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Quorum sensing and the population-dependent control of virulence

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One crucial feature of almost all bacterial infections is the need for the invading pathogen to reach a critical cell population density sufficient to overcome host defences and establish the infection. Controlling the expression of virulence determinants in concert with cell population density may therefore confer a significant survival advantage on the pathogen such that the host is overwhelmed before a defence response can be fully initiated. Many different bacterial pathogens are now known to regulate diverse physiological processes including virulence in a cell-density-dependent manner through cell–cell communication. This phenomenon, which relies on the interaction of a diffusible signal molecule (e.g. an *N*-acylhomoserine lactone) with a sensor or transcriptional activator to couple gene expression with cell population density, has become known as 'quorum sensing'. Although the size of the 'quorum' is likely to be highly variable and influenced by the diffusibility of the signal molecule within infected tissues, nevertheless quorum-sensing signal molecules can be detected *in vivo* in both experimental animal model and human infections. Furthermore, certain quorum-sensing molecules have been shown to possess pharmacological and immunomodulatory activity such that they may function as virulence determinants *per se*. As a consequence, quorum sensing constitutes a novel therapeutic target for the design of small molecular antagonists capable of attenuating virulence through the blockade of bacterial cell–cell communication.

Keywords: quorum sensing; *N*-acylhomoserine lactones; virulence; bacteria; infection; cell signalling

1. INTRODUCTION

A well-prepared army goes into battle with a knowledge of the opposition, significant numbers of troops and excellent lines of communication. In some respects, bacterial pathogens are no different and in recent years it has become clear that bacterial cells are capable of exhibiting much more complex patterns of multicellular behaviour than would perhaps be expected for simple unicellular micro-organisms. The ability of a single bacterial cell to communicate with its neighbours to mount a unified response that is advantageous to its survival in a hostile environment makes considerable sense. Such benefits may include improved access to complex nutrients or environmental niches, collective defence against other competitive micro-organisms or eukaryotic host defence mechanisms and optimization of population survival by differentiation into morphological forms better adapted to combating an environmental threat.

For many pathogens, the outcome of the interaction between host and bacterium is strongly affected by bacterial population density. Coupling the production of virulence factors with cell population size ensures the host has insufficient time to mount an effective defence against consolidated attack by the infecting bacterial population. Such a strategy inevitably depends on the capacity of an individual bacterial cell to sense other members of the same species and in response, differentially express specific sets of genes. The generic term 'quorum sensing' is now commonly used to describe the phenomenon whereby the accumulation of a diffusible, low molecular weight signal molecule (sometimes referred to as a 'pheromone' or 'autoinducer') enables individual cells to sense when the minimal population unit or 'quorum' of bacteria has been achieved for a concerted population response to be initiated (Fuqua *et al.* 1994). Quorum sensing is thus an example of multicellular behaviour and modulates a variety of physiological processes, including bioluminescence, swarming, swimming and twitching motility, antibiotic biosynthesis, biofilm differentiation, plasmid conjugal transfer and the production of virulence determinants in animal, fish and plant pathogens (for reviews, see Dunny & Winans 1999; Fuqua *et al.* 1996; Hardman *et al.* 1998; Salmond *et al.* 1995).

Signal transduction through quorum sensing depends on the direct or indirect (via a sensor) activation of a response regulator by a diffusible signal molecule and several chemically distinct families of such molecules have

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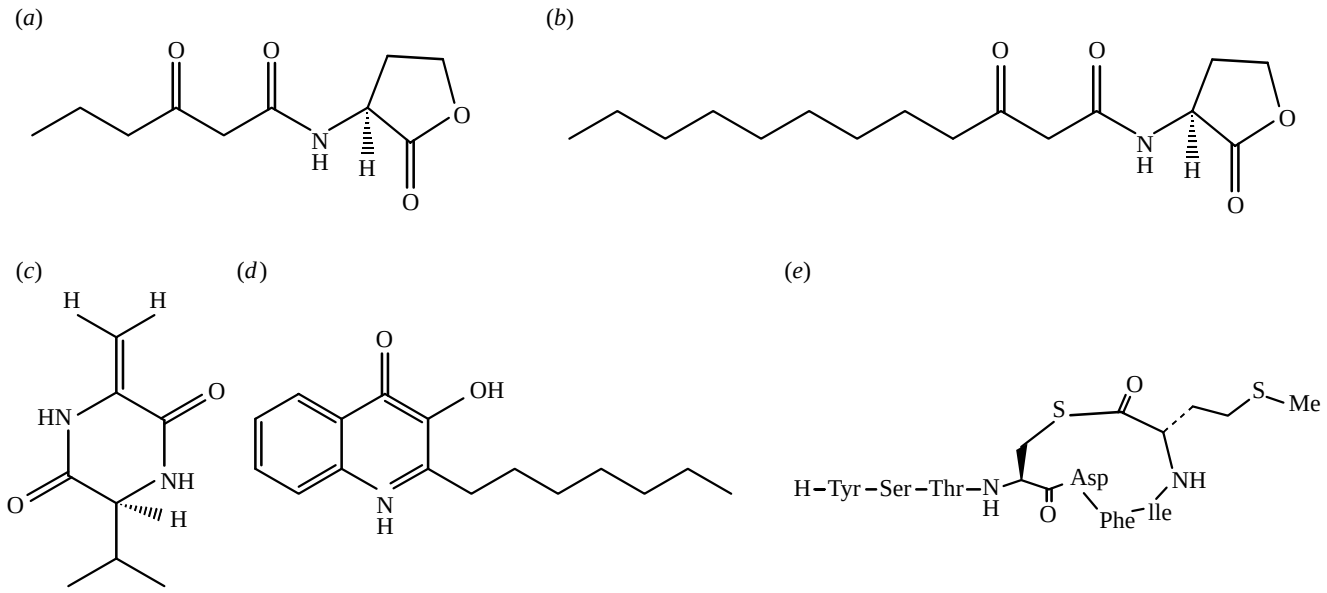

Figure 1. Structures of some representative quorum-sensing signal molecules. (*a*) *N*-(3-oxohexanoyl)-L-homoserine lactone; (*b*) *N*-(3-oxododecanoyl)-L-homoserine lactone; (*c*) cyclo(ΔAla-L-Val); (*d*) 2-heptyl-3-hydroxy-4-quinolone; and (*e*) group I *Staphylococcus aureus* cyclic peptide thiolactone.

now been identified. In Gram-negative bacteria, the most intensively investigated family of quorum-sensing signal molecules is the *N*-acylhomoserine lactones (AHLs; e.g. figure 1*a*,*b*), although non-AHL-dependent systems have also been identified. In contrast, Gram-positive bacteria employ quorum-sensing systems in which the signal molecule is often a post-translationally modified peptide (figure 1*e*; Kleerebezem *et al*. 1997; Mayville *et al*. 1999). With respect predominantly to the AHLs, the focus of this review will be (i) to discuss the role of quorum sensing in the control of virulence gene expression; (ii) to present evidence that quorum sensing occurs *in vivo* during infection; and (iii) to outline the potential of certain quorum-sensing signal molecules to function as virulence determinants *per se*.

2. QUORUM-SENSING SIGNAL MOLECULES IN GRAM-NEGATIVE BACTERIA

Production of the β-lactam antibiotic, 1-carbapen-2-em-3-carboxylic acid (carbapenem) by the terrestrial plant pathogenic bacterium *Erwinia carotovora* was discovered to be regulated by *N*-(3-oxohexanoyl)-L-homoserine lactone (3-oxo-C6-HSL; figure 1*a*; Bainton *et al*. 1992*a*,*b*). The significance of this finding lay in the fact that 3-oxo-C6-HSL and a related compound, *N*-(3-hydroxybutanoyl) homoserine lactone (3-hydroxy-C4-HSL), had until this time been exclusively known as 'autoinducers' of bioluminescence in the marine bacteria *Vibrio fischeri* and *Vibrio harveyi* (Eberhard *et al*. 1981; Cao & Meighen 1989). In *V. fischeri*, a symbiont found in the light organs of the sepiolid squid *Euprymna scolopes*, the structural and regulatory genes necessary for light production and synthesis of 3-oxo-C6-HSL (the *lux* regulon) are all located on a 9 kb DNA fragment (Engebrecht & Silverman 1984). The *lux* regulon is organized into two divergently transcribed units, separated by an intergenic regulatory region (Engebrecht & Silverman 1984; Stevens & Greenberg 1999). The leftward transcriptional unit consists of the *luxR* gene, encoding LuxR, a positive transcriptional regulator protein. The rightward operon, is comprised of the *luxI* gene, encoding the 3-oxo-C6-HSL synthase, followed by the *luxCDABE* structural genes, which encode the α- and β-subunits of the luciferase (*luxA* and *luxB*) and a fatty-acid reductase complex (*luxCDE*) necessary for light generation. The LuxI and LuxR proteins and a proposed LuxR binding site, a 20 bp inverted repeat called the *lux* box (Stevens *et al*. 1994; Stevens & Greenberg 1999), which is situated within the intergenic region, are required for the primary cell-density-dependent regulation of *lux* gene expression and are essential for the autoinduction response. LuxR is activated by binding 3-oxo-C6-HSL, which in turn induces transcription of *luxICDABE* (Stevens & Greenberg 1999). Homologues of LuxI and LuxR termed CarI (ExpI) and CarR were subsequently identified in *E. carotovora* (Swift *et al*. 1993; Pirhonen *et al*. 1993; McGowan *et al*. 1995). In contrast to the *lux* operon, *carI* (*expI*) is not linked to the *car* structural genes but is located elsewhere on the chromosome and is adjacent to a second LuxR homologue, ExpR (McGowan *et al*. 1995, 1996; Pirhonen *et al*. 1993).

The discovery that 3-oxo-C6-HSL was produced by both marine and terrestrial Gram-negative bacteria suggested that AHL-dependent quorum-sensing systems may be common throughout the bacterial kingdom. To explore this hypothesis, plasmid-based AHL biosensors have been constructed in which the accumulation of an AHL results in the expression of a specific phenotype. Such biosensors usually contain LuxR or a LuxR homologue together with an AHL-activated promoter fused to a reporter gene such as *lacZ* or *luxCDABE*, but lack an AHL synthase (Bainton *et al*. 1992*b*; Pesci *et al*. 1997; Piper *et al*. 1993; Swift *et al*. 1993; Winson *et al*. 1998). Since such strains do not produce any AHLs, expression of the reporter occurs only in the presence of exogenously added AHLs. Using a plasmid-based AHL biosensor carrying *luxRI′::luxAB*, spent culture supernatants were screened for the presence of AHLs. Positive results were

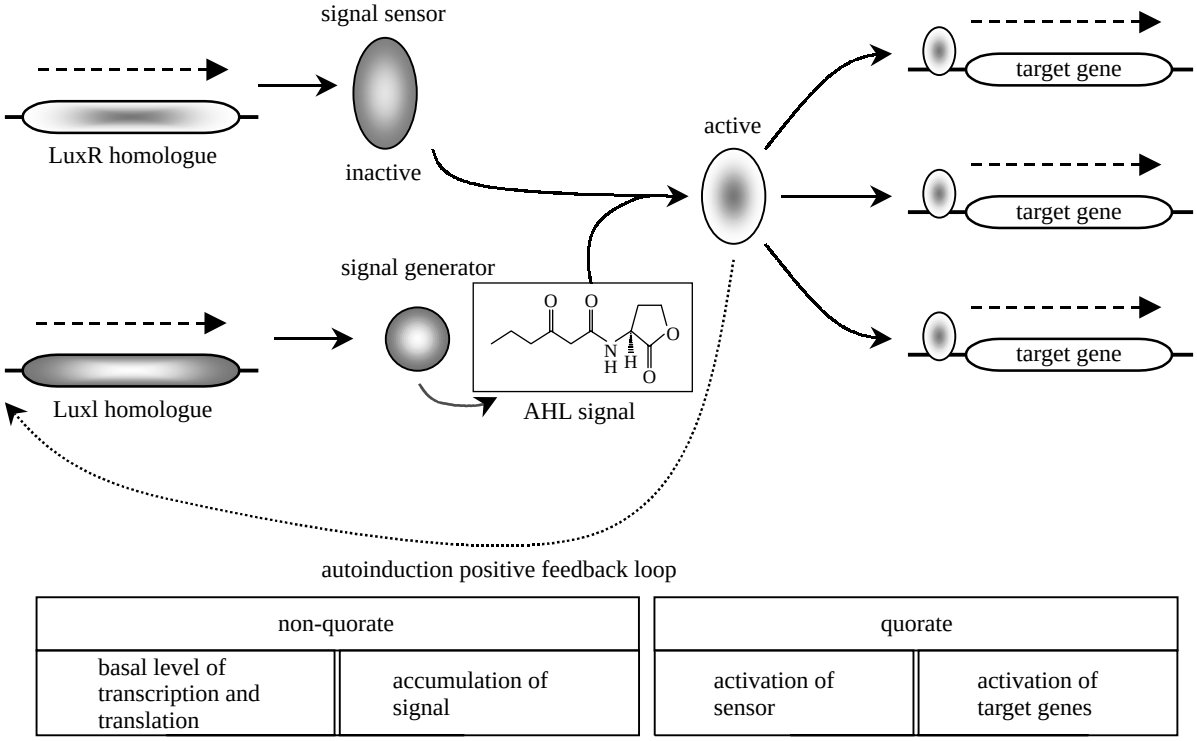

Figure 2. Schematic representation of AHL-dependent quorum sensing in Gram-negative bacteria.

obtained with supernatants from *Pseudomonas aeruginosa*, *Serratia marcescens*, *Erwinia herbicola*, *Citrobacter freundii*, *Enterobacter agglomerans* and *Proteus mirabilis* (Bainton *et al*. 1992*a*; Swift *et al*. 1993). For *P. aeruginosa*, *S. marcescens*, *E. herbicola* and *E. agglomerans*, the inducing compound was isolated and chemically confirmed as 3-oxo-C6-HSL (Bainton *et al*. 1992*a*). Elucidation of the structure, stereochemistry and synthesis of 3-oxo-C6-HSL produced by *E. carotovora* is fully described in Bainton *et al*. (1992*b*). These results confirmed, and extended, some earlier indications that the ability of bacteria to produce AHLs might extend beyond the bioluminescent vibrios (Greenberg *et al*. 1979). Since this work, other bacteria now known to produce AHLs include *Aeromonas hydrophila*, *Aeromonas salmonicida*, *Agrobacterium tumefaciens*, *Burkholderia cepacia*, *Chromobacterium violaceum*, *Nitrosomonas europoea*, *Obesumbacterium proteus*, *Pseudomonas aureofaciens*, *Pseudomonas fluorescens*, *Pseudomonas syringae*, *Pseudomonas putida*, *Rhanella aquatilis*, *Hafnia alvei*, *Ralstonia solanacearum*, *Rhizobium etli*, *Rhizobium leguminosarum*, *Rhodobacter spaeroides*, *Vibrio anguillarum*, *Vibrio logei*, *Vibrio metschnikovii*, *Xenorhabdus nematophila*, *Yersinia enterocolitica*, *Yersinia pestis* and *Yersinia pseudotuberculosis* (for reviews, see Dunny & Winans 1999; Hardman *et al*. 1998; Swift *et al*. 1999*a*,*b*). In these Gram-negative bacteria, AHLs have been identified with *N*-acyl side chains of 4, 6, 8, 10, 12 and 14 carbons with either an oxo- or hydroxy- or no substituent at the C3 position of the *N*-linked acyl chain. To date, only two compounds with acyl chains containing double bonds have been identified. These are 7,8-cis-*N*-(3-hydroxytetradecenoyl)homoserine lactone and 7,8-*cis*-*N*-(tetradecenoyl)homoserine lactone produced by *Rhizobium leguminosarum* (Schripsema *et al*. 1996; Gray *et al*. 1996) and *Rhodobacter sphaeroides* (Puskas *et al*. 1997), respectively. Figure 2 presents a schematic diagram illustrating AHL-dependent quorum sensing.

Interestingly, no Gram-positive bacteria have so far been shown to produce AHLs, and pathogenic Gram-negative bacteria which so far have been reported as AHL-negative include *Actinobacillus pleuropneumoniae*, *Escherichia coli*, *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Neiserria meningitidis*, *Salmonella* spp., *Vibrio cholerae*, *Campylobacter jejuni* and *Helicobacter pylori* (Swift *et al*. 1999*c*). While it is highly likely that these organisms are indeed AHL-negative, the lack of a response could also reflect the inability of the biosensor employed to respond to the AHL analogue produced. However, for some of these bacteria (e.g. *E. coli*, *V. cholerae* and *K. pneumoniae*) attempts to use chemical approaches such as electron impact mass spectrometry to detect characteristic fragment ions (e.g. the 102 molecular ion fragment corresponding to the homoserine lactone moeity; Camara *et al*. 1998) have proved negative (authors' unpublished data). In addition, perusal of the genome sequence databases for *H. influenzae*, *H. pylori* and *C. jejuni* reveals no obvious LuxI or LuxR homologues. Intriguingly, *E. coli* and *Salmonella* both possess a LuxR homologue called SdiA (suppressor of division inhibition), which is more than 60% identical to RhlR (VsmR) from *P. aeruginosa* (Wang *et al*. 1991; Ahmer *et al*. 1998; Garcia-Lara *et al*. 1996; Sitnikov *et al*. 1996; Latifi *et al*. 1995). In *E. coli*, SdiA has been proposed to regulate expression of the *ftsQAZ* gene cluster, which is required for cell division (Garcia-Lara *et al*. 1996; Sitnikov *et al*. 1996), while the *Salmonella* homologue has been implicated in the expression of several putative virulence determinants (Ahmer *et al*. 1998). Attempts to demonstrate that SdiA functionality depends on activation by an AHL have proved somewhat inconclusive. 3-oxo-C6-HSL, 3-hydroxy-C4-HSL and *N*-decanoyl-DL-homoserine lactone all weakly stimulate the SdiA-dependent P_2 promoter from the *ftsQAZ* gene cluster, although no

obvious structure–function relationship is apparent (Sitnikov *et al*. 1996). However, spent *E. coli* culture supernatant is more effective, implying the presence of a diffusible signalling molecule. Furthermore, Garcia-Lara *et al*. (1996) report that *sdiA* itself is regulated by an unidentified, extracellular factor present in spent growth medium.

Further evidence of a role for small, diffusible molecules in the control of cell division in *E. coli* has emerged from the work of Withers & Nordström (1999). They discovered that chromosomal replication could be inhibited by an extracellular factor present in the late logarithmic and early stationary phases of growth. This factor appears to exert its inhibitory activity during initiation of DNA replication directly at each replication fork and does not require transcription or translation. As a consequence, its mode of action must be fundamentally different from all other quorum-sensing systems so far described. This factor is also made by *Salmonella typhimurium* and in contrast to the AHLs, it does not partition into organic solvents, suggesting that it is a highly polar molecule. Coupling the initiation of DNA replication to quorum sensing may offer a bacterium an opportunity to sense the number of cells occupying the same niche such that it is able to slow down replication well in advance of stationary phase. This may be important since it takes time for ongoing replication to proceed to completion. Furthermore, cell survival in the stationary phase and the ability to respond rapidly to environmental changes may be compromised by the presence of stalled replication forks arising as a consequence of insufficient nutrients to complete ongoing replication once stationary phase has been reached (Withers & Nordström 1999).

The existence of an uncharged polar quorum-sensing signal molecule in *E. coli*, which may be related to that described by Withers & Nordström (1999), has been described by Surette & Bassler (1998). This molecule is produced in LB medium only when supplemented with glucose, maximum activity is found in the mid-logarithmic phase of growth and the molecule is rapidly degraded either as the glucose concentration becomes limiting or by the onset of stationary phase. Although no *E. coli* phenotype has been associated with this molecule, it was initially detected using a biosensor based on a quorum-sensing system involved in the regulation of bioluminescence in *Vibrio harveyi*. This free-living marine bacterium can be found in seawater and in the intestines of fish. Although the organization of the *lux* structural genes in *V. harveyi* is identical to that of *V. fischeri*, the mechanism of regulation is quite different (Freeman & Bassler 1999). Bioluminescence in *V. harveyi* is controlled by a sophisticated system involving two integrated quorum-sensing circuits that function via a pair of two-component sensor kinase-response regulator systems. The first of these systems responds to an AHL (3-hydroxy-C4-HSL), the synthesis of which depends not on a LuxI homologue but on LuxLM, an alternative AHL synthase (Bassler 1999; Freeman & Bassler 1999). Signal transduction through 3OH-C4-HSL occurs via LuxN, a membrane-located sensor kinase. The second regulatory system, which senses an, as yet unidentified, signal molecule (termed AI-2), is also sensed via a two-component regulatory pair (LuxP and LuxQ). Sensory information provided via either pathway is relayed to LuxO, a negative regulator of the *lux* structural genes. LuxR, a positive transcriptional activator is also required for *lux* gene expression in *V. harveyi*, but it should be noted that this LuxR protein shares no homology with the *V. fischeri* LuxR. The 3-hydroxy-C4-HSL or AI-2 pathways alone are sufficient for the induction *lux* gene expression in *V. harveyi*, although the kinetics and intensity of response to the two autoinducers are different (Freeman & Bassler 1999). By using *V. harveyi* mutants defective in their ability to respond to either 3-hydroxy-C4-HSL or AI-2, Bassler *et al*. (1997) concluded that the 3-hydroxy-C4-HSL system was highly species-specific, while AI-2 was non-species-specific since they were able to show that many species of bacteria, including laboratory and pathogenic *E. coli* (e.g. O157:H7 strains), *Vibrio cholerae* and *S. typhimurium* produce molecules capable of mimicking the activity of the *V. harveyi* AI-2 autoinducer. Since the chemical identity of these AI-2, *E. coli* or *Salmonella* molecules is not known, it is not clear whether they are identical or if they constitute a new family of quorum-sensing signal molecules. In this context, it is perhaps worth sounding a cautionary note since bacterially derived molecules (cyclic dipeptides) unrelated to AHLs have been identified in culture supernatants as a consequence of their ability to activate AHL biosensors (Holden *et al*. 1999). Interestingly, the *E. coli* laboratory strain DH5α does not produce AI-2-like activity and Surette *et al*. (1999) exploited this finding to clone, by complementation, a gene (termed *luxS*) responsible for AI-2 production common to *V. harveyi*, *S. typhimurium* and *E. coli*. LuxS is therefore presumed to be an AI-2 synthase and *luxS* homologues are present in a number of Gram-negative and Gram-positive bacteria for which genome databases are available (Surette *et al*. 1999). Whether AI-2 is involved in regulating or activating the *sdiA* gene or gene product, respectively, remains to be established, as does the nature of the signal-transduction system and target structural genes regulated via AI-2 in bacteria other than *V. harveyi*.

3. *N*-ACYL HOMOSERINE LACTONES AND THE REGULATION OF VIRULENCE GENES IN GRAM-NEGATIVE PATHOGENS

Among the AHL producers described above, are several organisms capable of infecting humans and animals. During infection, survival and multiplication in a hostile environment are clearly the priorities of a pathogen which must modulate expression of those genes necessary to establish the organism in a new niche. Parameters such as temperature, pH, osmolarity and nutrient availability are all known to function as environmental signals controlling the expression of coordinately regulated virulence determinants in bacteria (Williams 1988; Mekalanos 1992). A common feature of many bacterial infections is the need for the infecting pathogen to reach a critical cell population density sufficient to overwhelm host immune defences. It is therefore possible that AHLs may be involved in coordinating the control of virulence. The ability of a population of bacteria to coordinate their attack on the host may therefore be a crucial component in

the development of infection, particularly by opportunistic pathogens. Interestingly, most of the pathogenic AHL producers so far identified tend to be opportunists which can exist in multiple environments. In contrast, pathogens such as *H. influenzae*, *N. meningitidis* and *H. pylori*, which uniquely colonize and cause infections in humans, do not appear to employ AHL-mediated quorum sensing (Swift *et al*. 1999*c*). Among Gram-negative bacteria capable of causing infections in humans and animals, AHL-dependent quorum-sensing circuits have been described in *P. aeruginosa* (Latifi *et al*. 1996; Pesci *et al*. 1997), *B. cepacia* (Lewenza *et al*. 1999), *C. violaceum* (McClean *et al*. 1997), *Yersinia* spp. (Atkinson *et al*. 1999; Swift *et al*. 1999*a*; Throup *et al*. 1995), *Aeromonas* spp. (Swift *et al*. 1997) and *V. anguillarum* (Milton *et al*. 1997). The role of AHLs in controlling virulence gene expression is particularly well exemplified in *P. aeruginosa*.

(a) *Pseudomonas aeruginosa*

The pseudomonads exhibit considerable nutritional and metabolic versatility and are found in a wide variety of environmental niches ranging from soil and water to plants and animals (Passador & Iglewski 1995; Williams *et al*. 1996). Among the pathogenic members of this genus, *P. aeruginosa* is the most important human pathogen and is commonly responsible for respiratory tract infections in cystic fibrosis patients as well as blood, skin, eye and genito-urinary tract infections in patients immunocompromised by surgery, cytotoxic drugs or burn wounds (Passador & Iglewski, 1995; Williams *et al*. 1996). *P. aeruginosa* produces a wide variety of exoproducts many of which contribute to the virulence of this opportunistic pathogen. These include elastase, alkaline protease, exotoxin A, cytotoxic lectins and phospholipase (Passador & Iglewski 1995) in addition to the phenazine pigment pyocyanin (Hassett *et al*. 1992) and the siderophores, pyoverdin and pyochelin (Meyer *et al*. 1996). The synthesis of these exoproducts is not constitutive but is regulated by the prevailing growth environment with both nutrient deprivation (especially iron-deprivation) and growth rate influencing expression (Brown & Williams 1985). At the genetic level it is now apparent that regulation of the genes for many *P. aeruginosa* virulence determinants including elastase (*lasB*), the LasA protease (*lasA*), alkaline protease (*aprA*), rhamnolipids and exotoxin A (*toxA*), as well as type IV pilus-mediated twitching motility and synthesis of the siderophore pyoverdin, are mediated to varying degrees through quorum sensing (Glessner *et al*. 1999; Pesci & Iglewski 1999; Stintzi *et al*. 1998; Williams *et al*. 1996).

The discovery that *P. aeruginosa* possesses a LuxR homologue (LasR) which regulates *lasB* expression (Gambello & Iglewski 1991) and the isolation of 3-oxo-C6-HSL from *P. aeruginosa* cell-free supernatants by Bainton *et al*. (1992*a*) provided preliminary evidence of a role for quorum sensing in the regulation of virulence gene expression. Subsequent work from a number of laboratories established that *P. aeruginosa* has two pairs of LuxRI homologues, i.e. LasRI and RhlRI (also termed VsmRI) (Gambello & Iglewski 1991; Passador *et al*. 1993; Ochsner & Reiser 1995; Latifi *et al*. 1995), and four AHLs have been chemically characterized, namely 3-oxo-C6-HSL (Bainton *et al*. 1992*a*), *N*-3-oxododecanoyl-L-homoserine lactone (3-oxo-C12-HSL; figure 1*b*; Pearson *et al*. 1994), *N*-butanoyl-L-homoserine lactone (C4-HSL; Pearson *et al*. 1995; Winson *et al*. 1995) and *N*-hexanoyl-L-homoserine lactone (C6-HSL; Winson *et al*. 1995). The major signal molecules produced via LasI and RhlI respectively are 3-oxo-C12-HSL and C4-HSL, although in addition, LasI directs 3-oxo-C6-HSL synthesis and RhlI directs C6-HSL synthesis (Pearson *et al*. 1995; Winson *et al*. 1995). Furthermore LasR/3-oxo-C12-HSL is required for the expression of the *rhlRI* locus and *P. aeruginosa* employs a multilayered hierarchical quorum-sensing cascade linking LasR/3-oxo-C12-HSL, RhlR/C4-HSL and the alternative sigma factor RpoS to integrate the regulation of virulence determinants with survival in the stationary phase (Latifi *et al*. 1996). Intriguingly, expression of virulence genes such as that coding for the cytotoxic galactophilic lectin PA-I is directly dependent on both RhlR/C4-HSL and RpoS; mutations in *rhlR*, *rhlI* or *rpoS* all lead to a loss of lectin production, while *rpoS* mutants also exhibit reduced expression of elastase (K. Winzer, S. P. Diggle, C. Falconer, M. Camara and P. Williams, unpublished data). As would be predicted from the hierarchy, mutations in LasR should also lead to the loss of PA-I production. However, in a *lasR* mutant, lectin production was not abolished but substantially delayed when compared with the parent strain, suggesting that the *rhlRI* system is functional in the late stationary phase in the absence of *lasR* (Winzer, K., Diggle, S. P., Camara, M. and Williams, P. unpublished data). Another mechanism for bypassing *lasR* was described by Van Delden *et al*. (1998). They found that when elastase-negative *P. aeruginosa lasR* mutants are grown in a medium in which the sole carbon and nitrogen source provided was casein, spontaneous mutants are generated which suppress the *lasR* mutation such that *rhlRI* functionality and hence synthesis of elastase and other exoproducts is restored.

In *V. fischeri*, the *lux* box, a 20-nucleotide inverted repeat, situated within the intergenic region betweeen *luxR* and *luxI*, is required for the primary regulation of *lux* gene expression and is essential for the autoinduction response (Stevens *et al*. 1994). Similar motifs have been located upstream of the transcriptional start of a number of quorum-sensing-dependent genes in several different organisms (Salmond *et al*. 1995; Fuqua *et al*. 1996; Latifi *et al*. 1995; Pesci & Iglewski 1999) In *Agrobacterium tumefaciens*, *in vitro* experiments have unequivocally demonstrated that the LuxR homologue TraR binds to its target *lux* box only when activated by its cognate AHL, 3-oxo-C8-HSL (Zhu & Winans 1999). In *P. aeruginosa*, *lux* box-like sequences have been identified upstream of a number of genes including *lasB*, *rhlI*, *rhlA*, *lasI*, *lasA*, *rhlR*, *lasR* (Latifi *et al*. 1995; Pesci & Iglewski 1999) and *lecA* (K. Winzer, C. Falconer and P. Williams, unpublished data). Thus, these *lux* box sequences appear to be conserved regulatory elements and are likely to be the targets for either or both LasR, RhlR and/or other *P. aeruginosa* LuxR homologues present in the genome database (www.pseudomonas.com) but which have yet to be characterized experimentally. Since none of the *lux* box sequences are identical, *in vitro* binding studies with each purified *P. aeruginosa* LuxR homologue will be required to gain insights in DNA target specificity.

The LasRI and RhlRI systems have also been implicated in the regulation of exoenzyme secretion via the Xcp general secretory pathway and in the control of twitching motility. Chapon-Hervé *et al.* (1997) demonstrated that transcription of the *xcp* genes was reduced in both a *lasR* mutant and in an *rhlRI*-negative background. Control of secretion by quorum sensing presumably enables the organism to deal with the enormous induction of exoproduct (e.g. elastase, LasA protease, exotoxin A) synthesis as the bacterial population reaches a high cell density. Both the *las* and *rhl* quorum-sensing systems are required for type IV pilus-dependent twitching motility and infection by the pilus-specific phage D3112cts (Glessner *et al.* 1999). C4-HSL in particular appears to influence both the export and surface assembly of surface type IV pili, while 3-oxo-C12-HSL plays a role in maintaining cell–cell spacing and associations required for effective twitching motility (Glessner *et al.* 1999). Type IV pili have also been shown to be necessary for promoting the surface attachment of *P. aeruginosa* (O'Toole & Kolter 1998), a necessary pre-requisite to biofilm formation. Indeed, *P. aeruginosa* also employs quorum sensing to control biofilm formation; Davies *et al.* (1998) have shown that production of 3-oxo-C12-HSL via LasI is necessary for the formation of a normal biofilm.

In addition to the AHLs, two additional, chemically distinct, classes of putative quorum-sensing signal molecules have recently been described in *P. aeruginosa*. Holden *et al.* (1999) identified two cyclic dipeptides, cyclo(ΔAla-L-Val) (figure 1*c*) and cyclo(L-Pro-L-Tyr) present in cell-free culture supernatants via their capacity to weakly activate LuxR-dependent AHL biosensors and to inhibit AHL-mediated swarming in *Serratia liquefaciens*. Whether these cyclic dipeptides modulate AHL-dependent quorum sensing in the producer organism or in other organisms occupying the same ecological niche, or indeed whether they function as diffusible signal molecules *per se*, remains to be established. By studying the expression of *lasB* in a *lasR*-negative mutant, Pesci *et al.* (1999) discovered another *P. aeruginosa* quorum-sensing signal molecule unrelated to either the AHLs or cyclic dipeptides. This signal molecule, the synthesis and bioactivity of which is dependent on both the LasRI and RhlRI quorum-sensing systems, was chemically characterized as 2-heptyl-3-hydroxy-4-quinolone (figure 1*d*), a compound related to the well known class of 4-quinolone antibiotics (Pesci *et al.* 1999). Intriguingly, 4-quinolones such as nalidixic acid and ofloxacin have the capacity to induce high-level shiga toxin expression in enterohaemorrhagic *E. coli* by an as yet unidentified mechanism (Kimmit *et al.* 1999). It is therefore tempting to invoke the existence of a 4-quinolone signalling pathway in *E. coli* to account for these observations.

(b) *The pathogenic Yersiniae*

The genus *Yersinia* which belongs to the *Enterobacteriaceae* contains three species capable of causing disease in both humans and rodents (Cornelis & Wolf-Watz 1997; Swift *et al.* 1999*a*). *Yersinia pestis* is the causative agent of plague and is transmitted via the bite of an infected flea, while *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* are both food-borne pathogens capable of causing adenitis, septicaemia and gastrointestinal syndromes. Although plague and yersinosis have very different clinical manifestations, the three pathogenic species share many virulence determinants and a tropism for lymphoid tissues (Cornelis & Wolf-Watz 1997; Swift *et al.* 1999*a*). *Yersinia* virulence determinants include flagellins, lipopolysaccharide and siderophores, as well as gene products important for the attachment and penetration of the intestinal barrier, and a type III secretory pathway which allows the bacterium to inject Yop proteins directly into a target cell (e.g. macrophages and polymorphonuclear leucocytes) cytoplasm and thus evade the cellular immune response (Cornelis & Wolf-Watz 1997; Swift *et al.* 1999*a*). The regulation of virulence gene expression in the pathogenic Yersiniae is complex, with temperature playing a key role; *yop* gene expression, for example, depends on the temperature-dependent interplay between the transcriptional activator VirF, which itself is modulated by a histone-like protein, YmoA (Cornelis & Wolf-Watz 1997). All three pathogenic *Yersinia* species produce AHLs and each possesses at least two LuxRI pairs which, in contrast to the divergent genetic organization in *V. fischeri* and tandem arragement in *P. aeruginosa*, are transcribed convergently (Atkinson *et al.* 1999; Throup *et al.* 1995; Swift *et al.* 1999*a*). YenI, YpsI and YpeI from *Y. enterocolitica*, *Y. pseudotuberculosis* and *Y. pestis*, respectively, all direct the synthesis of 3-oxo-C6-HSL and C6-HSL in an approximately equimolar ratio (Throup *et al.* 1995; Atkinson *et al.* 1999; Swift *et al.* 1999*a*). Inactivation of *yenI* does not influence *yop* gene expression (Throup *et al.* 1995) and a role for quorum sensing in the regulation of virulence in *Y. enterocolitica* has remained elusive. In *Y. pseudotuberculosis* there are two quorum-sensing systems, YpsRI and YtbRI (Atkinson *et al.* 1999). Two phenotypes regulated by YpsRI quorum sensing have been observed; both are repressed in the parent strain and activated in a *ypsR* mutant. At 37 °C, but not at 22 °C, the *ypsR* mutant, but not the parent strain or the *ypsI* mutant, clump. This suggests that YpsR represses the production of a clumping factor in coordination with temperature changes. Additionally, the major flagellin subunit is overexpressed in the *ypsR* strain and, at 22 °C, this is reflected in (i) the hypermotility of the *ypsR* strain on swarm plates, and (ii) the induction of motility in the *ypsR* strain during the logarithmic phase of growth in broth (Atkinson *et al.* 1999). In comparison, the parent strain is not motile at 22 °C or 37 °C and the *ypsR* mutant is not motile at 37 °C. In contrast to the clumping factor, however, the effects on motility are mirrored in a *ypsI* strain. Thus, YpsR appears to act as a repressor of (i) a clumping factor, which is repressed at lower temperatures (i.e. 22 °C) and does not require acyl-HSL production for derepression. This may favour the aggregation of cells in a host where increasing numbers could improve (i) colonization and (ii) motility, which is repressed at higher temperatures (i.e. 37 °C), and requires acyl-HSL production for derepression to occur in the presence of YpsR. Hence this may facilitate the dispersal and dissemination of bacteria from high-density colonies when removed from the host. Since *Y. pestis* is characteristically non-motile, this poses an interesting question with respect to the function of the homologous quorum-sensing loci. For *Y. pestis*, mutation of the *ypeR* gene (equivalent to the *Y. pseudotuberculosis ypsR* gene) does not appear to influence the expression of major virulence factors including the

V antigen, pH6 antigen, Pla (which confers fibrinolytic and coagulase activities) or lipopolysaccharide (Swift *et al*. 1999*a*).

(c) *A. hydrophila, A. salmonicida and* V. anguillarum

While both *A. salmonicida* and *A. hydrophila* are pathogenic for salmonid fish (Fryer & Bartholomew 1996), only *A. hydrophila* causes disease in humans (gastroenteritis and septicaemia; Thornley *et al*. 1997). The virulence of *Aeromonas* is multifactorial, involving the combined activities of multiple surface-associated macromolecules and secreted exoproteins, including a number of serine and metalloproteases (Swift *et al*. 1999*b*). Many of these exoenzymes are only secreted at high cell densities in the stationary phase, invoking the possibility that they may be regulated via quorum sensing (Swift *et al*. 1999*b*). This has indeed proved to be the case and Swift *et al*. (1997) cloned a divergent pair of LuxRI homologues termed AhyRI and AsaRI in *A. hydrophila* and *A. salmonicida* respectively. In common with RhlI from *P. aeruginosa*, both AhyI and AsaI direct the synthesis predominantly of C4-HSL along with small amounts of C6-HSL (Swift *et al*. 1997). Mutagenesis of either *ahyI* or *ahyR* results in the loss of C4-HSL production in *A. hydrophila* and a substantial downregulation of both serine and metalloprotease activities, which can be restored in the *ahyI*-negative mutant by supplying exogenous C4-HSL (Swift *et al*. 1999*b*).

Apart from *V. fischeri*, and *V. harveyi*, there is little information on AHL-dependent quorum sensing in other non-luminous *Vibrio* species. When cell-free supernatants prepared from pathogenic vibrios including *V. cholerae*, *Vibrio parahaemolyticus*, *Vibrio vulnificus* and *V. anguillarum* were screened with AHL lux-based biosensors, only *V. anguillarum* gave a positive result (Milton *et al*. 1997; Swift *et al*. 1999*c*). This bacterium is responsible for a terminal haemorrhagic septicaemia known as vibriosis, which is of economic significance in the fish-farming industry. Although the first description of the disease was almost a century ago, not much is known of the pathogenesis of vibriosis and apart from a siderophore transport system, metalloprotease and chemotactic motility, few virulence determinants have been characterized (Crosa 1989; Milton *et al*. 1992, 1996). Milton *et al*. (1997) identified the major *V. anguillarum* AHL as *N*-(3-oxodecanoyl)-L-homoserine lactone (3-oxo-C10-HSL) and described a pair of LuxRI homologues termed VanRI. Mutagenesis experiments and the expression of *vanI* in *E. coli* confirmed that VanI was responsible for 3-oxo-C10-HSL synthesis and that *vanI* expression depended on VanR/3-oxo-C10-HSL. Interestingly, *V. anguillarum* produces an extracellular metalloprotease (EmpA) with 47% identity at the amino-acid level to the elastase of *P. aeruginosa*. Although the *empA* upstream sequence contains a potential *lux* box-like sequence, its expression does not appear to depend on the VanR/3-oxo-C10-HSL since *vanR*, *vanI* and *vanRI* mutants did not show any reduction in protease production (Milton *et al*. 1997). However, the EmpA protease of *V. anguillarum* is induced by gastrointestinal mucus to a level some ninefold greater than is observed in conventional broth (Denkin & Nelson 1999). It is therefore possible that the *vanIR* mutants may respond differently when exposed to mucus. Furthermore, the *vanI* mutant remained capable of weakly activating AHL biosensors suggesting the existence of additional layers of AHL-mediated regulatory hierarchy (Milton *et al*. 1997).

4. DOES QUORUM SENSING OCCUR *IN VIVO* DURING INFECTION?

Two main approaches can be taken to determine whether AHL-dependent quorum sensing occurs *in vivo* during infection. First, the virulence of mutants with defects in *luxI* or *luxR* homologues can be assessed in appropriate animal models. For mutants with defects in *luxI* homologues, it should be possible to restore virulence by the exogenous provision of the cognate AHL. Second, tissues from infected animals or humans can be extracted and analysed for the presence of AHLs and/or bacterial cells expressing quorum-sensing genes.

In plant pathogens such as *E. carotovora*, there is a direct relationship between virulence and AHL production in that mutants with defects in the quorum-sensing machinery are avirulent (Jones *et al*. 1993; Pirhonen *et al*. 1993) and virulence can be restored by the co-inoculation of 3-oxo-C6-HSL or by expression of a *luxI* homologue *in planta* (Fray *et al*. 1999). In *P. aeruginosa*, *lasR* mutants have been evaluated in a number of model infections. Using a neonatal mouse model of acute pneumonia, Tang *et al*. (1996) reported that a *lasR* mutant was much less virulent than the parent strain PAO1. In contrast, a *lasR*-negative mutant was as virulent as the wild-type in a murine model of corneal infection (Preston *et al*. 1997). More recently, the nematode *Caenorhabditis elegans* has been exploited to model mammalian bacterial pathogenesis and has been used to identify *P. aeruginosa* virulence factors (Mahajan Miklos *et al*. 1999). By screening *P. aeruginosa* Tn*phoA* mutants using this model, Tan *et al*. (1999) identified a *lasR* mutant which exhibited significantly reduced virulence in the *C. elegans* model, in an *Arabidopsis* leaf infiltration model and in a mouse full-thickness burn wound infection model. This mutant also failed to accumulate in the gut of *C. elegans*, suggesting that the establishment and/or proliferation of *P. aeruginosa* within the host may also be dependent on quorum sensing. For *P. aeruginosa*, the requirement for a functional LasR protein for virulence in plants, nematodes and mice suggests that at least for this pathogen, quorum sensing is a general feature of pathogenesis in all hosts. Although no data have been published on the virulence of *P. aeruginosa* strains with defects in other components of the quorum-sensing machinery (e.g. *lasI*, *rhlR* and *rhlI*), they are likely to exhibit reduced virulence given the nature of the regulatory hierarchy (Latifi *et al*. 1996; Pesci *et al*. 1997). Wang *et al*. (1996) developed an *in vivo* expression technology (IVET) system for *P. aeruginosa* which was applied to a neutropenic mouse infection model and recovered a number of novel genetic loci that were specifically induced *in vivo*. However, no known quorum-sensing regulatory elements were identified in this study, although only 22 loci were analysed and many more will clearly be involved in sustaining bacterial growth *in vivo*.

In addition to *P. aeruginosa*, two other Gram-negative pathogens with mutations in LuxR and LuxR homologues

have been evaluated in experimental animal models of infection. The LD_{50}s for rainbow trout infected either by immersion in seawater or by direct intraperitoneal injection with *V. anguillarum vanI* or *vanR* or *vanIR* mutants were no different to that of the wild-type (Milton *et al*. 1997). This implies that quorum sensing may not play a direct role in vibriosis, although there is a second quorum-sensing system in *V. anguillarum* analogous to that of *V. harveyi* (D. Milton, V. J. Hope, A. Hardman, M. Camara and P. Williams, unpublished data) such that it is possible that the *vanIR* mutations may be compensated for by this alternative system. However, when *V. anguillarum* transformed with a plasmid containing either a *vanR::lacZ* or a *vanI::lacZ* fusion are recovered from the kidneys of infected fish, it is clear from the β-galactosidase levels observed that both genes are being expressed during growth *in vivo* (D. Milton, V. J. Hope, A. Hardman, M. Camara and P. Williams, unpublished data).

Similarly, when BALB/c mice were challenged subcutaneously with either the wild-type *Y. pestis* or the isogenic *ypeR* mutant, the minimum lethal dose was the same for both strains (K. E. Isherwood, P. C. F. Oyston, S. Atkinson, P. Williams, G. S. A. B. Stewart and R. W. Titball, unpublished observations). There was, however, a small increase in time to death implying that the *ypeR* gene makes a contribution to *Y. pestis* pathogenicity, although it is again possible that the second quorum-sensing system compensates for mutations in the first (K. E. Isherwood, P. C. F. Oyston, S. Atkinson, P. Williams, G. S. A. B. Stewart and R. W. Titball, unpublished observations). Alternatively, it is possible that quorum sensing may be more important for the growth and survival of *Y. pestis* in the flea transmission vector than the host.

5. EVIDENCE FOR AHL PRODUCTION *IN VIVO*

The direct demonstration of quorum sensing *in vivo* could be achieved by the detection of AHLs in the tissues or body fluids of infected animals or humans using AHL biosensors. A number of *lux*-based plasmid AHL biosensors have been constructed (Winson *et al*. 1998). These are based on LuxR, LasR and RhlR, and their target promoters (P_{luxI}, P_{lasI} and P_{rhlI}, respectively) transcriptionally fused to the *luxCDABE* cassette from *Photorhabdus luminescens*. These sensors emit light in the presence of an activating AHL with sensitivities in the picomolar to nanomolar range. Although they are most sensitive to their natural ligands, they are capable of responding to a range of AHL analogues. Since AHLs are soluble in ethyl acetate and dichloromethane, these organic solvents can be used to extract and concentrate AHLs produced in tissues during growth *in vivo*.

During infection of rainbow trout (*Oncorhynchus mykiss*) with *A. salmonicida* or *A. hydrophila* or *V. anguillarum*, the bacteria become concentrated in the kidney, which can conveniently be removed, homogenized and extracted with dichloromethane. Using this approach, it has been possible to use AHL biosensors to demonstrate the presence of AHLs in infected but not in uninfected tissues (L. Fish, D. Milton, S. Swift and P. Williams, unpublished data). For *A. salmonicida*, the concentration of C4-HSL in the kidney detected using an *rhlRI′::lux* biosensor, was estimated to be approximately 20 nM although this may be an underestimate given that the efficiency of solvent extraction is unlikely to be 100%. In addition, the identity of the AHL is presumed since LuxR homologues will respond to a range of AHL analogues (Winson *et al*. 1998) and therefore definitive proof for the presence of a specific AHL depends on further chemical characterization. Although it was not possible to fully chemically characterize the AHL(s) produced *in vivo* by *A. salmonicida*, extraction of the kidney homogenates from *V. anguillarum* infected rainbow trout provided sufficient material for liquid-chromatography-mass spectrometry. This technique couples the resolving power of HPLC with mass spectrometry such that the mass of the molecular ion and its major component fragments can be determined for a compound with a given retention time. Using this approach, it was possible to unequivocally confirm the presence of 3-oxo-C10-HSL in infected kidneys, a finding which correlated with the activation of the *lasRlasI′::lux*-based AHL biosensor (V. Hope, D. Milton, A. Hardman, M. Camara and P. Williams, unpublished data).

In cystic fibrosis (CF) patients, chronic respiratory tract infection with *P. aeruginosa* or *B. cepacia* leads to destructive lung disease requiring aggressive antibiotic chemotherapy (Govan & Deretic 1996). Several studies have shown that *P. aeruginosa* virulence determinants such as elastase and alkaline protease are produced in the lungs of CF patients (Storey *et al*. 1998). The *lasR* transcript has also been detected in sputum samples (Storey *et al*. 1998), suggesting that AHL-mediated quorum sensing is occurring in the lungs of CF patients and that the sputum from such patients is likely to contain AHLs. By screening solvent-extracted sputum from CF patients infected with *P. aeruginosa*, with a range of AHL biosensors, we have shown that the sputum of patients infected with *P. aeruginosa* contain both short-chain AHLs (probably C4-HSL and C6-HSL) and a molecule which activates the *lasRI′::lux*-based AHL biosensor and co-migrates on thin layer chromatograms with 3-oxo-C12-HSL (B. Middleton, A. Hardman, H. Rogers, A. Knox, M. Camara and P. Williams, unpublished data). Interestingly, we have only been able to obtain mass spectrometry data to confirm the presence of C6-HSL, which is perhaps surprising given that it is produced *in vitro* via RhlI in much smaller quantities than C4-HSL (Winson *et al*. 1995) or 3-oxo-C12-HSL (Pearson *et al*. 1994). In contrast, sputum extracts from CF patients with *Staphylococcus aureus* infections did not activate any of the biosensors consistent with the lack of AHL production in these pathogens (B. Middleton, A. Hardman, H. Rogers, A. Knox, M. Camara and P. Williams, unpublished data). However, extracts from CF patients infected with *B. cepacia*, which produces C8-HSL (Lewenza *et al*. 1999), activated the *luxRI′::lux* biosensor much more strongly than the *lasRI′::lux* biosensor (B. Middleton, A. Hardman, H. Rogers, A. Knox, M. Camara and P. Williams, unpublished data), a result which would be predicted from structure–activity relationships using these biosensors with a range of AHL analogues (Winson *et al*. 1998). Additional preliminary evidence for the production of AHLs *in vivo* was presented by Stickler *et al*. (1998),

who tested sections of indwelling urethral catheters that had become colonized with bacterial biofilms. These were removed from patients undergoing long-term indwelling bladder catheterization and tested with an AHL biosensor-based on the *tra* system of *Agrobacterium tumefaciens* (Piper *et al.* 1993). Four out of nine catheter sections gave positive reactions, while unused catheters were negative, suggesting that AHLs were being produced *in situ* in the urinary tract (Stickler *et al.* 1998). However, neither the nature of the biofilm organisms present nor their *in vitro* AHL profiles were presented. Given that the *Agrobacterium* AHL biosensor also responds to molecules such as the cyclic dipeptides described by Holden *et al.* (1999), further work will be required to unequivocally demonstrate AHL production in catheter-associated biofilms.

6. DO AHLS FUNCTION AS VIRULENCE DETERMINANTS *PER SE*?

Although AHLs have so far largely been considered as effectors of prokaryotic gene expression, they are capable of influencing eukaryotic cell behaviour and potentially modulating disease processes. Cystic fibrosis is a genetic disease characterized by mucus hypersecretion and by chronic bacterial (particularly *P. aeruginosa*) infection and airway inflammation (Govan & Deretic 1996). CF patients carry mutations in a membrane protein termed the CF transmembrane conductance regulator (CFTR), which possesses cyclic AMP-dependent chloride channel activity and is defective in CF. Airway inflammation in CF patients is characterized by the influx and activation of large numbers of neutrophils, which play a major role in the pathology of CF lung disease (Govan & Deretic 1996). Interleukin-8 (IL-8) is a neutrophil-selective stimulus for chemoattraction, adhesion and elastase release and is considered to play an important role in CF (Palfreyman *et al.* 1997). Thus factors which induce IL-8 contribute to the inflammatory process and progressive lung deterioration. Interestingly, the *P. aeruginosa* quorum-sensing signal molecule 3-oxo-C12-HSL has been reported by some (Di Mango *et al.* 1995), but not others (Palfreyman *et al.* 1997), to stimulate the dose-dependent production of IL-8 by respiratory epithelial cells, albeit at higher levels than are normally produced by *P. aeruginosa* growing in laboratory media (approximately 5 μM; Pearson *et al.* 1995).

Submucosal tracheal gland serous (HTGS) cells are believed to play a major role in the pathophysiology of CF and secrete a number of antibacterial and anti-proteolytic proteins (including lactoferrin, lysozyme and secretory leucocyte proteinase inhibitor (SLPI)). Kammouni *et al.* (1997) have developed a CF HTGS cell line that has retained the CTFR defect. In this cell line, as in normal airway epithelial cells, it is possible to bypass the CTFR defect since calcium-dependent chloride channels remain functional and chloride secretion can be stimulated by nucleotides. ATP, UTP and their analogues have therefore been tested clinically and shown to improve mucociliary clearance in CF. Using both normal and CF HTGS cells, Saleh *et al.* (1999) sought to determine whether AHLs produced by *P. aeruginosa* influenced SLPI secretion. Although they had no effect on the secretion of SLPI in either normal or CF HTGS cells, 3-oxo-C6-HSL and 3-oxo-C12-HSL at picomolar concentrations inhibited the ATP- and UTP-dependent stimulation of SLPI secretion. Similar results were also obtained with C4-HSL and C6-HSL but at much higher concentrations (micromolar range) suggesting that efficient inhibition depends on the presence of a 3-keto substituent and may be independent of acyl chain length. Moreover, it is clear that AHLs such as 3-oxo-C12-HSL are not functioning as receptor antagonists but are able to potently downregulate expression of the nucleotide receptors P2Y2 and P2Y4. The signal transduction pathway involved is not known but may involve leucotrienes, since ibuprofen—an inhibitor of leucotriene production—blocked the action of 3-oxo-C12-HSL (Saleh *et al.* 1999). Furthermore, the high sensitivity of CF HTGS cells compared with normal HTGS cells to 3-oxo-C12-HSL and 3-oxo-C6-HSL suggests a CF-specific defect linked with arachidonate metabolism which, in CF, has long been known to be defective (Saleh *et al.* 1999). Alternatively, the ability of ibuprofen to inhibit the activation and translocation of NF-κB into the nucleus may lead to the downregulation of nucleotide receptor expression (Stuhlmeier *et al.* 1999) and account for the activities of the AHLs. Thus *P. aeruginosa* AHLs can potently modify the behaviour of a cell type (HTGS) believed to make a significant contribution to the pathophysiology of CF.

Apart from the lungs of CF patients, *P. aeruginosa* can cause infections in almost any other body site and Telford *et al.* (1998) sought to determine whether the immune system is capable of responding to AHLs. In murine and human leucocyte immunoassays *in vitro*, 3-oxo-C12-HSL but not 3-oxo-C6-HSL inhibited lymphocyte proliferation and tumor necrosis factor alpha production by lipopolysaccharide stimulated macrophages (figure 3*a*). Similarly, neither C4-HSL nor C6-HSL exhibited any inhibitory activity (D. Hooi, D. Pritchard, M. Camara, P. Williams and B. Bycroft, unpublished data). Furthermore, 3-oxo-C12-HSL potently downregulated the production of IL-12, a T helper cell 1 (Th-1) supportive cytokine (Telford *et al.* 1998). At high concentrations ($> 70\,\mu M$), 3-oxo-C12-HSL inhibited antibody production by keyhole limpet haemocyanin-stimulated spleen cells, but at lower concentrations antibody production was stimulated apparently by increasing the proportion of the immunoglobulin G1 (IgG1) isotype (figure 3*b*). 3-oxo-C12-HSL also promoted IgE production by interleukin-4-stimulated human peripheral blood mononuclear cells (Telford *et al.* 1998). Since T-cell responses constitute an important component of the host defence against *P. aeruginosa* (Stevenson *et al.* 1996) these data suggest that by modulating T-cell and macrophage functions, 3-oxo-C12-HSL is functioning as a virulence determinant *per se*. More specifically, by switching the T-helper-cell response from the antibacterial Th-1 response (characterized by the secretion of IL-12 and γ-interferon) to a Th-2 response, *P. aeruginosa* may modulate the host inflammatory response to promote its own survival and growth (figure 3*c*). Such immunomodulatory activity suggests that 3-oxo-C12-HSL and related compounds could find a therapeutic application in the modulation of autoimmune diseases and prevention of septic shock. The molecular basis for the immunomodulatory

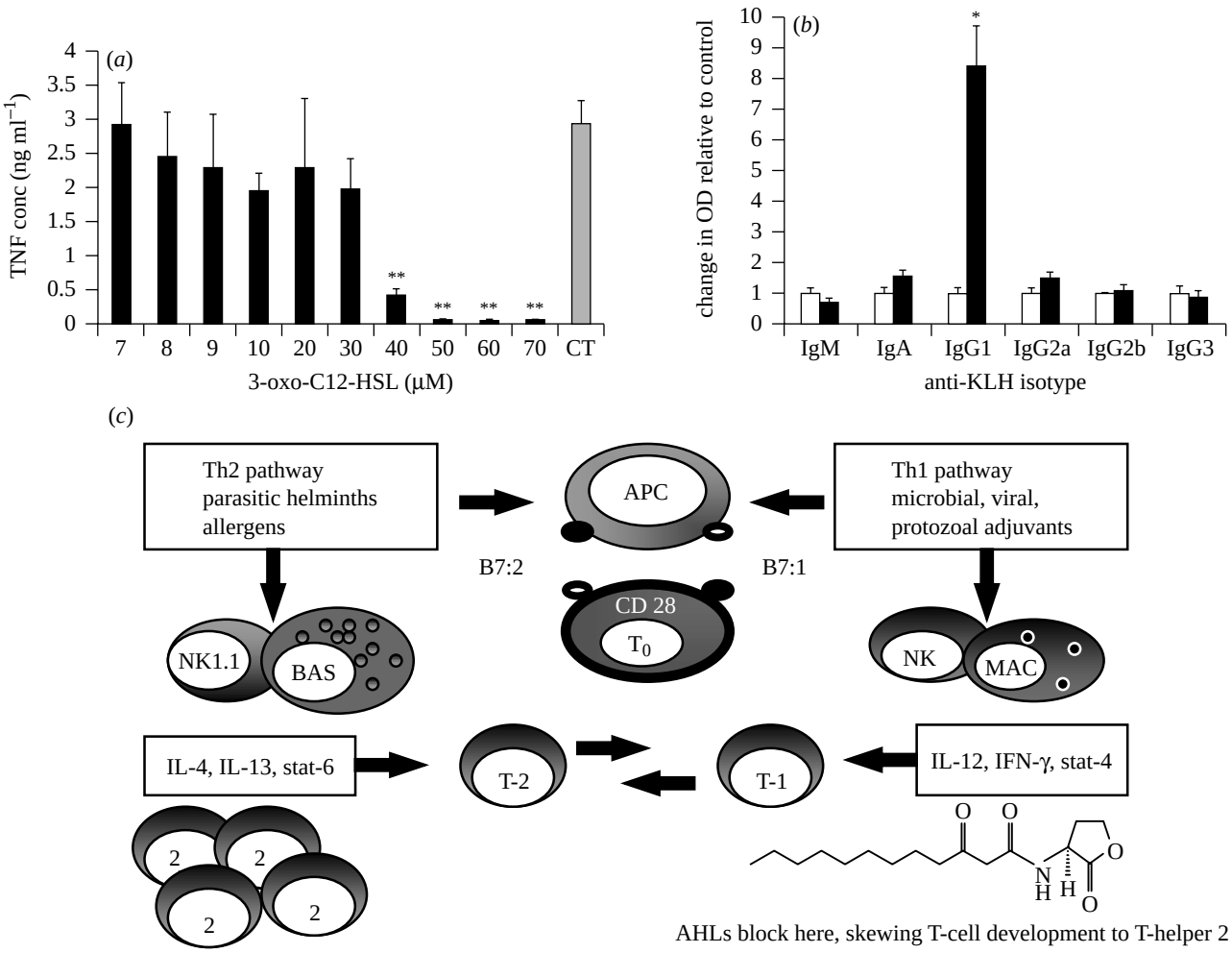

Figure 3. 3-oxo-C12-HSL suppresses mitogen-induced cell proliferation (Telford *et al*. 1998) and the ability of LPS-stimulated murine macrophages to secrete TNFα (*a*). Immune suppression may be selective, as murine B cells were stimulated to produce significantly more IgG1, an isotype characteristic of Th-2 activity, following stimulation with keyhole limpet haemocyanin (KLH); (*b*). Filled bars, +3-oxo-C12-HSL; open bars, −3-oxo-C12-HSL. These data suggest that 3-oxo-C12-HSL steers immune responses away from a possibly *Pseudomonas* protective Th-1 phenotype, as shown in (*c*). APC, antigen-presenting cell; T_0, undifferentiated T cell; NK, natural killer cell; BAS, basophil; MAC, macrophage; CT, control; stat, signal for transduction and activation of transcription; IL, interleukin; IFN, interferon.** $p < 0.01$; * $p < 0.05$.

activity of 3-oxo-Cl2-HSL is not known. However, it does not appear to be via phosphotyrosine kinases such as $p59^{fyn}$ or $p56^{lck}$, which are key enzymes in the initial activation of T lymphocytes, since neither enzyme is inhibited by 3-oxo-Cl2-HSL (Telford *et al*. 1998).

The modulation of T-cell, macrophage and CF HTGS-cell functions by 3-oxo-Cl2-HSL demonstrates that eukaryotic cells are influenced by AHLs. In addition, Lawrence *et al*. (1999) examined the effect of 3-oxo-Cl2-HSL and related compounds on the constrictor tone of porcine blood vessels. Over the range of 1–30 μM, 3-oxo-Cl2-HSL caused a concentration-dependent relaxation of U46619 (a thromboxane mimetic)-induced contractions of the coronary artery, but was markedly less effective in the pulmonary artery. Neither C4-HSL nor homoserine lactone exerted any activity, although the thiolactone analogue *N*-(3-oxododecanoyl)-L-homocysteine thiolactone was as quantitatively effective as 3-oxo-Cl2-HSL. *N*-3-oxododecanamide, which lacks the lactone ring, possesses only about 30% of the vasorelaxant activity of 3-oxo-Cl2-HSL (Lawrence *et al*. 1999). 3-oxo-Cl2-HSL appears to exert its inhibitory effect principally at the level of the smooth muscle, although its mechanism of action remains to be elucidated. This may involve activation of Na^+/K^+-ATPase and the subsequent hyperpolarization of vascular smooth muscle, a mode of action proposed to account for the vasorelaxant properties of the Cl8 polyunsaturated fatty acid, linoleic acid, on porcine coronary arteries (Pomposiello *et al*. 1998). Given that 3-oxo-Cl2-HSL is present in the lungs of CF patients chronically colonized with *P. aeruginosa*, the finding that it is a less potent vasorelaxant of pulmonary arterial smooth muscle is perhaps surprising but raises the possibility that other vascular beds may exhibit differential sensitivity to AHLs. Indeed a better indicator of the significance of this vasodilator activity may be obtained by investigating the effects of 3-oxo-Cl2-HSL on human resistance arteries and small veins rather than the much larger conduit arteries.

The qualitatively similar immunomodulatory and vasodilator activities of the AHLs appear to share a similar dependence on the presence of the Cl2 acyl chain given the inactivity of the shorter-chain compounds. This implies that their activities in this context differ from the

AHL-inhibited nucleotide-dependent stimulation of SLPI secretion in CF HTGS cells, where 3-oxo-C6-HSL was reported to be as active as 3-oxo-C12-HSL. When these observations are taken together, they suggest that 3-oxo-C12-HSL plays a role not only in regulating *P. aeruginosa* virulence gene expression but also the orchestration of eukaryotic cells to maximize the provision of nutrients via the bloodstream while downregulating host defence mechanisms.

7. BLOCKADE OF QUORUM SENSING AS A MEANS OF CONTROLLING INFECTION

As widespread resistance to conventional antibacterial agents continues to pose a major threat, the demand for novel therapeutic approaches to the treatment of infection is increasing. The ability to downregulate virulence gene expression may therefore offer a novel strategy for the treatment or prevention of infection. Our growing understanding that quorum sensing is a generic phenomenon and that a number of pathogens employ diffusible signal molecules to coordinate the control of multiple virulence and survival genes offers a novel chemotherapeutic target (Finch *et al.* 1998). Interference with transmission of the molecular message by a small molecule antagonist which competes for the autoinducer binding site of sensor or transcriptional activator proteins, thereby switching off virulence gene expression and attenuating the pathogen, is an attractive strategy. In this context, the ability of various AHL analogues to inhibit the action of the cognate AHL has been demonstrated (Eberhard *et al.* 1986; McClean *et al.* 1997; Passador *et al.* 1996; Schaefer *et al.* 1996*b*; Swift *et al.* 1997, 1999*b*). For example, C4-HSL-dependent exoprotease production in *A. hydrophila* can virtually be abolished by AHLs with acyl chains of 10, 12 or 14 carbons. Furthermore, Givskov *et al.* (1996) provided evidence that furanone compounds produced by the Australian macroalgae *Delisea pulchra* inhibit AHL-regulated processes including swarming in *S. liquefaciens* and bioluminescence in *V. fischeri*. As the inhibitory effect could be overcome by the addition of an excess of the cognate AHL, the furanones appear to be acting as competitive antagonists. The significant recent advances in defining the enzymatic activity and substrate requirements of LuxI homologues (Moré *et al.* 1996; Schaefer *et al.* 1996*a*; Jiang *et al.* 1998; Parsek *et al.* 1999) also emphasizes the potential of the AHL synthase as an antimicrobial target.

Quorum-sensing blocking agents developed for the prevention or treatment of infections due to AHL-producing Gram-negative bacteria are, however, likely to have a very narrow spectrum of activity since *P. aeruginosa* is the only major human pathogen known to employ AHLs for controlling virulence gene expression. Although many more Gram-negative and some Gram-positive pathogens possess the AI-2/LuxS-based system described by Surette *et al.* (1999), apart from bioluminescence, no phenotype has yet been associated with this quorum-sensing system. Consequently, there is no evidence to suggest that it may constitute a novel antibacterial target. This is not, however, the case for the extracellular factor described by Withers & Nordström (1999) since this molecule is capable of directly inhibiting DNA replication without any requirement for transcription or translation. It is therefore likely that elucidation of the structure of this class of quorum-sensing signal molecules may offer opportunities to design potent antibacterial analogues that are refractory to the relatively rapid turnover observed for the natural molecule. Although no AHL quorum-sensing blocking agents have yet been evaluated *in vivo* in experimental animal models of infection, blockade of the cyclic thiolactone peptide-dependent quorum-sensing system, which controls virulence gene expression in *Staphylococcus aureus*, has been shown to attenuate infection in a mouse skin abscess model (Mayville *et al.* 1999). Given the importance of methicillin-resistant *S. aureus* (MRSA), which are resistant to virtually all clinically available antibiotics including vancomycin (Speller *et al.* 1997), this offers a real possibility for the development of novel anti-staphylococcal chemotherapy that will not be susceptible to conventional antibiotic-resistance mechanisms. However, while such agents would have obvious value in prophylaxis, their therapeutic potential for the treatment of established infections, especially in immunocompromised patients, is less apparent, since an intact host defence may well constitute a necessary prerequisite for clearing the infection.

We dedicate this article to the memory of the late Professor Gordon S. A. B. Stewart, who made many major contributions to the study of quorum sensing. Research in the authors' laboratories has been supported by grants and studentships from the Biotechnology and Biological Sciences Research Council, the Medical Research Council, the Wellcome Trust and the European Union, and these are gratefully acknowledged.

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