Finding gene-expression patterns in bacterial biofilms.

Christophe Beloin, Jean-Marc Ghigo

Finding gene expression patterns in bacterial biofilms

Christophe BELOIN and Jean-Marc GHIGO*

Groupe de Génétique des Biofilms

Institut Pasteur

CNRS URA 2172

25 rue du Dr. Roux, 75724 Paris CEDEX 15, France

** Corresponding author:*

E-mail address: jmghigo@pasteur.fr

Tel: 33 (0)1 40 61 39 17.

Fax: 33 (0)1 45 68 87 90.

- **Abstract**

Biofilm is a bacterial lifestyle that is thought to require or involve a differential gene expression compared to that of planktonic bacteria. Recently, we have witnessed a change of focus from the simple hunt for hypothetical essential biofilm genes to the identification of late and more complex biofilm functions. However, finding common bacterial biofilm gene expression patterns through global expression analysis is still difficult. Owing to the apparently minimal overlap between functions involved in biofilm formation by different bacteria, exploring the biofilm lifestyle could prove to be a case-by-case task for which global approaches show their limits.

The study of biofilms is still a wide-open area of investigation, influenced by the hypothesis that the phenotypic changes observed in microorganisms as they attach to surfaces are due to the differential expression of genes within biofilms [1].

Genetic analyses have revealed the diversity of genetic factors participating in biofilm formation and there are undoubtly multiple pathways to build a biofilm [2]. These factors, especially when they are involved in the early stages of biofilm formation, can often be functionally replaced or overridden by others, depending on the media and growth conditions [3]. Therefore, although the study of initial attachment probably still holds some surprises, the quest for an essential adhesion step may be in vain.

Recently we witnessed a change of focus from the simple hunt for genes involved in the initial step of adhesion toward the identification, through global analysis, of late biofilm functions.

Evidence for differential gene expression in biofilms

Early evidence of differential gene expression within a bacterial biofilm came from gene fusion studies which suggested that the expression of up to 38% of the *E. coli* bacterial genome may be affected by biofilm formation [4]. However, it is likely that the extent of gene expression required to induce the formation of a biofilm may not be of that large of a magnitude, nor require genetic re-programming, as the most recent DNA array analyses performed with different bacterial biofilm models show that only a small proportion of the genome (1 to 15%) undergoes a significant change in expression compared with a non-biofilm mode of growth [5-9].

These studies have generated the hope that it may be possible to identify a common universal gene expression pattern within bacterial biofilms. This postulate has received a lot

of attention because the identification of such a pattern could allow one to monitor, or control, this lifestyle in situations of economic or clinical relevance.

The smallest common denominator in biofilm gene expression: hardly a trend

Significant progress has been made toward the understanding of biofilm gene expression. However, due to the absence of experimental gold standards, extracting a biofilm gene expression pattern from the available data is still difficult. Below are briefly presented what constitute, in our opinion, the strongest trends, or the very smallest common denominator between all the studies that have been done on bacterial biofilms.

The switch from a planktonic to an attached lifestyle

Whereas the requirement of flagellar motility in the early stages of biofilm formation remains controversial [10,11], different reports show that flagella might not be required within a mature biofilm. Accordingly, genes encoding components of the flagellum are repressed soon after the bacteria reach the surface [4,8,12]. Therefore, the repression of flagellar gene expression may be one of the first and best documented examples of genetic "reprogramming" leading to the sessile lifestyle.

Expressing genes for polysaccharide production

Rich in water, the matrix is a complex milieu implicated in air-liquid pellicle formation, as well as solid surface-associated biofilm formation. Many biofilm matrix polysaccharidic components have been identified recently. Beside the PIA/PNAG polymer encoded by the *icaABCD* locus in *Staphylococcus aureus* and *epidermidis*, Gram-negative bacteria components such as colanic acid (*E. coli*), alginate, glucose and mannose rich Pel and Pls matrix components (*P. aeruginosa*), cellulose and β-1,6-GlcNac polymer (*Salmonella* and *E. coli*) have been reported to play important roles for biofilm formation [13-18]. These extracellular polysaccharides are key elements that shape and provide structural support for

bacterial biofilms. However, most of the questions regarding the temporal and spatial regulation of exopolysaccharide production are still unanswered.

The stationary phase-like character of the biofilm

Biochemical and genetic evidence support the hypothesis that bacteria probably face different conditions within a biofilm as compared to during planktonic growth [4,19,20]. Most of the biofilm population that is not in direct contact with the nutrient fluids will likely be subjected to progressive microaerobic conditions, increased osmotic pressure, pH variation and decreased nutrient accessibility. In *E. coli*, a significant part of the biofilm response involves stationary phase induced genes [6,7]. In wild type *B. subtilis,* among the 121 biofilm induced genes that have a known function, 60% of them are activated during sporulation, a phenomenon that is induced by starvation conditions encountered in stationary phase [21]. However, depending on the experimental conditions, the expression of the stationary phase sigma factor, *rpoS*, has been shown to be either repressed by 2-3 fold, or slightly activated, in biofilms in *P. aeruginosa* [9,22] and the role of *E. coli rpoS* in biofilms remains much debated [6,23,24]. Nevertheless, biofilm conditions often have strong similarities with conditions that prevail in stationary phase (planktonic) cultures.

Activation of stress-induced pathways within biofilms

In contrast to the notion that biofilms may represent a protection against environmental stresses, there is now ample evidence that bacteria develop stress responses within biofilms (see Table 1). While this could suggest that living in a biofilm has a cost, it also constitutes one of the major genetic signatures of the biofilm lifestyle. The activation of some stress pathways, like the *cpx* or *rcs* pathways, has been associated with functions such as surface-sensing through the perception of membrane perturbation [7,25,26]. Membrane stress, triggered by bacteria-surface and bacteria-bacteria interactions, could therefore constitute a

natural signal for the activation of several regulatory pathways that would promote stabilization and/or maturation of the biofilm. However, the exact role of these stress-responses in the formation and physiology of mature biofilms remains an open question.

The prevalence of genes of unknown function in biofilm differentially expressed genes

Biofilms are considered to be environments where new, or previously unrecognized, biological properties could be expressed. Thus, it was initially expected that many genes with unknown function could play a role in this lifestyle. Global analyses of gene expression confirmed that genes of unknown function often represent the largest group of genes differentially expressed in biofilms (30 to 50%) [7-9]. However, this proportion is not overwhelming and, more often than not, even slightly lower than the overall percentage of such genes in the corresponding bacterial genome. Therefore, although it is likely that new aspects of bacterial biology are expressed during biofilm formation, so far, the harvest of totally new biofilm-related functions has been relatively meager.

The regulatory circuits involved in the biofilm lifestyle: everybody for themselves?

So far, the search for a unifying biofilm gene expression pattern has been rather unsuccessful. This is particularly the case when it comes to key regulatory pathways. Indeed, compared with some expectations, the numbers of new regulatory pathways that have been associated with the biofilm lifestyle are relatively modest and none of them has been demonstrated to be specific nor required in all biofilm situations (see Table 2). This may indicate that key proteins in putative biofilm signaling pathways are yet to be discovered because they are maybe modified in quality rather than in quantity. For example, some key components could be regulated through phosphorylation cascades that are not detected in global expression analyses. The detailed determination of the biofilm phosphoproteome may give us a clearer view of key biofilm regulatory pathways. However, highly transient

regulatory events may still remain elusive unless independent knowledge of the network of co-regulated genes provides us with some clues to identify the regulator itself.

Is each biofilm unique?

Why is it so difficult to find a trend among all the studies that have been performed, even with the same bacteria (*P. aeruginosa, E. coli)* in reasonably similar experimental models? It was to be expected that a biofilm formed in a stream would be different from one formed on a medical implant. However, it comes as quite a surprise that three recent transcriptome analyses on genes overexpressed in *E. coli* biofilms share only 2 genes in common [5-7]. Hence, not only is what is true for *P. aeruginosa* not true for *E. coli* but what is true for *E. coli* K-12 in one experimental model may not be true for *E. coli* K-12 in another experimental model. If this is confirmed by further studies, one has to seriously consider the possibility that each biofilm may be a world of its own.

Dealing with biofilm complexity

Think locally?

Whether there are patterns to be found or not, the heterogeneity that prevails within a biofilm often precludes drawing very insightful conclusions from global analyses. While this intrinsic heterogeneity represents a known major difficulty, one also has to acknowledge that the approaches used so far are probably not well adapted to reveal the spatial complexity of the biofilm lifestyle. Functions resulting from localized (niche within heterogeneous biofilms) or transient gene expression may prove to play a greater role than currently recognized. For example, phase variation seems to be a common phenomenon that regulates different processes such as adhesion [27].

Exploring the function of these genes may require physiologically relevant alternatives to traditional molecular biology methods. Systematic over-expression studies may offer new

insights into the role of genes with unknown functions in biofilm formation [28]. Strategies adapted to the study of highly heterogeneous environments such as *in vivo* or promoter-trap based strategies (STM, IVET, RIVET) [29] or gene-targeted *gfp* fusions have been under-used and will also certainly be helpful identifying genes only transiently expressed within biofilm sub-populations.

Minding the timing?

Whereas the spatial complexity of the biofilm has often been fully acknowledged, the dynamic dimension of gene expression within biofilm is a relatively new area of study. The transcriptome and proteome analyses performed on biofilms taken at different ages demonstrated that gene expression is changing along time. For example, in *B. subtilis* more than 55% of the differentially expressed genes in biofilm versus planktonic cultures were actually expressed at only one time point [8]. The observation of the protein content of a *P. putida* biofilm after 4, 6, 12 and 24h of growth also clearly demonstrated that protein expression changed overtime [12]. By analogy with the cell structure information obtained by 3D reconstitution out of 2D slice cell imaging, the temporal dimension of gene expression within biofilm suggests that thorough temporal analysis, rather that genome-wide transcription snap shot could be most informative, all other experimental conditions than time being equal.

Conclusions

The field is progressively leaving infancy and is now dealing with functions expressed after the biofilm is formed. However, the existence of a universal biofilm gene expression pattern is still questionable and the search for a single gene whose inhibition would lead to biofilm control may be hopeless. Exploring the biofilm lifestyle could then prove to be a case-by-case task and, because building a shared gold standard may be a sensible but unpractical

approach, restricted in scope and significance to specific bacteria in specific situations. Furthermore, success in dealing with the diversity at hand in biofilms is likely to require new approaches. Our capacity to use these new developments to decipher pure culture biofilm will also condition our capacity to understand even more complex microbial environment such as mixed species biofilms.

Acknowledgments

We thank the referees for their very constructive suggestions. We thank S. Da Re, B. Molles and B. Lakowski for helpful comments and critical reading of the manuscript. J.-M. G. and C. B. are supported by the Institut Pasteur, Paris, France and CNRS URA2172 grants.

Table 1: Example of stress responses induced within biofilms

Function	Genes/Proteins	Organism	Refs
Prophages	Pf1	*P. aeruginosa*	[9]
	PBSX	*B. subtilis*	[8,21]
Proteases	Clp proteins	*L. monocytogenes*	[30]
DNA repair	RecO	*L. monocytogenes*	[31]
SOS response	RecA, DinI, SulA	*E. coli*	[7]
Chaperons	DnaK, DnaJ	*E. coli*	[7]
Heat shock	HtpX, HtpG	*E. coli*	[7]
Oxydation stress	Sod proteins, CysK	*L. monocytogenes*	[31]
	SodB	*P. aeruginosa*	[32]
	SoxS	*E. coli*	[5]
Envelope stress	*cpx* and *rpoE* pathways	*E. coli*	[7,25]
	psp pathway	*E. coli* and *S. typhimurium*	[7,33]
Sigma factor	σ^W-mediated response	*B. subtilis*	[8]

Table 2: Regulatory circuits involved in biofilm formation

Regulator	Bacterial species	Function in biofilm formation	Refs
Gram negative bacteria			
barA/uvrY	*E. coli*	Activates biofilm formation	[36]
cpxRA	*E. coli*	Senses surface perturbation and required for optimal cell to cell interactions	[7,25]
crp	*E. coli*	Represses biofilm formation (catabolite repression)	[34]
csrAB	*E. coli*	Represses biofilm formation and activates detachment	[34]
hns	*E. coli*	Reduces adhesion in anoxic conditions	[40]
ompR/envZ	*E. coli*	Increases attachment *via* curli and cellulose gene activation	[20]
rcsB-yojN-rcsC	*E. coli*	Activates biofilm formation *via* remodeling of cell surface composition	[26]
rpoS	*E. coli* *P. aeruginosa*	Reduces or increases depth of the biofilm Reduces depth of the biofilm	[6,23,24] [9,11,22]
crc	*P. aeruginosa*	Required for normal biofilm development (activation of type IV motility)	[35]
gacAS	*P. aeruginosa*	Required for microcolonies formation	[37]
mvaT	*P. aeruginosa*	Reduces adhesion to abiotic surfaces *via cup* gene repression	[41]
rpoN	*P. aeruginosa* *V. fisheri*	Role in initial adhesion and biofilm architecture Role in biofilm architecture	[38] [39]
Gram positive bacteria			
abrB	*B. subtilis*	Represses biofilm formation	[21]
ccpA	*B. subtilis* *S. mutans*	Reduces depth of the biofilm (catabolite repression) Necessary for full biofilm maturation	[8] [42]
spo0A	*B. subtilis*	Required for mature biofilm formation (repress *abrB*)	[8,43]
spo0H	*B. subtilis*	Required for mature biofilm formation	[43]
arlRS	*S. aureus*	Reduces primary adherence to polystyrene	[48]
rbf	*S. aureus*	Required for mature biofilm formation on abiotic surfaces	[50]
sarA	*S. aureus*	Activates biofilm formation *via* PIA/PNAG activation	[44,45]
sigmaB	*S. epidermidis*	Activates biofilm formation	[46]
bfrAB	*S. gordonii*	Activates PVC and saliva-coated hydroxyapatite biofilm formation	[47]
hk11/rr11	*S. mutans*	Role in biofilm architecture	[49]
brpA	*S. mutans*	Required for mature biofilm formation on abiotic surfaces	[42]

Quorum-sensing issues have been deliberately omitted (see P. Greenberg's opinion in this issue)

References

1 O'Toole, G. et al. (2000) Biofilm formation as microbial development. *Annu Rev Microbiol* 54, 49-79

2 O'Toole, G.A. (2003) To build a biofilm. *J Bacteriol* 185 (9), 2687-2689

3 Vallet, I. et al. (2001) The chaperone/usher pathways of *Pseudomonas aeruginosa*: identification of fimbrial gene clusters (*cup*) and their involvement in biofilm formation. *Proc Natl Acad Sci U S A* 98 (12), 6911-6916

4 Prigent-Combaret, C. et al. (1999) Abiotic surface sensing and biofilm-dependent regulation of gene expression in *Escherichia coli*. *J Bacteriol* 181 (19), 5993-6002

5 Ren, D. et al. (2004) Gene expression in *Escherichia coli* biofilms. *Appl Microbiol Biotechnol* 64 (4), 515-524

6 Schembri, M.A. et al. (2003) Global gene expression in *Escherichia coli* biofilms. *Mol Microbiol* 48 (1), 253-267

7 Beloin, C. et al. (2004) Global impact of mature biofilm lifestyle on *Escherichia coli* K-12 gene expression. *Mol Microbiol* 51 (3), 659-674

8 Stanley, N.R. et al. (2003) Identification of catabolite repression as a physiological regulator of biofilm formation by *Bacillus subtilis* by use of DNA microarrays. *J Bacteriol* 185 (6), 1951-1957

9 Whiteley, M. et al. (2001) Gene expression in *Pseudomonas aeruginosa* biofilms. *Nature* 413 (6858), 860-864

10 Pratt, L.A. and Kolter, R. (1998) Genetic analysis of *Escherichia coli* biofilm formation: roles of flagella, motility, chemotaxis and type I pili. *Mol Microbiol* 30 (2), 285-293

11 Heydorn, A. et al. (2002) Statistical analysis of *Pseudomonas aeruginosa* biofilm development: impact of mutations in genes involved in twitching motility, cell-to-cell signaling, and stationary-phase sigma factor expression. *Appl Environ Microbiol* 68 (4), 2008-2017

12 Sauer, K. and Camper, A.K. (2001) Characterization of phenotypic changes in *Pseudomonas putida* in response to surface-associated growth. *J Bacteriol* 183 (22), 6579-6589

13 Solano, C. et al. (2002) Genetic analysis of *Salmonella enteritidis* biofilm formation: critical role of cellulose. *Mol Microbiol* 43 (3), 793-808

14 Wang, X. et al. (2004) The *pgaABCD* locus of *Escherichia coli* promotes the synthesis of a polysaccharide adhesin required for biofilm formation. *J Bacteriol* 186 (9), 2724-2734

15 Matsukawa, M. and Greenberg, E.P. (2004) Putative Exopolysaccharide Synthesis Genes Influence *Pseudomonas aeruginosa* Biofilm Development. *J Bacteriol* 186 (14), 4449-4456

16 Jackson, K.D. et al. (2004) Identification of *psl*, a Locus Encoding a Potential Exopolysaccharide That Is Essential for *Pseudomonas aeruginosa* PAO1 Biofilm Formation. *J Bacteriol* 186 (14), 4466-4475

17 Friedman, L. and Kolter, R. (2004) Two Genetic Loci Produce Distinct Carbohydrate-Rich Structural Components of the *Pseudomonas aeruginosa* Biofilm Matrix. *J Bacteriol* 186 (14), 4457-4465

18 Friedman, L. and Kolter, R. (2004) Genes involved in matrix formation in *Pseudomonas aeruginosa* PA14 biofilms. *Mol Microbiol* 51 (3), 675-690

19 Huang, C.T. et al. (1998) Spatial patterns of alkaline phosphatase expression within bacterial colonies and biofilms in response to phosphate starvation. *Appl Environ Microbiol* 64 (4), 1526-1531

20 Prigent-Combaret, C. et al. (2001) Complex regulatory network controls initial adhesion and biofilm formation in *Escherichia coli* via regulation of the *csgD* gene. *J Bacteriol* 183 (24), 7213-7223

21 Ren, D. et al. (2004) Gene expression in *Bacillus subtilis* surface biofilms with and without sporulation and the importance of *yveR* for biofilm maintenance. *Biotechnol Bioeng* 86 (3), 344-364

22 Xu, K.D. et al. (2001) Gene expression and protein levels of the stationary phase sigma factor, RpoS, in continuously-fed *Pseudomonas aeruginosa* biofilms. *FEMS Microbiol Lett* 199 (1), 67-71

23 Adams, J.L. and McLean, R.J. (1999) Impact of *rpoS* deletion on *Escherichia coli* biofilms. *Appl Environ Microbiol* 65 (9), 4285-4287

24 Corona-Izquierdo, F.P. and Membrillo-Hernandez, J. (2002) A mutation in *rpoS* enhances biofilm formation in *Escherichia coli* during exponential phase of growth. *FEMS Microbiol Lett* 211 (1), 105-110

25 Otto, K. and Silhavy, T.J. (2002) Surface sensing and adhesion of *Escherichia coli* controlled by the Cpx-signaling pathway. *Proc Natl Acad Sci U S A* 99 (4), 2287-2292

26 Ferrieres, L. and Clarke, D.J. (2003) The RcsC sensor kinase is required for normal biofilm formation in *Escherichia coli* K-12 and controls the expression of a regulon in response to growth on a solid surface. *Mol Microbiol* 50 (5), 1665-1682

27 Danese, P.N. et al. (2000) The outer membrane protein, antigen 43, mediates cell-to-cell interactions within *Escherichia coli* biofilms. *Mol Microbiol* 37 (2), 424-432

28 Tenorio, E. et al. (2003) Systematic characterization of *Escherichia coli* genes/ORFs affecting biofilm formation. *FEMS Microbiol Lett* 225 (1), 107-114

29 Finelli, A. et al. (2003) Use of in-biofilm expression technology to identify genes involved in *Pseudomonas aeruginosa* biofilm development. *J Bacteriol* 185 (9), 2700-2710

30 Helloin, E. et al. (2003) Carbon starvation survival of *Listeria monocytogenes* in planktonic state and in biofilm: a proteomic study. *Proteomics* 3 (10), 2052-2064

31 Tremoulet, F. et al. (2002) Comparison of protein patterns of *Listeria monocytogenes* grown in biofilm or in planktonic mode by proteomic analysis. *FEMS Microbiol Lett* 210 (1), 25-31

32 Sauer, K. et al. (2002) *Pseudomonas aeruginosa* displays multiple phenotypes during development as a biofilm. *J Bacteriol* 184 (4), 1140-1154

33 Wang, Q. et al. (2004) Gene expression patterns during swarming in *Salmonella typhimurium*: genes specific to surface growth and putative new motility and pathogenicity genes. *Mol Microbiol* 52 (1), 169-187

34 Jackson, D.W. et al. (2002) Biofilm formation and dispersal under the influence of the global regulator CsrA of *Escherichia coli*. *J Bacteriol* 184 (1), 290-301

35 O'Toole, G.A. et al. (2000) The global carbon metabolism regulator Crc is a component of a signal transduction pathway required for biofilm development by *Pseudomonas aeruginosa*. *J Bacteriol* 182 (2), 425-431

36 Suzuki, K. et al. (2002) Regulatory circuitry of the CsrA/CsrB and BarA/UvrY systems of *Escherichia coli*. *J Bacteriol* 184 (18), 5130-5140

37 Parkins, M.D. et al. (2001) *Pseudomonas aeruginosa* GacA, a factor in multihost virulence, is also essential for biofilm formation. *Mol Microbiol* 40 (5), 1215-1226

38 Thompson, L.S. et al. (2003) The alternative sigma factor RpoN regulates the quorum sensing gene rhlI in *Pseudomonas aeruginosa*. *FEMS Microbiol Lett* 220 (2), 187-195

39 Wolfe, A.J. et al. (2004) Vibrio fischeri sigma54 controls motility, biofilm formation, luminescence, and colonization. *Appl Environ Microbiol* 70 (4), 2520-2524

40 Landini, P. and Zehnder, A.J. (2002) The global regulatory hns gene negatively affects adhesion to solid surfaces by anaerobically grown *Escherichia coli* by modulating expression of flagellar genes and lipopolysaccharide production. *J Bacteriol* 184 (6), 1522-1529

41 Vallet, I. et al. (2004) Biofilm formation in *Pseudomonas aeruginosa*: fimbrial cup gene clusters are controlled by the transcriptional regulator MvaT. *J Bacteriol* 186 (9), 2880-2890

42 Wen, Z.T. and Burne, R.A. (2002) Functional genomics approach to identifying genes required for biofilm development by Streptococcus mutans. *Appl Environ Microbiol* 68 (3), 1196-1203

43 Hamon, M.A. and Lazazzera, B.A. (2001) The sporulation transcription factor Spo0A is required for biofilm development in *Bacillus subtilis*. *Mol Microbiol* 42 (5), 1199-1209

44 Beenken, K.E. et al. (2003) Mutation of *sarA* in *Staphylococcus aureus* limits biofilm formation. *Infect Immun* 71 (7), 4206-4211

45 Valle, J. et al. (2003) SarA and not sigmaB is essential for biofilm development by *Staphylococcus aureus*. *Mol Microbiol* 48 (4), 1075-1087

46 Knobloch, J.K. et al. (2004) RsbU-dependent regulation of *Staphylococcus epidermidis* biofilm formation is mediated via the alternative sigma factor sigmaB by repression of the negative regulator gene *icaR*. *Infect Immun* 72 (7), 3838-3848

47 Zhang, Y. et al. (2004) Identification of a novel two-component system in *Streptococcus gordonii* V288 involved in biofilm formation. *Infect Immun* 72 (6), 3489-3494

48 Fournier, B. and Hooper, D.C. (2000) A new two-component regulatory system involved in adhesion, autolysis, and extracellular proteolytic activity of *Staphylococcus aureus*. *J Bacteriol* 182 (14), 3955-3964

49 Li, Y.H. et al. (2002) Novel two-component regulatory system involved in biofilm formation and acid resistance in *Streptococcus mutans*. *J Bacteriol* 184 (22), 6333-6342

50 Lim, Y. et al. (2004) Control of glucose- and NaCl-induced biofilm formation by *rbf* in *Staphylococcus aureus*. *J Bacteriol* 186 (3), 722-729