BACTERIAL CROSSTALK – COMMUNICATION BETWEEN BACTERIA, PLANT AND ANIMAL CELLS

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INTRODUCTION

Prokaryotes have evolved elaborate signal transduction mechanisms to perceive sensory information and so facilitate adaptation to the prevailing growth environment. Many of the intracellular events which take place in response to changes in osmolarity, pH, temperature, and nutrient availability have been the subject of much molecular scrutiny. In this context, a major recurring theme is the so-called 'two-component' system in which information is relayed via phosphoryl transfer from sensor to regulator proteins (Hoch & Silhavy, 1995; see Frankel, this volume). Such systems closely resemble the signal transduction mechanisms operating in higher organisms, implying that bacteria are capable of exhibiting more complex patterns of multicellular behaviour than would perhaps be predicted for unicellular microorganisms. Bacteria respond to, and process, external signals from neighbouring cells whether they are of bacterial, plant or animal origin. Such intercellular communication may involve small diffusible signal molecules and secreted proteins as well as surface-associated macromolecules. With respect to the former, the last decade has seen a tremendous growth in our understanding of the mechanisms bacteria use to coordinate their behaviour in concert with their own population size. 'Quorum sensing' as this cell density sensing mechanism has become known, is now accepted as a generic phenomenon within the bacterial kingdom (for reviews, see Fuqua *et al.*, 1994, 1996; Salmond *et al.*, 1995; Swift *et al.*, 1996; Williams *et al.*, 1992; Williams, 1994). Essentially, quorum sensing enables a bacterium to function in a multicellular manner by coupling gene expression to the attainment of a critical ('quorate') population size. Quorum sensing depends on the activation of a response regulator by a 'self-generated' diffusible signal molecule, and several chemically distinct families of such molecules have now been identified. Moreover, some quorum sensing systems are themselves controlled by external host-derived signals, as in the case of *Agrobacterium*

tumefaciens where plant derived opines are required to trigger the quorum sensing-dependent conjugal transfer of Ti plasmids (Zhang *et al.*, 1993). Conversely, bacterial quorum sensing signal molecules *per se* can influence the host immune response as exemplified by the immunomodulatory properties of the *Pseudomonas aeruginosa* autoinducer *N*-(3-oxododecanoyl) homoserine lactone (Telford *et al.*, 1998). Furthermore, several pathogens and symbionts are capable of manipulating eukaryotic host cell signal transduction pathways either by targeting host-cell receptors, whose function is to receive hormonal, cytokine or other signals or via the direct delivery of bacterial proteins into eukaryotic cells by surface-attached bacteria.

In this chapter, an overview of this intercellular information flow and its potential exploitation will be presented which, in the case of bacterial cell–cell communication, has largely become apparent through identifying the signal molecules involved in systems where their effect can easily be detected. In the sections on bacterial–plant (see McGowan & Salmond, this volume) and bacterial–animal cell (see also Neyt & Cornelis, this volume) communication, examples have been chosen to illustrate different themes that are found within this cross-talk. Parallels can be drawn between the ways in which different classes of signalling molecules are recognized by plant and animal cells, although often such communication is a consequence of the eukaryotic cells identifying bacterial components in order to defend themselves against pathogenic attack. This is particularly true with regard to recognition of proteins secreted by bacteria and there are very close similarities between the mechanisms used by bacteria to deliver proteins to (or into) both plant and animal cells. It is beyond the scope of this chapter to review all of the literature related to prokaryotic–eukaryotic interactions, but specific examples of the types of signalling events that occur will be given, while a more extensive treatment of the subversion of host cell signal transduction pathways by pathogenic *Yersinia* spp. can be found in the chapter by Neyt and Cornelis.

THE 'LANGUAGES' OF BACTERIAL CELL–CELL COMMUNICATION

Bacteria, like higher differentiated organisms, are likely to derive considerable survival benefits by coordinating the behaviour of their populations, whether in pure or mixed cultures (Shapiro, 1988). Such benefits may include improved access to complex nutrients or environmental niches, collective defence against other competitive microorganisms or eukaryotic host defence mechanisms, and optimization of population survival by differentiation into morphological forms better adapted to combating an environmental threat. Examples of the latter include the coordinated differentiation and migration of groups of highly specialized swarmer cells in the rapid colonization of a surface, sporulation, the formation of multicellular fruiting bodies and adoption of the biofilm mode of growth.

Similarly, with regard to conjugal plasmid transfer, the ability of a potential donor to monitor the availability of recipients contributes to the decision of whether or not to make the energetically expensive investment required for cell–cell conjugation.

The coordination of this multicellular behaviour in prokaryotes frequently centres upon the generation of small diffusible signal molecules, which are sometimes referred to as microbial 'hormones' or 'pheromones' or 'auto-inducers' (Wirth *et al.*, 1996; Swift *et al.*, 1996), which constitute one of the few ways in which an individual bacterial cell can obtain information about the status of other members of the same species. By far the most intensively investigated family of bacterial quorum sensing systems utilizes *N*-acyl-homoserine lactones (AHLs) as signalling molecules, although it is now apparent that alternative signalling molecule 'languages' exist (Table 1).

Discovering AHL-mediated cell–cell communication in Gram-negative bacteria

In 1992 Bainton *et al.* (1992*a,b*) discovered that a diffusible signal molecule present in cell-free stationary phase culture supernatants regulated the production of the β-lactam antibiotic 1-carbapen-2-em-3-carboxylic acid in the plant pathogen *Erwinia carotovora*. This molecule was identified as *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL; Fig. 1) and its significance relates to the fact that OHHL had been identified many years earlier as the 'autoinducer' of bioluminescence in *Vibrio fischeri* (Eberhard *et al.*, 1981). In this Gram-negative marine bacterium, OHHL synthesis requires the *luxI* gene product and the *luxICDABE* genes are transcribed in a cell density-dependent manner by the transcriptional activator protein, LuxR (Sitnikov *et al.*, 1995; see Greenberg, this volume). LuxR is activated by binding OHHL, and 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; McGowan & Salmond, this volume). In contrast to the *lux* operon, *carI* is not linked to the *car* structural genes but is located elsewhere on the chromosome (McGowan *et al.*, 1995; 1996). Furthermore, mutations in *carI(expI)* downregulate exoenzyme production thus rendering the organism avirulent *in planta* unless supplied with the exogenous OHHL (Jones *et al.*, 1993; Pirhonen *et al.*, 1993).

These findings stimulated the development and use of biosensors to screen cell-free culture supernatants from a wide range of bacterial species for the presence of OHHL or related AHLs (Bainton *et al.*, 1992*a*; Pearson *et al.*, 1994; Pearson *et al.*, 1995; Shaw *et al.*, 1997; Swift *et al.*, 1993; Throup *et al.*, 1995; Winson *et al.*, 1995; Winson *et al.*, 1998). Such biosensors usually contain an AHL-activated promoter fused to a reporter gene(s) such as *lacZ* or *luxCDABE*, together with a LuxR homologue but lack the AHL synthase. Consequently, activation of the reporter depends on the presence of an exogenous AHL. Using these approaches, Gram-negative bacterial species

Table 1. *Some examples of diffusible bacterial cell–cell signalling molecules and the phenotypes they control*

Bacterial species	Signal molecule	Phenotype
Aeromonas salmonicida	BHL/HHL	Exoproteases
Aeromonas hydrophila	BHL/HHL	Exoproteases, biofilms
Agrobacterium tumefaciens	OOHL	Ti plasmic conjugation
Bacillus subtilis	ComX peptide, CSF	Competence
Bacillus subtilis	Oligopeptide	Sporulation
Chromobacterium violaceum	HHL	Violacein pigment, HCN, exoenzymes
Erwinia carotovora	OHHL	Carbapenem antibiotic, exoenzymes
Erwinia stewartii	OHHL	Exopolysaccharide
Enterococcus faecalis	Oligopeptides	Conjugal plasmid transfer
Myxococcus xanthus	A-signal	Fruiting body formation
Pseudomonas aeruginosa	BHL/HHL/OdDHL	Exoenzymes, HCN, pyocyanin, secretion, RpoS, biofilms
Pseudomonas aureofaciens	HHL	Phenazine antibiotics
Ralstonia solanacearum	3-OH PAME	Exopolysaccharide, exoenzymes
Rhizobium leguminosarum	7,8-*cis*-HtDHL	Growth inhibition
Rhodobacter sphaeroides	7,8-*cis*-tDHL	Aggregation
Serratia liquefaciens	BHL/HHL	Swarming
Staphylcoccus aureus	Cyclic octapeptides	Exotoxins, exoenzymes, coagulase, protein A, cell wall proteins
Streptococcus pneumoniae	Heptadecapeptide (CSP)	Competence
Vibrio anguillarum	ODHL	Haemolysin
Vibrio fischeri	OHHL	Bioluminescence
Vibrio harveyi	HBHL	Bioluminescence, polyhydroxybutyrate
Xanthomonas campestris	DSF	Exoenzymes; exopolysaccharide
Yersinia pseudotuberculosis	OHHL/HHL/OHL	Motility

The abbreviations used are: OHHL, *N*-(e-oxohexanoyl)homoserine lactone; HBHL, *N*-(3-hydroxybutanoyl)homoserine lactone; HHL, *N*-hexanoyl homoserine lactone; BHL, *N*-butanoylhomoserine lactone; OOHL, *N*-(3-oxooctanoyl)homoserine lactone; OHL, *N*-octanoylhomoserine lactone; ODHL, *N*-(3-oxodecanoyl)homoserine lactone; OdDHL, *N*-(3-oxododecanoyl)homoserine lactone; 7,8-*cis*-HtDHL, 7,8-*cis*-*N*-(3-hydroxytetradecenoyl) homoserine lactone; 7,8-*cis*-tDHL, 7,8-*cis*-*N*-(tetradecenoyl)homoserine lactone. DSF, an uncharacterized diffusible extracellular factor; CSP, competence stimulating peptide; CSF, competence stimulating factor oligopeptide; 3-OH PAME, 3-hydroxypalmitic acid methyl ester.

belonging to the genera *Agrobacterium, Erwinia, Enterobacter, Pseudomonas, Vibrio, Yersinia, Aeromonas, Serratia, Chromobacterium, Hafnia, Rahnella*, and *Obesumbacterium* (Bainton *et al.*, 1992*a*; McClean *et al.*, 1997; Milton *et al.*, 1997; Pearson *et al.*, 1994, 1995; Shaw *et al.*, 1997; Swift *et al.*, 1993, 1997; Throup *et al.*, 1995; Winson *et al.*, 1995, 1998) have all given positive results with LuxR-based biosensors. However, although LuxR proteins respond most sensitively to their natural AHL, they are sufficiently flexible to detect a range of related AHL analogues such that the identity of the activating molecule(s) cannot be deduced without further chemical analysis. This usually requires the partitioning of the active compound(s) into an organic solvent prior to purification by preparative reverse phase HPLC followed by mass spectrometry and nuclear magnetic resonance spectroscopy. Elucida-

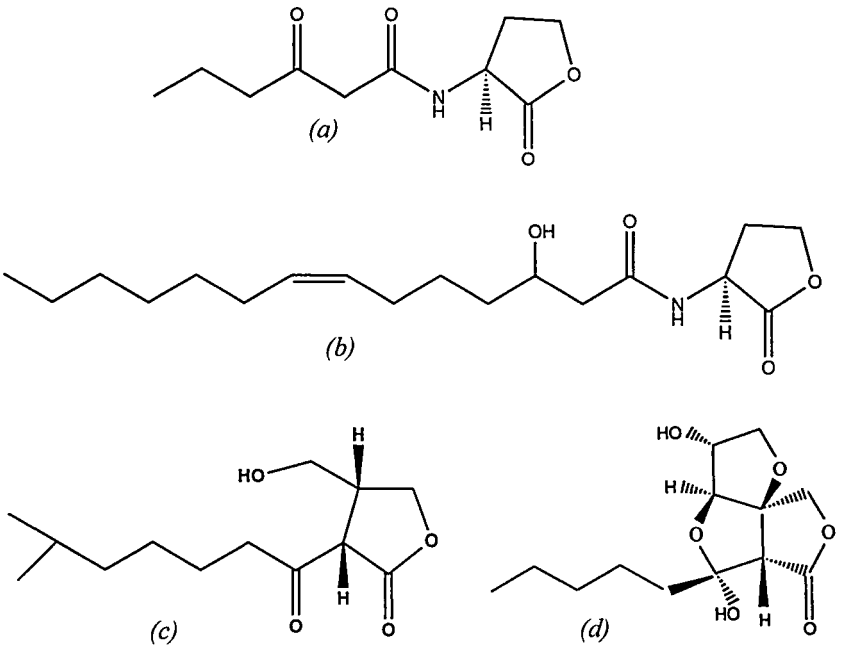


Fig. 1. Structures of (a) *N*-(3-oxohexanoyl)homoserine lactone; (b) 7,8-*cis*-*N*-(3-hydroxytetradecenoyl)homoserine lactone; (c) A-factor and (d) Syringolide-1.

tion of the structure, stereochemistry and synthesis of the AHL responsible for regulating the production of the carbapenem antibiotic in *Erwinia carotovora* is comprehensively presented in Bainton *et al.* (1992*b*).

Using the approaches described above, 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 (Fig. 1) 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. The former compound has recently also been identified in *Pseudomonas fluorescens* (B. Laue, G. S. A. B. Stewart & P. Williams, unpublished data).

This AHL-based quorum sensing 'language' family is involved in the regulation of diverse cell-density associated phenotypes including antibiotic biosynthesis, plasmid conjugal transfer, swarming, cessation of cell growth, aggregation, nodulation, capsular polysaccharide production, protein secretion, biofilm maintenance and differentiation, in the production of exoenzyme virulence determinants and cytotoxins in human, plant and animal pathogens (Fuqua *et al.*, 1994, 1996; Williams *et al.*, 1992, 1994; Salmond *et*

al., 1995; Swift *et al.*, 1996). More detailed descriptions of these AHL-dependent regulatory circuits in *V. fischeri, P. aeruginosa, E. carotovora, Aeromonas* and *Yersinia* spp. will be presented in the following chapters by Greenberg, Pesci and Iglewski, McGowan and Salmond and Swift *et al.*

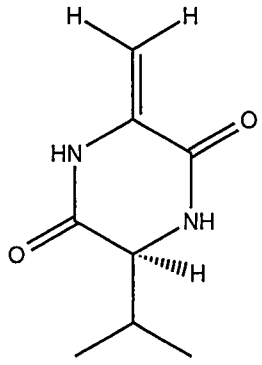
Non-AHL mediated Gram-negative bacterial cell–cell signalling

Cell density-dependent gene regulation, mediated via small diffusible signalling molecules appears to be a common theme throughout the bacterial kingdom. Although AHLs constitute only one such 'quorum sensing language' and appear to be restricted to Gram-negative bacteria, lactones feature as components of other bacterial cell–cell signalling molecules. In certain *Streptomyces* species for example, the γ-butyrolactones (Fig. 1) have long been recognized as autoregulatory signal molecules involved in the control of antibiotic biosynthesis, resistance and differentiation (Horinouchi & Beppu, 1992; see Yamada, this volume). Butyrolactones with antifungal activity, have also been isolated from spent culture supernatants of *Pseudomonas aureofaciens* (Gamard *et al.*, 1997) a Gram-negative bacterium which employs AHL-mediated quorum sensing to control the synthesis of the antifungal phenazine antibiotics (Wood *et al.*, 1997). Furthermore, the plant pathogen *Ralstonia solanacearum* possesses a AHL-based quorum sensing circuit which is itself regulated by the LysR-type transcriptional activator, PhcA and a novel volatile signalling molecule, 3-hydroxypalmitic acid methyl ester (3-OH PAME; Flavier *et al.*, 1997*a,b*). 3-OH PAME is required for the expression of PhcA-regulated virulence factors and for virulence *in planta* The volatility of this signalling molecule is particularly interesting, especially since plants have been suggested to use volatile signalling molecules such as methyl-jasmonate, ethylene and methylsalicylate in the generation of intra- and inter-plant systemic responses following wounding or infection (Mur *et al.*, 1997).

In the cabbage pathogen *Xanthomonas campestris* pv. *campestris*, mutations in the *rpf* gene cluster result in down-regulation of exoenzyme and exopolysaccharide synthesis and reduced virulence (Barber *et al.*, 1997). The phenotype of mutants in one of these genes, *rpfF*, was restored by a diffusible extracellular factor (DSF) present in the culture supernatants of the parent strain. Although DSF appears not to be an AHL, and *rpfF* mutants do not respond to the presence of exogenous AHLs, it may be a fatty acid derivative, since decanoic and dodecanoic acids and their hydroxy derivatives, acid hydrolysates of lipopolysaccharide and chloroform–methanol extracted lipids all restore, to some extent, endoglucanase activity in the *X. campestris rpfF* mutant (Barber *et al.*, 1997). DSF is however distinct from the 3-OH PAME produced by *R. solanacearum*, since it is not volatile and the corresponding methyl esters of decanoic and dodecanoic acids do not restore exoenzyme production in *X. campestris*. Apart from other xantho-

monads and a strain of *Erwinia herbicola*, culture supernatants or chloroform-methanol cell extracts from other phytopathogenic bacteria including *Erwinia, Pseudomonas* and *Ralstonia* strains failed to induce endoglucanase synthesis. DSF may therefore represent a novel class of signalling molecule which awaits full chemical characterization. However, DSF is clearly not the only diffusible signal molecule produced by *X. campestris* since a second diffusible factor has been implicated in the regulation of both extracellular polysaccharide and the yellow pigment xanthomonadin indicating the existence of two separate intercellular signalling systems with overlapping roles (Poplawsky *et al.*, 1998).

Although biosensors containing a LuxR homologue, together with an AHL-activated promoter fused to a reporter gene, have proved extremely valuable in the search for AHLs, a second class of putative cell–cell signalling molecules capable of activating *luxRI'::luxCDABE* biosensors has been identified during the screening of Gram-negative bacterial cell-free supernatants for AHLs (S. R. Chhabra, M. T. G. Holden, P. Stead, N. J. Bainton, P. J. Hill, G. P. C. Salmond, G. S. A. B. Stewart, B. W. Bycroft & P. Williams, unpublished observations). Elucidation of the structures of these molecules revealed that they constitute a family of diketopiperazines (DKPs; Fig. 2) i.e. cyclic dipeptides including cyclo(ΔAla-L-Val), and cyclo(L-Pro-L-Tyr). While both of these DKPs are produced by *P. aeruginosa*, cyclo(ΔAla-L-Val) is also made by *Proteus mirabilis, Citrobacter freundii* and *Enterobacteragglomerans*. A third DKP, cyclo(L-Phe-L-Pro) has also been identified in *P. fluorescens* and *Pseudomonas alcaligenes* (S. R. Chhabra, M. T. G. Holden, P. Stead, N. J. Bainton, P. J. Hill, G. P. C. Salmond, G. S. A. B. Stewart, B. W. Bycroft & P. Williams, unpublished observations). Although they only weakly activate LuxR-based biosensors, these compounds are capable of antagonizing OHHL-mediated induction of bioluminescence, suggesting that they may compete for the same binding site on the LuxR protein target. The detection of these molecules via the LuxR-based AHL biosensor probably represents fortuitous cross-talk between distinct signalling systems rather than stimulation by an additional quorum sensing signal. However, it has been shown (R. de Nys, K. Yamamoto, M. Givskov, N. Kumar, R. Read, R. Utsumi & S. Kjelleberg; personal communication) that DKPs such as cyclo(L-Pro-L-Tyr) are produced by *E. coli* and can influence the transcription of specific stationary phase (RpoS) regulated genes such as *fic* and *bolA*, suggesting that this class of molecules has a signal transducing function. Cyclo(L-Phe-L-Pro) and cyclo(L-Pro-L-Tyr) possess significant phytotoxic activity on spotted knapweed (*Centaurea maculosa*) and are produced not only by the pseudomonads but also by the phytopathogenic fungus *Alternaria alternata* (Stierle *et al.*, 1988; Bobylev *et al.*, 1996). DKPs have also been shown to elicit pharmacological effects in humans and other mammals, acting on the central nervous system where they modulate a remarkable range of behaviours (Prasad, 1995). This is perhaps not that



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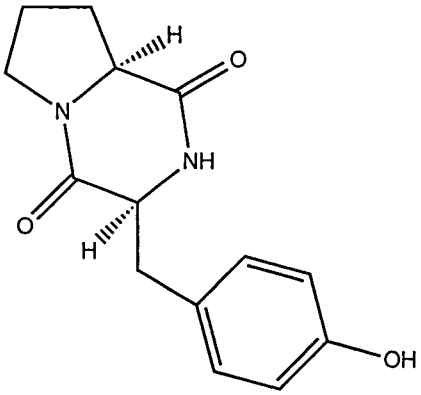

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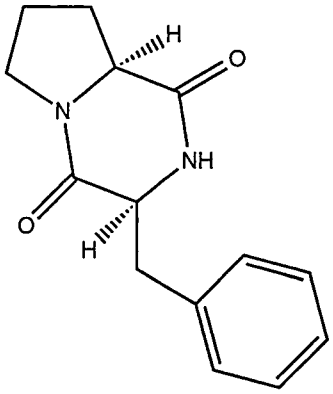


(c)

Fig. 2. Structures of (a) Cyclo(ΔAla-L-Val); (b) Cyclo(L-Pro-L-Tyr) and (c) Cyclo(L-Phe-L-Pro).

surprising considering the structural similarity shared between some cyclic dipeptides and endogenous signalling peptides such as thyrotropin-releasing hormone (Prasad, 1995). It is therefore conceivable that these bacterially generated cyclic dipeptides directly influence host–bacterial pathogen interactions.

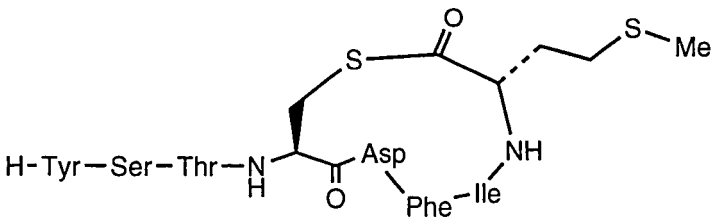
Despite the significant advances made in understanding cell–cell communication in diverse Gram-negative bacterial genera, the existence of such signalling mechanisms in bacteria such as *E. coli* and *Salmonella typhimurium* have remained enigmatic. Although the *E. coli* genome sequence does not contain any known AHL synthases (i.e. members of either the LuxI or any LuxM/AinS families), both *E. coli* and *Salmonella* possess a LuxR homologue, SdiA, which is over 50% identical to RhlR (VsmR) from *P. aeruginosa*

(Ahmer *et al.*, 1988; 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 a number of genes including several putative virulence determinants (Ahmer *et al.*, 1998). The AHLs, OHHL, *N*-(3-hydroxybutanoyl) homoserine lactone and *N*-decanoylhomoserine-DL-lactone all weakly stimulate the SdiA-dependent P_2 promoter of 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, *sdiA* itself has been reported to be regulated by an extracellular factor present in spent growth medium (Garcia-Lara *et al.*, 1996). More recently, Surette and Bassler (1998) have provided further evidence for their existence of a cell–cell signalling molecule(s) in both *E. coli* and *S. typhimurium*, based on the capacity of spent culture supernatants to stimulate bioluminescence in a *V. harveyi* mutant defective in the production of an as yet unidentified polar autoinducer molecule. The chemical identity and relationship between these putative *E. coli* and *Salmonella* signalling molecules and those responsible for activation of *sdiA* and the SdiA-driven *ftsQAZ* P_2 promoter remain to be established.

Cell–cell communication in Gram-positive bacteria

As yet, no AHLs have been identified in any Gram-positive bacterial genus, although several Gram-positives have been reported to weakly activate LuxR-based AHL biosensors (Williams, 1994). However, such activating molecules may well be DKPs. A number of Gram-positive organisms are, however, known to employ extracellular signalling molecules. These generally appear to be small, modified oligopeptides which interact with two-component histidine protein kinase signal transduction systems (Kleerebezem *et al.*, 1997; Wirth *et al.*, 1996; see Dunny, this volume). These systems regulate, for example, the development of genetic competence in *Bacillus subtilis* and *Streptococcus pneumoniae*, conjugation in *Enterococcus faecalis* and virulence in *Staphylococcus aureus*. In the latter, the expression of a number of cell-density dependent virulence factors is regulated by the global regulatory locus, *agr* (**a**ccessory **g**ene **r**egulator), which consists of two transcriptional units (Ji *et al.*, 1995, 1997*b*). The smaller unit encodes δ-haemolysin and RNAIII, while the larger encodes a sensor (*agrC*) and a response regulator (*agrA*) together with two genes (*agrD* and *agrB*) responsible for the generation of the quorum sensing signal molecule. This is an octapeptide cleaved from the middle of the *agrD* gene product and exported into the extracellular medium in a process which appears to involve AgrB (Ji *et al.*, 1997*b*). Furthermore, *S. aureus* strains can be divided into at least three

groups on the basis of the ability of their peptide signalling molecules to cross-activate or -inhibit *agr* expression, i.e. the cognate peptides of one *S. aureus* group inhibit expression of *agr* in another group of strains (Ji *et al.*, 1997*b*). Although the peptide sequences for the three *S. aureus* groups are known, the linear molecules are inactive. By comparing the physical, spectroscopic and biological properties of the purified native *S. aureus* group I octapeptide and a series of synthetic analogues, the structure of the *S. aureus* pheromones have been unequivocally established as cyclic thiolactones in which a central cysteine residue is covalently linked to the carboxy terminus of the C-terminal amino acid (Z. Affas, P. W. McDowell, W. C. Chan, B. W. Bycroft, G. S. A. B. Stewart & P. Williams, unpublished observations; Fig. 3). In addition, synthetic group III cyclic thiolactone (Fig. 3) has been shown to effectively inhibit α-toxin and TSST-1 synthesis in group I *S. aureus* (Z. Affas, P. W. McDowell, W. C. Chan, B. W. Bycroft, G. S. A. B. Stewart & P. Williams, unpublished observations). While coagulase-negative staphylococci such as *Staphylococcus epidermidis* also make these cyclic thiolactone signalling molecules (Otto *et al.*, 1998), their contribution to virulence gene regulation has not been established, and it is conceivable that their function is to facilitate competition with *S. aureus* for particular ecological niches.



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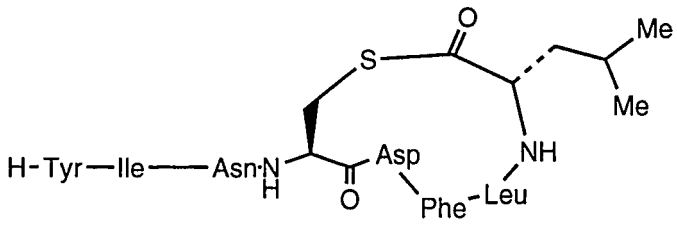

(b)

Fig. 3. Structures of (a) Group I and (b) Group III *S. aureus* cyclic thiolactone peptide pheromones.