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REPORT

Bacterial conversations: talking, listening and eavesdropping. A NERC Discussion Meeting held at the Royal Society on 7 December 2005

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1. PRIMITIVE CHATTERING CLASSES: BACKGROUND TO QUORUM SENSING

One fascinating recent discovery in microbiology is that bacteria can communicate with each other—and there is increasing evidence that bacterial communication is also very important for higher organisms. The process of communication is termed 'quorum sensing (QS)'; it is a density-dependent process that involves the production of extracellular, diffusible signal molecules that coordinate gene expression. The term 'quorum sensing' was coined by Steve Winans, one of the speakers at the meeting. The name encapsulates the essence of the process—that it is density-dependent and sufficient bacteria have to be present for the process to be effective. It is analogous to the legal context of a quorum, when enough voting members must be present at a meeting before a decision can be taken. By acting in concert, microbial population behaviour can be considered to be analogous to that of a multicellular organism and QS systems are the mechanisms that allow concerted action. QS is now known to be very widespread and it modulates many physiological processes in bacteria that are associated with humans, plants, animals, soils and marine and fresh waters.

Quorum sensing is a topic that crosses the responsibilities of different UK Research Councils. It is important in medical research because, as we enter the post-antibiotic era, there is great interest in blocking QS as a mechanism to reduce virulent disease. Quorum sensing is important in agriculture, because of its role in food spoilage and in maintaining healthy plants in the soil. There are engineering problems relating to biofilms and bioremediation of contaminants, we now known, is depend on bacterial QS. For these reasons, the Natural Environment Research Council (NERC) took the initiative to organize this discussion meeting on QS, involving scientists from all research communities.

There has been an explosion in the number of publications on QS in the last decade and QS is increasingly seen as central to the success of bacteria. It has been estimated that between 5 and 25% of the genes within the bacterial genomes sequenced to date are controlled by QS regulatory networks. The role of QS in disease has received considerable attention, in both plants and animals. The evidence for the involvement of QS is strong, because mutants of pathogenic bacteria that are defective in QS show greatly reduced virulence. So there is an expectation that better understanding of the role of QS in pathogenicity will offer novel opportunities to combat bacteria that cause human, animal and plant diseases. With the widespread increase in antibiotic resistant bacteria, it is essential that we develop alternative procedures to neutralize pathogenic bacteria.

QS is increasingly seen to have wider implications than for bacterial alone. Evidence is accumulating that animals and plants may 'listen' to bacterial signals and utilize these signals in complex ways. This should not be surprising; bacteria are ubiquitous and have existed for close to 3.7 Gyr; for the first 2 Gyr of the Earth's existence, bacteria were the only life form on the planet. Higher organisms evolved from and by interacting with bacteria. If, as now seems likely, QS is a very important factor in bacteria success, then it should not be surprising that eukaryotic organisms are also capable of exploiting QS in a variety of ways. There are examples of the production of molecules by plants and animals that mimic QS compounds and so confuse QS regulation in bacteria. That is, blocking of QS has evolved as a successful strategy to resist infection by pathogenic bacteria. Although it is common to consider bacteria as only having a negative effect on higher organisms, the reverse is actually the case. Symbiosis is widespread and is involved in processes as diverse as nitrogen fixation in the rhizosphere to light generation in deep-sea fishes. QS is essential for maintaining these interactions between host and bacteria. In addition, QS appears to be involved in more subtle processes that impinge on the ecological success of higher organisms. For example, the zoospores of seaweeds 'eavesdrop' on bacterial communication by utilizing QS molecules to select surfaces for attachment and colonization. Other seaweeds protect themselves from biofouling by producing antibacterial molecules that inhibit QS. It is becoming clear that QS is an important factor in the interaction between bacteria and higher organisms.

This was the justification for NERC deciding to organize a major discussion meeting on QS. The field is developing very rapidly and there has been an almost exponential increase in publications on QS in the last

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5 years. QS impinges on all aspects of microbiology, from human diseases through to microbial ecology. It is a fundamentally interesting process that explains the interactions between organisms and how they are controlled. QS also has implication for the development of ecological theory.

2. A SHORT HISTORY OF QUORUM SENSING IN GRAM-NEGATIVE AND GRAM-POSITIVE BACTERIA

The history of QS was outlined in an introductory lecture by Paul Williams (University of Nottingham). He pointed out that bacteria are much more complex than the classical view of them as autonomous unicellular organisms with little capacity for collective behaviour. They are highly interactive and possess a broad repertoire of chemical mechanisms for cell-to-cell communication. One of first examples of QS came from the bioluminescent bacteria that inhabit the light organs of deep-sea fish and squid. It was found that the amount of light produced by *Vibrio fischeri* was not correlated with the number of bacteria present but there appeared to be a threshold above which light was produced. It was initially thought that there must be an inhibitor present that inhibited bioluminescence and that the inhibition was relieved at higher cell densities. However, Nealson *et al.* (1970) demonstrated that light production was activated by the production of an autoinducing substance. We now know in detail the mechanism of light production, that it is controlled by QS and that the mechanism is cell-to-cell communication, controlling gene expression through the LuxR–LuxI system Although this early research, and many subsequent investigations, has been in the context of density dependence, we now know that some QS processes are not heavily dependent on the number of bacteria present in a population. QS appears to be a versatile mechanism allowing quite subtle interactions between bacteria.

Paul Williams explained how chemical signal molecules control cell-to-cell communication. Small molecules (which are sometimes referred to as autoinducers—or pheromones, by analogy to the insect system of communication) are synthesized and diffuse in and out of bacterial cells. As the number of bacteria increases, more QS signal molecules are produced and their concentration in the external environment rises. On achieving a critical threshold concentration, a target sensor kinase or response regulator is activated (or repressed), resulting in the expression of QS-dependent genes. However, the size of the quorum varies and QS is dependent on a number of abiotic factors, such as the viscosity (Reynolds number) of the growth environment, which controls the rate of diffusion of the molecules. Other important factors controlling QS are the balance between synthesis, accumulation and turnover of the signal molecules.

Like most forms of signalling, QS relies on the diffusion of signal molecules, so enabling a bacterial population to act cooperatively and hence gain a competitive advantage. It is postulated that an assemblage may benefit if it is able to exploit specific environmental niches. Or concerted action may also provide collective defence against other prokaryotic competitors, or against eukaryotic defence mechanisms. For example, survival could be enhanced through differentiation into different morphological forms, such as swarmer cells or biofilms, which are better able to resist changes in the local environment; biofilm development and swarming are both QS-controlled function.

It is known that QS regulates a wide range of physiological processes and involves a number of different signal molecules. Activities under QS control include secondary metabolite production, motility, symbiosis, nodulation, conjugal plasmid transfer, biofilm maturation and virulence in numerous bacterial genera (Swift *et al.* 2001). A number of chemically distinct families of QS signal molecules have been identified. The most intensively investigated are the *N*-acylhomoserine lactone (AHL) family in Gram-negative bacteria and the peptide autoinducers of Gram-positive bacteria. QS contributes to environmental adaptation in many bacteria in a number of different ways. For example, in *Pseudomonas*, AHLs are involved in the elaboration of virulence determinants in pathogenic species, and in the development of plant biocontrol characteristics in beneficial species. In addition, AHLs have been thought to be important in biofilm formation and stability, by controlling the interchange between biofilm and planktonic growth phase of *Pseudomonas*, so enabling motile cells to escape from a biofilm. However, another lecture by Søren Molin (Technical University of Denmark) demonstrated that biofilm development and stability is more complex than this simple paradigm (Beloin *et al.* 2004).

QS also crosses the prokaryotic–eukaryotic boundary and QS signal molecules can also influence the behaviour of eukaryotic organisms in both the plant and mammalian worlds. Certain QS signal molecules, for example, possess potent immune modulatory, pharmacological and antibacterial activities, which may result in enhanced bacterial survival by promoting an advantageous lifestyle within a range of environmental niches.

3. QS AND BACTERIAL INTERACTION WITH PLANTS AND ANIMALS

Bacterial interactions with higher organisms are important at two basic levels. They can be detrimental, largely as agents of disease in both plants and animals, but they can also be beneficial, acting as symbionts. Both processes have been shown to involve QS.

In the area of symbiosis, Allan Downie (John Innes Centre, Norwich, UK) described how QS influences symbiotic nitrogen fixation by *Rhizobium leguminosarum*. This bacterium enters into a nitrogen-fixing symbiosis with legumes, such as pea and vetch. Part of that relationship involves a change to the plant structure with the induction of nodule development on the roots. QS is involved in *Rhizobium* activity (Danino *et al.* 2003). Downie has identified four *luxI* type genes involved in AHL production and six

LuxR-type regulators, which regulate gene expression in response to AHLs. The regulation is complex and the AHL-dependent regulatory systems operate as a network, with cross-regulation, which is controlled by different AHL molecules. At the top of the network are chromosomal genes that encode a LuxR-type regulator and an AHL synthase. There are also two QS regulated loci on the symbiotic plasmid, one of which (*rhi*) appears to play a role in nodule formation of peas. A novel gene has been identified that is under QS regulation in free-living culture. It is induced by root exudates and is strongly expressed by bacteria in infection threads but is not expressed in the mature zone of the nodule. Another QS system on the symbiotic plasmid is involved in a novel mechanism of plasmid transfer that is induced by the recipient via a QS relay. Various QS regulatory systems also influence other phenotypes in *Rhizobium* including adaptation to stress, processing of exopolysaccharides and biofilm growth.

Stephen Winans (Cornell University, USA) discussed cell-to-cell communication in the plant pathogen *Agrobacterium tumefaciens* (Pappas *et al.* 2004), which induces the formation of crown gall tumours at wound sites on host plants by directly transforming plant cells. This disease strategy benefits the bacteria because the infected plant tissue produces novel nutrients, called opines, that the colonizing bacteria use as nutrients. Winans showed that almost all of the genes that are required for virulence, and all the opine-uptake and utilization genes, are carried on large tumour-inducing (Ti) plasmids. It has been known for more than 25 years that specific opines are required for Ti plasmid conjugal transfer. This knowledge led to the discovery of a cell-to-cell signalling system on these plasmids that is similar to the LuxR–LuxS system first described in *V. fischeri*. All Ti plasmids that have been described to date carry a functional LuxI-type AHL synthase (TraI) and a LuxR-type signal receptor and transcription regulator called TraR. The *traR* genes are expressed only in the presence of specific opines called conjugal opines. The TraT–TraI system provides an important model for LuxR–LuxS type systems, especially those found in the agriculturally important Rhizobiaceae family.

Another plant pathogen, *Erwinia carotovora* was the subject for George Salmond (University of Cambridge, UK). *Erwinia carotovora* belongs to the Family Enterobacteriaceae and causes soft-rot diseases, such as soft rot and blackleg of potato. QS is involved in the development of virulence factors (Coulthurst *et al.* 2006). The main virulence factors under QS control are multiple pectinases, cellulases and proteases, which degrade the host plant cell walls. A number of secondary metabolites produced by *E. carotovora* were also shown to be under the control of QS.

QS is increasingly seen as an important factor in bacterial infections of humans and animals (Shi *et al.* 2005). Gary Dunny (University of Minnesota, USA) described a QS-inducible conjugation system in *Enterococcus faecalis*. Enterococci are opportunists that cause urinary tract infections and wound infections. They are economically very important and are one of the leading causes of secondary infections acquired in hospitals, where antibiotic resistance is an increasing problem. The transfer of antibiotic resistance from bacterium to bacterium involves conjugation and a large number of gene products are required for conjugative transfer of the antibiotic resistance plasmid. The process is controlled by cell-to-cell communication between recipient cells, which do not contain the plasmid, and the plasmid-carrying donor cells. In this case, QS involves a peptide signal molecule. Dunny described recent experiments that have determined, at the molecular level, how the QS response is initiated in donor cells, and how donor cells endogenously produce the signal molecule to control self-induction. Dunny believes that the system may serve as a useful bacterial model to address the evolution of biological complexity and evolution of complex systems.

4. NOVEL WAYS TO COMBAT INFECTION BY BLOCKING QS

It has recently been shown that it is possible to block QS in pathogenic microbes. There is considerable interest in determining if this approach might the basis of future antimicrobial strategies. Michael Givskov (BioCentrum-DTU, Denmark) described research from his laboratory into blocking QS. This approach to controlling bacterial infections is inherently very different to conventional antibiotics, which are effective because they target growth or metabolic activity of bacterial cells and lead to cell death. However, as society knows to its cost, this mode of antibiotic action results in the survival of resistant bacteria and multi-antibiotic resistance is now a major worldwide problem. An alternative approach to killing bacteria with antibiotics is to reduce their pathogenicity or virulence (Hentzer & Givskov 2003). Givskov described how *Pseudomonas aeruginosa*, and a number of other pathogenic bacteria, control their virulence through QS. This has recently been shown to be involved in the development of tolerance to various antimicrobial treatments and immuno-modulation. But it is relevant to ask why virulence should be regulated. It is believed that the ability to switch-off virulence factors could provide a bacterium with a strategy to overwhelm host defence and so cause disease. In a density-dependent process, when virulence is only expressed at high cell densities, a bacterium may be able to grow within a host without detection by the immune system. With QS control, once bacterial density is sufficiently high, virulence factors would be activated, so offering the bacteria an advantage over the host defence. Therefore, an effective approach to combat bacterial infection might be to develop a drug capable of blocking cell–cell signalling, so resulting in an infectious bacterium becoming susceptible to the host's defence. That is, an approach of using signal blockers to attenuate bacterial pathogenicity, rather than directly killing the bacteria, could be an attractive strategy for controlling bacterial infections.

Lian-Hui Zhang (The National University of Singapore, Singapore) also considered how to block bacterial infection—a process, which he termed

quorum quenching. Again, the approach relies on the fact that QS pathogens produce and respond to signal molecules to synchronize expression of virulence genes. Zhang has looked for enzymes that can degrade or inactivate AHL signals, with the aim of controlling bacterial infection by quenching QS. AHLs have been targeted because they are the most characterized QS signals, being produced by over 70 bacterial species. Three types of novel AHL-degradation enzymes have been identified and characterized. The first group is AHL-lactonases, which are widely conserved in *Bacillus* species and in *A. tumefaciens* (Zhang *et al.* 2002). A second group is AHL-acylases, found in *Ralstonia* sp. and in *P. aeruginosa* and a third group are paraoxonases, which are increasingly found in mammalian species, and are potent AHL degrading enzymes. Quorum quenching mechanisms are present in many prokaryotic and eukaryotic organisms and, interestingly, quorum-quenching enzymes have also recently been detected in mammalian species (Yang *et al.* 2005). This is an important finding that highlights the importance that quorum quenching may play in the innate defence of the host against infectious diseases.

Zhang has shown that AHL-lactonase, encoded by the *aiiA* gene of *Bacillus thuringiensis*, is a specific enzyme that can hydrolyse both short and long chain AHL signal molecules but shows no or little enzyme activity on other types of lactones and esters. When expressed in transgenic tobacco and potato plants, the enzyme effectively quenched the QS system of the phytopathogen, *E. carotovora*, and conferred a high level of disease resistance. Transgenic tobacco leaf tissues that express AHL-lactonase were also resistant to infection; using GFP-labelled *E. carotovora*, the pathogenic cells were found to be unable to penetrate into the leaf tissues. In contrast, in the wild type tobacco control, bacteria were found in all the leaf tissues that showed soft-rot symptoms. Quorum quenching also has the potential to be effective in animal systems. Research on the nematode model-system (*Caenorhabditis elegans*) has also shown that, when the human pathogen *P. aeruginosa* expresses AHL-acylase, it was much less effective in its ability to kill the animal.

Zhang then described new regulatory proteins that control bacterial QS signalling. By using transposon mutagenesis, he found that mutation of the *pprB* and *vqsM* genes of *P. aeruginosa*, that encode for a response regulator and a global transcription factor, respectively, resulted in reduced AHL signal biosynthesis and production of the QS-dependent virulence factor. Microarray analysis showed that most genes in QS regulon, including the genes encoding for AHL biosynthesis, were down-regulated in these two mutants. Addition of long-chain AHL molecules did not restore the mutant phenotypes, suggesting that these two regulatory proteins may be implicated in long chain AHL signal influx. Investigations are continuing to establish the roles of these proteins in QS as well as the feasibility of using them as new targets to develop novel quorum-quenching strategies.

5. INTERACTIONS IN THE NATURAL ENVIRONMENT: EAVESDROPPING BY HIGHER ORGANISMS

The green seaweed *Ulva* is a prominent seaweed on the shore throughout the world; it is also economically important because it is a major marine biofouling organism. Ian Joint (Plymouth Marine Laboratory, UK) described how cells signalling can be effective across the prokaryotic/eukaryotic boundary—specifically how *Ulva* utilizes AHL molecules, produced by bacteria, for the selection of sites for attachment (Joint *et al.* 2002). *Ulva* reproduces by releasing motile zoospores that swim away from the parent plant, attach to a surface and develop into a new plant. The numbers of zoospores released are truly immense and thousands swim away from the tip of a mature plant each day. But what are the factors that influence the selection of surfaces for attachment? The initial observation was that more *Ulva* zoospores settled on surfaces colonized by bacteria than on clean surfaces and that the numbers of attached zoospores were proportional to the size of the bacterial population. This suggested a specific selection mechanism—which turned out to involve AHL signal molecules. The evidence was based on four types of experiments. Using biofilms of the marine bacterium, *Vibrio anguillarum*, it was found that the wild type attracted zoospores but mutants that were defective in AHL production did not attract. Zoospore settlement assays using *Escherichia coli* expressing recombinant AHL synthase genes also showed that settlement was only enhanced when AHLs were produced. Synthetic AHLs were also found to attract zoospores at environmentally realistic concentrations. Finally, the attraction to AHL-producing bacteria was inactivated by quorum quenching and there was no attraction of zoospores to *V. anguillarum* expressing a recombinant gene (*aiiA*) from *Bacillus* for AHL-lactonase that inactivates AHL molecules. The mechanism of attraction of zoospores to AHL does not appear to be chemotactic because they do not swim directly towards a source of AHLs. However, the swimming behaviour does change when AHLs are detected and swimming speed decreases dramatically. This effect is called chemokinesis. It results in the accumulation of zoospores at the site of AHL production because the rapid, random movement of every zoospore changes as it enters the zone of elevated AHL concentration and it slows down completely. The physiology of the response has been investigated and it appears that calcium is employed as a second messenger. AHLs causes an influx of calcium and it is postulated that the reduction in swimming speed occurs through calcium-dependant modulation of the flagellar beat pattern. Preliminary experiments using the manganese quench technique have produced evidence for calcium influx in the presence of the AHL signal molecules. This is the first example of a calcium-signalling event in a eukaryote in response to bacterial QS signals. The characterization of the signal transduction pathway in *Ulva* may aid in the identification of similar signalling pathways in other eukaryotes.

Zoospores are not attracted to all bacteria and a number of isolates that inhibit zoospore attachment, rather than causing an attraction. The production of AHLs is not constant during the growth phase of a bacterial biofilm and the relationship between QS, biofilm age and composition has proved to be very complex. What benefit might seaweed get from attaching to certain bacterial species? That is not yet clear. It is known that bacteria are essential for the normal development of seaweeds; that is, certain bacteria have to be present for normal morphology. However, there does not appear to be a close correlation between the species of bacteria that attract zoospore and those that are essential for normal morphological development. The case of *Ulva* eavesdropping on bacterial communication is a novel example of cell-to-cell communication. It is not yet clear how common this phenomenon of communication across the prokaryote/eukaryote boundary is likely to be. It will be interesting to discover how many organisms have evolved mechanisms to exploit bacterial communication to enhance their ecological success.

In the natural environment, microbial communities are very complex. The impact of QS on microbial communities is not well understood but is likely to affect species diversity and function. Andrew Whiteley (Centre for Ecology and Hydrology, Oxford) described how QS can modify microbial community activity in an industrial wastewater treatment system. Seven Proteobacterial strains isolated from the treatment plant all produced AHL-like compounds (Valle *et al.* 2004). Three of these belong to genera, which had not previously been shown to include AHL-producing species. This finding highlights the current lack of knowledge about how widespread AHL production is likely to be within the microbial world. The treatment system was designed to degrade waste that contained phenol; but bacterial activity and assemblage composition within the system could be very variable. However, addition of AHLs to sludge samples resulted in changes to both community function (i.e. the efficiency of phenol degradation) and of bacterial assemblage composition, as determined by length-heterogeneity PCR and denaturing gradient gel electrophoresis. Phenol degradation was more stable as a result of the AHL augmentation and there were large changes in the dominant bacteria. AHL addition caused a functional member of the *Thauera* genus to be replaced by a member of the *Comomonas* genus—a specific and direct response to AHL addition. These observations suggest that QS plays an important role in maintaining microbial community structure, not only in the reduced microbial assemblages of wastewater treatments, but also within the complex natural assemblages found in soils, freshwater and the oceans. QS has clear implications for ecosystem function in general and also for industrial wastewater treatment.

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