N-Acylhomoserine Lactones, Quorum Sensing, and Biofilm Development in Gram-negative Bacteria

Steve Atkinson, Miguel Cámara, and Paul Williams

Abstract
Many different Gram negative bacteria employ N-acylhomoserine lactones (AHLs) as diffusible signal molecules which enable bacterial populations to co-ordinate gene expression as a function of cell population density. Such co-ordinated community behavior, termed “quorum sensing” (QS) regulates diverse physiological processes including secondary metabolite production, motility, DNA transfer and pathogenicity. AHLs are produced during the biofilm mode of growth and AHL-dependent QS influences the development, integrity and architecture of biofilm communities as well as orchestrating the optimal timing and production of secondary metabolites to combat predators and host defense mechanisms. In most cases the identity of the QS-regulated target structural genes which contribute to biofilm development have not yet been identified. However, the increased susceptibility of biofilms formed by Pseudomonas aeruginosa QS mutants to conventional antibacterial agents and host defenses highlights the utility of AHL-dependent QS as a novel antimicrobial target.

Introduction
Bacteria have evolved multiple integrated sensory systems to facilitate adaptation to environmental challenges at both the individual cell and population levels. The perception and processing of chemical information forms a pivotal component of the regulatory mechanisms necessary for such population-dependent adaptive behavior. Many bacteria employ self-generated small diffusible signal molecules to control gene expression as a function of cell population density. In this process, termed “quorum sensing” (QS), the concentration of a signal molecule which accumulates in the extracellular environment reflects the cell number such that the perception of a threshold concentration of signal molecule determines when the population is “quorate” and ready to make a collective behavioral adaptation (Salmond et al., 1995; Williams et al., 2000; Swift et al., 2001; Cámara et al., 2002).

QS signal molecule diffusion between spatially separated bacterial subpopulations may also convey information about their physiological state, their numbers, and the specific environmental conditions being encountered. The notion that a QS signal molecule can accumulate is based on the assumption that there will be a diffusion barrier over which more molecules will accumulate than are lost from a given micro-habitat (Winzer et al., 2002;
Redfield, 2002). This has been suggested as a “compartment” or “diffusion” sensing system where QS signal molecule accumulation is both the measure for the degree of compartmentalization and the means to distribute this information among the community (Redfield, 2002; Williams et al., 2007). QS may therefore be considered as an alternative version of diffusion sensing (quorum diffusion sensing might be a more appropriate term), where the threshold concentration of the QS signal eliciting a response can only be achieved by a population rather than by a single cell. Consequently, the size of the quorum is not fixed but will depend on the relative rates of synthesis and loss of QS signal molecule. There are also situations where a single bacterium can switch from the non-quorate to quorate state. For example individual Staphylococcus aureus cells trapped intracellularly within an endosome in mammalian endothelial cells are unable to escape and replicate within the cytoplasm unless they are able to express their agr QS system (Qazi et al., 2001). In such situations, the QS system is clearly functioning in the “compartment” not “population” sensing mode. It is also important to consider QS as only one of multiple environmental parameters which a bacterial cell must integrate in order to make a behavioral decision (Withers et al., 2001). Consequently QS systems have been identified as integral components of global gene regulatory networks in diverse bacterial genera.

The QS signal molecule classes so far identified are chemically diverse and include N-acylhomoserine lactones (AHLs), γ-butyrolactones, 2-alkyl-4-quinolones, furanones, fatty acid derivatives and peptides. No “universal” QS language has been discovered and only the autoinducer-2/LuxS QS system appears to be shared by both Gram negative and Gram-positive bacteria (Winzer et al., 2002; Winzer and Williams, 2003; Vendeville et al., 2005) (see also Chapters 7 and 8). However, many more chemically distinct QS signal molecules are likely to exist and it has been argued that the majority of extracellular bacterial metabolites including compounds with antibiotic activity are likely to function as signal molecules (Yim et al., 2006). Consequently, a number of criteria have been proposed to distinguish between QS signal molecules and other extracellular metabolites. Firstly, the biosynthesis of the QS signal should occur during a specific stage of growth, under certain physiological conditions, or in response to particular environmental conditions. Secondly, the QS signal must accumulate in the extracellular milieu, attain a critical threshold concentration and be recognized by a specific bacterial receptor. Thirdly, the cellular response to the molecule should extend beyond the physiological changes required to metabolize or detoxify the molecule (Winzer et al., 2002). These criteria, in conjunction with the concept of diffusible signal molecule-mediated density dependent QS, define the boundary from which the role of diffusible signal molecules in co-ordinating bacterial behavior can be conceptualized.

In this chapter the contribution of N-acylhomoserine lactone (AHL)-dependent QS to the biofilm lifestyle of Gram-negative bacteria will be explored.

**AHL-dependent quorum sensing**

AHLs are employed as QS signal molecules by Gram negative proteobacteria belonging to α, β and γ subdivisions. So far no AHL-producing Gram positive bacteria have been identified (Swift et al., 1998; Withers et al., 2001; Cámara et al., 2002; Chhabra et al., 2005). Structurally AHLs consist of a homoserine lactone ring covalently linked via an amide bond to an acyl side chain (ranging between 4 and 18 carbons) which may be saturated or
unsaturated and with or without a hydroxy-, oxo- or no substituent on the carbon at the 3 position of the N-linked acyl chain (Figure 6.1). AHLs are usually synthesized by enzymes belonging to the LuxI family of AHL synthases, around 100 of which are currently present in the genome databases. Studies of the crystal structures of EsaI and LasI have revealed that this large protein family belongs to the GCN5-related N-acetyltransferase protein family (Watson et al., 2002; Gould et al., 2004). Most LuxI proteins share low homologies although ten invariant amino acid residues have been identified which are essential for activity and are localized within the N-terminal domain of the protein (Fuqua et al., 2001). Three members of a second family of AHL synthases, the LuxM family, has also been identified in the genus Vibrio (Hanzelka et al., 1999; Milton et al., 2001). Both LuxI and LuxM proteins employ the same substrates for AHL synthesis, namely S-adenosylmethionine (SAM) and the appropriately charged acyl-acyl carrier protein (acyl-ACP; (Hanzelka et al., 1999)). To date it has not been possible to predict the nature of the AHL(s) produced by a given LuxI or LuxM protein from bioinformatic analyses alone. However, the availability of several different biosensors have greatly facilitated the detection and preliminary identification of AHLs although unequivocal chemical identification requires mass spectrometry and/or NMR spectroscopy (Swift et al., 1999; Chhabra et al., 2005). From such experiments it has become clear that many LuxI homologues direct the synthesis of a variety of different AHLs although in most cases 1–2 different compounds predominate (Ortori et al., 2006).

Once synthesized, AHLs accumulate extracellularly and diffuse into neighboring bacterial cells where they usually interact with members of the LuxR family of transcriptional regulators (Fuqua et al., 1996; Swift et al., 2001; Cámara et al., 2002). AHLs bind to, and activate LuxR homologous proteins and the resulting LuxR protein/AHL complex activates or represses the relevant target structural gene(s) (Swift et al., 2001; Zhang et al., 2002; Cámara et al., 2002). LuxR proteins bind to a region within the promoter/operator

![Figure 6.1 General structures of 3-oxo-AHL, N-(3-oxoacyl)homoserine lactone; 3-hydroxy-AHL, N-(3-hydroxyacyl) homoserine lactone and AHL, N-acylhomoserine lactone, where R ranges from C1 to C15. The acyl side chains may also contain one or more double bonds.](image-url)
of the target genes which contains a region of dyad symmetry, a 20 base pair palindrome called the lux box (Fuqua et al., 2001; Zhang et al., 2002). Such regulatory regions are often found upstream of the transcriptional start site for genes coding for LuxI-type AHL synthases such that AHL synthesis is subject to auto-amplification and hence AHLs are often referred to as “autoinducers.” Furthermore, a LuxRI pair can be considered as a quorum sensing “module” several of which may be linked as for example in Pseudomonas aeruginosa where LasRI regulates the rhlRI genes (Latifi et al., 1996).

The LuxR/AHL paradigm forms the basis of AHL-mediated multicellular behavior in Gram-negative bacteria and is responsible for controlling a variety of population-dependent phenotypes including bioluminescence, swarming, swimming and sliding motility, antibiotic biosynthesis, plasmid conjugal transfer and the production of virulence determinants in animal, fish and plant pathogens (for reviews see (Swift et al., 1999; Swift et al., 2001; Williams, 2002). It is also apparent that AHL-mediated QS systems are expressed during the biofilm mode of growth and contribute to the development and dispersal of surface-associated bacterial biofilm communities.

**AHL production during the biofilm mode of growth**

The possibility that bacterial cell-to-cell communication might contribute to the biofilm lifestyle was proposed in 1993 (Williams and Stewart, 1993). One of the earliest experimental indications that AHL-dependent QS contributed to the biofilm mode of growth came from studies of the recovery rates of the ammonia oxidizer, Nitrosomonas europaea (Batchelor et al., 1997). Planktonic cells starved of ammonia exhibited long lag phases prior to nitrite production following supplementation with ammonium. In contrast, biofilm populations of N. europaea colonizing sand or soil particles in continuous flow, fixed column reactors exhibited no lag phase prior to nitrite production even after long term ammonium starvation. Since supplementation of starved planktonic cells with both ammonium and N-(3-oxohexanoyl)homoserine lactone (3-oxo-C6-HSL) reduced the lag phase by a factor of five and as AHLs were detected in N. europaea culture supernatants, the authors suggested that the rapid recovery of high-density biofilm populations was due to the production and accumulation of AHLs to levels, not possible in low density cell suspensions, where they may be responsible for controlling metabolism or other properties of the biofilm community (Batchelor et al., 1997).

Preliminary evidence for AHL production in natural biofilms was provided by McLean et al. (1997) who reported that AHLs could be detected in aquatic biofilms growing on submerged stones but not from rocks lacking a biofilm. Similarly, AHLs have been detected in microbial mats (Bachofen and Schenk, 1998). These are coherent macroscopic accumulations of micro-organisms which have formed layered structures on solid surfaces and sediments and are found in habitats such as thermal springs, pond bottoms, tidal regions and in algal blooms. However in both studies, neither the identity of the biofilm organisms present nor their in vitro AHL profiles were presented. Stickler et al. (1998) screened bacterial isolates taken from biofilms growing on indwelling urethral catheters for AHL production using an AHL biosensor. Of the 19 isolates obtained, 14 P. aeruginosa strains were reported to be AHL positive and a further five isolates (Providencia stuartii, Proteus mirabilis, Morganella morganii, Escherichia coli, and Klebsiella pneumoniae) were either weakly positive or negative.
Indwelling urethral catheters which had become colonized with bacterial biofilms were also removed from patients undergoing long-term bladder catheterization and cut into sections. Four of nine catheter sections gave positive biosensor reactions for AHLs while unused catheters were negative suggesting that AHLs were being produced in situ in the catheter associated biofilms (Stickler et al., 1998). *P. aeruginosa* biofilms were also shown to produce AHLs in the catheter in both an in vitro bladder model and ex vivo in catheters which had been freshly removed from the patient. These data suggest that AHLs are present in biofilms and produced in vivo during a clinically significant infection. However, the use of AHL biosensors alone can give rise to false positive results given that AHL biosensors also can respond to unrelated molecules such as the cyclic dipeptides (Holden et al., 1999). Consequently this work needs to be interpreted with some caution and requires further chemical characterization of the compounds responsible for AHL biosensor activation.

For *P. aeruginosa*, the major AHLs produced are N-(3-oxododecanoyl)homoserine lactone (3-oxo-C12-HSL) and N-butanoylhomoserine lactone (C4-HSL). These AHLs are synthesized via LasI and RhlI and activate LasR and RhlR respectively (Pearson et al., 1994; Winson et al., 1995). To unequivocally establish that AHLs are produced during the growth of *P. aeruginosa* biofilms in flow cells, Charlton et al. (2000) devised a quantitative method for 3-oxo-AHLs using gas chromatography (GC) coupled with mass spectrometry. 3-oxo-AHLs such as 3-oxo-C12-HSL but not un-substituted AHLs such as C4-HSL can be assayed sensitively after derivatization with pentafluorobenzyloxime (PFBO) which converts the β-ketone of the 3-oxo-AHL to an oxime (Charlton et al., 2000). Using this approach, the concentration of 3-oxo-C12-HSL present in the *P. aeruginosa* biofilm was found to be in excess of 600 μM and around 14 μM in the biofilm effluent. *P. aeruginosa* was also found to produce significant amounts of N-(3-tetradecanoyl) homoserine lactone (3-oxo-C14-HSL), N-(3-oxodecanoyl) homoserine lactone (3-oxo-C10-HSL) and N-(3-oxo-octanoyl) homoserine lactone (3-oxo-C8-HSL) (Charlton et al., 2000). Although the biological significance of these additional AHLs is not known, 3-oxo-C12-HSL levels in biofilms appear to be far higher than those previously estimated to be present in cell free supernatants prepared from planktonic cultures of *P. aeruginosa* (1 μM) (Pearson et al., 1995). The high biofilm concentration of 3-oxo-C12-HSL may reflect the partitioning and equilibration of the hydrophobic 3-oxo-C12-HSL molecule between the intra- and extracellular compartments or the restricted diffusion of 3-oxo-C12-HSL possibly as a consequence of interactions between the QS signal molecule and extracellular biofilm matrix components. Since AHLs are rapidly inactivated through lactonolysis at alkaline pHs (Yates et al., 2002), it is also possible that conditions within the biofilm matrix are less likely to promote ring opening. Thus the concentration of 3-oxo-C12-HSL which accumulates is a balance between production and turnover within the biofilm. 3-oxo-C12-HSL production will clearly be dependent on substrate availability, the levels of LasI and LasR and also RsaL, which negatively regulates lasI expression (Rampioni et al., 2006).

*P. aeruginosa* chronically colonizes the lungs of individuals with cystic fibrosis (CF) (Lyczak et al., 2002). Both AHLs, lasR and lasI transcripts have been detected directly in CF sputum obtained from patients colonized with *P. aeruginosa* (Middleton et al., 2002; Erickson et al., 2002). Using an indirect method to assay for AHL production in CF sputum, Singh et al. (2000) incubated 14C-methionine (which is incorporated into AHLs via SAM...
during AHL biosynthesis) to show that de novo AHL synthesis occurs in sputum incubated ex vivo for 4 hours. The method was subsequently used to determine the relative ratios of 3-oxo-C12-HSL and C4-HSL produced by laboratory and CF isolates of P. aeruginosa in broth, biofilms and CF sputum. From the profiles obtained, the authors concluded that the similarity in the ratios obtained from biofilms and CF sputa in contrast to broth (where 3-oxo-C12-HSL was produced at a rate between 3 and 10 times that of C4-HSL) supports the hypothesis that P. aeruginosa forms biofilms in CF sputum (Singh et al., 2000).

**AHL-dependent quorum sensing and biofilm development**

Biofilms are generally considered to develop in a stepwise manner from initial attachment to cell migration, aggregation and microcolony stages through to maturation and dispersal (O’Toole et al., 2000; Kjelleberg and Molin, 2002; Sauer et al., 2002; Klausen et al., 2003; Webb et al., 2003) (see also MacEachran and O’Toole, this volume; Pamp et al., this volume). Intuitively gene regulation in such a complex community would be predicted to be tightly regulated and to involve communication between the individuals within the population such that a concerted response can be elicited. The detection of AHL production within biofilms raises questions with respect to the contribution of QS to each stage of biofilm development and the identity of any AHL-regulated biofilm specific genes. These questions have been addressed experimentally by examining the biofilm phenotypes of strains with mutations in genes which code for AHL synthesis (e.g. luxI homologues) or mediate the AHL-response (luxR homologues). AHL synthase mutants are especially useful in this context since the parental phenotype can be restored by the exogenous provision of the cognate AHL as well as by supplying an intact copy of the mutated gene. Here we review the evidence for the contribution of specific QS-dependent genes to adherence, aggregation, biofilm maturation and dispersal in AHL-producing Gram-negative bacteria. Table 6.1 summarizes the current literature with respect to the key organisms in which AHL-dependent QS is known to play a role in biofilm development.

### Adherence and aggregation

Bacterial cell attachment to biotic or abiotic surfaces constitutes the initial phase of biofilm development (see also MacEachran and O’Toole, this volume). However there are few examples currently in the literature where AHL-dependent QS is essential for this process. The timing of QS-controlled exopolysaccharide (EPS) production does however play a major role in the surface adherence and colonization potential of *Pantoea stewartii* (see also Eberl and Fuqua, this volume). This plant pathogen causes vascular wilt and leaf blight in sweet corn and maize. The EPS, stewartan, represents a major virulence factor in *P. stewartii*, which accumulates in the plant xylem vessels post-infection, and by blocking the free flow of water leads to the characteristic wilting condition (Koutsoudis et al., 2006). The *cps* operon responsible for stewartan biosynthesis is negatively regulated via a QS system consisting of the LuxRI homologues EsaRI and 3-oxo-C6-HSL (von Bodman et al., 1998).

A *P. stewartii esal* mutant which is both AHL and EPS negative produces dense bacterial mats in *vitro* and tightly packed bacterial agglomerates in *vivo* when compared with the parent which exhibits low levels of adhesion. The addition of exogenous synthetic AHLs
restores the parental phenotype in a concentration dependent manner. However, the esaRI double mutant was unable to adhere to surfaces but could form aggregates (flocs) in liquid culture (Koutsoudis et al., 2006). In the parent P. stewartii strain, during the initial attachment event, negative regulation of EPS enhances the probability of attachment taking place in an EPS-free environment. In contrast, the esaI mutant is locked into the adhesion phase of biofilm development while the double esaRI mutant is locked into the high cell density mode (Koutsoudis et al., 2006). In P. stewartii the QS system does not actually promote attachment per se but prevents the expression of EPS which would otherwise inhibit attachment at the early stages of biofilm development. As QS systems activate their targets when a population achieves a particular size, the cell numbers present during the initial attachment stages of biofilm development will presumably not be sufficient to elicit a response. In P. aeruginosa, for example, differences in attachment between QS mutants and the parental strains have only been observed in vitro in nutrient-rich growth media (Davies et al., 1998; De Kievit et al., 2001; Shih and Huang, 2002). It is possible therefore that inverse coordinate regulatory systems such as the one described for P. stewartii, which de-repress as the cell population reaches a threshold level may allow early stage attachment to proceed prior to biofilm maturation.

Burkholderia cenocepacia is ubiquitous in the environment and is frequently isolated from the sputum of cystic fibrosis sufferers where, in common with P. aeruginosa, it causes chronic respiratory infections (Isles et al., 1984). The QS systems of B. cenocepacia, which are termed cepIR and cciIR respectively employ N-octanoyl homoserine lactone (C8-HSL) and N-hexanoyl homoserine lactone (C6-HSL) (Huber et al., 2001; 2002; Tomlin et al., 2005). Both QS systems regulate virulence factor expression and interestingly the cciR locus is located within the B. cenocepacia pathogenicity island associated with epidemic strains (Huber et al., 2001; 2002; Tomlin et al., 2005). A detailed quantitative analysis of biofilm attachment and maturation indicated that the cepIR system was not involved in the regulation of attachment in strain B. cenocepacia H111 (Huber et al., 2001;2002). However, in B. cenocepacia strain K56-2, the cciR and cciR cepIR mutants but not the cciI or cciI cepI mutants were reported to show significant reductions in a simple attachment assay (Tomlin et al., 2005). In flow cell chambers however the K56-2 cepR and cepI mutants were attachment-impaired. Thus in B. cenocepacia, the regulation of attachment by AHL-dependent QS is both strain and growth environment dependent.

Floc formation and aggregation in liquid cultures has been correlated with an increased likelihood of biofilm development (Parsek and Greenberg, 2005). The properties of flocs are similar to those of static biofilms with the component cells embedded within an extracellular matrix (Parsek and Greenberg, 2005). The enteropathogen Yersinia pseudotuberculosis possesses two pairs of LuxRI homologues (YpsRI and YtbRI) and produces multiple AHLs (Atkinson et al., 1999). Mutation of ypsR resulted in severe flocculation in liquid culture, upregulation of flagellin synthesis and a hypermotile phenotype on semi-solid agar plate assays (Atkinson et al., 1999). Interestingly, mutation of the AHL synthase gene, ypsI did not promote cellular aggregation although the same motility phenotype is apparent. Since ypsI expression and hence AHL production is not YpsR-dependent (Atkinson et al., 1999) it is possible that YpsR, in common with EsaR from E. stewartii, represses the target structural genes required for cellular aggregation. Consequently flocculation may be repressed at
Table 6.1  Gram-negative bacteria in which AHL-dependent QS contributes to biofilm development

<table>
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<tr>
<th>Organism</th>
<th>Major AHL(s) produced</th>
<th>QS loci</th>
<th>AHL regulated biofilm phenotype</th>
<th>AHL regulated structural genes contributing to the biofilm phenotype</th>
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<td>EsaR/I</td>
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<td>C8-HSL</td>
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<td>AhyR/I</td>
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low cell densities but becomes activated by AHL-dependent de-repression of YpsR as the population becomes “quorate.” Y. pseudotuberculosis is very closely related to Yersinia pestis, the causative agent of bubonic and pneumonic plague which possesses a closely related AHL-dependent quorum sensing system (Atkinson et al., 2006). This pathogen is transmitted mainly via the bites of infected fleas and forms dense aggregates within the flea gut. Physical blockage of the flea proventriculus which connects the midgut to the esophagus by bacterial biofilm-like aggregates impede the passage of a blood meal into the mid-gut such that Y. pestis is conveyed to the bite site by regurgitation when blocked fleas next attempt to feed, so transmitting the pathogen. Given that Y. pseudotuberculosis ypsR mutants aggregate it is possible that AHL-dependent quorum sensing is involved in biofilm formation in the flea gut. However, Jarrett et al. (2004) have shown that a Y. pestis strain with mutations in both AHL synthases as well as in luxS was still able to produce a biofilm on a glass surface and infect and block fleas as well as the parent strain. However, this does not rule out a role for QS in flea blockage if the Y. pestis QS system negatively regulates aggregation in a manner analogous to that of YpsR and EsaR. The aggregation phenotype would only become apparent on mutating the corresponding luxR homologue.

Flocculation is also QS-controlled in the free living photoheterotroph Rhodobacter sphaeroides which possesses a QS system consisting of the LuxRI homologues CerRI and 7,8-cis-N-(tetradecanoyl)homoserine lactone (C14:1-HSL). When grown in liquid culture R. sphaeroides cerI mutants flocculate but the aggregates can be dispersed by adding an exogenous source of C14:1-HSL. The cerI mutant also produces up to 30 times more exopolysaccharide than the parent and these data, when taken together, indicate that the QS system of R. sphaeroides is involved in repressing a biofilm-associated phenotype (Puskas et al., 1997). AHL-dependent QS therefore regulates cell aggregation in both Yersinia and Rhodobacter.

Maturation
Although insights into the contribution and role of AHL-dependent QS to biofilm maturation have primarily been derived from studies with P. aeruginosa, the impact of QS on biofilm maturation has also been investigated in Aeromonas, Burkholderia and Serratia.

Pseudomonas
The LasRI/RhlRI QS system of P. aeruginosa is the most extensively investigated and understood QS cascade in Gram-negative bacteria. Transcriptomic studies of planktonic cells have revealed that the LasRI- and RhlRI-regulated genes (over 500) are scattered throughout the chromosome supporting the view that the P. aeruginosa quorum sensing circuitry constitutes a true global regulatory system (Hentzer et al., 2003; Schuster et al., 2003; Wagner et al., 2003). P. aeruginosa readily forms biofilms on both biotic and abiotic surfaces which are problematic in both industrial and healthcare settings. Initial work on the impact of QS on biofilm formation in P. aeruginosa indicated that LasRI but not the RhlRI system was an integral part of the biofilm maturation process and governed microcolony formation (Davies et al., 1998). This was because lasI mutants formed flat undifferentiated biofilms which were much more susceptible to dispersal by detergents such as sodium dodecyl sulfate than the parent strain. These interesting data suggested that
AHL-dependent QS played an important role in determining the architecture of a biofilm. However, other laboratories reported that *P. aeruginosa* parent and QS mutant biofilms were either architecturally indistinguishable or showed only minor differences (Heydorn et al., 2002; Bjarnsholt et al., 2005a; Bjarnsholt et al., 2005b). These discrepancies are probably the consequence of the different *P. aeruginosa* PAO1 strains, environmental conditions (e.g. carbon source) and biofilm fermenter systems employed. Interestingly, both Hentzer et al. (2003) and Bjarnsholt and Givskov (2007) suggest that the structural stability of *P. aeruginosa* biofilms may be compromised in QS mutants. Furthermore, it has been suggested that one possible explanation for the contradictory results on the role of QS in biofilm formation in *P. aeruginosa* may be the potential accumulation of secondary mutations in QS mutants (Beatson et al., 2002). If this is the case a biofilm population will be heterogeneous and might actually be composed of a series of distinct niches populated by clonally compartmentalized bacteria (Parsek and Greenberg, 2005; Bjarnsholt and Givskov, 2007).

To determine whether, when and where the AHL synthase genes *lasI* and *rhlII* are expressed during *P. aeruginosa* biofilm growth, both *gfp* and *lacZ* reporter gene fusions have been employed (De Kievit et al., 2001; Sauer et al., 2002). During the course of an 8 day biofilm flow cell experiment, *lasI* expression was found to decrease progressively over time whereas *rhlI* expression did not change appreciably. For both genes, spatial analysis indicated that expression was maximal in cells located at the substratum but decreased with increasing biofilm height (De Kievit et al., 2001). In a complementary study which employed *lasB* and *rhlA* reporter fusions respectively as markers for *las* and *rhl* activity, Sauer et al. (2002) reported that the *las* system was expressed before *rhl* during the early irreversible attachment stage whereas the *rhl* system was not induced until the early stages of maturation when cell clusters became progressively layered. Interestingly using alcian blue as a stain for acidic EPS, *lasI* mutant cells, in contrast to the parent PAO1 cells, were observed to be more closely packed together within the biofilm and not separated by EPS (Sauer et al., 2002).

Scanning confocal microscopy of *P. aeruginosa* biofilms formed in continuous flow chambers has revealed a complex architecture where the bacteria are embedded in an extracellular matrix containing open channels which facilitate the flow of fresh nutrients and bacterial metabolites including QS signals throughout the biofilm. Since QS has been linked to the formation of structured biofilms, it is likely that the products of certain QS-regulated genes are essential for this process. In this context, rhamnolipid biosurfactants appear to be required for the maintenance but not formation of microcolonies and open channels in *P. aeruginosa* biofilms. This is because mutation of the rhamnolipid biosynthesis gene *rhlA* results in a strain which retains the ability to form but not maintain channels surrounding *P. aeruginosa* biofilm macrocolonies (Davey et al., 2003). This finding was confirmed by the exogenous provision of rhamnolipids to the *rhlA* mutant which partially restored the biofilm architecture to that of the parent strain. Furthermore, *rhlA::gfp* expression coincided with microcolony formation and continued throughout biofilm development where cell density was high. Since *rhlA* is controlled by RhlR/C4-HSL, these findings establish a direct link between QS and biofilm functionality. Indeed the importance of the timing of *rhlA* expression was further highlighted by experiments in which inappropriate overproduction or supply of rhamnolipids disrupted biofilm development (Davey et al., 2003). These
findings were extended by Lequette and Greenberg (2005) who showed that expression of a rhlA-gfp reporter in _P. aeruginosa_ biofilms was low during early biofilm development even in the presence of exogenously supplied C4-HSL but was induced once microcolonies of > 20 μm had formed. They also noted that rhlA was preferentially expressed in the stalks rather than caps of mature microcolonies.

In common with _P. aeruginosa_, biosurfactant production in _Pseudomonas fluorescens_, _Serratia liquefaciens_ and _B. cenocepacia_ is regulated by AHL-dependent QS. In _Pseudomonas putida_ PCL1445 the LuxRI pair, PpuRI control the production of two cyclic lipodepsipeptides termed putisolivins I and II respectively in conjunction with long chain AHLS such as 3-oxo-C10-HSL and 3-oxo-C12-HSL (Dubern _et al._, 2006). Mutation of either _ppuR_ or _ppuI_ abolishes putisolvin production and results in the formation of dense biofilms on wells in PVC microtiter plates. Putisolvin production in the _ppuI_ mutant can be restored by the addition of the long chain AHLS which consequently no longer develops the characteristic dense biofilm on PVC (Dubern _et al._, 2006).

Other AHL-regulated _P. aeruginosa_ genes which are involved in biofilm development are _lecA_ and _lecB_ which code for lectins which preferentially bind hydrophobic galactosides and L-fucose respectively Diggle _et al._, 2006; Tielker _et al._, 2005). Both genes are regulated by RhlR/C4-HSL as well as by the alternative sigma factor RpoS (Winzer _et al._, 2000). By using a _lecA-gfp_ translation fusion and immunoblot analysis of the biofilm extracellular matrix, Diggle _et al._ (2006) showed that _lecA_ is expressed in biofilm grown _P. aeruginosa_ cells. In static biofilm assays on both polystyrene and stainless steel, biofilm depth and surface coverage was reduced by mutation of _lecA_. Biofilm surface coverage on steel coupons was also inhibited by growth in the presence of either isopropylthio-D-galactoside (IPTG) or _p_-nitrophenyl-α-D-galactoside (NPG). Furthermore, mature wild-type biofilms formed in the absence of these hydrophobic galactosides could be dispersed by the addition of IPTG. In contrast, addition of _p_-nitrophenyl-α-L-fucose (NPF) which has a high affinity for the _P. aeruginosa_ LecB lectin, had no effect on biofilm formation or dispersal. These data suggest that the observed effects on biofilm formation were due to the competitive inhibition of LecA-ligand binding. Similar results were also presented for _P. aeruginosa_ biofilms grown under dynamic flow conditions on steel coupons, suggesting that LecA contributes to _P. aeruginosa_ biofilm architecture under different environmental conditions. Mutation of _lecB_ has also been reported to result in defective biofilm formation (Tielker _et al._, 2005). Thus both lectins can contribute to biofilm formation although only LecA specific ligands appear to be capable of disrupting pre-formed biofilms. As yet, the nature of the carbohydrate ligands recognized by LecA and LecB and present in _P. aeruginosa_ biofilms have not been identified.

In _P. aeruginosa_, it has recently been established that extracellular, genomic DNA is a major component of the biofilm matrix (Whitchurch _et al._, 2002) (see also Pamp _et al._, this volume). The DNA released appears to be to be localized primarily in the stalks of microcolonies (Allesen-Holm _et al._, 2006). Biofilm formation in flow chambers was also inhibited by DNase I which was also shown to be capable of dissolving pre-formed biofilms in a biofilm age-dependent manner (Whitchurch _et al._, 2002). The mechanism by which this DNA is released may involve the lysis of a subpopulation of the culture although other mechanisms dependent on phage or membrane vesicles cannot be ruled out. DNA release
in *P. aeruginosa* does however involve AHL-dependent quorum sensing. This is because the level of extracellular DNA released by a *lasI rhlI* double mutant was significantly reduced compared with the parent strain and could be restored by supplying C4-HSL and 3-oxo-C12-HSL (Allesen-Holm *et al.*, 2006). The differences noted between the biofilms formed by the parent and *lasI rhlI* double mutant with respect to the amount of extracellular DNA in the region of the biofilm closest to the substratum also correlated with the spatial expression of *lasI* and *rhlI* in *P. aeruginosa* biofilms as reported by De Kievit *et al.* (2001). Although the AHL-dependent target genes involved in mediating DNA release have yet to be identified, a *pqsA* mutant defective in the production of the 4-quinolone QS signal molecule, 2-heptyl-3-hydroxy-4-quinolone (PQS), in common with the *lasI rhlI* mutant, generates low amounts of extracellular DNA (Allesen-Holm *et al.*, 2006). Furthermore in a static biofilm model, Diggle *et al.* (2003) have shown that the addition of PQS to a wild-type *P. aeruginosa* strain greatly enhances biofilm surface coverage. Since AHL-dependent QS and PQS signaling are intricately linked, the target genes involved may well be co-regulated by both QS systems.

### Aeromonas

The fish pathogen *Aeromonas hydrophila* which readily forms biofilms in a variety of environmental niches employs an AHL-based QS system consisting of the LuxR homologue, AhyR and the LuxI homologue AhyI which directs the synthesis of C4-HSL (Swift *et al.*, 1997; Lynch *et al.*, 2002). Mutation of either *ahyR* or *ahyI* result in the loss of extracellular serine- and metalloproteases which can be restored in an *ahyI* mutant by the addition of C4-HSL. *A. hydrophila* readily forms complex three dimensional biofilms on stainless steel with microcolonies covering up to 50% of the available surface area (Lynch *et al.*, 2002). In a continuous flow chamber, C4-HSL and the AhyI protein have both been detected in the developing biofilm while mutation of *ahyI* resulted in the formation of immature biofilms lacking microcolonies. The parenteral biofilm architecture could be partially restored by the provision of C4-HSL. Mutation of *ahyR* does not result in the loss of C4-HSL biosynthesis, since *ahyR* is not absolutely required for *ahyI* expression, and does not influence biofilm depth or microcolony formation. However, biofilm surface coverage in the *ahyR* mutant increased to around 80% of the available surface area (Lynch *et al.*, 2002). While these data support a role for AHL-dependent QS in *A. hydrophila* biofilm maturation, the specific QS dependent genes involved have not yet been identified.

### Burkholderia

From a transposon insertion library, Huber *et al.* (2001) obtained a number of *B. cenocepacia* H111 mutants defective in biofilm maturation on polystyrene surfaces one of which was also AHL-negative. Mapping of the insertion site revealed that the transposon had inserted into the *cepR* gene. Since CepR positively regulates *cepI*, this would also account for the loss of AHL production. Both *cepI* and *cepR* mutants exhibited biofilm maturation defects in that both were arrested at the microcolony stage and, in contrast to the parent strain, failed to colonize the entire surface. The parental biofilm phenotype was restored to the *cepI* mutant by providing exogenous C8-HSL firmly establishing a role for QS in *B. cenocepacia* biofilm maturation (Huber *et al.*, 2001). Similar architectural defects in *B.
cenoepectacia QS mutant biofilms have also been noted by Tomlin et al. (2005). Although the QS-regulated structural genes responsible for biofilm maturation in B. cenocepacia have not yet been characterized, three higher level regulators (yciR, subB and yciL) of the cep QS system have been identified in strain H111 as a consequence of their biofilm phenotype and reduced C8-HSL synthesis (Huber et al., 2002). However, neither exogenous C8-HSL addition nor provision of cepR in trans restored the biofilm defects in any of these mutants. Consequently while the mechanism by which they influence cep-dependent QS remains to be elucidated it is clear that the cep quorum-sensing system is a major checkpoint for biofilm maturation in B. cenocepacia in strains H111 and K56-2. To determine whether AHL-dependent QS contributed generally to biofilm maturation in the Burkholderia cepacia complex, Wopperer et al. (2006) introduced an AHL-degrading enzyme (AiiA) via a plasmid into a large number of different strains. Although dramatic differences in the biofilm forming capacity of these strains on polystyrene was noted, it was apparent that the expression of aiiA reduced biofilm biomass in many but not all strains.

Serratia

Serratia liquefaciens MG1 (also termed Serratia marcescens MG1) has a well characterized AHL-driven QS system which governs surface colonization through swarming motility by regulating the expression of the biosurfactant, serrawettin (Eberl et al., 1996). This strain forms an unusual biofilm when grown in flow chambers which consists of cell aggregates, chains of cells and elongated filamentous cells. Disruption of the AHL synthase swrI resulted in the loss of C4-HSL synthesis and the formation of thin, immature biofilms lacking aggregates and filamentous cells. Restoration of wild-type biofilm architecture to the swrI mutant was achieved by providing C4-HSL exogenously (Labbate et al., 2004). Two C4-HSL-dependent genes termed bsmA and bsmB were identified from a screen of transposon mutants for poor biofilm producers. Both mutants failed to form the biofilm cell aggregates and cell chains characteristic of the parent strain unless genetically complemented. Both BsmA and BsmB appear to be involved in cellular aggregation since the bsmA mutant exhibited excess aggregation while the bsmB mutant failed to aggregate, data which implies that cellular aggregation is likely to be a highly regulated process in S. liquefaciens. Although the functions of BsmA and BsmB are not known, BsmA has been suggested to be an adhesin given the excess adhesion and aggregation observed when bsmA was expressed in multicopy (Labbate et al., 2004). Interestingly, Serratia biofilm morphology is also dependent on nutrient cues such that the contribution of QS to biofilm architecture can be over-ridden by the growth medium used to culture the organism (Rice et al., 2005).

Dispersal

As biofilms develop to maturity there will be dispersal of aggregates from the main body of the multicellular structure. Dispersal has been considered as a passive process which is a consequence of the immediate environmental conditions and has usually been considered to occur by erosion, sloughing, abrasion or predator grazing (Bryers, 1988). While passive dispersal will undoubtedly be a factor in the dissemination of biofilm aggregates, active dispersal driven by the individuals within the population is accepted as an alternative strategy which bacteria employ to control dissemination of single cells or clusters of cells still...
bound by the biofilm matrix. Different types of dispersal mechanism have been reported in *Actinobacillus actinomycetemcomitans* *Staphylococcus epidermidis* and *P. aeruginosa* (Kaplan et al., 2003; Purevdorj-Gage and Stoodley, 2004). An active dispersal mechanism could conceivably be triggered at a predetermined point such as the attainment of a critical mass. Such a mechanism could be QS-mediated. While this is an attractive notion, dispersal driven by AHL-mediated QS is poorly understood. Purevdorj-Gage et al. (2005) examined this process in *P. aeruginosa* and found that although there was no difference in biofilm development and coverage between the parent strain and a *lasI rhlI* double mutant, seeding dispersal was severely impaired in the mutant. Furthermore, this phenomenon could not be attributed to swimming motility since both the double mutant and parent remained motile on semi-solid agar plate assays. The authors suggested that QS was likely to be involved in the differentiation process possibly by sensing cell population density and nutrient depletion within the periphery of biofilm clusters (Purevdorj-Gage et al., 2005).

As noted previously, biosurfactant production in a variety of Gram-negative bacteria is AHL-dependent and required for swarming motility since biosurfactants promote population migration by reducing surface tension (Daniels et al., 2004). In biofilms, coupling the production of biosurfactants with high cell population densities may promote biofilm dispersal. While rhamnolipid synthesis is involved in biofilm channel maintenance in *P. aeruginosa*, rhamnolipids do not appear to be required for seeding dispersal (Purevdorj-Gage et al., 2005). In *P. putida*, putisolvins inhibit biofilm formation and can promote the breakdown of existing biofilms (Kuiper et al., 2004). Consequently it has been suggested that high *P. putida* biofilm population densities stimulate the release of bacterial cells from the biofilm as a dispersal mechanism for escaping nutrient depletion and encouraging the colonization of other environmental niche(s) (Dubern et al., 2006).

AHL-dependent QS is also clearly involved in the dispersal of *S. marcescens* biofilms (Rice et al., 2005). After 70 to 80 hours of growth in a flow cell, the mature filamentous biofilm formed by the wild-type strain sloughed off from the surface. Similar results were obtained with an *S. marcescens swrI* mutant supplied with C4-HSL. However, no sloughing was observed in the *swrI* mutant grown in the absence of C4-HSL. Since the sloughing profile of a biosurfactant-deficient *S. marcescens* strain was the same as the parent, this suggests that the QS-mediated dispersal is likely to involve a range of gene products (Rice et al., 2005).

**Transcriptomic and proteomic investigations of the biofilm phenotype**

Rapid developments in microarray technology and mass spectrometry have facilitated the genome wide analysis of bacterial adaptations to different growth environments at both the transcriptional and proteomic levels. However, the application of these technologies to biofilm grown bacteria have so far had to treat biofilms largely as homogeneous entities and consequently the profiles obtained only show the average across a biofilm grown to a specific developmental stage. Different cells within distinct areas of a biofilm will experience differential access to nutrients and oxygen and this in turn results in a variety of micro-environmental conditions which will dictate diversity in gene expression patterns (Werner et al., 2004) (see also Kjelleberg and Givskov, this volume; Webb, this volume).
Genome-wide studies of biofilms have been viewed either by treating biofilms as a single entity in which gross phenotypic changes emerge as particular genes are mutated or alternatively from the perspective of a specific developmental stage, i.e. attachment, maturation or dispersal. In each situation, there have been conflicting views as to whether a particular biofilm architecture is governed by a designated set of genes which constitute the “biofilm regulon.” In a microarray study by (Whiteley et al., 2001) in which the transcriptomes of planktonic and continuous culture biofilm P. aeruginosa cells were compared, only 1% of the genes (73 about of 5570) were found to be differentially expressed (2-fold or greater). The most highly activated genes in the biofilm array were those of a temperate bacteriophage related to Pf1 while genes for pili and flagellar biosynthesis were strongly downregulated a finding which correlates with their role in the early but not later stages of biofilm maturation (Whiteley et al., 2001). No differences in the expression of the las or rhl QS systems was reported. In a comparable study by Hentzer et al. (2005) the gene expression profiles of biofilm grown P. aeruginosa cells were noted to most closely resemble those of stationary phase cells. Similar findings were also reported by Waite et al. (2005). These studies generally support the notion that biofilm development does not depend on a unique genetic program, i.e. the “biofilm regulon,” but instead depends on a series of adaptive responses to the prevailing micro-environmental conditions. Hentzer et al. (2005) did however identify a subset of AHL-regulated genes in biofilm grown P. aeruginosa that were differentially expressed compared with planktonic cells. These included, for example, the lectins, LecA (PA-1L) and LecB, the mutation of which results in biofilm-defective strains (Tielker et al., 2005; Diggle et al., 2006) the rhamnolipid biosynthetic gene, rhlC, genes coding for pyocyanin and hydrogen cyanide biosynthesis and pvdQ (Hentzer et al., 2005). While the physiological function of the latter is not known, it is required for synthesis of the siderophore pyoverdine and also has AHL-inactivating activity. PvdQ is an amidase/acylase which cleaves the amide bond of AHLs with fatty acid side chains ranging from 11 to 14 carbons (Sio et al., 2006) and may therefore represent a post-translational mechanism for modulating 3-oxo-C12-HSL levels within biofilms.

Proteomic approaches have also provided useful data highlighting the multiple phenotypes displayed by P. aeruginosa during biofilm development (Sauer et al., 2002). The levels of proteins involved in motility, alginate production and quorum sensing were observed to vary significantly with over 50% of the proteome reported to exhibit a six-fold or greater change in expression level when planktonic cells were compared with mature biofilm cells (Sauer et al., 2002). When a lasI mutant was compared with the parental strain, las-dependent QS was observed to be important in late but not early biofilm stages (Sauer et al., 2002). Apart from P. aeruginosa, proteomics has only been used to investigate the role of QS in biofilm development in P. putida (Arevalo-Ferro et al., 2005) where it has emerged that a major set of QS-regulated proteins overlaps with those identified as differentially regulated in sessile compared with planktonic cells. Interestingly, the majority of surface-induced proteins were negatively regulated by QS in biofilm cells, i.e. were upregulated in the ppuI mutant compared with the parent strain (Arevalo-Ferro et al., 2005).

As post-genomic and imaging technologies are further refined particularly with respect to investigating gene expression in single bacterial cells, the spatial and temporal impact of AHL-dependent QS on surface adaptation and the nature of the AHL-regulated structural
genes involved in determining biofilm architecture in pseudomonads and other AHL-producers should become more apparent (see also Kjelleberg and Givskov, this volume).

**QS in multi-species biofilms**

Much of the work on AHL-dependent QS and biofilms has focused on single-species biofilms. However, given that natural biofilms are often polymicrobial and that many different Gram-negative bacteria produce the same or different AHLs, it is possible that AHL-dependent QS also contributes to the maturation of mixed species biofilms. Although LuxR homologues preferentially respond to a specific AHL, they are capable of responding to a range of structurally related AHLs albeit less sensitively (McClean et al., 1997; Winson et al., 1998). Furthermore, bacteria such as Escherichia coli, Klebsiella and Salmonella which do not themselves produce AHLs, nevertheless possess a LuxR homologue termed SdiA which responds to AHLs produced by other bacteria (Ahmer, 2004). In Salmonella enterica SdiA regulates a number of genes including several involved in adherence to host cells, extracellular matrix proteins and resistance to complement-mediated killing (Ahmer, 2004). In E. coli, SdiA regulates genes involved in glutamate metabolism including gadA which codes for a glutamate decarboxylase which plays an important role in acid resistance. Interestingly C6-HSL was reported to enhance the tolerance of E. coli to acid in an sdiA-dependent manner (Houdt et al., 2006). Consequently such AHL signal interception could also play a role in the maturation of biofilms in mixed communities containing AHL-producers and bacteria such as E. coli or S. enterica.

When dialysis tubing containing spent culture supernatants with or without AHLs was placed in lakewater, McLean et al. (2005) noted that the nature of the adherent population changed with a notable reduction in the adherent population biodiversity. Exogenously supplied AHLs have also been shown to influence bacterial sludge community composition (Valle et al., 2004). In laboratory studies, AHL-mediated communication between P. aeruginosa and B. cenocepacia in mixed biofilms has been investigated and reported to be unidirectional in that B. cenocepacia responds to the P. aeruginosa AHLs but not vice versa (Riedel et al., 2001). Interestingly, differences in biofilm architecture with respect both to the spatial distribution of microcolonies and the formation of mixed microcolonies was observed when B. cenocepacia H111 (AHL positive) was grown with either a P. aeruginosa AHL producer or a non-producer (Riedel et al., 2001). QS has also been reported to influence interactions between P. aeruginosa and Agrobacterium tumefaciens in mixed cultures (An et al., 2006). Although P. aeruginosa, in both planktonic and biofilm modes of growth, rapidly outgrows A. tumefaciens, P. aeruginosa quorum sensing mutants showed reduced growth yields in planktonic co-cultures. In older biofilms, while the amount of A. tumefaciens biomass decreased in co-culture with the wild-type P. aeruginosa, it remained constant with P. aeruginosa lasR rhlR mutant (An et al., 2006). These experiments provide a useful basis from which to begin dissecting the role of QS in promoting competitive interactions within microbial communities.

**AHLs, biofilms, and interactions with higher organisms**

Apart from their role as signal molecules in QS, certain AHLs and notably 3-oxo-C12-HSL, have a broad range of biological activities which can influence the interactions...
between AHL-producing bacteria and other organisms. For example, 3-oxo-C12-HSL inhibits both growth and agr-mediated quorum sensing in S. aureus (Qazi et al., 2006), filamentation in Candida albicans (Hogan et al., 2004) (see also Hogan et al., this volume), has immune modulatory activity (Telford et al., 1998; Chhabra et al., 2003) and elicits both pro- and anti-inflammatory responses (Smith et al., 2002; Pritchard et al., 2005). 3-oxo-C12-HSL also influences smooth muscle contraction in blood vessels (Lawrence et al., 1999) and exerts a marked bradycardia in rats (Gardiner et al., 2001). Thus, for P. aeruginosa, an opportunistic pathogen, 3-oxo-C12-HSL not only appears to function as a QS signal molecule controlling expression of key virulence determinants but also as a means to gain a competitive survival advantage in the presence of other organisms occupying the same ecological niche.

Zoospore settlement

There is also evidence that higher organisms can “tune-in” to AHL signaling between planktonic and biofilm bacterial communities. An intriguing example of this involves the intertidal green macroalga Ulva which can reproduce asexually via the release of vast numbers of motile, asexual zoospores which initiate a process of surface selection involving the sensing of a surface followed by temporary adhesion. If a surface is suboptimal, the zoospore will detach. One important factor which influences zoospore surface-selection is the presence of a bacterial biofilm and there is a positive correlation between zoospore settlement and bacterial biofilm density (Joint et al., 2000). This observation led to the discovery that AHLs produced by bacterial biofilms are involved in the attraction of Ulva zoospores to surfaces. From investigations of the response of Ulva zoospores to (a) Vibrio anguillarum mutants defective in AHL production or expressing an AHL-inactivating enzyme, (b) E. coli strains expressing AHL synthases from recombinant plasmids and (c) synthetic AHLs, it emerged that zoospore attraction to a surface is, at least in part, mediated via zoospore-mediated AHL detection (Joint et al., 2002; Tait et al., 2005). Image analysis using GFP-tagged V. anguillarum biofilms revealed that zoospores settle directly on bacterial cells and in particular on microcolonies which are sites of concentrated AHL production (Tait et al., 2005) (Figure 6.2). The mechanism of attraction of zoospores does not appear to involve a chemotactic orientation towards the AHLs but instead a chemokinesis in which spore swimming speed rapidly decreases in the presence of AHLs such that they accumulate at the AHL source. Zoospore swimming speed for example decreased more rapidly over wild-type V. anguillarum biofilms when compared with those of an isogenic AHL negative mutant. AHL detection by zoospores causes an influx of calcium and it has been suggested that the reduction in swimming speed occurs through calcium-dependent modulation of the flagellar beat pattern (Joint et al., 2002). Ulva zoospores can sense a range of different AHLs (Tait et al., 2005) although the chemoresponse was reported to be most marked towards 3-oxo-C12-HSL (Wheeler et al., 2006), an interesting finding given the broad biological activity of this AHL molecule and its capacity to modulate mammalian host cell responses through calcium signaling (Shiner et al., 2006).

The reason(s) why Ulva zoospores target bacterial biofilms as a preferred site for attachment remains unclear but bacterial biofilms are clearly an important factor in biofouling. Many studies have shown that microbial biofilms influence the settlement of
Figure 6.2 Attraction of Ulva zoospores to AHL-producing Vibrio anguillarum biofilm microcolonies. (A) AHL production in biofilm microcolonies of V. anguillarum carrying the gfp-based AHL biosensor luxR-Pluxl-RBSII::gfpmut3*-T0 (B) DAPI-stained biofilm showing zoospores settled on a V. anguillarum microcolony. Images are transmission images overlaid with the fluorescent color image. Reproduced with permission from Tait et al. (2005). A color version of this figure is available at http://www.horizonpress.com/hsp/supplementary/biofilm/ch6fig2.jpg.
marine invertebrates. It has been assumed that grazing organisms can exploit bacterial biofilms as a food source but this is unlikely to be a factor in the attraction of an alga. Consequently, the presence of a bacterial biofilm may signal that the environment is benign for *Ulva* zoospores. However, given that the interaction is very specific, with zoospores settling directly on bacterial cells in the biofilm, rather than merely attaching in the vicinity, there are likely to be other reasons why zoospores prefer attaching to bacterial biofilms. For example, many green algae do not develop normal morphology when grown under axenic conditions (Provasoli and Pintner, 1980). Recently, Matsuo et al. (2003) have shown that differentiation in the green alga *Monostroma oxypermum* depends on the presence of specific bacterial strains, i.e. normal morphology depends on particular bacteria and not on bacteria in general. Consequently the preference of *Ulva* zoospores for settlement on AHL-producing bacterial biofilms may facilitate a close association between the developing thallus and certain essential bacteria (Tait et al., 2005).

**Protozoan grazing**

The growth and survival of bacteria in many different natural environments are constrained to some extent by the activities of predatory protozoa. Adoption of the biofilm mode of growth and the ability to produce toxic secondary metabolites are important bacterial defense mechanisms against grazing by protozoans although the effectiveness of the resistance mechanism depends on the protozoan feeding type (Matz and Kjelleberg, 2005; Weitere et al., 2005) (see also Matz, this volume). Selective predation by protozoa has been suggested to drive the evolution of bacterial grazing resistance strategies including biofilm adaptations, cell-to-cell signaling and the emergence of pathogenic traits (Matz and Kjelleberg, 2005).

In contrast to stationary phase bacterial cultures, predation is not likely to be such a problem for exponentially growing cultures since the high rates at which bacterial cell numbers are increasing should be sufficient to compensate for grazing losses. In situations where nutrients are limiting and bacterial growth is restricted, biofilm formation is a useful mechanism for combating predation. Whether bacteria are growing in planktonic cultures or as biofilms, the co-ordination of secondary metabolite production as a function of cell population density through QS is likely to constitute a useful strategy for ensuring that sufficient local concentrations of these toxic metabolites are attained. QS may therefore facilitate resistance to predation by controlling the architecture of biofilms and by regulating the production of anti/protozoal secondary metabolites. Consequently it can be predicted that mutants with defective AHL-dependent QS systems might be more susceptible to predation. So far, this has been shown to be the case for *S. marcescens*, *Chromobacterium violaceum* and *P. aeruginosa* (Matz and Kjelleberg, 2005).

The differentiated biofilm phenotype of *S. marcescens* MG1 has been described earlier in this chapter. Under static growth conditions, MG1 forms biofilms with microcolony-like structures but in flow cells forms highly differentiated filamentous biofilms consisting of cell chains and clusters (Labbate et al., 2004). Experiments with a suspension feeder flagellate (*Bodo saltans*) and a surface feeder (*Acanthamoeba polyphaga*) have revealed that while QS is not involved in the grazing resistance of *S. marcescens* microcolony-type biofilms, the QS-controlled biofilm specific differentiation into filaments and cell chains does confer effective
resistance against protozoan grazing (Queck et al., 2006). While there was no apparent role for QS in the development of resistance against protozoan grazing during the early stages of biofilm development by S. marcescens, QS clearly places a key role in protection of the late stage differentiated biofilm. Comparable findings have also been obtained for P. aeruginosa where the surface feeding flagellate Rhynchomonas nasuta induced microcolony formation in both parent and QS mutant (lasR and lasR/rhlR) strains although the latter showed reduced resistance to grazing (Matz et al., 2004). The lasR/rhlR mutant however showed a distinct lack of toxicity against the flagellates during late biofilm development. Hence AHL-dependent QS clearly contributes to the resistance of Gram-negative bacteria to protozoan gazing particularly with respect the production of inhibitors which confer protection against a wide range of protozoan feeding types (Weitere et al., 2005). A detailed account of bacterial biofilm-protozoan interactions is provided by Matz, this volume.

**AHL-dependent QS in biofilms as an antibacterial target**

Bacterial biofilm-centered infections are notoriously difficult to treat and conventional antibiotic therapy often fails as a consequence of the tolerance of biofilm bacteria (Davies, 2003). When compared with planktonic cells, biofilm bacteria almost always exhibit a large (up to 1000 fold) increase in resistance to diverse antimicrobial agents (see also Kjelleberg and Givskov, this volume). While the mechanisms underlying tolerance are not well understood, they are clearly multifactorial and include slow growth and metabolic rates, restricted penetration and specific resistance mechanisms (Davies, 2003). Given that more than 80% of bacterial infections in humans have been proposed to involve biofilms (Davies, 2003), there is an urgent need to discover novel targets against which effective therapeutic agents for the treatment of biofilm-centered chronic infections can be developed. In this context, AHL-dependent QS is an attractive target. This is because biofilms formed by P. aeruginosa QS mutants exhibit enhanced susceptibility to biocides such as sodium dodecyl sulfate and hydrogen peroxide and to antibiotics such as tobramycin (Davies et al., 1998; Bjarnsholt et al., 2005a). Agents which block QS either by inhibiting AHL synthesis, by inactivating AHLS or preventing the response to AHLS are likely to render P. aeruginosa biofilms susceptible to conventional antibiotics and to aid clearance by host defense mechanisms (Williams, 2002; Rasmussen and Givskov, 2006). Proof of this principle both in vitro and in vivo in experimental animal infection models has been presented for natural products such as furanones, patulin and penicillic acid and garlic extracts (Hentzer et al., 2003; Bjarnsholt et al., 2005b; Rasmussen et al., 2005b; Rasmussen and Givskov, 2006). Using transcriptomic approaches, many of these natural products clearly downregulate the las and rhl regulons (Hentzer et al., 2003; Rasmussen et al., 2005a). Further development of QS blockade as an anti-biofilm strategy will clearly depend on drug discovery programs uncovering safe effective QS inhibitors and in identifying the QS target structural genes which are responsible for the antibiotic tolerance of P. aeruginosa biofilms. Whether the inhibition of QS will render the biofilms of other AHL producers more susceptible to antimicrobials remains to be established although B. cenocepacia biofilms formed by cepI and cepR mutants have been reported to be more susceptible to sodium dodecyl sulfate while a cepI cciI double mutant biofilm was more sensitive to ciprofloxacin that the parent strain (Tomlin et al., 2005).
Concluding remarks
Among Gram-negative bacteria known to co-ordinate gene expression via AHL-dependent QS, there is now convincing evidence that AHLs are produced during the biofilm mode of growth in both laboratory and natural environments. It is also apparent that AHL-dependent QS is neither essential nor is it a dominant gene regulatory pathway required for facilitating bacterial adaptation to the biofilm lifestyle. There is also no evidence that biofilm development depends on a genetically programmed series of events driven by QS or indeed any other master regulatory system. QS is clearly a useful strategy for co-ordinating bacterial behavior within a population and undoubtedly can influence the structural development, integrity and architecture of a biofilm community as well as orchestrating the optimal timing and production of toxic secondary metabolites by the biofilm community to combat predators and host defense mechanisms. With the exception of the rhamnolipids which play a role in biofilm channel maintenance, the identity of the QS-regulated structural genes which contribute to biofilm development in *P. aeruginosa* or indeed to any other AHL-producing Gram-negative bacterium has yet to be uncovered. However, it is strikingly apparent that the loss by mutation of AHL-dependent QS in *P. aeruginosa* renders the biofilm population more susceptible to antimicrobial agents and host defenses. Consequently, the inhibition of QS offers an exciting target for the development of novel antibacterial agents for the treatment of chronic biofilm-centered infections.

Acknowledgments
Research in the authors’ laboratories has been funded by the Biotechnology and Biological Sciences Research Council, UK, the Medical Research Council UK, the Natural Environmental Research Council, the Wellcome Trust and the European Union which are gratefully acknowledged.

References


