. aeruginosa*, the synthesis of colanic acid, a major matrix exopolysaccharide, was induced in the biofilm-grown cells. They also observed that the synthesis of the flagella was stopped in the attached cells by downregulation of the *fliC* gene encoding the flagellar structural protein.

Proteome and transcriptome analysis in *P. putida* and *P. aeruginosa* (Table 1.1) recently confirmed the deep physiological changes induced upon bacterial contact with a surface (Sauer and Camper, 2001; Whiteley et al., 2001). These studies also gave further insights into the differences between free-living cells and those in biofilms (discussed later). Analyses of protein and gene expression at different time points suggested that the colonising bacteria undergo a succession of physiological states, which could be

Table 1.1: Selected genes and proteins differentially expressed in biofilms developed on abiotic surfaces

Gene or Locus Number	Protein	Function	Expression[a]	Organism	Reference
Exopolysaccharide and lipopolysaccharide production					
algC		Alginate synthesis	Up	*P. aeruginosa*	Davies, Chakrabarty, and Geesey, 1993
wcaB		Colanic acid synthesis	Up	*E. coli*	Prigent-Combaret et al., 1999
mucC		Negative regulator for alginate synthesis	Up	*P. putida*	Sauer and Camper, 2001
lpxD	UDP-3-0[hydroxylauroyl] glucosamine *N*-acetyltransferase	Lipopolysaccharide synthesis	Up	*P. putida*	Sauer and Camper, 2001
wbpG		Lipopolysaccharide synthesis	Up	*P. putida*	Sauer and Camper, 2001
Motility and attachment					
fliC	Flagellin	Flagellar synthesis	Down	*E. coli*	Prigent-Combaret et al., 1999
csgA	Curlin	Curli synthesis	Up	*E. coli*	Prigent-Combaret, 2000
fleN		Flagellar synthesis regulator	Down	*P. putida*	Sauer and Camper, 2001
flgG	Flagellar basal body rod protein	Flagellar synthesis	Down	*P. putida*	Sauer and Camper, 2001
pilC		Type IV fimbrial synthesis	Up	*P. putida*	Sauer and Camper, 2001
pilK	Methyltransferase CheR homolog	Chemotactism	Up	*P. putida*	Sauer and Camper, 2001
PA2128	Probable fimbrial protein	Fimbrial synthesis	Down	*P. aeruginosa*	Whiteley et al., 2001
pilA	Pilin protein	Fimbrial synthesis	Down	*P. aeruginosa*	Whiteley et al., 2001
flgD		Flagellar basal body modification	Down	*P. aeruginosa*	Whiteley et al., 2001

(*continued*)

Table 1.1 (*continued*)

Gene or Locus Number	Protein	Function	Expression[a]	Organism	Reference
PA2129	Probable pili assembly chaperone	Fimbrial synthesis	Down	*P. aeruginosa*	Whiteley et al., 2001
PA1092	Flagellin type B	Flagellar synthesis	Down	*P. aeruginosa*	Whiteley et al., 2001
fliD	Flagellar capping protein	Flagellar synthesis	Down	*P. aeruginosa*	Whiteley et al., 2001
flgE	Flagellar hook protein	Flagellar synthesis	Down	*P. aeruginosa*	Whiteley et al., 2001
Membrane proteins, secretion, and transport					
ompC		Porin	Up	*E. coli*	Prigent-Combaret et al., 1999
proU	Transport system of glycine betaine	Adaptation to osmotic changes	Up	*E. coli*	Prigent-Combaret et al., 1999
nikA		Transport of nickel	Up	*E. coli*	Prigent-Combaret et al., 1999
nlpD	Outer membrane lipoprotein		Up	*P. putida*	Sauer and Camper, 2001
potB		ABC transporter	Up	*P. putida*	Sauer and Camper, 2001
ybaL		Probable K^+ efflux transporter	Up	*P. putida*	Sauer and Camper, 2001
xcpS	General secretion pathway protein F		Up	*P. putida*	Sauer and Camper, 2001
tatA		Translocation protein	Up	*P. aeruginosa*	Whiteley et al., 2001
tatB		Translocation protein	Up	*P. aeruginosa*	Whiteley et al., 2001
tolA		Involved in lipopolysaccharide (LPS) synthesis	Up	*P. aeruginosa*	Whiteley et al., 2001
omlA	Outer membrane lipoprotein		Up	*P. aeruginosa*	Whiteley et al., 2001

PA3038		Probable porin	Down	*P. aeruginosa*	Whiteley et al., 2001
PA1710		Type III secretion central regulator	Down	*P. aeruginosa*	Whiteley et al., 2001
PA3234		Probable sodium:solute symporter	Down	*P. aeruginosa*	Whiteley et al., 2001
Carbon and nitrogen catabolism					
pepT	Tripeptidase T		Up	*E. coli*	Prigent-Combaret et al., 1999
PA2015	Probable acyl-coenzyme A dehydrogenase		Down	*P. putida*	Sauer and Camper, 2001
chiC	Chitinase		Up	*P. putida*	Sauer and Camper, 2001
PA4867	Urease β subunit		Up	*P. aeruginosa*	Whiteley et al., 2001
PA3584	Glycerol-3-phosphate dehydrogenase		Down	*P. aeruginosa*	Whiteley et al., 2001
PA0108	Cytochrome *c* oxidase, subunit III		Down	*P. aeruginosa*	Whiteley et al., 2001
PA0105	Cytochrome *c* oxidase, subunit II		Down	*P. aeruginosa*	Whiteley et al., 2001
PA0106	Cytochrome *c* oxidase, subunit I		Down	*P. aeruginosa*	Whiteley et al., 2001
PA3418	Leucine dehydrogenase		Down	*P. aeruginosa*	Whiteley et al., 2001
Transcription and translation					
asnB	Probable asparagine synthetase		Down	*P. putida*	Sauer and Camper, 2001
leuS	Leucyl-tRNA synthase		Down	*P. putida*	Sauer and Camper, 2001
PA5316	50S ribosomal protein L28		Up	*P. aeruginosa*	Whiteley et al., 2001
PA3742	50S ribosomal protein L19		Up	*P. aeruginosa*	Whiteley et al., 2001

(*continued*)

Table 1.1 (*continued*)

Gene or Locus Number	Protein	Function	Expression[a]	Organism	Reference
PA4262	50S ribosomal protein L4		Up	*P. aeruginosa*	Whiteley et al., 2001
PA4247	50S ribosomal protein L18		Up	*P. aeruginosa*	Whiteley et al., 2001
PA4261	50S ribosomal protein L23		Up	*P. aeruginosa*	Whiteley et al., 2001
PA4267	30S ribosomal protein S7		Up	*P. aeruginosa*	Whiteley et al., 2001
PA4744	Translation initiation factor IF-2		Up	*P. aeruginosa*	Whiteley et al., 2001
PA3049		Ribosome modulation factor	Down	*P. aeruginosa*	Whiteley et al., 2001
PA2620	ATP-binding protease component ClpA		Down	*P. aeruginosa*	Whiteley et al., 2001
rpoH		σ-factor	Up	*P. aeruginosa*	Whiteley et al., 2001
rpoS		σ-factor	Down	*P. aeruginosa*	Whiteley et al., 2001
Drug resistance					
ampC	β-lactamase	Ampicillin resistance	Up	*P. putida*	Sauer and Camper, 2001
mexB		Multidrug efflux pump	Up	*P. putida*	Sauer and Camper, 2001
str	Streptomycin 3′-phosphotransferase	Streptomycin resistance	Up	*P. putida*	Sauer and Camper, 2001

[a] Up means activated in biofilm. Down means repressed in biofilm.

compared to a sequential process of development. During the early stages, the individual microorganisms have to sense physicochemical differences at the solid–liquid interface. Later, as the matrix develops and the attached population multiplies, intercellular communications can become progressively operative.

A hallmark of a mature biofilm is its ordered architecture consisting of large mushroom-shaped colonies interspersed among less dense channels in which liquid flow has been measured (for a review, see Costerton et al., 1994). Such organisation suggests that the intercellular signalling takeover is a key to the last episode of biofilm development. In natural conditions, the construction of these ordered multicellular structures involves collective properties, such as positive and negative tropisms, cell aggregation and dispersion, cell-to-cell activation, and repression of subsets of the genome. These processes are obviously very complex and could require an unexpected number of genes and functions.

To date, only one type of signalling process – quorum sensing – has been described in biofilms. In a seminal study, Davies and co-workers (1998) demonstrated that the quorum-sensing molecules, typically acylated homoserine lactones (acyl-HSLs), are involved in biofilm maturation. These signal molecules accumulate in the bacterial environment as a function of cell number and mediate population-density-dependent gene expression. A *P. aeruginosa* mutant defective for the *lasI* system of acyl-HSL production, although still capable of early cell–surface interactions, did not develop the structural organisation of a wild-type biofilm. The *lasI* mutant biofilm was much thinner, more crowded, and sensitive to the biocide sodium dodecyl sulphate (SDS). Addition of the acyl-HSL normally produced by the *lasI* system restored wild-type biofilm architecture and SDS resistance. Furthermore, the loss of SDS resistance by the *lasI* mutant occurred despite the lack of any change in exopolysaccharide production, indicating that the biocide resistance in wild-type biofilms is a result of cellular adaptation rather than a limitation of SDS diffusion in the polymer matrix. Acyl-HSLs have also been detected in natural biofilms developed on urethral catheters removed from patients (Stickler et al., 1998) and on immersed stones from the San Marcos River in Texas (McLean et al., 1997).

3 SURFACE-MODULATED FUNCTIONS

Examples of differential gene and protein expression in biofilms and free-living cells are summarized in Table 1.1. A clear trend is the repression of flagellum synthesis observed in *E. coli, P. putida,* and *P. aeruginosa.* In *E. coli,* the

loss of flagella in the attached cells was confirmed by electron microscopy (Prigent-Combaret et al., 1999). A second trend is the activation of functions involved in the colonisation process itself, such as exopolysacharide production and oversynthesis of adhesion structures (curli and lipopolysaccharide). These common features strongly suggest that the biofilm-forming bacteria are equipped with specialised recognition structures that enable them to perceive their contact with abiotic materials.

Many genes in Table 1.1 are involved in growth functions (such as assimilation, transport, and ribosome building), indicating strong differences between the planktonic and the biofilm growth modes. However, because of heterogeneous conditions within the biofilms, these differences have probably been underestimated. A complete understanding of the construction of a mature biofilm architecture will require considerably more research. There is no doubt that gradients or local limitations of nutrients and oxygen are responsible for a large number of different gene expression patterns. Interesting processes will be occurring only in particular niches and therefore are impossible to investigate with global methods. The identification of the switching mechanisms allowing bacteria to leave the biofilm and return to the planktonic status as individual cells is an example of an important challenge for future research.

Nevertheless, the results presented in Table1.1 give interesting insights into one of the most detrimental properties of biofilm-grown cells – increased resistance to biocides and antibiotics. As mentioned previously, the SDS resistance of *P. aeruginosa* biofilms has been associated with quorum-sensing mechanisms. In *P. putida*, the expression of *mexB* (encoding a component of the antibiotic efflux system), a streptomycin resistance *str* gene, and the expression of the β-lactamase *ampC* gene were found to be surface induced (Table 1.1). In *P. aeruginosa*, the major aminoglycoside-resistance mechanism is impermeability to antibiotic entrance. This impermeability involves several factors, including the *tolA* gene and terminal electron transport proteins (Whiteley et al., 2001). The *tolA* gene product affects lipopolysaccharide structure and aminoglycoside affinity for the outer membrane. Since mutants that underproduce TolA are hypersensitive to aminoglycoside, *tolA* activation in *P. aeruginosa* biofilms (Table 1.1) could contribute to aminoglycoside resistance. Moreover, repression of cytochrome *c* oxidase in biofilms (Table 1.1) could be regarded as an additional factor contributing to increased resistance to aminoglycosides (Whiteley et al., 2001). Several other surface-modulated functions in Table 1.1, for example, porin synthesis, might also be candidates for antibiotic resistance factors.

4 ABIOTIC SURFACE SENSING

The construction and the physiology of the ordered structure of mature biofilms has to be understood mainly in terms of cell-to-cell signalling. In contrast, the initial stages of surface contamination concern individual cells and have to be described at the level of the intracellular signalling events. As mentioned previously, a pioneer bacterium must sense its contact with the material in order to trigger its transformation from a swimming cell to a surface colonising cell. Two questions have to be answered in this process. What physicochemical parameters are sufficiently different to enable the bacterium to discriminate the liquid phase and the interface? And what cellular structures are able to recognise these differences and transmit the information to the genome?

As it is well established that in *E. coli* there is a quasilinear relationship between the osmolarity of the external medium and the intracellular concentration of potassium (Epstein and Schultz, 1965), Prigent-Combaret and coworkers (1999) compared the intracellular K^+ concentration of planktonic and biofilm cell populations. Ten hours after inoculation into the culture vessel, the attached bacteria displayed a significantly higher internal K^+ concentration than the planktonic cells. This result indicates that the osmolarity of the microenvironment surrounding the two types of bacteria was higher around the attached cells. In my opinion, this observation can have two non-exclusive explanations. First, the electric charges present at the surface and on the organic molecules adsorbed on it decrease water activity (that is, the proportion of water molecules acting as pure solvent) at the liquid–solid interface. This layer of 'different water' is actually very thin with regard to the dimensions of the bacterium and its appendages, but could activate some sensors located on that region of the cell envelope, which faces the abiotic surface. Second, a bacterium that becomes immobilised on a surface is subjected to considerably lower convective forces than a swimming cell. It is therefore conceivable that fimbrial breaking and dispersion could be reduced in these confinement conditions. As a result, fimbrial density would increase around the cell. This situation may have two important consequences: an additional decrease of water activity in the immediate vicinity of the cell, due to the electric charges existing on the fimbriae, and saturation of the processes of fimbrial construction, with subsequent accumulation of monomers in the bacterial periplasm. In all cases, the osmolarity of the periplasm and the microenvironment of the cell would increase.

Recent genetic studies may support these hypotheses and answer the second question about the cellular recognition structures. The curli-mediated adherence of *E. coli* depends on the integrity of two signal transduction systems:

EnvZ/OmpR (Vidal et al., 1998) and CpxA/CpxR (Dorel et al., 1999). Through a complex regulatory network, these systems control the expression of several genes, including those encoding curli (Prigent-Combaret et al., 2001), in response to two types of signals: medium osmolarity and periplasmic accumulation of non-secreted proteins.

Increasing osmolarity of the environment activates the sensor protein EnvZ and leads to improved phosphorylation of OmpR, resulting in modulation of its binding ability to the regulatory sequences of the target genes. In addition to curli synthesis, flagellum and colanic acid production is also controlled by the EnvZ/OmpR two-component system (Prigent-Combaret et al., 1999). Sensing of interfacial osmolarity changes through EnvZ/OmpR could therefore constitute a major part of the transition process that the bacterium undergoes upon contact with a surface.

Although clearly established (Dorel et al., 1999), the role of the CpxA/CpxR sensor-regulator system in colonisation is more difficult to understand. By unknown sensing mechanisms, this system is involved in recognition of periplasm saturation with 'useless' proteins, such as non-secreted monomers or non-addressed outer membrane proteins, and is able to trigger an appropriate scavenging response. For example, the outer membrane lipoprotein NlpE is known to activate the Cpx pathway when overproduced from a multicopy plasmid (Danese et al., 1995). Dorel and co-workers (1999) have observed that transposon insertions in the *cpxA* gene, as well as NlpE overproduction, strongly reduced curli gene expression and adherence. It is therefore conceivable that the CpxA/CpxR system constitutes another part of the *E. coli* surface-sensing machinery and could be activated by periplasmic accumulation of monomers when the external density of fimbriae on the surface of the immobilised cell is increased by electrical and mechanical interactions that remain to be explored.

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