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Cross-kingdom signalling: exploitation of bacterial quorum sensing molecules by the green seaweed *Ulva*

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The green seaweed *Ulva* has been shown to detect signal molecules produced by bacteria. Biofilms that release *N*-acylhomoserine lactones (AHLs) attract zoospores—the motile reproductive stages of *Ulva*. The evidence for AHL involvement is based on several independent lines of evidence, including the observation that zoospores are attracted to wild-type bacteria that produce AHLs but are not attracted to mutants that do not produce signal molecules. Synthetic AHL also attracts zoospores and the attraction is lost in the presence of autoinducer inactivation (AiiA) protein. The mechanism of attraction is not chemotactic but involves chemokinesis. When zoospores detect AHLs, the swimming rate is reduced and this results in accumulation of cells at the source of the AHL. It has been demonstrated that the detection of AHLs results in calcium influx into the zoospore. This is the first example of a calcium signalling event in a eukaryote in response to bacterial quorum sensing molecules. The role of AHLs in the ecology of *Ulva* is discussed. It is probable that AHLs act as cues for the settlement of zoospores, rather than being directly involved as a signalling mechanism.

Keywords: cross-kingdom signalling; *Ulva*; *N*-acylhomoserine lactones; chemokinesis; calcium ion influx

1. INTRODUCTION

Microbial populations in the natural environment are abundant, diverse and complex. A litre of seawater in the surface of the ocean will typically contain 10^9 bacteria and the total number of bacteria species, although currently unknown, has been estimated to be as many as 1 million (Curtis *et al.* 2002). How is this huge diversity maintained and how do bacteria interact? Quorum sensing (QS) is an excellent hypothesis to explain how bacteria might operate in concert. In this paper, we will consider the implications of QS for bacterial assemblages in the marine environment. In particular, we will discuss how a higher organism, the green alga *Ulva*, appears to exploit the chemicals that bacteria use for cell-to-cell communication. This paper will review the evidence for the involvement of bacterial signal molecules in the settlement of *Ulva* zoospores onto marine surfaces, the mechanisms that zoospores use to detect signal molecules. Finally, we discuss whether this cross-kingdom activity is truly QS or just the exploitation of signal molecules as cues.

2. QUORUM SENSING BY BACTERIA IN NATURAL ENVIRONMENTS

Early ideas about bacteria considered them as very simple organisms—little more than bags of enzymes. However, evidence gradually accumulated that bacteria, although morphologically simple, are capable of complex interactions. Once the concept was accepted that bacteria were not merely independent entities, it then became interesting to understand how they might interact. An obvious starting point was to consider metabolism and the idea developed that bacteria could act together in consortia, with the metabolism of one species resulting in the production of a compound, or series of compounds, that would be metabolized by another species. In one of the first demonstrations of concerted action, Slater & Bull (1982) used continuous culture to isolate a bacterial population from soil that was capable of breaking down the herbicide 2-chloroproprionamide. The interesting observation was that different experiments always resulted in the isolation of the same six species of bacteria. Slater & Bull (1982) used the term 'consortium' to describe this stable community. The herbicide was broken down as a result of co-metabolism by four of the bacteria in the consortium, but the other two species appeared to play no role in the catabolism of the herbicide. They appeared to exist on the by-products of metabolism of the core set of four species. This experiment demonstrated that bacteria were capable of concerted action—in this case, apparently driven by the different metabolic capabilities of six different species.

These experiments by Slater & Bull (1982) were done in chemostat cultures. In the natural environment of the soil, an obvious question is how can bacteria maintain a consortium in an environment where dispersion is likely—either by physical process of dilution by rainwater or grazing by protozoa and nematodes? If bacteria grow as single cells, then similar problems are likely to occur in every environment where they grow; for

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One contribution of 12 to a Theme Issue 'Bacterial conversations: talking, listening and eavesdropping'.

example, in the oral cavity, saliva is continually diluting the bacterial assemblage and in freshwater and marine environments, physical processes tend to lead to the dispersion of any pelagic assemblage. The problem of how to maintain a functional consortium is partially solved if bacteria are attached to a surface, through the biofilm mode of growth.

3. BACTERIAL BIOFILMS: WHAT MECHANISMS MIGHT BE IMPORTANT FOR THEIR FORMATION?

Attachment means that dilution is less of a problem for the maintenance of microbial assemblages, since most microbial biofilms adhere very securely to surfaces and are not easily dislodged. Growth as a biofilm does not solve the problem of cell losses due to grazing; indeed, it may exacerbate that problem by providing a concentrated source of food for the grazing organisms. Nevertheless, biofilms are clearly a very successful strategy for microbial growth and any surface immersed in water (freshwater or seawater) will quickly become colonized by a bacterial biofilm. The biofilm mode has other properties that appear to contribute to the success of this growth strategy. For example, biofilm bacteria are much less susceptible to treatment with chemically diverse biocides and antibiotics than the same planktonic cells; this causes many problems in medical (Bjarnsholt & Givskov 2007) and industrial fields (Costerton *et al.* 1999). Bacteria within biofilms also appear to have a very different physiology from that in the plankton phase of growth (Costerton *et al.* 1995). DNA microarray data have shown biofilms expressing unique patterns of genes, including those involved in adhesion and autoaggregation, and those for anoxic conditions (Schembri *et al.* 2003). However, it is suspected that these genes are not unique to biofilm bacteria and will be expressed under different planktonic conditions; there must presumably be factors that control what mode of growth is selected.

Biofilms in the natural environment are very complex entities that potentially consist of many hundreds of different species. There are real challenges in understanding how different bacteria interact with their own and other species. If metabolic consortia are common in biofilms, how do they maintain their structural integrity, so that appropriate species can develop in close proximity to one another? One mechanism might be by the process of co-aggregation that maintains cell–cell contact. Co-aggregation is when two or more genetically distinct bacteria interact by specific cell–cell recognition (Palmer *et al.* 2003) and is now a well-recognized phenomenon, particularly in oral plaque-forming bacteria. Co-aggregation between oral bacteria generally involves specific recognition between protein adhesins (lectins) and sugar receptors (saccharides). More than 300 taxa of dental plaque bacteria have been shown to co-aggregate (Palmer *et al.* 2003) and each strain may have few or many co-aggregating partners. Co-aggregation is an important process in the establishment and maintenance of the biofilms that comprise dental plaque, but is also known to occur among biofilm-forming freshwater bacteria and in all probability, in all biofilms (Rickard *et al.* 2000).

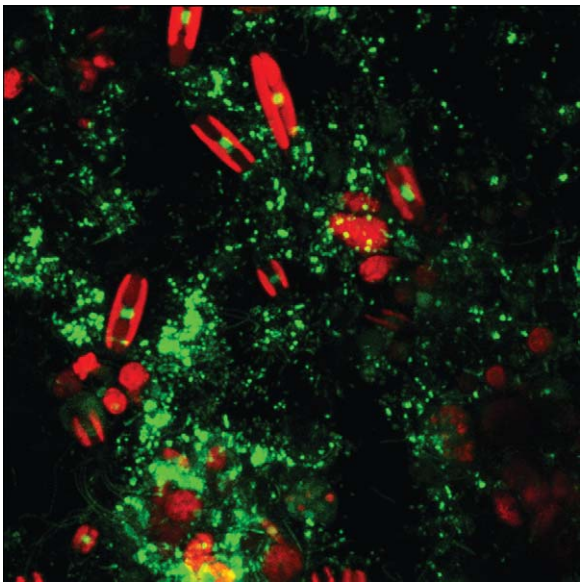

Figure 1. Epifluorescence image of the surface of a rock from an intertidal pool on the South Devon coast.The sample was taken in December 2004 and was stained with SYBR Green I and viewed with a Bio-Rad 1024 confocal laser microscope. The bacteria are stained green and the red fluorescence is due to the autofluorescence of chloroplasts within microalgae.

Although metabolic consortia and co-aggregation are plausible mechanisms to explain the development of the biofilm mode of growth, it is now known that other factors are involved. In particular, it has been established that QS is an important process in maintaining the attachment of bacteria to surfaces and the biofilm mode of growth—at least single species biofilms of many bacteria—for example, in *Pseudomonas aeruginosa*, *Aeromonas hydrophila* and *Vibrio cholerae* (Parsek & Greenberg 2005). Owing to their densely packed nature and limited capacity for diffusion, QS might be an ideal way to control different bacterial cells within a biofilm because they are densely packed and distances are small for the diffusion of signal molecules. However, the role of QS in multispecies biofilms is much less well understood. In this paper, we deal with an even greater level of complexity within a biofilm and that is how eukaryotes might interact with prokaryotes.

Marine biofilms are very complex structures that comprise both bacteria and eukaryotic microbes (figure 1). In addition to the intrinsic constituents of the microbial assemblage, other larger organisms interact, usually through the attachment of seaweeds and invertebrates, such as barnacles and limpets. This interaction between the biofilm and higher plants and animals is of interest at several levels. There is an obvious effect of grazing organisms in removing the microbial biofilm (Thompson *et al.* 2004) and the potential for both seaweeds and invertebrates to supply organic nutrients, through the excretion of organic matter, to the heterotrophic bacteria in the biofilm. Then, there is the question of how these larger organisms select surfaces for attachment, which inevitably involves some interaction with bacteria, because all marine surfaces are colonized by a microbial biofilm. Most of the invertebrates and all of the seaweeds attach during one stage in the reproductive cycle. In the case of

most biofouling invertebrates, motile larvae are released into the plankton (the holoplankton), where they are dispersed from the parent organism to colonize a new surface. Most seaweeds also produce a motile phase that is released into the water column before attaching to a surface and developing into a new plant. Of particular interest here are the mechanisms that zoospores of the green macroalga, *Ulva*, use to detect a suitable surface for attachment—a process that involves bacterial QS molecules (Joint *et al.* 2002).

4. EVIDENCE FOR CROSS-KINGDOM (PROKARYOTE/EUKARYOTE) DETECTION OF QUORUM SENSING MOLECULES

QS mechanisms in bacteria are now well established as a density-dependent process to control population activity. Small, freely diffusible chemical signal molecules diffuse from the bacterial cells. As the population increases, signal molecules accumulate until a critical threshold concentration is reached that is high enough to effect a response in the whole population by activating target genes (Swift *et al.* 2001). A wide range of small signal molecules are involved. Gram-positive bacteria generally use post-translationally processed peptides, whereas within Gram-negative bacteria, the *N*-acylhomoserine lactone (AHL) family of signalling molecules is most commonly used. AHLs vary in the length of the acyl side chain, which ranges from 4 to 18 carbon atoms, may be saturated or unsaturated, and may have hydroxyl- or oxo substituents on the third carbon atom of the *N*-linked acyl chain (Chhabra *et al.* 2005). When the critical threshold concentration is reached, AHL activation involves the formation of a complex with members of the LuxR or LuxN family proteins; the complex then drives the expression of multiple target genes (Lazdunski *et al.* 2004). The targets include genes required for AHL synthesis, and a wide range involved in secondary metabolism, virulence and biofilm development (Williams *et al.* 2007).

Our interest is specifically in the detection of AHLs by *Ulva* zoospores. In a study of the factors that influence the attachment of zoospores to marine surfaces, Joint *et al.* (2000) found a correlation between the number of zoospores that attached to a surface and the number of bacteria on that surface—specifically, more zoospores were present with increasing numbers of bacteria. In a detailed statistical analysis, Joint *et al.* (2000) found clear evidence that the zoospores were not just associating with the bacterial biofilm but were also actually attaching to bacteria. The bacterial density 'under' the zoospores (i.e. the bacteria that were not visible when viewed with a microscope because they were hidden by a settled zoospore) was nearly 25 times higher than if the zoospores had attached in a random matter across the biofilm. This observation suggested a direct interaction between the bacteria and the zoospores. When mutants of *Vibrio anguillarum*, defective in AHL production, were tested for their ability to attract zoospores, no attraction was detected. Surprisingly, QS appeared to be a plausible explanation for the attraction of zoospores to marine bacterial biofilms.

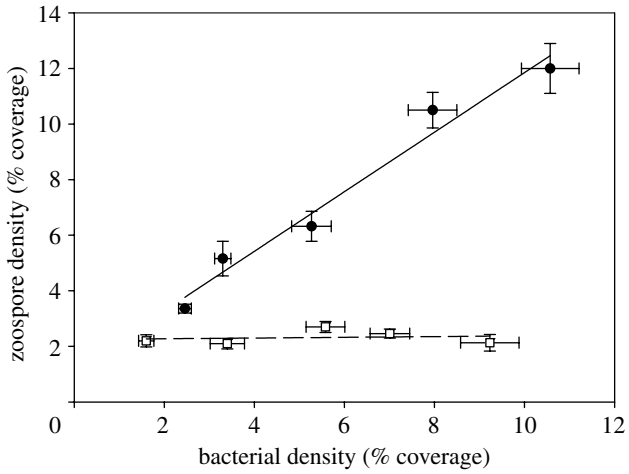

Figure 2. The relationship between the density of bacteria (*Vibrio anguillarum*) in a biofilm and the number of zoospores that attach to the biofilm. The response of the wild-type (●), which produces three AHL molecules (C6-HSL, 3-hydroxy-HSL and 3-oxo-C10-HSL) is compared with a *vanIM* mutant (□) that produces no AHLs.

A number of different approaches finally established that AHLs played a role in the attraction of zoospores to bacterial biofilms. As with mixed natural bacterial assemblages, biofilms of a pure culture of *V. anguillarum* wild-type (WT) also showed a positive correlation between the numbers of zoospores attaching and the bacterial cell density (Joint *et al.* 2002).Two QS circuits, termed VanI/R (homologous to the *Vibrio fischeri* LuxR/I), with the cognate signal molecule *N*-(3-oxodecanoyl)-L-homoserine lactone (3-oxo-C10-HSL), and VanM/N (homologous to the *V. harveyi* LuxM/N), with the cognate signal molecules *N*-hexanoyl-L-homoserine lactone (C6-HSL) and *N*-(3-hydroxyhexanoyl)-L-homoserine lactone (3-hydroxy-C6-HSL), regulate target gene expression in a hierarchical manner (Milton *et al.* 2001). There was no stimulation of attachment with a *vanM* mutant, which does not produce C6-HSL or 3-hydroxy-C6-HSL. Since the AHLs produced by *vanM* are also required for the production of 3-oxo-C10-HSL, the *V. anguillarum vanM* mutant is in fact deficient for a third AHL, 3-oxo-C10-HSL. An unequivocal confirmation of the involvement of AHLs came from the lack of response by zoospores to a double *vanIM* mutant, which produces none of the three AHLs that are associated with *V. anguillarum* (figure 2).

These experiments strongly suggested that *Ulva* zoospores could sense and respond to AHLs produced by *V. anguillarum*; but was it possible that other unidentified phenotypes of these mutants might affect zoospore settlement? To exclude this possibility, settlement experiments were also done with *Escherichia coli* expressing the recombinant AHL synthase genes *vanI* or *vanM*. Biofilms of *E. coli* strains expressing the recombinant *V. anguillarum* AHL synthases VanI (producing 3-oxo-C10) and VanM (producing C6-HSL and 3-hydroxy-C6-HSL) caused significantly higher zoospore settlement (Joint *et al.* 2002). No stimulation of zoospore attachment was found on biofilms of the control *E. coli* strains that produced no AHLs. This was a second set of evidence that

supported the conclusions of the *V. anguillarum* mutant experiments that AHLs produced by bacterial biofilms were sensed by *Ulva* zoospores. Both experimental approaches suggested that *Ulva* zoospores were capable of sensing all the three AHLs produced by *V. anguillarum*, i.e. 3-oxo-C10-HSL, 3-hydroxy-C6-HSL and C6-HSL.

The third independent piece of evidence for the detection by zoospores of AHLs came from the action of the autoinducer inactivation enzyme AiiA, a lactonase enzyme that has been shown to degrade AHLs (Dong *et al.* 2000; Carlier *et al.* 2003). QS signalling properties in bacteria are destroyed if the homoserine lactone ring is open. Tait *et al.* (2005) found that zoospores were not attracted to biofilms of *V. anguillarum* expressing the recombinant *Bacillis* lactonase-coding gene *aiiA*, suggestion that the signalling properties of AHLs were lost. We also investigated whether AHLs could be detected in the medium covering biofilms of both WT and recombinant types. Using thin layer chromatography (TLC), Tait *et al.* (2005) visualized 3-oxo-C10-HSL by overlaying the TLC plate with a long-chain AHL biosensor, *E. coli* (pSB1075). The WT *V. anguillarum* was shown to produce AHLs when growing both as a biofilm and as a cell suspension in seawater. However, 3-oxo-C10-HSL production was completely abolished by the *V. anguillarum* NB10 expressing *aiiA*. By using an alternative AHL biosensor (*E. coli*, pSB401), Tait *et al.* (2005) also showed that C6-HSL and 3-hydroxy-C6-HSL were produced by WT *V. anguillarum*; again, these AHLs could not be detected in strain NB10 expressing *aiiA*. Therefore, the opening of the homoserine lactone ring meant that no AHL production could be detected in the strain expressing the lactonase. Significantly, this also abolished the ability of *V. anguillarum* to attract *Ulva* zoospores.

The final evidence for the involvement of AHL in zoospore settlement was provided by attraction of zoospores to synthetic AHLs (Joint *et al.* 2002). When 3-oxo-C10-HSL was incorporated into agarose films, there was a significant enhancement of zoospore attachment. Since the AHL was diffusing out from the agarose film, this suggested that zoospores were detecting a gradient of AHLs diffusing from the agarose. To test this, Joint *et al.* (2002) added dissolved AHLs to the seawater over a biofilm of AHL-producing *V. anguillarum*, with the aim of masking the gradient in AHL concentration diffusing from the biofilm. The presence of AHL in the medium in which the zoospores were swimming significantly reduced zoospore attraction by this bacterium. We assumed that this indicated that the reduction in settlement was because the zoospores could not detect any gradient in AHL concentration, and so any chemotactic response was nullified. Using this method, we were able to demonstrate that zoospores were attracted to all but the smallest AHL, *N*-butanoyl-L-homoserine lactone (C4-HSL; Tait *et al.* 2005).

All four lines of evidence are consistent with the hypotheses that the *Ulva* zoospores alter their behaviour when they detect AHLs. This is clear evidence for the direct involvement of AHLs in the surface selection by zoospores. But was this specifically due to QS or was it a less specific effect?

5. ZOOSPORES ATTACH DIRECTLY TO BACTERIAL CELLS

With biofilms of both mixed assemblages of natural marine bacteria (Joint *et al.* 2000), and with single species cultures (Joint *et al.* 2002), the number of zoospores attaching is usually positively correlated with the density of bacteria in a biofilm. We presented strong statistical evidence that the zoospores were attaching directly to bacterial cells rather than to the surrounding substratum (Joint *et al.* 2000). Although the evidence was strong that AHLs were involved, it was also possible that other factors were also involved in some way in the selection of surfaces for attachment by zoospores. For example, zoospores could respond to some properties relating to the surface itself. Callow *et al.* (2002) showed that zoospores preferentially attached within crevices and to micrometre scale imperfections in steel surfaces. Therefore, it was possible that some of the interactions between zoospores and bacterial biofilms were due to variations in surface topography.

Tait *et al.* (2005) tested this hypothesis by comparing the results obtained by the statistical approach of Joint *et al.* (2000) with a direct visual method using *V. anguillarum*—as mutants and with a *gfp*-based AHL reporter system. The procedure of Joint *et al.* (2000) is microscope-based and compares the number of bacteria that are visible after zoospores have attached (i.e. on the surface between the zoospores) with an identical biofilm to which no zoospores have attached. If settlement is a random process, then the number of bacteria visible per unit area should be the same in both cases. However, experiments with AHL-producing bacteria always show that the density of visible bacteria in the presence of zoospores is much less than in control biofilms (Patel *et al.* 2003). In other words, more bacteria are covered by zoospores than would be expected by a random process.

Using biofilms of WT *V. anguillarum*, Tait *et al.* (2005) also found that a large proportion of the bacteria were covered by zoospores. Experiments with biofilms of the *vanM* mutant (which does not produce AHLs) showed no difference in density of visible bacteria in the presence and absence of zoospores. That is, there was no density-dependent stimulation of settlement and the zoospores were not attaching preferentially to bacterial cells. We then demonstrated, using a green fluorescent protein (*gfp*)-based AHL reporter system, that this difference in settlement was indeed related to AHL production (Tait *et al.* 2005). The plasmid (pDM42), carrying a *luxR*-P_{luxI}-RBSII::*gfp*mut3*-T_0 gene fusion, was mobilized into the WT and *vanM* mutant strains of *V. anguillarum*; this gene fusion expresses the *gfp* in the presence of AHLs. We showed that green fluorescence only occurred in biofilms of the WT but not of the *vanM* mutant. This is further evidence that the zoospores exploit signal molecules rather than some features of surface topography, because, with very minor differences, the surface properties of the WT and mutant strains were the same (Tait *et al.* 2005). This conclusion was further supported by killing bacterial cells without affecting the physical integrity of the biofilm surfaces. Biofilms exposed to either UV light or 100 $\mu g\ ml^{-1}$

chloramphenicol for 30 min had significantly reduced zoospore attachment, again demonstrating that surface topography is not the dominant factor in zoospore settlement on biofilms (Tait *et al.* 2005).

We have also investigated a number of Gram-positive strains that also enhanced the settlement of zoospores. These bacteria do not produce AHL molecules, but cell-to-cell signalling is mediated by signal peptides (Williams *et al.* 2007). We isolated several actinobacteria (actinomycete) that form filamentous biofilms. In this case, surface topography appeared to be an important factor and the 'rough' surface of the actinobacteria was ideal for zoospore settlement (figure 3*a*). Indeed, roughness was the most important factor with these bacterial isolates because when these filamentous strains were treated with UV or with antibiotics to kill the biofilms, the number of zoospores that settled was not significantly different from the live biofilms (figure 3*b*), in contrast to AHL-producing bacteria (figure 3*c*).

A number of different factors are therefore involved in the success of attachment of *Ulva* zoospores—the physical nature of the surface, as well as the presence of bacterial biofilms. However, chemical signalling, through AHL production by bacterial biofilms, appears to be a very important process that significantly enhances the number of *Ulva* zoospores that attach to a surface in the marine environment.

6. SPECIFICITY OF ZOOSPORE ATTRACTION TO DIFFERENT *N*-ACYLHOMOSERINE LACTONES

Our initial evidence for the involvement of QS molecules in zoospore settlement was based in part on the fact that zoospores could detect and respond to synthetic AHLs in very similar ways to *V. anguillarum* and other bacterial biofilms (Joint *et al.* 2002). In that study, three different synthetic AHLs were tested, which differed in the length of the acyl side chain. In a more detailed assessment of AHL specificity, Tait *et al.* (2005) tested a wide range of AHLs, both synthetic and those produced by *E. coli* and *A. hydrophila* biofilms. The synthetic AHLs tested were unsubstituted C4-, C6-, C8-, C10- and C14-HSLs, as well as the 3-oxo- and 3-hydroxyl-substituted series of the same AHLs. Zoospore settlement was enhanced by all AHLs except for the shortest side chain C4-HSL. The greatest effect was seen with longer *N*-acyl side chains, the 3-oxo or 3-hydroxy substituents tended to attract more zoospores than the unsubstituted AHLs. In terms of zoospore settlement, very similar results were obtained with natural AHL and synthetic molecules. A range of AHLs were synthesized by *E. coli* biofilms expressing different recombinant *luxI* homologues. 3-oxo-C10-HSL was produced by *vanI* from *V. anguillarum*, 3-oxo-C6-HSL by *luxI* from *V. fischeri* and both C4-HSL and C6-HSLs by *rhlI* from *P. aeruginosa*. As with synthetic molecules, these natural AHL molecules significantly enhanced the attachment of *Ulva* zoospores. In all cases, the integrity of the homoserine lactone ring is crucial. If the ring is opened by altering the pH, the attraction of zoospores is eliminated (Joint *et al.* 2002). This is also the case in bacterial QS, where signalling is abolished with open-ring AHLs (Yates *et al.* 2002). Hence, zoospores detect and respond to AHLs with a wide range of *N*-acyl side chains—but a closed homoserine lactone ring is an essential requirement.

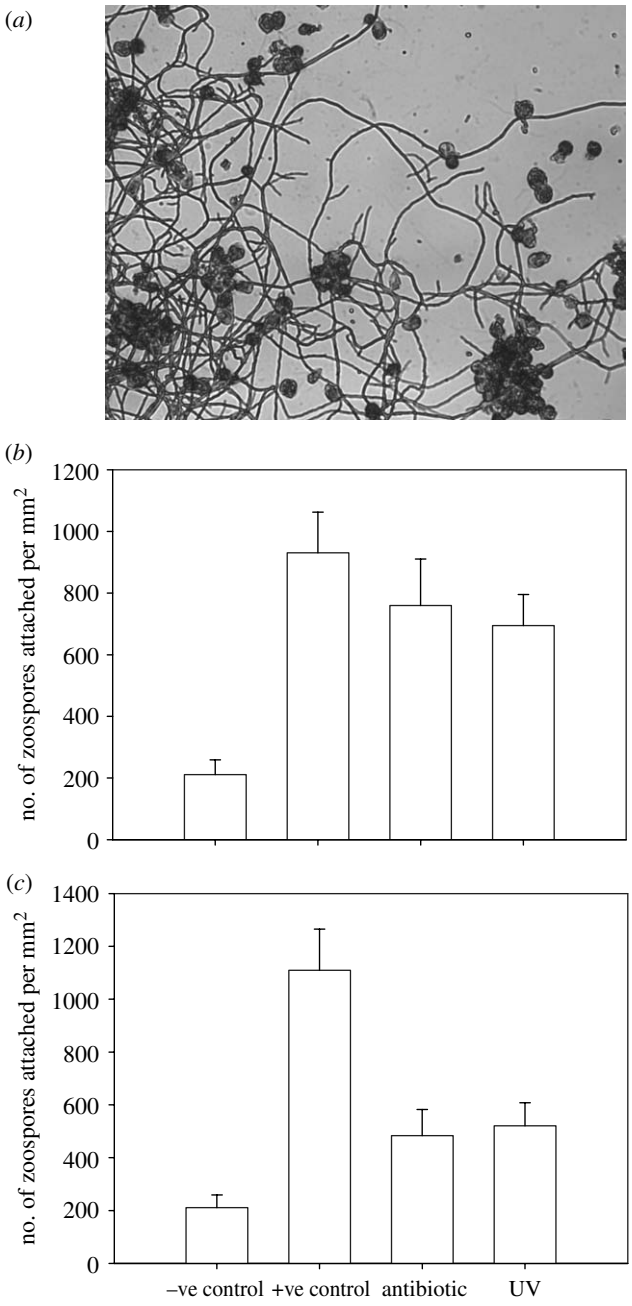

Figure 3. Attraction of zoospores to biofilm surface roughness. (*a*) Transmission image of zoospores settled on a filamentous actinobacteria biofilm (scale bar, 25 µm). Zoospore settlement on biofilms of (*b*) a filamentous actinobacteria is compared with (*c*) an AHL-producing strain. Treating the biofilms with UV (1 h) or antibiotics (5 units ml^{-1} penicillin, 30 min) caused no significant reduction in settlement on the filamentous biofilm, but reduced settlement on the AHL-producing biofilm.

7. HOW DOES *ULVA* ZOOSPORE BEHAVIOUR RESPOND TO *N*-ACYLHOMOSERINE LACTONES?

Our initial assumption was that a chemotactic response must be used by zoospores in responding to AHL-producing biofilms. This conclusion appeared to be

supported by experiments where the addition of AHLs to the medium covering a biofilm would abolish any attraction to the biofilm (Joint *et al.* 2002). This observation appeared to be consistent with the idea that the added AHL abolished a gradient in AHL concentration, and hence any chemotactic response of the zoospores would be compromised. However, although AHLs may be acting as specific chemoattractants for *Ulva* zoospores, the mechanism through which they act turned out to be somewhat unexpected.

A point source of AHL will rapidly result in the accumulation of *Ulva* zoospores and the density becomes so high that they are obvious to the naked eye. Wheeler *et al.* (2006) showed accumulation around a point source of *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) when the AHL was present as a dried spot on the surface of a glass slide. The zoospores aggregated beyond the dimensions of the point source, suggesting that they were responding to a diffusion gradient. The magnitude of the accumulation and the speed with which zoospores aggregated suggested that 3-oxo-C12-HSL is evidently a strong chemoattractant. However, zoospores did not accumulate around the end of a micropipette filled with 3-oxo-C12-HSL, at concentrations up to 125 $\mu mol\,l^{-1}$ (Wheeler *et al.* 2006). Nor, in a standard assay for chemotaxis, did zoospores swim preferentially into capillary tubes filled with different AHLs; the number entering was no different from that in a control. How can this apparent discrepancy—the strong chemoattraction to a dried spot of 3-oxo-C12-HSL, but a lack of chemotaxis—be resolved?

There are, of course, many ways in which a motile organism may respond to a chemoattractant. Chemotaxis is one mechanism that specifically alters the directional orientation of an organism in response to a chemical stimulus. However, chemoattractants may alter the distribution of motile organisms in other ways, e.g. through the modulation of swimming speed or turning frequency. Such a response is termed a chemokinesis, the best-characterized example of which is the biased random walk of flagellated bacteria due to the regulation of tumbling frequency.

Video motion analysis of swimming *Ulva* zoospores demonstrated that there was a distinct chemokinesis following exposure to AHLs. The addition of 3-oxo-C12-HSL in excess of 25 $\mu mol\,l^{-1}$ to the seawater surrounding *Ulva* zoospores caused an immediate decrease in their swimming speed, i.e. mean swimming speed was reduced up to 73% within 4 min (Wheeler *et al.* 2006). A comparison of different AHLs indicated that 3-oxo-C12-HSL, followed by 3-oxo-C10-HSL, were the most effective signal molecules in altering the swimming behaviour of *Ulva* zoospores. Similar effects were observed with bacterial biofilms. Analysis of zoospore swimming speeds over *V. anguillarum* biofilms demonstrated that mean speed decreased much more rapidly over WT biofilms (producing two AHL molecule types) than over biofilms of a *vanM* mutant in which AHL synthesis has been disrupted (Wheeler *et al.* 2006). This is a clear indication that the AHL-mediated chemokinesis occurs over microbial biofilms and contributes to the observed increase in settlement.

The lower threshold concentration for the chemokinesis was measured at 25 $\mu mol\,l^{-1}$, which is much higher than the concentration required to activate many AHL-regulated QS circuits in bacteria. However, it must be considered that the two responses are very different, as the AHL-mediated chemokinesis enables a fast-moving eukaryote to locate a mature bacterial biofilm. The concentration of 3-oxo-C12-HSL present in a biofilm of *P. aeruginosa* has been estimated to be in excess of 600 $\mu mol\,l^{-1}$ (Charlton *et al.* 2000), suggesting that certain signal molecules may accumulate to a very high degree within a mature biofilm environment. 3-oxo-C12-HSL concentrations were much lower in the aqueous phase, suggesting that there is a very significant concentration gradient at the aqueous/biofilm interface. Thus, it may be that the *Ulva* chemoresponse will only be effective in the immediate vicinity of a mature biofilm because dispersion might be expected to dilute the concentration of AHLs to the point at which they cannot function in QS. However, recently, Miller *et al.* (2005) have detected a large area of bioluminescence in the open ocean of the northwestern Indian Ocean. In fact, the bioluminescence was so strong that it could even be detected by satellite from space. Since bacterial bioluminescence is under the control of QS, and is dependent on a sufficient AHL concentration, this raises the possibility that dispersion processes do not necessarily lead to the dilution of signal molecules to the point at which they are ineffective.

This rapid alteration of swimming speed provides a possible mechanism to explain an attraction to AHLs that may occur without chemotaxis. A random, fast-moving population will accumulate at a point if their swimming velocity is reduced every time a zoospore enters a zone of elevated AHL concentration. We found strong support for such a mechanism when both zoospore swimming speed and direction were analysed around point sources of AHL (Wheeler *et al.* 2006). Swimming speed decreased in proximity to sources of both 3-oxo-C10-HSL and 3-oxo-C12-HSL, but there was no directional orientation towards either point source. It is important to note that these measurements were performed on zoospores swimming very close to the surface of the microscope slide. Zoospore swimming speed was not reduced in free-swimming zoospores, i.e. those not directly in contact with a solid substrate. The requirement for surface contact therefore ensures that a cell does not completely cease swimming before a solid substrate for settlement is located. An alternative, but unlikely, explanation for these behavioural changes could be that there are toxic effects of AHL. However, if AHLs were mediating an adverse effect on zoospore physiology, the ability of the propagule to sense and irreversibly attach to a substrate would also be compromised. As we observed no inhibition of this settlement process following exposure to 3-oxo-C12-HSL, the AHL molecules appear to exert a specific effect on zoospore swimming behaviour.

8. CELLULAR MECHANISMS INVOLVED IN *N*-ACYLHOMOSERINE LACTONE-MEDIATED CHEMOKINESIS

In bacteria, AHLs regulate gene expression by binding directly to transcriptional activators or receptor kinases involved in complex signal transduction cascades (Williams *et al.* 2007). We do not know the exact cellular mechanisms that enable *Ulva* zoospores to detect AHLs, but it is highly probable that the signal transduction pathway employs calcium as a second messenger. Calcium signalling has previously been implicated in the swimming responses of motile algae, notably in the phototactic and chemotactic responses of *Chlamydomonas* and also in the chemotactic responses of male *Ectocarpus* gametes (Harz & Hegemann 1991; Maier & Calenberg 1994; Ermilova *et al.* 1998). Phototactic orientation of *Chlamydomonas* occurs via the opening of light-gated cation channels, known as channelrhodopsins, leading to an influx of H^+ and Ca^{2+} ions (Nagel *et al.* 2002, 2003). Calcium is also involved in flagellar movement, and changes in intraflagellar calcium regulate the flagella by controlling dynein-dependent microtubule sliding (Smith 2002). Cellular orientation is proposed to occur as a result of the differential sensitivities of the two flagella to calcium (Kamiya & Witman 1984).

Based on the *Chlamydomonas* model, we predicted that AHLs should cause an influx of calcium, followed by a corresponding increase in cytosolic calcium. This would then mediate a decrease in swimming speed by a calcium-dependent modulation of the flagellar beat pattern. We examined whether a calcium influx occurs in the AHL signal transduction pathway in swimming zoospores using the manganese quench technique. Briefly, when a cell is stimulated in the presence of excess manganese, the opening of calcium channels allows Mn^{2+} ions to enter the cytosol. Mn^{2+} binds irreversibly to calcium indicator dyes (such as calcium orange), quenching the fluorescence (Taylor *et al.* 1996). Thus, the quench is indicative of the opening of calcium channels and the corresponding calcium influx. Zoospores loaded with the calcium indicator dye, calcium orange, demonstrated a reproducible decrease in dye fluorescence on exposure to 3-oxo-C12-HSL, at concentrations up to 175 $\mu mol\,l^{-1}$. This indicates calcium influx (figure 4*a*). Little or no quenching of dye fluorescence was observed in untreated zoospores, implying calcium influx is directly linked to the presence of the AHL signal molecule (figure 4*b*).

This is the first example of a calcium signalling event in a eukaryote in response to bacterial QS signals. The calcium influx in response to 3-oxo-C12-HSL supports the hypothesis that the chemokinesis in *Ulva* zoospores can occur by a similar mechanism to the tactic responses of *Chlamydomonas*. However, in order to determine the precise role of calcium in regulating flagellar beat patterns, it will be important to determine how the calcium influx influences cytosolic calcium concentrations in *Ulva* zoospores. In particular, it will be of great interest to differentiate the calcium signalling events that mediate chemokinesis in *Ulva* zoospores and chemotaxis in *Chlamydomonas*. The characterization of this signal transduction pathway may aid in the identification of similar signalling pathways in other eukaryotes.

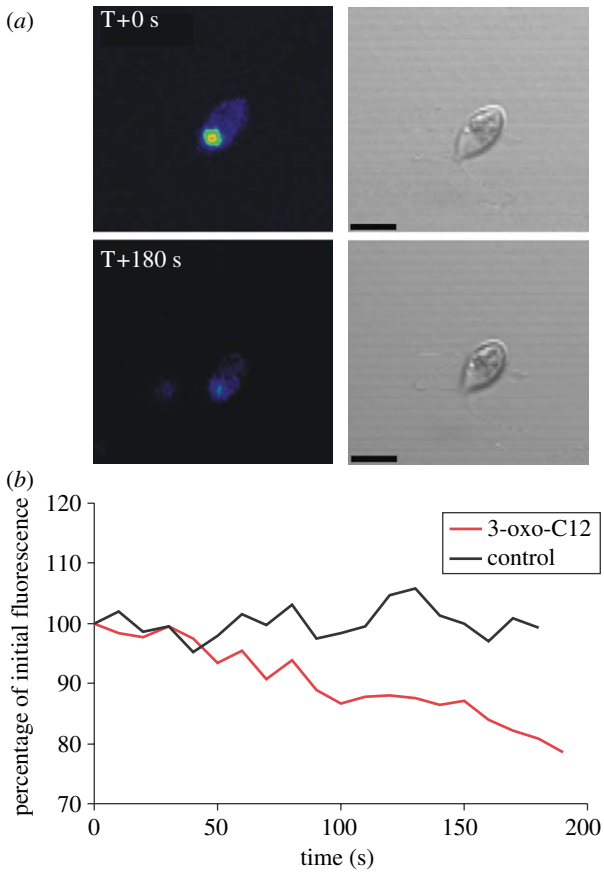

Figure 4. Exposure to 3-oxo-C12-HSL induces a calcium influx in *Ulva* zoospores. (*a*) False colour image of an *Ulva* zoospore loaded with the fluorescent calcium indicator dye, calcium orange AM, and viewed by confocal laser microscopy. Calcium influx was determined by the manganese quench technique. *Ulva* zoospores were exposed to 175 $\mu mol\,l^{-1}$ 3-oxo-C12-HSL after prior incubation in seawater containing 100 $\mu mol\,l^{-1}$ $MnCl_2$ for 5 min. Fluorescence was imaged following excitation at 568 nm, using a Bio-Rad 1024 confocal laser microscope. Only zoospores that had not undergone settlement and retained flagella throughout the time-course were measured. (*b*) Mean cellular fluorescence in *Ulva* zoospores expressed as a percentage of initial fluorescence on addition of 3-oxo-C12-HSL ($n=9$, control $n=5$). Scale bar is 5 μm.

9. POTENTIAL ECOLOGICAL BENEFITS FOR *ULVA* OF DETECTING *N*-ACYLHOMOSERINE LACTONE SIGNAL MOLECULES

The nature of the *Ulva* chemoresponse also begins to shed some light on the ecological relevance of this process and its role in the settlement process. Chemokinesis is only one of the processes involved in the settlement of a motile zoospore into a hard substrate. Zoospores are strongly negatively phototactic, orientating their swimming away from the light and thus maximizing their chances of locating a suitable substrate for settlement. Therefore, light appears to mediate a longer range orientation of the zoospores. AHL chemokinesis would allow the zoospore to locate a suitable bacterial biofilm once a solid substrate has been located. AHLs are just one of the many factors influencing the selection of a suitable substrate for selection. The chemistry, wettability and

topography of the surface are also important (Callow & Callow 2000). This may explain why the response to AHLs is a chemokinesis rather than a chemotaxis. The chemokinesis locks the zoospore into the vicinity of a bacterial biofilm, but still allows these other factors to influence the final destination for settlement.

It is relevant to ask what evolutionary selection might have been involved in the exploitation of bacterial signal molecules by *Ulva* zoospores. Why do zoospores target bacterial biofilms as a preferred site for attachment? It may be that the presence of an active bacterial biofilm could indicate that a surface is benign, since bacteria are less likely to grow well and establish stable biofilms on toxic or unfavourable surfaces. However, this hardly seems a strong selection factor and does not explain the specificity of the interaction—with zoospores preferentially settling on top of bacterial microcolonies, rather than settling in the vicinity, as might be expected if the bacteria were merely indicating an acceptable substratum. Might the interaction be of primary benefit to the bacteria, rather than the alga? Certainly, bacteria will gain from the presence of a primary producer, which would be a source of organic matter for heterotrophic bacteria. However, a benefit to the bacteria does not involve any evolutionary advantage to the seaweed and it is difficult to envisage any section pressure on the *Ulva* that results from more active bacterial biofilms.

It may be that more complex interactions between bacteria and *Ulva* are involved and that bacteria provide something of benefit to the alga which has ensured that mechanisms have evolved that influence swimming and settlement behaviour of the zoospore. It has been known for many years that some green macroalgae, including *Ulva*, show aberrant morphology when grown axenically. Provasoli & Pinter (1980) found that the thallus did not develop normally in the absence of bacteria, and normal growth and sporogenesis were only restored on the introduction of bacteria (Stratmann *et al.* 1996). More recently, Matsuo *et al.* (2003) demonstrated that specific bacterial strains were involved in the differentiation of the green alga *Monostroma oxyspermum*. That is, the normal morphology of the seaweed depends on particular bacteria and not on bacteria in general. Matsuo *et al.* (2003) suggested that this function is restricted to a small group of bacteria in the Bacteriodetes phylum, specifically *Cytophaga* and *Flavobacterium* spp.

The control on morphology occurs at very low densities of bacteria, and extracellular substances produced by bacteria appear to initiate the alteration. Matsuo *et al.* (2003) demonstrated that supernatants of bacterial cultures have the ability to restore the natural morphology of the alga. Interestingly, extracts of brown and red algae were also able to restore normal growth in *M. oxyspermum* (Tatewaki *et al.* 1983). Matsuo *et al.* (2005) went on to identify and characterize an exogenous growth factor, thallusin, which is produced by bacteria belonging to the Bacteriodetes group. They suggested that thallusin is an essential factor for normal growth of *M. oxyspermum* and that this function derives from a very small group of bacterial species.

If specific bacteria are involved in normal morphogenesis, then that might be a plausible explanation for why algal zoospores use AHL molecules as cues for settlement. If the essential bacteria also use AHL as QS molecules, then that provides a mechanism to ensure that zoospores settle in the vicinity of the specific bacteria that they require for normal development, growth and survival to the next generation. Marshall *et al.* (2006) recently attempted to test this hypothesis by characterizing a number of bacterial strains for both their ability to attract zoospores and their influence on plantlet development.

Marshall *et al.* (2006) isolated bacteria from intertidal rocks that are colonized by *Ulva* and identified the strains from their 16S sequences. Interestingly, and in contrast to the findings of Matsuo *et al.* (2005), bacteria other than the Bacteriodetes stimulated growth and morphology of *Ulva*. The majority of the isolates had some effect on the morphology of developing plantlets of *Ulva*, with only 7 out of 20 unique isolates having no effect on morphology. There was no clear specificity because the isolates that altered morphology were distributed over several bacterial taxa. The ability of these isolates to attract zoospores was also tested, but no overall correlation was found between isolates that altered morphology of *Ulva* and those that enhanced zoospore settlement. Only a few isolates (3 out of 20) both stimulated settlement and altered plant morphology.

Unfortunately, these experiments may not provide a definitive answer to the question of whether those bacteria that attract zoospores are also the bacteria that are required for normal growth. First, the experiments of Marshall *et al.* (2006) were done with bacteria that were readily isolated by standard microbiological methods on nutrient agar plates. Many more bacteria are present in natural biofilms that are not readily cultivated and it is not known if these bacteria may be involved in morphogenesis control. Second, the finding of Tait *et al.* (2005) that the production of AHLs by some strains depends on the growth phase of the bacteria indicates that AHL production is not necessarily continuous. Therefore, it remains a possibility that AHLs provide an excellent cue to swimming zoospores that they are in the vicinity of bacteria which are likely to result in normal morphological growth. We believe that further experiments are required before the hypothesis can be rejected.

10. POTENTIAL EXPLOITATION OF QUORUM SENSING TO CONTROL MARINE BIOFOULING

This involvement of bacterial QS in zoospore settlement opens up opportunities to develop novel approaches to the control of marine biofouling. The growth of organisms on ships and marine structures is a multi-billion dollar problem, and current control methods rely on toxic chemicals. Some bacteria appear to be toxic to zoospores. Hölmstrom *et al.* (1996) and Patel *et al.* (2003) isolated strains capable of inhibiting *Ulva* zoospore settlement. These strains were most closely related to *Pseudoalteromonas*, bacteria noted for the production of antifouling compounds (Egan *et al.* 2001).

The reverse process also occurs and eukaryotes produce compounds that have adverse effects on bacteria. It has been known for many years that some marine organisms remain relatively free from fouling. For marine invertebrates such as sponges, this is largely due to the production of deterrents by the endosymbiont community. Other marine organisms themselves produce deterrents to discourage colonization of their surfaces. For example, the red macroalga *Delisea pulchra* controls biofilm formation on its surface by production of halogenated furanones, compounds structurally similar to short-chain AHL molecules (Manefield *et al.* 2002). These halogenated furanones have been shown to inhibit AHL-dependent gene expression in *Serratia liquefaciens* (Rasmussen *et al.* 2000), *Erwinia carotovora* (Manefield *et al.* 2001) and *P. aeruginosa* (Hentzer *et al.* 2003). Concentrations of halogenated furanones have been measured at the surface of the alga in sufficient quantities to inhibit attachment of bacteria (Dworjanyn *et al.* 1999) and are thought to be the cause of the prevalence of Gram-positive bacteria on marine algal surfaces—whereas the marine environment is usually dominated by Gram-negative bacteria.

AHLs are thought to modulate cellular concentrations of LuxR by binding and protecting the protein from proteolytic degradation. Manefield *et al.* (2002) speculated that the mode of action of the furanone produced by *D. pulchra* is to destabilize LuxR regulatory proteins, reducing their concentration within the cell. Thus, the half-life of LuxR was reduced 100-fold in the presence of furanones. Recent research has indicated that furanones, at concentrations not toxic to mammalian cells, can also inhibit growth, swarming and biofilm formation of Gram-positive bacteria, but through a different mechanism (Ren *et al.* 2004). Full DNA microarrays were used to examine differential gene expression of *Bacillus subtilis* exposed to 5 $\mu g\ ml^{-1}$ furanone. Induced genes included those involved in stress responses.

Other macroalgae may also control colonization of their surfaces by interfering with AHL signalling in bacteria. Borchardt *et al.* (2001) discovered that 3-oxo group AHLs could be rapidly inactivated by exposure to oxidized halogen compounds, agents used routinely to control microbial growth in industrial systems. Those AHLs without 3-oxo substitution retained their activity. The brown alga *Laminaria digitata* produces bromoperoxidase, which can catalyse the reaction of bromide to HOBr in the presence of H_2O_2. Experiments were conducted to demonstrate the loss of 3-oxo-C6-HSL activity upon exposure to *L. digitata*.

11. DOES THE USE OF *N*-ACYLHOMOSERINE LACTONES BY *ULVA* CONSTITUTE CELL SIGNALLING (QUORUM SENSING) OR IS IT MERELY RESPONDING TO A CUE?

Is it clear that *Ulva* zoospores can detect AHLs and that their behaviour is altered in the presence of AHLs. In addition, we have demonstrated that calcium ion flux is a specific response to the detection of AHLs, with the possibility that this is a second signal that will lead to modulation of flagellar movement. However, does this mean that AHL-mediated zoospore settlement represents a specific signalling mechanism between the alga and the bacteria, or merely that the zoospores exploit AHL molecules as settlement cues?

There is increasing discussion about QS within an evolutionary context. Keller & Surrette (2006) point out that signalling pathways might sometimes have evolved for reasons other than cell-to-cell signalling. And in this issue, Diggle *et al.* (2007) discuss communication and cooperation as some of the greatest problems in evolutionary biology. In particular, communication between bacterial species (let alone across kingdoms) is difficult to explain in the context of current understanding of evolutionary theory. Diggle *et al.* (2007) suggest that in order to demonstrate that a substance is a signal, rather than a cue, requires the development of a hypothesis that the process evolved specifically owing to the response that it elicits. If there is no such selection pressure, it is very likely that an organism will merely use the signal molecules as a 'cue', rather than a true signal. Even within bacterial assemblages, the exploitation by one bacterium of a signal molecule produced by another may not be true signalling if there is no direct benefit to the cell that produces the signal. This is probably the case for *Ulva* zoospores.

In terms of defining a selection pressure, it is possible to postulate that the AHL signal ensures that a zoospore will attach in the vicinity of a bacterium that produces an essential factor for normal morphology and development of the alga. A plant that develops aberrant morphology is unlikely to reproduce as efficiently as normal plants; if this process depends on encountering the relevant bacterial species, this could confer a selective advantage and would ensure that the trait was retained over evolutionary time. There is little or no cost to the zoospore in using the AHL as a signal. They swim actively and are negatively phototactic. The metabolic cost in detecting AHL would be small, particularly if calcium ion influx initiates a signal cascade that is similar to the phototactic response.

Although AHL molecules must be intact to elicit a response, the attraction of zoospores to AHLs appears to be a rather unspecific response. Almost all AHL molecules tested, with the exception of C4-HSL (Tait *et al.* 2005), are effective in attracting zoospores. This lack of specificity contrasts with the very specific role of different AHL molecules in bacterial QS. Also to date, although we have identified a definite behavioural response, *viz.* a reduction in swimming speed, we have not identified a specific gene or gene product that might be under AHL control. This would be necessary before we could claim that the zoospores' response is due to specific signalling between the bacteria and the alga.

QS is a density-dependent process. The attraction of zoospores to bacteria is independent of the number of zoospores—the potential beneficiaries of AHL detection. The interaction will be influenced by the biofilm density and the presence of more bacteria may result in higher AHL concentration; but there can be no direct effect of zoospore density. Unlike QS, there are no apparent mechanisms to feedback. The detection of an AHL molecule by a zoospore cannot result in the

production of more AHL by the bacterial biofilm—at least not directly.

Could there be any evolutionary pressures on bacteria to produce AHLs specifically for the purpose of attracting zoospores? While, as discussed previously, the ability to detect and respond to AHLs could confer a clear selective advantage to *Ulva*, similar evolutionary pressure is not apparent for the bacteria. There would seem to be no ecological benefit to the bacteria that would result in the adaptation of its QS mechanisms for the explicit purpose of promoting zoospore settlement. Indirectly, organic matter excreted by the settled zoospores and developing plants might enhance the activity of the bacterial biofilm, and perhaps result in more AHL production. But any such increase in AHL production by bacteria is unlikely to be a mechanism to attract more zoospores to the biofilm because this increase in AHL would be of benefit to the bacteria, not to the algae. This scenario does not provide a mechanism for such a process to evolve or be maintained in *Ulva*.

Therefore, it is much more probable that *Ulva* zoospores are only using AHLs as cues for the selection of a suitable surface for attachment, rather than as a density-dependent mechanism to modulate the activity of the *Ulva* assemblage. In other words, the effect is at the level of the individual zoospore, rather than the whole assemblage.

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