# UNDERSTANDING BIOFILM RESISTANCE TO ANTIBACTERIAL AGENTS

#### David Davies

According to a public announcement by the US National Institutes of Health, "Biofilms are medically important, accounting for over 80% of microbial infections in the body". Yet bacterial biofilms remain poorly understood and strategies for their control remain underdeveloped. Standard antimicrobial treatments typically fail to eradicate biofilms, which can result in chronic infection and the need for surgical removal of afflicted areas. The need to create effective therapies to counter biofilm infections presents one of the most pressing challenges in anti-bacterial drug development. In this article, the mechanisms that underlie biofilm resistance to antimicrobial chemotherapy will be examined, with particular attention being given to potential avenues for the effective treatment of biofilms.

MICROSCOPY A microscopy technique which uses scanning laser light to excite fluorescent dyes within a thick sample, such as a biofilm. The image is collected in two dimensions and several images can be combined in an image stack to produce a cross sectional image through a sample or to create a threedimensional rendering of the sample. CLSM is particularly useful for imaging the positioning of biological structures within a three

dimensional space.

CONFOCAL LASER SCANNING

Department of Biological Sciences, State University of New York, Binghamton, New York 13902, USA. e-mail: dgdavies@binghamton.edu doi:10.1038/nrd1008 A biofilm is a population or community of bacteria living in organized structures at a liquid interface. Early Confocal Laser Scanning Microscopy (CLSM) of single-species biofilms<sup>1,2</sup> revealed that biofilm bacteria live in cellular clusters or Microcolonies that are encapsulated in a matrix composed of an extracellular polymeric substance (EPS), separated by open water channels that act as a primitive circulatory system for the delivery of nutrients and the removal of metabolic waste products. Fluid flow within the water channels was discovered using nuclear magnetic resonance (NMR) imaging and serial CLSM observations of inert particles; the rate and direction of this flow having been determined in several systems<sup>3,4</sup>.

Within a biofilm, each bacterium occupies a specific microenvironment, which is determined by surrounding cells, proximity to a channel (both of which determine the pH and availability of nutrients and oxygen) and the EPS matrix. The structuring of biofilms in microcolonies and water channels has been shown to be influenced by fluid flow, nutrient composition and intercellular small messenger molecules, or quoromones (acylated homoserine lactones, AHLs), that are used for bacterial communication<sup>5-7</sup> (see BOX 1).

Knockout experiments have shown that the genes that control the biosynthesis of these messengers are involved in the the formation of a differentiated *Pseudomonas aeruginosa* biofilm, and the knockout phenotype develops a biofilm of densely packed cells a few layers thick<sup>5</sup>. A representative diagram of a biofilm formed by the pathogen *P. aeruginosa* is depicted in FIG. 1.

#### **Biofilms and human disease**

Biofilms have been increasingly recognized as being important in human disease. The number of diseases associated with bacterial biofilms is considered to be quite large, with colitis, vaginitis, urethritis, conjunctivitis and otitis being just a short list of common examples. Biofilm infections have been known to be problematic in the oral cavity, and GINGIVITIS serves as an example of the prevalence of such infections. It has been reported that 24% of adults have lost at least 4 mm of periodontal attachment, and 60% of 15-year-olds and 40–50% of adults have some form of gingival (biofilm) infection<sup>8,9</sup>. Biofilms are also important as colonizers of medical devices, including urinary, venous and arterial catheters<sup>10</sup> and shunts. In a study of 4,000 infants given

#### Box 1 | Quorum sensing

Recent advances in research on cell-cell communication in bacteria have demonstrated a roll for chemical signalling in bacterial biofilms. This research has shown that small, diffusible molecules — members of the class of N-acylated homoserine lactones (AHLs) — are released by biofilm bacteria into their local environment, where they can interact with neighbouring cells. In all cases, AHLs are known to associate with a cognate DNA-binding protein that is homologous to LuxR in Photobacterium fischeri, which causes a conformational change in the protein that facilitates DNA-polymerase binding and initiates transcription of target genes. As bacterial densities increase with growth, these AHLs can accumulate to a threshold concentration and induce the transcription of specific genes throughout the population. This process couples the transcription of specific genes to bacterial-cell density89. Regulation of this type has been referred to as 'quorum sensing', because it suggests the requirement for a 'quorate' population of bacterial cells that is necessary for the activation of AHL-responsive genes<sup>90</sup>. Regulation of this type enables the coordination of bacterial behaviour at the population level, and ensures that the bacteria respond as a group to carry out special functions. For instance, quorum sensing has been shown to be responsible for the release of degradative extracellular enzymes and cytotoxins in a number of bacterial species. It is advantageous for bacteria to act as a group, rather than as autonomous individuals. The role of quorum sensing in bacterial infections is still incompletely characterized; however, great effort is currently being put into the investigation of this phenomenon. These studies are expected to yield important dividends in the development of new anti-infective chemotherapies.

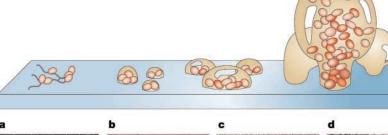
cerebrospinal-fluid shunts, 15–20% were found to be infected by a biofilm<sup>11</sup>. In addition, respirators, sigmoidoscopes, contact lenses, artificial implants (for example, heart valves, pacemakers, ventricular assist devices, synthetic vascular grafts and stents), urinary prostheses and orthopaedic prostheses (such as artificial joints and pins), have all been shown to be infected with biofilms. It has even been speculated that breast implantation-associated medical problems might arise primarily from biofilm infections on the implant material rather than from the implant itself (G. Ehrlich, personal communication).

Although numbers are difficult to evaluate critically, it is taken for granted within the biofilm research community that many undiagnosed chronic diseases in humans might be of biofilm origin.

Problems associated with human biofilm infections result from two distinct characteristics of all biofilms. First, biofilms are highly resistant to immune killing and clearance, and to treatment with antimicrobial agents<sup>12,13</sup>. Second, protected biofilms might be capable of shedding individual bacteria and sloughed pieces of biofilm into surrounding tissues and the circulatory system. Such shed cells might be responsible for acute illness, which might recur despite vigorous antimicrobial treatments.

### **Biofilm resistance to antimicrobial agents**

Owing to the compact nature of biofilm structures, the presumed reduced rates of cellular growth and respiration of biofilm bacteria and the protection conferred by biofilm matrix polymers, natural and artificial chemical agents are unable to adequately attack and destroy infectious biofilm populations<sup>10,11</sup>. Increased antibiotic resistance is a general trait associated with biofilm bacteria. When attached, bacteria show a profound resistance, rendering biofilm cells 10-1,000-fold less susceptible to various antimicrobial agents than the same bacterium grown in PLANKTONIC (free-floating) culture. For instance, chlorine (as sodium hypochlorite) — an oxidizing biocide that is considered to be one of the most effective antibacterial agents — requires a 600-fold increase in concentration to kill biofilm cells of Staphylococcus aureus compared with planktonic cells of the same species14. Several factors have been suggested to account for the extraordinary resistance of biofilm bacteria to antibiotics: the reduced metabolic and growth rates shown by biofilm bacteria, particularly



GINGIVITIS
Infection of the gingival crevice
(periodontal pocket) of the oral
cavity with a variety of
microorganisms, causing
inflammation of the periodontal
tissue and bone loss. Caused by
members of the genus
Capnocytophaga, Porphyromonas,
Rothia and others.

A microscopic aggregation of cells in a biofilm.

MICROCOLONY

PLANKTONIC Organisms that are free-floating in a fluid environment.



Figure 1 | **Five stages of biofilm development.** Biofilm maturation is a complex developmental process that involves several stages, each with unique characteristics that should be considered when designing strategies for biofilm treatment with antibiotics. Each stage of development in the diagram is paired with a photomicrograph of a developing *Pseudomonas aeruginosa* biofilm. All photomicrographs are shown at the same scale. Modified with permission from REF. 58 © (2002) American Society for Microbiology.

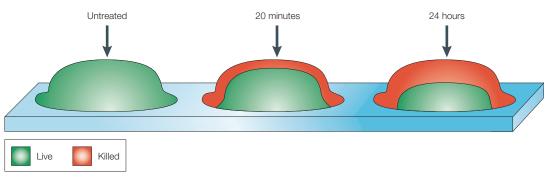


Figure 2 | **Biofilm resistance to anibiotic addition.** Treatment of biofilms with antibiotics often results in incomplete killing, allowing unaffected bacteria to act as a nucleus for the spread of infection following the withdrawal of antibiotic therapy.

those deep within the biofilm, might make them inherently less susceptible to antibiotics; the biofilm EPS matrix might act as an adsorbent or reactant, thereby reducing the amount of agent available to interact with biofilm cells (additionally, the biofilm structure might physically reduce the penetration of antimicrobial agents by walling off access to regions of the biofilm); and biofilm cells are physiologically distinct from planktonic bacteria, and express specific protective factors, such as MULTIDRUG EFFLUX PUMPS and stressresponse REGULONS<sup>15–22</sup>. As detailed molecular studies emerge, it is becoming apparent that each of these factors is important in the unusual resistance of biofilms to antimicrobials. FIGURE 2 represents the activity of antibiotics against a typical biofilm population. Initial treatment is usually effective in killing bacteria only at the margins of biofilm microcolonies. Bacteria deep within these microcolonies are not always killed by the antibacterial agents<sup>23,24</sup>, and can potentially form a NIDUS for continued dissemination of the infection.

There are growing concerns that the antibiotic treatment of NOSOCOMIAL infections and wound infections is driving the evolution of antibiotic-resistant microorganisms, and concerns regarding the over-prescription of antibiotics are increasingly being raised<sup>25,26</sup>. These concerns are reflected in a publication by Rastegar *et al.*<sup>27</sup>, which reported the identification of *P. aeruginosa* as the most common causes of wound infection in burn patients, with a frequency of 73.9%. The frequency of *P. aeruginosa* that is resistant to gentamicin, carbenicillin, co-trimoxazole, ceftizoxime and tetracycline was more than 95%. These results exemplify the need to develop alternate anti-infective strategies, including new antibiotics and vaccines, which can be used against *P. aeruginosa* and other pathogens.

The resistance of biofilms to antibiotic treatments depends on the microorganism under consideration and the antibiotic used. In a study by Olson and colleagues<sup>28</sup>, the Calgary Biofilm Device (a continuous-flow device that is used for the culture of biofilms) was used to assess the susceptibility of planktonic versus biofilm cultures of a number of different bacterial species and several different antibiotics. The antibiotics tested included ampicillin, ceftiofur, cloxacillin, oxytetracycline, penicillin G, streptomycin, tetracycline, enrofloxacin, erythromycin,

gentamicin, tilmicosin and trimethoprim-sulphadoxine. All of these antibiotics were effective in the treatment of planktonic cultures of *Actinomyces pyogenes, Corynebacterium renale, C. pseudotuberculosis, Staphylococcus aureus, S. hyicus* and *Streptococcus agalactiae*; however, biofilms formed by all of these organisms were resistant to all of the antibiotics tested. Some bacteria were shown to have biofilm susceptibilities that were equal to planktonic cultures, including *Pasteurella multocida, Mannheimia haemolytica, Streptococcus suis* and *S. dysgalactiae. Salmonella* sp. and *Pseudomonas aeruginosa* were susceptible to enrofloxacin, gentamicin, ampicillin, oxytetracycline and trimethoprimsulphadoxine as planktonic cultures, but were sensitive as biofilms only to enrofloxacin.

Biofilms also show enhanced resistance to hostdefence mechanisms. In chronic infections, such as the bronchopulmonary P. aeruginosa infection in cystic fibrosis (CF) patients (see BOX 2), bacteria persist despite an intact host immune defence and frequent antibiotic treatment. An important reason for the persistence of the bacteria is their capacity for the biofilm mode of growth. Biofilm-grown P. aeruginosa showed reduced activation of COMPLEMENT compared with planktonic bacteria<sup>29</sup>. Furthermore, the aggregation of bacteria into EPS-coated biofilms might make them less susceptible to phagocytosis<sup>30–33</sup>. Biofilm bacteria have also been reported to be resistant to certain aspects of the HUMORAL IMMUNE SYSTEM<sup>29,34,35</sup>. The accessibility is presumed to be reduced due to the protective EPS. Persistence of biofilms results in damage to the host as phagocytic cells will release indiscriminately their oxidative burst, resulting in collateral tissue damage.

#### **Antibiotic penetration**

One of the factors that is generally conceded to have a role in antibiotic resistance by biofilms is the inability of the antibiotic to penetrate to all areas of the biofilm. Several studies have been carried out in which antibiotic penetration has been assessed by detecting the concentration of the antibiotic at the base of the biofilm. In one such series of experiments, the penetration of the antibiotic ciprofloxicin was investigated for its ability to pass through biofilms of *P. aeruginosa* to a germanium crystal substratum in an infrared (IR) field. Germanium

MULTIDRUG EFFLUX PUMP A molecular pump integrated into the cell envelop of certain bacteria which is able to transport antibiotics into and out of the cell.

#### REGULON

A set of operons that are controlled by a single regulatory protein.

#### NIDUS

Latin for nest, but in this context a place or point in a host where a pathogen can develop and breed.

#### NOSOCOMIAL

Something acquired or originating in a hospital, such as a nosocomial infection.

#### COMPELEMENT

A complex of blood serum proteins of the immune system that interact sequentially with antibody—antigen complexes.

HUMORAL IMMUNE SYSTEM Extracellular branch of the immune system mediated by antibodies MINIMUM INHIBITORY CONCENTRATION The minimum concentration of a substance required to prevent growth of a microoganism.

EXOPOLYMERIC MATRIX A network of long-chain polymers produced by microorganisms of a biofilm which supports the structure of the biofilm. crystal is transparent to IR radiation which passes, unimpeded, through the crystal to create an evanescent field extending 0.2 µm above the surface. The IR signature of a material (such as an antibiotic) that is located within the evanescent field is, therefore, detectable and can be monitored *in situ* and in real time. Results from these experiments established that the biofilm was able to significantly reduce, but not entirely block, antibiotic penetration<sup>36</sup>. These and subsequent results also showed that penetration rates through biofilms depended on the antibiotic used and are not directly correlated with antibiotic susceptibility<sup>37</sup>.

Wild-type *Klebsiella pneumoniae* grown on filter discs were shown to have reduced antibiotic penetration for ampicillin compared with ciprofloxicin. However,  $\beta$ -lactamase-deficient *K. pneumoniae* biofilms — in which ampicillin was shown to penetrate completely — were still resistant to treatment, with a log reduction of 0.18 for the mutant strain, compared with 0.06 for the wild type and > 4 for the wild type in planktonic culture<sup>38</sup>. These results indicated that reduced antibiotic penetration might be important in protection for certain antibiotics, but that this reduction could not account for the overall resistance of biofilms to antibiotic treatment.

Although numerous studies have described the penetration of antibiotics into biofilms, the manner in which these studies were performed should be a consideration when interpreting these results and in assessing their significance. It is typical to grow a biofilm on a permeable support or a substratum through which the antibiotic can be detected directly. In such studies, the ability of the antibiotic to reach the substratum might be a function of biofilm surface coverage in addition to biofilm penetration. FIGURE 3 illustrates this problem, and shows that when antibiotic is added to a biofilm culture, it can pass through gaps in the interstices between microcolonies and lead investigators to conclude that the antibiotic concentration beneath the biofilm is equivalent to the concentration within the biofilm. Ideally, penetration studies should be performed by taking measurements at the centre of microcolonies.

In an effort to address the question of antibiotic penetration into dense cell aggregates of biofilms, Matin and colleagues used direct microscopic observation of

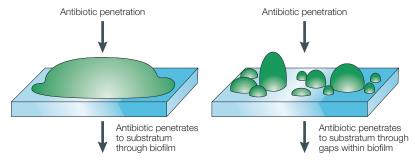


Figure 3 | **Antibiotic penetration.** Antibiotic penetration through biofilms is commonly determined by how much antibiotic is detectable beneath the biofilm following addition of antibiotic. These measurements are influenced by the amount of biomass and percent surface coverage by the biofilm.

tetracycline penetration into biofilms formed by *Escherichia coli*. In this study, it was shown that biofilms formed over two days on a polystyrene surface were less susceptible to the antibiotic than were planktonic cells; however, the biofilms showed tetracycline-mediated fluorescence distributed throughout the entire biofilm following exposure to the antibiotic for 7.5–10 minutes<sup>39</sup>. Although this study did not provide quantitative data on the concentration of tetracycline within the biofilm, it nonetheless demonstrated that the antibiotic was able to penetrate to all observable areas of the biofilm.

Biofilm populations are typically found at markedly higher cell densities than planktonic bacteria. It has been noted that the higher cell density of a biofilm population might account, in part, for their enhanced resistance to antimicrobial treatment. Larsen<sup>40</sup> has reported that the minimum inhibitory concentrations (MICs) of the antibiotics amoxicillin, doxycycline and metronidazole were all shown to increase markedly when planktonic populations of *Porphyromonas gingivalis* were tested at cell densities equal to those found in biofilm populations (10<sup>7</sup>–10<sup>8</sup> cells ml<sup>-1</sup>). Although this result indicated that an inoculum effect is part of the explanation for the increased resistance of biofilm bacteria, it does not completely explain differences between biofilm and planktonic susceptibilities. Larsen also noted that biofilm populations were still at least two to eight times more resistant to amoxicillin and doxycycline compared to equivalent numbers of planktonic bacteria.

The presence of the exopolymeric matrix of biofilms has long been held to have a role in limiting the penetration of antimicrobials to cells deep within biofilms. Roques and colleagues<sup>41</sup> have provided support for this asumption by showing that increasing the size of the hydrophobic side chains of selected quaternary ammonium compounds reduces the susceptibility of *S. aureus* to treatment with antibiotics when these bacteria are embedded in a hydrophobic matrix of EPS. When the matrix was washed from the cells, the susceptibility rose to 90% of the susceptibility of planktonic bacteria<sup>41</sup>. Resistant biofilm bacteria become susceptible to antimicrobial treatments following dispersion or disaggregation of the biofilm — an observation that further supports the idea that the EPS matrix might impart protection to the biofilm by limiting transport (D. G. Davies, unpublished observations).

One promising solution to the problem of antibiotic penetration has emerged from the manipulation of electrical fields that surround bacteria in a biofilm. This 'Bioelectric Effect' — a term coined by J. W. Costerton and colleagues — has been postulated to electrically alter the configuration of the EPS matrix surrounding biofilm bacteria, and perhaps also to enhance the penetration of antimicrobial agents across the bacterial-cell envelope<sup>42</sup>. Using alternating-current densities of less than 100 micro-Amperes per cm², it was found that the antibiotic concentrations required to kill biofilm cells were greatly reduced compared with untreated biofilm bacteria. These concentrations, however, were still higher than those needed to kill planktonic bacteria of the same species<sup>43,44</sup>.

AUXOTROPH

An organism that has acquired a nutritional requirement through the process of mutation.

SPORULATION

The production an endospore by bacteria of the genera *Clostridia* and *Bacillus*.

SIGMA FACTOR

Any of several bacterial DNA-binding proteins that direct the binding of DNA-directed RNA-polymerase to the promoter of an operon.

#### Reduced growth in biofilms

Microbiologists have long known that non-dividing bacteria escape the killing effects of antibiotics targeted against growth-specific factors. For example, early penicillin selection experiments to obtain E. coli AUXOTROPHS relied on the differential replication rates of wild-type and nutrient-dependent organisms. Many investigators accept that biofilm bacteria have reduced growth rates, and that this is a contributing factor to the unusual resistance of biofilms to effective antibiotic treatment. Yet evidence for reduced growth in biofilms is limited and many reports of reduced activity arise primarily from unpublished observations. One reason for the dearth of direct information relating to divisional activity within biofilms is the difficulty of making such measurements. Soren Molin's research group at the Danish Technical University has been one of the leaders in the study of metabolic activity within biofilms, and work in this lab using fluorescent tags for specific metabolic markers has shown that cells in the centres of the largest microcolonies in biofilms do, in fact, have reduced metabolic rates compared with cells at or near the surface (FIG. 4). In some instances, cells in small microcolonies also show reduced metabolic activity. These observations indicate that nutrient availability is a crucial factor influencing metabolic activity and, if properly supplied, cells within even large microcolonies continue to be active at rates equivalent to those seen for planktonic cultures<sup>45</sup>. These researchers pointed out that during the initial phases of biofilm development, all cells at the colonization surface are highly active, with ribosomal promoter activity corresponding to that of rapidly growing cells. Following primary colonization and the formation of small microcolonies, activity gradually decreases, initially in the central parts of the microcolony and eventually also at the surface<sup>45</sup>. In related work, DeBeer et al.4 have directly measured oxygen concentrations at various depths of biofilms using microelectrodes. This work has shown that the oxygen levels are depleted by as much as 30-fold near the centre of larger microcolonies. This work indicates that other nutrients, such as organic carbon, will likewise be depleted towards the centre of microcolonies. These studies have been performed in continuous culture, but it is expected that the nutritional status of biofilm bacteria in an infection will have considerably less nutrient available and, as such, growth rates are expected to be correspondingly lower under these conditions.

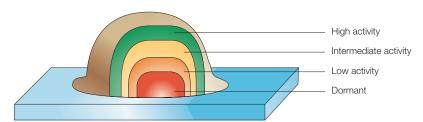


Figure 4 | **Metabolic activity in a biofilm mirocolony.** Metabolic activity in a biofilm cell cluster is a function of depth within the biofilm and is influenced by nutrient transport. Cells at the edges of a microcolony at the bulk liquid interface are the most active. Cells deep within the microcolony can potentially remain domant and provide a nucleus for regrowth following antibiotic treatment.

The lowered metabolic activity of at least some biofilms might therefore account for their enhanced resistance to treatment with antibiotics that are active against growth factors in planktonic bacteria. Observations of the partial killing of bacteria within biofilms by such antibiotics can be explained by this type of resistance. Biofilms that are treated with antibiotics can potentially sustain mortality on the periphery while the deeper organisms persist and form a nucleus for regrowth, and, through the use of metabolic stains for the detection of respiratory activity, this has been shown to be the case<sup>23,24</sup>. Cells at the bulk water interface of biofilms are rapidly killed, whereas deep within cell clusters near the substratum bacteria remain active and apparently unaffected by the treatment.

Antibiotics with activity against non-growing cells have been shown to have enhanced activity against biofilm bacteria compared with antibiotics that do not depend on rapid growth for activity. When imipenem and ciprofloxicin were used on *E. coli* grown as a biofilm, their effectiveness was greater when compared with antibiotics (such as  $\beta$ -lactams) that were only effective against growing bacteria, but less effective than the same antibiotics used against planktonic *E. coli*<sup>46</sup>. Similar results have been reported for *P. aeruginosa*, which, as a biofilm, shows greater susceptibility to fluoroquinolone antibiotics compared with  $\beta$ -lactam antibiotics<sup>47-49</sup>.

In another study, Spoering and Lewis<sup>50</sup> compared the slow growth of sub-populations of *P. aeruginosa* biofilm cells with stationary phase cells. The authors reported that the antibiotic resistance of these two populations was similar, and concluded that slow growth is potentially a factor in resistance. However, caution should be exercised when interpreting these results, as these data were not normalized and biofilm cells and stationary-phase cells might have other attributes in common in addition to lowered metabolic rates, such as the activation of stress-response regulons and the activation of efflux mechanisms.

## Unique biofilm physiology

Research on biofilms has demonstrated differences between planktonic and attached bacteria that imply physiological alterations following attachment to a surface. Early observations from a wide variety of laboratories<sup>51–54</sup> stimulated the development of the hypothesis that biofilm bacteria were potentially physiologically distinct from planktonic bacteria.

Building on this earlier work, it has been established that biofilm bacteria display unique gene-expression patterns, and, furthermore, that these patterns are not observed in free-living bacterial cells<sup>18,55–59</sup>. The attachment of bacteria to a surface initiates the expression of biofilm-specific genes, culminating in what has been described by Costerton<sup>33</sup> as a 'biofilm phenotype'. In terms of bacterial gene-expression mechanisms, this major phenotypic change is analogous to sporulation or starvation/survival. These phenotypic changes can occur as a result of the induction of a series of RNA-polymerase-associated SIGMA FACTORS<sup>60</sup>, which results in

### Box 2 | Biofilms and the cystic fibrosis lung

Cystic fibrosis (CF) is the most prevalent lethal genetic disease among people of European descent. In the United States, approximately 30,000 children and adults are afflicted. CF is inherited as an autosomal recessive trait at a rate of 1 in 2,000 live births among Caucasians<sup>91</sup>. The hallmark of CF is the progressive loss of pulmonary function caused by chronic bacterial infection, typically with mucoid Pseudomonas aeruginosa. In such infections, the bacteria persist despite an intact host immune defence and frequent antibiotic treatment. An important reason for the persistence of the bacteria is their capacity for the biofilm mode of growth 32,33. Direct examination by confocal scanning light microscopy (CSLM) of cystic fibrosis sputum has confirmed the presence of biofilm-like structures encased in a polymeric matrix 92. CSLM can be used on live specimens without altering the natural structure of the biofilm. Furthermore, extracellular quorum-sensing signalling molecules (extracellular chemical signals that cue cell-density-dependent gene expression) were detected in sputum<sup>90</sup> that were shown by Davies et al.5 to coordinate biofilm formation.

> cally distinct from their free-floating counterparts<sup>32</sup>. In a recent review, O'Toole et al.61 described biofilm formation as a process of microbial development similar to that seen in cell-cycle-controlled SWARMER-TO-STALK cell transition in Caulobacter crescentus, sporulation in Bacillus subtilis and fruiting-body formation by Myxococcus xanthus. From a structural point of view, it is similar to the formation of tissue from a variety of individual cells. This view is gaining increasing acceptance as studies on initial events in biofilm development reveal alterations in bacterial-cell physiology that hint at changes that can occur throughout the developmental cycle<sup>55,58,62-74</sup>. The concept of a unique biofilm phenotype is crucial to identifying new targets for controlling bacterial infections.

> Recent investigations have been directed at deter-

biofilm bacteria that are morphologically and biochemi-

mining the degree to which gene regulation during biofilm development controls the switch from planktonic to biofilm growth. By monitoring changes in global gene-expression patterns in attached Pseudomonas aeruginosa cells, Brözel and colleagues<sup>75</sup> found that the expression levels of at least 11 proteins were altered during various stages of attachment. Genevaux and colleagues<sup>71</sup> screened a library of *E. coli* Tn10-insertion mutants with altered adhesion abilities: fifty adhesiondeficient mutants were isolated that showed less than 40% attachment compared with the wild type, and 22 mutants were found with an attachment of 40-75% compared with the wild type. The majority of these mutants showed defects in motility. Using a screen in E. coli K-12, similar to the approach of Genevaux et al.<sup>71</sup>, Prigent-Combaret and colleagues<sup>56</sup> revealed major changes in the patterns of gene expression during the switch from planktonic to attached growth. Attachmentdependent regulation of gene expression was seen in 38% of the generated lacZ gene fusions (out of 446 clones). Using gene-chip technology, Whitely et al. detected 72 genes that showed differential expression in biofilm populations of P. aeruginosa compared with planktonic cultures<sup>57</sup>. More recently, it has been shown that in Pseudomonas putida more than 30 genes and 40 gene products were altered within 6 hours following

attachment18. In work on Streptococcus mutans, Svensater and colleagues<sup>76</sup> detected increased concentrations for 57 out of 694 proteins analysed in biofilm populations, compared with planktonic populations. Thirteen proteins in biofilm cells were not detected in planktonic cultures and nine proteins were found only in planktonic cultures. Chemostat-grown P. aeruginosa planktonic cells, compared with mature biofilm cells grown in silicon tubing in identical media, reveal more than 800 proteins that have a sixfold or greater change in expression level (more than 50% of the proteome). This difference was higher than when planktonic P. aeruginosa were compared with planktonic cultures of *P. putida*<sup>58</sup>. These results indicate that physiological changes in the transition from planktonic to attached cells are profound and undoubtedly complex. An example of comparative twodimensional polyacrylamide gels (2D PAGE) for biofilm and planktonic populations of P. aeruginosa is shown in FIG. 5. These gels highlight the numerous differences in cellular protein profiles that give rise to the differences in physiology between biofilm and planktonic forms of the

The differences in gene expression and protein profiles that are seen between planktonic and biofilm populations indicate that several factors are likely to be responsible for the differences observed in the resistance to antibiotics by these two modes of bacterial growth. Recent studies have shown that the disruption of the expression of potential multidrug efflux pumps in biofilms of P. putida using KT 2410 TRANSPOSON insertion results in attachment-defective mutants<sup>77</sup>. This indicates that several biofilm-associated traits are under unified regulatory control and that numerous mechanisms, which are associated with antibiotic resistance, might be operative at the same time within a biofilm.

It is interesting to note that the bacteriacidal activity of biocides, such as chlorine and glutaraldehyde, cannot be adequately neutralized by specific resistance mechanisms, such as efflux pumps, which are effective against antibiotics. Chlorine and glutaraldehyde are, therefore, able to kill biofilm bacteria if their concentration is sufficiently high. These biocides were tested against P. aeruginosa embedded in calcium alginate beads, and it was found that these 'biofilm' organisms were less susceptible to treatment with chlorine and glutaraldehyde compared with planktonic bacteria, but complete killing was achievable if the concentration of these biocides was sufficiently high<sup>78</sup>. This indicates that the overriding factor contributing to biofilm resistance to specific antibiotics might be the specific genes that are activated (and deactivated) during biofilm growth, rather than nonspecific mechanisms for protection, which should also provide protection against biocides such as chlorine.

#### **Biofilm detachment**

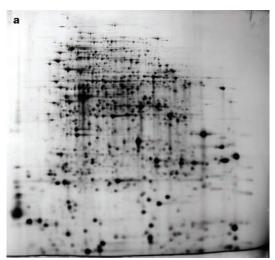
The process of AUTODISPERSION or disaggregation of biofilm cells is of considerable interest as a means of increasing the antibiotic sensitivity of biofilm bacteria. The ability to induce dispersion has potential in controlling biofilms directly; alternatively, it might be used as an adjuvant to existing antimicrobial therapies to enhance

SWARMER-TO-STALK CELL TRANSITION Upon exhaustion of nutrients. members of the group of fruiting myxobacteria swarmer cells migrate together and undergo differentiation into stalk cells, forming a vertical structure rising above a surface.

FRUITING-BODY A structure of the fruiting myxobacteria at the end of a stalk composed of differentiated cells which are converted to myxospores (resting bodies).

TRANSPOSON A mobile segment of DNA that has the ability to integrate into a chromosome. Transposons usually carry genes that are used in transposition as well as other genes, often selectable markers, such as for antibiotic resistance.

AUTODISPERSION The disaggregation of a biofilm or biofilm microcolony as a result of physiological activity of the resident microorganisms.



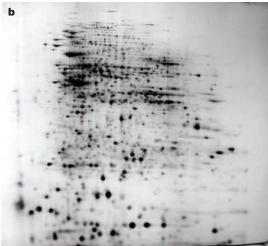


Figure 5 | **2D PAGE gels of Pseudomonas aeruginosa. a** | Protein profile from plantonic bacteria. **b** | Climax stage, six-day-old biofilm. Modified with permission from REF. 58 © (2002) American Society for Microbiology.

their cidal activity. In 1998, Allison and co-workers observed that spent medium from P. fluorescens cultures was able to induce dispersion of biofilms formed by this bacterium<sup>79</sup>; the same phenomenon has been observed with biofilms formed by P. aeruginosa<sup>5,58</sup>. Vats and Lee<sup>80</sup> described the discovery that surface-protein-releasing enzyme (SPRE) produced by Streptococcus mutans is actively involved in the degradation of attachment polymers on tooth surfaces, which causes the release of bacteria from the tooth surface. In this work, SPRE was shown to result in a 20% increase in detachment compared with control samples. In a more recent paper, Jackson et al.81 describe the discovery that the RNAbinding protein CsrA (carbon storage regulator) acts as an activator of biofilm dispersal in E. coli. The effects of CsrA are proposed to be mediated by regulation of intracellular glycogen biosynthesis and catabolism. Biofilm dispersion is an almost untouched area of research, but one which has the promise of providing significant opportunities for alternative approaches to biofilm control in the coming years.

### Targets for novel biofilm antibacterial agents

Recent studies on the pathogenesis of Pseudomonas infections have begun to take advantage of presently available technology to identify virulence genes and mRNA transcripts from infections in humans, mice and a number of animal models, including fruit flies, Caenorhabditis elegans and the greater wax moth Galleria mellonella<sup>82,83</sup>. For instance, insect models of acute-stage infections of the HAEMOLYMPH by P. aeruginosa pathogenesis have been generated, allowing the examinination of free-floating bacteria (F. M. Ausubel, personal communication). A P. aeruginosa mutant, defective in the gene ybtQ, was shown to have attenuated virulence in both the wax moth and the burned-mouse model, but not in *C. elegans*. Previous studies of P. aeruginosa mutants that were tested in both C. elegans and G. mellonella have shown that several mutants showed attenuated pathogenesis in

one species but not in another <sup>84–86</sup>. These results indicate that hosts differ in their response to different virulence factors and that positive results for mutants of bacteria attenuated in a particular virulence gene should not be automatically extrapolated to alternate hosts.

Another area of pathogenesis research that requires additional attention considers which specific proteins are produced during a biofilm infection. In a recent publication, Drenkard and Ausubel<sup>59</sup> identified a regulatory protein, PvrP, in *P. aeruginosa* that controls the conversion between antibiotic-resistant and susceptible forms. This gene has been shown to be actively transcribed by biofilm bacteria; compounds that inhibit the expression or activity of PvrP might prove to be useful in treatment of *P. aeruginosa* biofilm infections. It is expected that many bacteria will be found to produce specific resistance or virulence gene products, which can act as targets for anti-infective treatments.

### **Future directions of biofilm research**

One of the main difficulties in treating biofilm infections arises from a lack of understanding of the characteristics of the biofilm mode of growth. So far, no comprehensive investigation has taken place to examine the phenotypic characteristics of the entire growth cycle of a biofilm-forming bacterial pathogen. Understanding when and how to treat biofilm infections requires knowledge of whether the bacterial population is of one phenotype or of many. If separate phenotypes are shown to exist, then antibiotic treatments might not be effective against the entire infection, leaving cells behind to recolonize and debilitate the host. The presence of such sub-populations of cells has been noted by Lewis and colleagues who refer to these resistant bacteria as 'persister' cells<sup>50,87</sup>. A number of suggestions have been put forward to explain the increased resistance of persister cells, including mutations or alterations in the cellular machinery that is responsible for preprogrammed cell death or apoptosis88.

HAEMOLYMPH
The body fluid that bathes tissues of invertebrates having an open circulatory system.

Evidence, however, points to several factors that could be important in such resistance, and continued research is necessary to identify these specific resistance traits. For instance, biofilm bacteria could present stages when stress-response proteins are produced, conferring increased resistance to environmental conditions (including immune attack, as well as the presence of antibiotics).

The future of treatment strategies for biofilm infections seems to rest with specifically targeting unique biofilm characteristics, either in combination with conventional antibiotic therapy or to tailor treatment regimens to target biofilm infections when they are most susceptible to antibiotics. To develop these novel methods, research in the future needs to be directed towards more rigorous studies of the physiological status of biofilm bacteria during an infection.

For instance, biofilms could be found to produce markers that are associated with the release of shed bacteria from the biofilm population. These markers could then be identified with stages in biofilm development

and used to diagnose the condition of a biofilm infection and to direct the administration of an appropriate treatment strategy. As an example, antibiotic therapy would be more effective if administered during a stage of rapid bacterial growth. Additionally, specific biofilm markers might indicate when it is necessary to administer antibiotics prophylactically to prevent bacteraemia or infection of remote sites. By understanding the physiology of biofilm development, chemotherapeutic agents could be developed to promote or prevent transitioning from one stage of biofilm maturation to the next by targeting unique biofilm regulatory or signalling molecules. Finally, specific agents might by discovered or developed which will interfere with the production of virulence factors, or promote (or inhibit) the shedding of biofilm bacteria (to coincide with antibiotic therapy) as a particular case requires. Overall, the characterization of biofilm development is a crucial component to understanding the diagnosis, treatment and management of biofilm infections.

 Lawrence, J. R., Korber, D. R., Hoyle, B. D., Costerton, J. W. & Caldwell, D. E. Optical sectioning of microbial biofilms. J. Bacteriol. 173, 6558–6567 (1991).

## One of the first descriptions of the 3-dimensional structure of natural biofilms.

- Lawrence, J. R. & Neu, T. R. Confocal laser scanning microscopy for analysis of microbial biofilms. *Methods Enzymol.* 310, 131–144 (1999).
- Lewandowski, Z., Stoodley, P., Altobelli, S. & Fukushima, E. Hydrodynamics and kinetics in biofilm systems — recent advances and new problems. Proceedings of the Second IAWQ International Specialized Conference on Biofilm Reactors. Paris, France, pp. 313–319 (1993).
- DeBeer, D., Stoodley, P., Roe, F. & Lewandowski, Z. Effects of biofilm structure on oxygen distribution and mass transport. *Biotech. Bioeng.* 43, 1131–1138 (1994).
- Davies, D. G. et al. The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science 280, 295–298 (1998).

## The first paper to link biofilm development with intercellular communication.

- Martinelli, D., Bachofen, R. & Brandl, H. Effect of medium composition, flow rate, and signaling compounds on the formation of soluble extracellular materials by biofilms of *Chromobacterium violaceum. Appl. Microbiol. Biotechno.* 59, 278–283 (2002).
- Huber, B. et al. Genetic analysis of functions involved in the late stages of biofilm development in *Burkholderia cepacia* H111. Mol. Microbiol. 46, 411–426 (2002).
- National Institute of Dental Research. The Oral Health of United States Adults. The National Survey of Dental Caries in US Employed Adults and Seniors: 1985–1986. DHHS Pub. No. (NIH) 87–2868. (US Department of Health and Human Services, Bethesda, Maryland 1987).
- National Institute of Dental Research. Oral health of United States children. The National Survey of Dental Caries in US School Children, 1986–1987. DHHS Pub. No. (NIH) 89–2247. (Department of Health and Human Services, Bethesda, Maryland 1989).
- Passerini, L., Lam, K., Costerton, J. W. & King, E. G. Biofilms on indwelling vascular catheters. *Crit. Care Med* 20, 665–673 (1992).
   Pople, I. J., Bayston, R. & Hayward, R. D. Infection of
- Pople, I. J., Bayston, R. & Hayward, R. D. Infection of cerebrospinal fluid shunts in infants: a study of etiological factors. *J. Neurosurg.* 77, 29–36 (1992).
- Costerton, J. W. et al. Bacterial biofilms in nature and disease. Annu. Rev. Microbiol. 41, 435–464 (1987).
- Hoiby, N. et al. in Microbial Biofilms (eds Lappin–Scott, H. M. & Costerton, J. W.) (Cambridge University Press, Cambridge, 1995).
- Luppens, S. B., Reij, M. W. van der Heijden, R. W., Rombouts, F. M. & Abee, T. Development of a standard test to assess the resistance of Staphylococcus aureus biofilm cells to disinfectants. Appl. Environ. Microbiol. 68, 4194–4200 (2002).

- Brown, M. R., Allison, D. G. & Gilbert, P. Resistance of bacterial biofilms to antibiotics: a growth-rate related effect? J. Antimicrob. Chemother. 22, 777–783 (1988).
- Anwar, H., Strap, J. L. & Costerton, J. W. Establishment of aging biofilms: possible mechanism of bacterial resistance to antimicrobial therapy. *Antimicrob. Agents Chemother.* 36, 1347–1351 (1992).
- Mah, T. F. & O'Toole, G. A. Mechanisms of biofilm resistance to antimicrobial agents. *Trends Microbiol.* 9, 34–39 (2001).
- Sauer, K. & Camper, A. K. Characterization of phenotypic changes in *Pseudomonas putida* in response to surface-
- associated growth. J. Bacteriol. 183, 6579–6589 (2001).
   Stewart, P. S. Mechanisms of antibiotic resistance in bacterial biofilms. Int. J. Med. Microbiol. 292, 107–113 (2002).

# An excellent review from an engineering perspective on antibiotic resistance in bacterial biofilms.

- Donlan, R. M. & Costerton, J. W. Biofilms: survival mechanisms of clinically relevant microorganisms. Clin. Microbiol. Rev. 15, 167–193 (2002).
  - A comprehensive review outlining many organisms of medical importance and what is known about their resistance to specific antibiotics.
- 21 Gilbert, P., Maira-Litran, T., McBain, A. J., Rickard, A. H. & White, F. W. The physiology and collective recalcitrance of microbial biofilm communities. *Adv. Microb. Physiol.* 46, 202-256 (2002)

# A thoughtful and insightful look at the mechanisms underlying the resistance of biofilms to antibiotic treatments.

- Gilbert, P., Allison, D. G. & McBain, A. J. Biofilms in vitro and in vivo: do singular mechanisms imply cross-resistance?.
   J. Appl. Microbiol. Suppl. 98, S–110S (2002).
- Yu, F. P. & McFeters, G. A. Rapid in situ assessment of physiological activities in bacterial biofilms using fluorescent probes. J. Microbiol. Methods 20, 1–10 (1994).
- Huang, C.-T., Yu, F. P., McFeters, G. A. & Stewart, P. S. Nonuniform spatial patterns of respiratory activity within biofilms during disinfection. *Appl. Environ. Microbiol.* 61, 2252–2256 (1995).

# A classic paper that visually demonstrates the effect of an antimicrobial agent against a biofilm in situ. Onarheim, H. et al. Outbreak of multiresistant

- Onarheim, H. et al. Outbreak of multiresistant Acenetobacter baumannii infection. Tidsskr. Nor. Laegeforen. 120, 1028–1033 (2000).
- Still, J., Law, E., Friedman, B., Furman, S. & Newton, T. Vancomycin resistant organisms in a burn unit. South Med. J. 94, 810–812 (2001).
- Rastegar, L. A., Bahrami, H. H. & Alaghebandan, R. Pseudomonas infections in Tohid Burn Center, Iran. Burns 24, 637–641 (1998).
- Olson, M. E., Ceri, H., Morck, D. W., Buret, A. G. & Read, R. R. Biofilm bacteria: formation and comparative susceptibility to antibiotics. *Can. J. Vet. Res.* 66, 86–92 (2002)

- Jensen, E. T. et al. Complement activation by Pseudomonas aeruginosa biofilms. Microb. Pathol. 15, 377–388 (1993).
- Rogers, J., Phillip, F. & Olliff, C. The effects of extracellular slime from Staphylococcus epidermidis on phagocytic ingestion and killing. FEMS Immunol. Med. Microbiol. 9, 109–115 (1994)
- Moran, F. J. et al. Phagocytosis and killing of slimeproducing Staphylococcus epidermiclis by polymorphonuclear leukocytes. Effects of sparfloxacin. Rev. Esp. Quimioter. 11, 52–57 (1998).
- Costerton, J. W., Lewandowski, Z., Caldwell, D., Korber, D. & Lappin-Scott, H. M. Microbial biofilms. *Annu. Rev. Microbiol.* 49, 711–745 (1995).
- Costerton, J. W., Stewart, P. S. & Greenberg, E. P. Bacterial biofilms: a common cause of persistent infections. *Science* 284, 1318–1322 (1999).
- Meluleni, G. J., Grout, M., Evans, D. J. & Pier, G. B. Mucoid Pseudomonas aeruginosa growing in a biofilm in vitro are killed by opsonic antibodies to the mucoid exopolysaccharide capsule but not by antibodies produced during chronic lung infection in cystic fibrosis patients. J. Immuno. 155, 2029–2038 (1995).
- Dasgupta, M. K. Biofilm causes decreased production of interferon-y. J. Am. Soc. Nephrol. 7, 877–882 (1996).
- Suci, P. A., Mittelman, M. W., Yu, F. P. & Geesey, G. G. Investigation of ciprofloxacin penetration into Pseudomonas aeruginosa biofilms. Antimicrob. Agents Chemother. 38, 2125–2133 (1994).

# An excellent paper describing a novel in situ method for determining antibiotic penetration into biofilms.

- Vrany, J. D., Stewart, P. S. & Suci, P. S. Comparison of recalcitrance to ciprofloxacin and levofloxacin exhibited by Pseudomonas aeruginosa bofilms displaying rapidtransport characteristics. Antimicrob. Agents Chemother. 41, 1352–1358 (1994).
- Anderl, J., Franklin, M. J. & Stewart, P. S. Role of antibiotic penetration limitation in *Klebsiella pneumoniae* biofilm resistance to ampicillin and ciprofloxicin. *Antimicrob. Agents Chemother.* 44, 1818–1824 (2000).
- Stone, G., Wood, P., Dixon, M., Keyhan, M. & Matin, A. Tetracycline rapidly reaches all the constituent cells of uropothogenic *Escherichia coli* biofilms. *Antimicrob. Agents Chemother.* 46, 2458–2461 (2002).
- Larsen, T. Susceptibility of Porphyromonas gingivalis in biofilms to amoxicillin, doxycycline and metronidazole. Oral Microbiol. Immunol. 5, 267–271 (2002).
- Campanac, C., Pineau, L., Payard, A., Baziard-Mouysset, G. & Roques, C. Interactions between biocide cationic agents and bacterial biofilms. *Antimicrob. Agents Chemother.* 46, 1469–1474 (2002).
- Stoodley, P., deBeer, D. & Lappin-Scott, H. M. Influence of electric fields and pH on biofilm structure as related to the bioelectric effect. Antimicrob. Agents Chemother. 41, 1876–1879 (1997).

- 43. Costerton, J. W., Ellis, B., Lam, K., Johnson, F. & Koury, A. E. Mechanism of electrical enhancement of efficacy of antibiotics in killing biofilm bacteria. Antimicrob. Agents Chemother. 38, 2803-2809 (1994).
  - Introduction of a novel strategy using electrical fields to enhance the effectiveness of antibiotics against
- McLeod, B. R., Fortun, S., Costerton, J. W. & Stewart, P. S. Enhanced bacterial biofilm control using electromagnetic fields in combination with antibiotics. Methods Enzymol. 310, 656-670 (1999).
- Sternberg, C. et al. Distribution of bacterial growth activity in flow-chamber biofilms. Appl. Environ. Microbiol. 65 4108-4117 (1999).

#### A landmark paper demonstrating the activity and positioning of single cells in biofilms in real time.

- Ashby, M. J., Neale, J. E. & Critchley, I. A. Effect of antibiotics on non-growing planktoinic cells and biofilms of Escherichia coli. J. Antimicrob. Chemotherapy **33**, 443–452 (1994)
- Shigeta, M. G. et al. Permeation of antimicrobial agents through Pseudomonas aeruginosa biofilms: a simple
- method. *Chemotherapy* **43**, 340–345 (1997). Shigeta, M., Komatsuzawa, M., Sugai, M., Suginaka, H. & Usui, T. Effect of the growth rate of Pseudomonas aeruginosa biofilms on the susceptibility to antimicrobial agents. Chemotherapy 43, 137-141 (1997).
- Tanaka G. et al. Effect of the growth rate of Pseudomonas aeruginosa biofilms on the susceptibility to antimicrobial agents: beta-lactams and fluoroquinolones. Chemotherapy 45, 28-36 (1999).
- Spoering A. L. & K. Lewis, K. Biofilms and planktonic cells of Pseudomonas aeruginosa have similar resistance to killing by antimicrobials. *J. Bacteriol.* **183**, 6746–6751 (2001). An examination of bacterial resistance based on defects in preprogrammed cell death. This paper has stimulated considerable debate regarding the mechanism of biofilm resistance.
- McCoy, W. F. & Costerton, J. W. Fouling biofilm development in tubular flow systems. *Dev. in Indust. Microbiol.* **23**, 551–558 (1982).
- Morisaki, H. Effect of solid-liquid interface on metabolic activity of E. coli. J. Gen. Appl. Microbiol. 29, 195-204
- Jeffrey, W. H. & Paul, J. H. Activity of an attached and free-living *Vibrio* sp. As measured by thymidine incorporation, p-iodonitrotetrazolium reduction, and ATP/ADP ratios. Appl. Fnviron. Microbiol. 51, 150-156 (1986).
- Davies, D. G. & McFeters, G. A. Growth and comparative physiology of Klebsiella oxytoca attached to granular activated carbon particles and in liquid media. Microb. Ecol. 15, 165-175 (1988).
- Davies, D. G. & Geesev, G. G. Regulation of the alginate biosynthesis gene algC in Pseudomonas aeruginosa during biofilm development in continuous culture. *Appl. Environ. Microbiol.* **61**, 860–867 (1995).
- Prigent-Combaret, C., Vidal, O., Dorel, C. & Lejeune, P. Abiotic surface sensing and biofilm-dependent regulation of gene expression in E. coli. J. Bacteriol. 181, 5993-6002
- Whiteley, M. et al. Gene expression in Pseudomonas
- aeruginosa biofilms. Nature **413**, 860–864 (2001). Sauer, K., Camper, A. K., Ehrlich, G. D., Costerton, J. W. & Davies, D. G. Pseudomonas aeruginosa displays multiple phenotypes as a biofilm. J. Bacteriol. 184. 1140-1154 (2002).
  - A paper describing the different phenotypic stages of biofilm development using primarily a proteomics approach. It was the first paper to outline the complete life-cycle of a biofilm-forming pathogen.
- Drenkard, E. & Ausubel, F. M. Pseudomonas biofilm formation and antibiotic resistance are linked to phenotypic variation. *Nature* **416**, 740–743 (2002).

#### An important paper linking phenotypic differences in biofilms to antibiotic susceptibility.

- Schurr, M. J., Martin, D. W., Mudd, M. H. & Deretic, V. Gene cluster controlling conversion to alginate-overproducing phenotype in Pseudomonas aeruginosa: functional analysis in a heterologous host and role in the instability of mucoidy. J. Bacteriol. 176, 3375–3382 (1994).
- O'Toole, G. A., Kaplan, H. B. & Kolter, R. Biofilm formation as microbial development, Annu, Rev. Microbiol, 54, 49-79
- Belas B. Simon M. & Silverman, M. Bequiation of lateral flagella gene transcription in Vibrio parahaemolyticus.
- J. Bacteriol. 167, 210–218 (1986).
  Dagastino, L., Goodman, A. E. & Marshall, K. C Physiological responses induced in bacteria adhering to surfaces. *Biofouling* **4**, 113–119 (1991).

  Santos, R., Callow, M. E. & Bott, T. R. The structure of
- Pseudomonas fluorescens biofilms in contact with flowing systems. Biofouling 4, 319–336 (1991).
- Davies, D. G., Chakrabarty, A. M. & Geesey, G. G. Exopolysaccharide production in biofilms: substratum activation of alginate gene expression in Pseudomonas aeruginosa, Appl. Environ, Microbiol, 59, 1181–1186 (1993), First report of the the detection of specific gene activity in bacteria at the single-cell level, in real time and in situ.
- Dalton, H. M. & March, P. E. Molecular genetics of bacterial attachment and biofouling. Biotechnology 9, 252-255 (1998)
- Dekkers, L. C. et al. Role of the O-antigen of lipopolysaccharide, and possible roles of growth rate and of NADH:ubiquinone oxidoreductase (nuo) in competitive tomato root-tip colonization by *Pseudomonas fluorescens* WCS365. *Mol. Plant Microbe Interact.* **11**, 763–771 (1998).
- O'Toole, G. A. & Kolter, R. Flagellar and twitching motility are necessary for Pseudomonas aeruginosa biofilm development. Mol. Microbiol. 30, 295–304 (1998).
- O'Toole, G. A. & Kolter, R. Initiation of biofilm formation in Pseudomonas fluorescens WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. *Mol. Microbiol.* **28**, 419–461 (1998).
- DeFlaun, M. F., Oppenheimer, S. R., Streger, S., Condee, C. W. & Fletcher, M. Alterations in adhesion, transport, and membrane characteristics in an adhesiondeficient pseudomonad. Appl. Environ. Microbiol. 65, 759-765 (1999).
- Genevaux, P., Bauda, P., DuBow, M. S. & Oudega, B. Identification of Tn10 insertions in the rfaG, rfaP, and galU genes involved in lipopolysaccharide core biosynthesis that affect Escherichia coli adhesion. Arch. Microbiol. 172, 1-8 (1999).
- Pratt, L. A. & Kolter, R. Genetic analysis of Escherichia coli biofilm formation: roles of flagella, motility, chemotaxis and type I pili. Mol. Microbiol. 30, 285-293 (1998).
- Rodriguez-Herva, J. J., Reniero, D., Galli, E. & Ramos, J. L Cell envelope mutants of *Pseudomonas putida*: physiological characterization and analysis of their ability to survive in soil. Environ. Microbiol. 1, 479-488 (1999).
- Steyn B, Oosthuizen, M. C., MacDonald, R., Theron, J. & Brozel, V. S. The use of glass wool as an attachment surface for studying phenotypic changes in *Pseudomonas* aeruginosa biofilms by two-dimensional gel electrophoresis. Proteomics 1 871-879 (2001)
- Brözel, V. S., Strydom, G. M. & Cloete, T. E. E. A method for the study of *de novo* protein synthesis in *Pseudomonas* aeruginosa after attachment. *Biofouling* **8**, 195–210 (1995).
- Svensater, G., Welin, J., Wilkins, J. C., Beighton, D. & Hamilton, I. R. Protein expression by planktonic and biofilm cells of Streptococcus mutans. FEMS Microbiol. Lett. 205, 139-146 (2001).
- Espinosa-Urgel, M., Salido, A. & Ramos, J. L. Genetic analysis of functions involved in adhesion of *Pseudomonas* putida to seeds. *J. Bacteriol.* **182**, 2363–2369 (2000).

- 78. Grobe, K. J., Zahller, J. J. & Stewart, P. S. Role of dose concentraion in biocide efficacy against Pseudomonas aeruginosa. J. Indust. Microbiol. Biotechnol. 29, 10-15 (2002).
- Allison, D. G., B. Ruiz, B., SanJose, C., Jaspe, A. & Gilbert, P. Extracellular products as mediators of the formation and detachment of Pseudomonas fluorescens biofilms. FEMS Microbiol. Lett. 167, 179-184 (1988).
- Vats, N. & Lee, S. F. Active detachment of Streptococcus mutans cells adhered to epon-hydroxylapatite surfaces coated with salivary proteins in vitro. Arch. Oral Biol. 45,

#### An interesting and significant report on the regulation of autodispersion of biofilms.

- Jackson, D. W. et al. Biofilm formation and dispersion under the influence of the global regulator CsrA of Escherichia coli. J. Bacteriol. 184, 290-301 (2002).
- Choi, J. Y. et al. Identification of virulence genes in a pathogenic strain of Pseudomonas aeruginosa by representational difference analysis. J. Bacteriol. 184, 952-961 (2002).
- Hubank, M. & Schatz, D. G. Identifying differences in mRNA expression by representational difference analysis of cDNA. *Nucleic Acids Res.* **22**, 5640–5648 (1994).
- Jander, G., Rahme, L. G. & Ausubel, F. M. Positive correlation between virulence of Pseudomonas aeruginosa mutants in mice and insects. J. Bacteriol. 182, 3843-3845 (2000).

#### A well-conducted investigation of the differences in virulence of an important pathogen in different model hosts

- Tan, M. W., Mahajan-Miklos, S. & Ausubel, F. M. Killing of Caenorhabditis elegans by Pseudomonas aeruginosa used to model mammalian bacterial pathogenesis. Proc. Natl Acad. Sci. USA **96**, 715–720 (1999). Tan, M. W., Rahme, L. G., Sternberg, J. A., Tompkins, R. G.
- & Ausubel, F. M. Pseudomonas aeruginosa killing of Caenorhabditis elegans used to identify P. aeruginosa virulence factors. Proc. Natl Acad. Sci. USA **96**, 2408–2413
- Brooun, A., Liu, S. & Lewis, K. A dose-response study of antibiotic resistance in Pseudomonas aeruginosa biofilms. Antimicrob. Agents Chemother. 44, 640-466 (2000).
- Lewis, K. Riddle of biofilm resistance. Antimicrob. Agents
- Chemother. **45**, 999–1007 (2001). Latifi, A., Foglino, M., Tanaka, K., Williams, P. & Lazdunski, A. A hierarchical quorum-sensing cascade in Pseudomonas aeruginosa links the transcriptional activators LasR and RhIR (VsmR) to expression of the stationary-phase sigma factor RpoS. Mol. Microbiol. 21, 1137–1146 (1996).
- Fuqua, W. C., Winans, S. C. & Greenberg, E. P. Quorum sensing in bacteria: the LuxR-Luxl family of cell density responsive transcriptional regulators. J. Bacteriol. 176, 269-275 (1994).
- May, T. B. et al. Alginate synthesis by Pseudomonas aeruginosa: a key pathogenic factor in chronic pulmonary infections of cystic fibrosis patients. Clin. Microbiol. Rev. 4, 191–206 (1991).
- Singh, P. et al. Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. Nature **407**, 762–764 (2000).

#### Online links

### **FURTHER INFORMATION**

Encyclopedia of Life Sciences: www.els.net

US National Institutes of Health Guide: SBIR/STTR Study and Control of Microbial Biofilms:

http://grants2.nih.gov/grants/guide/pa-files/PA-99-084.html Access to this interactive links box is free online