Biofilms strike back

George A O'Toole & Philip S Stewart

Sublethal concentrations of antibiotics increase bacterial drug tolerance by promoting biofilm formation.

© 2005 Nature Publishing Group http://www.nature.com/naturebiotechnology

Excessive use of antibiotics has increased resistance of many microbes to these drugs. In a recent issue of *Nature*, Hoffman *et al.*[1] show that too little antibiotic can also be detrimental. They demonstrate that subinhibitory levels of the aminoglycoside-class antibiotic tobramycin increase biofilm formation by various isolates of the bacterial pathogen *Pseudomonas aeruginosa*. This finding may lead to new therapies against persistent infections stemming from biofilm formation.

A biofilm is a dense aggregation of microbial cells bound together by a slimy extracellular matrix of polysaccharide and protein. When bacteria group together in these multicellular communities, they are able to tolerate antimicrobial challenges that no rmally eradicate free-floating individual cells[2]. Given the safe haven offered by biofilm formation, it is perhaps not surprising that bacteria have evolved to respond to low concentrations of biocides or antibiotics by inducing biofilm formation. Hoffman *et al.* provide molecular clues regarding the mechanisms underlying this response.

P. aeruginosa is a primary culprit in infections associated with cystic fibrosis, burn wounds and the use of catheters. The authors discovered that low levels of tobramycin stimulated biofilm formation by surface-attached bacteria. Other aminoglycoside antibiotics also increased the number of bacterial cells in the biofilm. Furthermore, similar effects were seen with *Escherichia coli*, suggesting that this effect was not specific to *P. aeruginosa*.

In fact, previous studies have shown that several bacteria respond to antibiotic treatment by increasing polysaccharide synthesis or biofilm formation[3]. Perhaps this response is an evolutionary adaptation of bacteria that must defend against various microbially produced antibiotics (**Fig. 1**). Although clinical use of antibiotics has been routine for only a few decades, bacteria have been subject to a continual onslaught of antimicrobial agents produced by fungi and other bacteria throughout their evolutionary history. One response to the pressure of surviving these antibiotic wars may be the formation of protected biofilm communities.

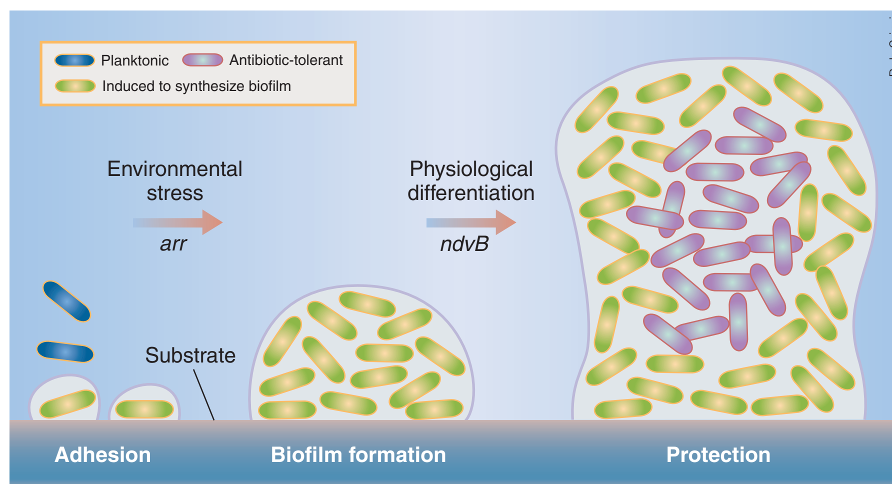

Figure 1 Biofilm formation as a stress response. Environmental stress induces planktonic bacteria (blue) to form a biofilm. In the model system of *P. aeruginosa* challenged with tobramycin, the aminoglycoside antibiotic is the stress. Of the cells induced to produce biofilm (green), a subpopulation (purple) develops tolerance of chemical challenges by virtue of physiological differentiation and/or the altered microenvironment. In *P. aeruginosa*, *arr* is required to respond to low levels of tobramycin. The *ndvB* gene product contributes to the protection of cells.

To gain insight into the mechanism of biofilm induction, Hoffman *et al.* investigated the role of cyclic-di-GMP in this process. A number of recent studies have implicated cyclic-di-GMP in multicellular behaviors of bacteria, especially in the synthesis of extracellular polysaccharides that may mediate biofilm or pellicle formation[4]. Benziman and colleagues first described this small metabolite, synthesized from two molecules of GTP, in their study of cellulose synthesis by *Gluconacetobacter xylinus* (formerly *Acetobacter xylinum*)[5]. Cyclic-di-GMP was shown to stimulate the activity of the cellulose synthase enzyme activity up to 200-fold. Proteins that catalyze the synthesis of cyclic-di-GMP have a conserved core GGDEF amino acid motif and are referred to as diguanylate cyclases. Enzymes that degrade this metabolite to either a linear molecule or two GMP molecules, called phosphodiesterases, are characterized by the presence of an EAL triamino acid motif.[5]

The *P. aeruginosa* genome has ~40 proteins with either a GGDEF or EAL motif, or both. The availability of a library of transposon mutants disrupted in every nonessential gene of *P. aeruginosa* [6] allowed Hoffman *et al.* to test mutants lacking each of these GGDEF/EAL-containing proteins for reduced biofilm synthetic capacity in response to sub-inhibitory levels of tobramycin. A mutation in one such gene product, a predicted inner membrane protein called Arr (PA2818), resulted in a *P. aeruginosa* strain in which biofilm formation was no longer increased in the presence of low levels of aminoglycoside antibiotics. This mutant makes a normal biofilm in the absence of antibiotics. Membranes isolated from a strain lacking Arr showed ~50% less phosphodiesterase activity than the wild-type strain, and a mutation in the key E residue of the EAL domain rendered the protein nonfunctional. Taken together, these data suggested that Arr does indeed participate in cyclic-di-GMP metabolism.

Here it is worth remarking that the connection between biofilm formation and

George A. O'Toole is in the Department of Microbiology and Immunology, Dartmouth Medical School, 505 Vail Building, North College Street, Hanover, New Hampshire 03755, USA and Philip S. Stewart is at the Center for Biofilm Engineering and Department of Chemical and Biological Engineering, Montana State University, 366 EPS Building, Bozeman, Montana 59717, USA.
email: phil_s@erc.montana.edu

cyclic-di GMP observed by Hoffman *et al.* is the opposite of that observed in most prior studies. The prevailing, though not exclusive view, is that elevated intracellular levels of cyclic-di-GMP are associated with increased synthesis of extracellular polysaccharides and enhanced autoaggregation or biofilm accumulation. Hoffman and colleagues acknowledge this discrepancy and suggest that the regulatory function of cyclic-di-GMP is complex. Given this complexity, it may be premature to prescribe therapeutic manipulation of cyclic-di-GMP for managing infections.

How might Arr contribute to biofilm formation and biofilm biology? A mutation in *arr* does not appear to influence biofilm development via effects on flagella or pili, two types of surface appendages involved in biofilm formation by *P. aeruginosa*. Nor does *arr* appear to alter the rate of formation of small colony variants, another factor that can contribute to biofilm formation and biofilm antibiotic resistance[7]. The authors demonstrated no apparent contribution of the polysaccharide alginate, but they did not rule out the possibility that Arr regulates the production of extracellular polysaccharide matrix encoded by two recently discovered loci[8,9]. It is also possible that tobramycin stimulates a previously uncharacterized biofilm production pathway in *P. aeruginosa*—a hypothesis that remains to be tested.

Mutation of *arr* has no apparent effect on the ability of *P. aeruginosa* to form a biofilm, at least as assessed in a static model of biofilm formation. Interestingly, in addition to the lack of stimulation in the *arr* strain upon treatment with low levels of antibiotic, the biofilm produced by an *arr* mutant was ~100-fold more susceptible to killing by high levels of tobramycin than the biofilm formed by the wild-type bacterium. There was no difference in planktonic resistance of the wild-type bacterium and the *arr* mutant. Therefore, *arr* provides a second example of a gene involved in a biofilm-specific pathway of antibiotic resistance[10]. In contrast with the *ndvB* locus, which encodes a glucosyltransferase required for the synthesis of periplasmic glucans, it is not clear how Arr mediates this resistance.

The widespread use of implants in modern medicine means that addressing the issue of biofilm-based infections is of increasing importance. Although little is known regarding mechanisms of biofilm tolerance, it is becoming increasingly clear that the mechanisms underlying this protection are distinct from classical mechanisms of antibiotic resistance described for planktonic cells. As long as large pharmaceutical companies continue to abandon their anti-infective programs and medical device companies are reluctant to invest in novel anti-infective coatings, it will remain the purview of small, innovative biotechnology companies to discover new approaches to eliminating biofilm-based infections.

1. Hoffman, L.R. *et al.* Aminoglycoside antibiotics induce bacterial biofilm formation. *Nature* **436**, 1171–1175 (2005).
2. Costerton, J.W., Stewart, P.S. & Greenberg, E.P. Bacterial biofilms: a common cause of persistent infections. *Science* **284**, 1318–1322 (1999).
3. Rachid, S., Ohlsen, K., Witte, W., Hacker, J. & Ziebuhr, W. Effect of subinhibitory antibiotic concentrations on polysaccharide intercellular adhesin expression in biofilm-forming *Staphylococcus epidermidis*. *Antimicrob. Agents Chemother.* **44**, 3357–3363 (2000).
4. Jenal, U. Cyclic di-guanosine-monophosphate comes of age: a novel secondary messenger involved in modulating cell surface structures in bacteria? *Curr. Opin. Microbiol.* **7**, 185–191 (2004).
5. Ross, P., Mayer, R. & Benziman, M. Cellulose biosynthesis and function in bacteria. *Microbiol. Rev.* **55**, 35–58 (1991).
6. Jacobs, M.A. *et al.* Comprehensive transposon mutant library of *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. USA* **100**, 14339–14344 (2003).
7. Drenkard, E. & Ausubel, F.M. *Pseudomonas* biofilm formation and antibiotic resistance are linked to phenotypic variation. *Nature* **416**, 740–743 (2002).
8. Friedman, L. & Kolter, R. Two genetic loci produce distinct carbohydrate-rich structural components of the *Pseudomonas aeruginosa* biofilm matrix. *J. Bacteriol.* **186**, 4457–4465 (2004).
9. Matsukawa, M. & Greenberg, E.P. Putative exopolysaccharide synthesis genes influence *Pseudomonas aeruginosa* biofilm development. *J. Bacteriol.* **186**, 4449–4456 (2004).
10. Mah, T.F. *et al.* A genetic basis for *Pseudomonas aeruginosa* biofilm antibiotic resistance. *Nature* 426, 306–310 (2003).

A classic assembly of nanobiomaterials

Andreas Mershin, Brian Cook, Liselotte Kaiser & Shuguang Zhang

A recent multidisciplinary conference in Crete underscored advances in protein and peptide self-assembly in a variety of biotechnological applications.

In antiquity, Crete was known as the crossroads of the Mediterranean. Minoan culture was enriched and invigorated by its diverse mix of cultures and thriving commerce with Africa, Europe and Asia. This ancient crossroads was somewhat fitting therefore as a backdrop for a conference that sought to bring together scientists from several different disciplines—biology, chemistry, physics and engineering—and from different continents to discuss diverse aspects of self-assembling biomaterials[1]. The result was very much more than the sum of the parts. And despite the nascent nature of the field, already several tangible applications appear within reach in areas as diverse as materials and biological patterning, adhesives, antimicrobial agents and hydrogels.

In order for peptide self-assembly[2] to be better understood, it will be important to understand how small proteins fold *in vivo* and *in vitro*. One means of accomplishing this is to obtain atomic resolution structures of the entire folding pathway from the denatured state, through to the intermediate and transition states, to the final, native state by incorporating nuclear magnetic resonance (NMR), systematic mutational analysis and molecular dynamics simulation. This combination of methods makes it possible to ascertain protein folding conformations even though most transitional states are fluctuating rapidly and can be so short-lived that they cannot be observed by NMR alone. Using this multidisciplinary approach, the structure of an unfolded protein can be determined under physiological (nondenaturing) conditions—an essential step for understanding how proteins unravel in the cell (Alan Fersht, University of Cambridge, Cambridge, UK). Because most diseases involving protein misfolding result from a native protein that first unfolds and then refolds incorrectly or aggregates, the application of this approach to small self-assembling peptides may facilitate the design of drugs that inhibit unfolding and therefore stabilize the native form of the protein. Thus far, there has been excellent agreement between experimental benchmarks and previous simulations[3]. In a related area, parallel studies on folding and assembly of natural triple-β-stranded proteins extracted from viruses have led to the

Andreas Mershin, Brian Cook, Liselotte Kaiser and Shuguang Zhang are at the Center for Biomedical Engineering, Laboratory of Self-assembly, 500 Technology Square, Room NE47-376, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA.
e-mail: Shuguang@mit.edu

© 2005 Nature Publishing Group http://www.nature.com/naturebiotechnology