J. Pharm. Pharmacol. 1994, 46: 252–260
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Compromising Bacterial Communication Skills*

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The progressive differentiation of a single fertilized egg through multicellular stages during the development of a vertebrate animal is controlled by sophisticated systems which depend on communication between cells. The initial steps in the formation of the central nervous system, the heart, lungs, liver, kidneys and limbs all depend on the transmission of molecular signals between cells. In the simplest of organisms, the prokaryotes, reproduction occurs by division of a single bacterial cell into two without formation of a zygote. Consequently, most bacteria are considered as non-differentiating and non-co-operative uni-cellular microorganisms. Although the potential for cell–cell communication amongst the prokaryotes is not well established, bacteria are known to employ intracellular sensing mechanisms which relay information within an individual cell via phosphoryl transfer from sensor to regulator proteins (Dorman & Ní Bhriain 1992). These systems facilitate adaptive responses to changes in the external growth environment by altering patterns of gene expression. Their close resemblance to signal transduction mechanisms operating in higher organisms suggests that bacteria may be capable of exhibiting more complex patterns of co-operative behaviour than generally recognized. Amongst the few established examples of bacteria known to exchange intercellular signals or pheromones are the myxobacteria, once misclassified as fungi, which form multicellular fruiting bodies. In *Enterococcus faecalis*, peptide pheromones signal donor cells to produce adhesions which promote the cell–cell contact required for transfer of sex plasmids from donor to recipient. A-Factor (Fig. 1, II) is a pheromone which controls streptomycin biosynthesis, streptomycin resistance and sporulation in the certain members of the genus *Streptomyces*. In the marine symbiont *Vibrio fischeri*, the induction of bioluminescence is controlled by the pheromone *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL; Fig. 1, I) (Stephens 1986; Kaiser & Losick 1993). Until recently, no other bacteria were thought to employ OHHL as a signalling molecule to co-ordinate the control of gene expression in a population of bacterial cells. However, our studies of the biosynthetic pathway to the β-lactam antibiotic 1-carbapen-2-em-3-carboxylic acid (Fig. 2, I) in the Gram-negative bacterium *Erwinia carotovora* have uncovered an unexpected role for OHHL-mediated control of gene expression in diverse Gram-negative bacteria including many organisms pathogenic to man, animals and plants. In this article, I will review our current understanding of this pheromone-mediated bacterial sensory system in the regulation of bacterial secondary metabolism and virulence and suggest why compromizing bacterial communication skills could lead to the design of novel therapeutic agents.

Pheromone-Mediated Control of Bacterial Bioluminescence

Luminous organisms are widely distributed in terrestrial, freshwater and marine environments and include bacteria, fungi, fish and squid. Light emission is catalysed by enzymes collectively known as luciferases although their only common feature is their requirement for oxygen (Meighen 1991; Stewart & Williams 1992). This property was first noted in the seventeenth century by Robert Boyle who discovered that light emission from rotting wood (presumably contain-

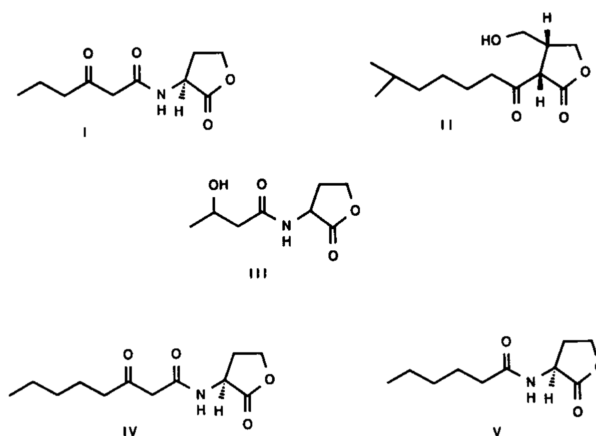


FIG. 1. Structures of I, *N*-(3-oxohexanoyl)homoserine lactone; II, A-factor, (2*S*,3*R*)-2-(6-methylheptanoyl)-3-hydroxymethyl-4-butanolide; III, *N*-(3-hydroxybutyryl)homoserine lactone; IV, *N*-(3-oxo-octanoyl)homoserine lactone: V, *N*-hexanoyl homoserine lactone.

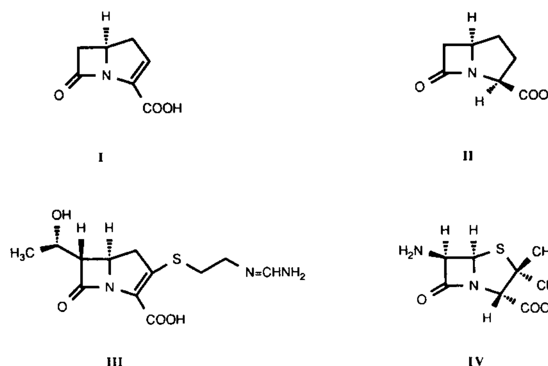


FIG. 2. Structures of I, (5*R*)-carbapen-2-em-3-carboxylic acid; II, (3*S*,5*R*)-carbapenam-3-carboxylic acid; III, imipenem; IV, 6-amino-penicillanic acid.

* Conference Science Medal 1992 lecture presented at the British Pharmaceutical Conference, University of Reading, 5 September 1993.

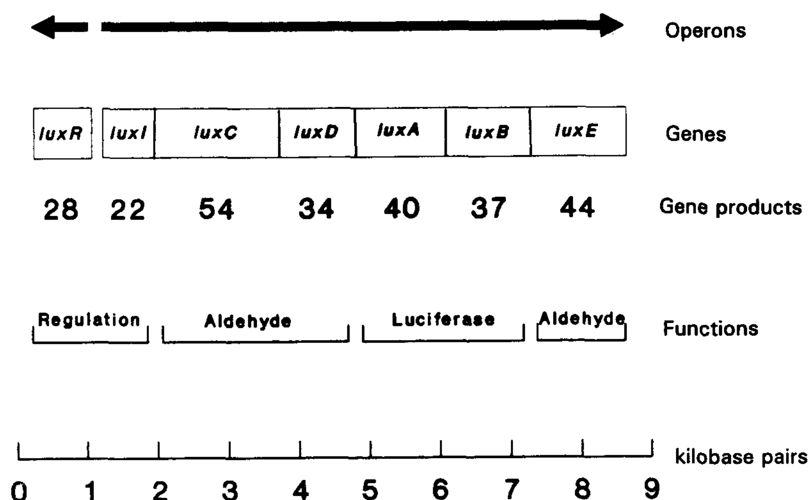
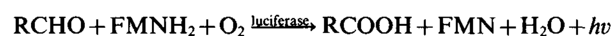


FIG. 3. Organization and function of the *lux* genes cloned from *V. fischeri*. Arrows denote operons containing *lux* genes; the leftward arrow marks operon L (containing *luxR*) and the rightward arrow marks operon R (containing the genes *luxICDABE*). The molecular masses (kDa) of the *lux* gene protein products and their functions are shown below the gene designations (adapted from Silverman et al 1989).

ing luminescent bacteria or fungi) required air (Boyle 1668). Bioluminescent bacteria can be found as free-living organisms, as symbionts, parasites or saprophytes. Some symbiotic luminescent marine bacteria such as *V. fischeri* and *Photobacterium phosphoreum* are found in the specialized light organs of shallow and deep water fish, respectively, this relationship presumably assisting fish to lure other prey, attract sexual partners or distract predators through the emission of light, whilst in return offering the bacteria nutrients and a protected niche.

The emission of light by luminous bacteria involves the intracellular oxidation of a long-chain aliphatic aldehyde (e.g. tetradecanal) and reduced flavin mononucleotide ($FMNH_2$) by luciferase:



$$RCHO + FMNH_2 + O_2 \xrightarrow{\text{luciferase}} RCOOH + FMN + H_2O + h\nu$$

In this reaction, the excess free energy is liberated as blue-green light. Bacteria such as *Escherichia coli* are dark since they lack the luciferase and the multi-enzyme fatty acid reductase complex required for the synthesis of the aldehyde substrate. However, since *E. coli* can supply $FMNH_2$, all that is required to make this dark bacterium bioluminescent is the transfer of the genes for luciferase and aldehyde synthesis. By screening for bioluminescent colonies, a 9 kilobase-pair (kbp) DNA fragment was isolated from *V. fischeri* which encoded all the functions necessary for light production and regulation (Engebrecht & Silverman 1984). The α and β subunits of the luciferase are coded by the *luxA* and *luxB* genes respectively, whilst the reductase, transferase and synthetase which make up the fatty acid reductase complex are coded by *luxC*, *luxD* and *luxE*, respectively. The organization of this regulon i.e. a group of genes which share a common regulator is shown in Fig. 3. Upstream of *luxC* are two further genes, *luxI* and *luxR* which are involved in the control of lux gene expression.

In *V. fischeri*, bioluminescence only occurs in dense bacterial cultures as light emission is controlled by the synthesis of a pheromone or autoinducer. Nealson et al (1970) were the first to observe that although *V. fischeri* only emits light at high cell densities, it produced an extracellular substance which could induce luminescence in cultures of low cell density. Consequently, the accumulation of this sensory molecule in the growth medium was suggested to induce the synthesis of the enzymes required for bioluminescence. Therefore, it is not cell density itself which influences the activation of bioluminescence but the accumulation of the pheromone to a critical threshold concentration at which light emission can increase by up to 10 000-fold per cell. Thus, in a natural habitat such as sea-water, where free-living *V. fischeri* exist at low cell densities, autoinduction of light emission would not occur. However, where *V. fischeri* is found as a symbiont in the light organs of fish where it grows to high cell densities in a relatively closed environment, accumulation of the pheromone can occur leading to induction of bioluminescence (Meighen 1991). The *V. fischeri* pheromone was first isolated by Eberhard et al (1981) and chemically characterized as *N*-(3-oxohexanoyl)homoserine lactone (OHHL; Fig. 1, I), a molecule which is sufficiently hydrophobic to diffuse freely across cell membranes. OHHL thus provides a mechanism by which a strong co-ordinated response can be achieved by a population of bacterial cells, i.e. it represents an intercellular communication device.

OHHL is generated in *V. fischeri* via the *luxI* gene product (Meighen 1991; Stewart & Williams 1992). OHHL binds to and activates the LuxR protein (the product of the *luxR* gene) such that the LuxR-OHHL complex stimulates transcription of the operon containing *luxI* and the structural genes *luxCDABE*. *LuxI* is the first gene in this operon and so this leads to increased levels of the LuxI protein and thus more OHHL which further activates LuxR setting up an autoinduction cascade (Fig. 4). Since OHHL is freely diffusible, the induction of one cell leads directly to the induction of surrounding cells, creating a positive feedback circuit that can generate a large and rapid response to a small initial stimulus. The OHHL-LuxR complex therefore constitutes a chemical communication system that enables

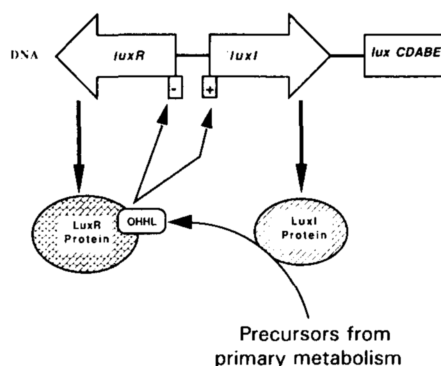


FIG. 4. Regulation of the *V. fischeri lux* regulon. The pheromone OHHL is produced at a low constitutive rate during the early stage of growth. When a sufficient level has accumulated, the interaction ot OHHL with the LuxR protein stimulates transcription of the rightward operon which contains *luxI* followed by the *lux* structural genes (*luxCDABE*). This leads to additional LuxI production and hence additional OHHL, thus creating a positive feedback loop.

V. fischeri to sense its own population density. Although OHHL was considered to be species specific in inducing bioluminescence only in *V. fischeri* and *V. logei*, a structurally related autoinducer, *N*-(3-hydroxybutyryl)homoserine lactone (Fig. 1, III) is utilized by *V. harveyi* (Cao & Meighen 1989). However, in this luminous bacterium, an equivalent of *luxR* has yet to be identified. Thus, systems analogous to the LuxR-OHHL response regulator may be assumed to control bioluminescence in other marine bacteria although the molecular components of most of these have yet to be characterized. Until recently, attempts to identify a wider spectrum of non-luminous bacteria capable of synthesizing and responding to the *lux* autoinducer family had not been successful (Greenberg et al 1979).

Carbapenem Antibiotics—Biosynthesis and Regulation

Since Alexander Fleming's discovery of penicillin in 1929, many different classes of antibiotics have been isolated, many of which have proved extremely effective in the fight against infection. However, the conflict between man and pathogenic bacteria has not been a one-sided battle; bacterial resistance has rendered ineffective entire groups of antimicrobial agents fueling the ongoing search for new compounds. In the 1970s, a new and diverse group of β-lactam antibiotics, the carbapenems was discovered. Members of this class of antibiotics have the broadest antibacterial spectra of all the β-lactams. Their apparent usefulness is further enhanced by their high intrinsic resistance to β-lactamases, the β-lactam inactivating enzymes responsible for bacterial resistance to penicillins and cephalosporins. More than 40 structurally diverse carbapenems have been isolated from the actinomycetes, although the simplest antibacterially active carbapenem has rather surprisingly been isolated from strains of the Gram-negative genera *Erwinia* and *Serratia* (Bycroft et al 1988). Despite their promise as potent chemotherapeutic agents, the development of the carbapenems has been hampered by the low titre and instability problems associated with attempts to produce them by fermentation from the *Streptomyces*. Consequently, the only currently licensed carbapenem, imipenem (Fig. 2, III), is produced by the expensive method of total chemical synthesis.

To exploit fully this potent class of antibiotics requires a plentiful supply of basic building blocks analogous to 6-aminopenicillanic acid (Fig. 2, IV) which revolutionized the design of semi-synthetic penicillins. With this aim in mind, we chose to focus on the genetically amenable carbapenem-producing strains of *Serratia* and *Erwinia* to begin our attempt to unravel the detailed biosynthetic pathway to 1-carbapen-2-em-3-carboxylic acid (Fig. 2, I). Earlier biochemical studies (Bycroft et al 1988) indicated that in these Gram-negative bacteria, this simple carbapenem was derived from acetate and glutamate, possibly via a novel carbapenam-3-carboxylic acid (Fig. 2, II). This putative precursor, although antibacterially inactive, is stable and if overproduced could conceivably function as a suitable building block for novel semi-synthetic carbapenem antibiotics.

To gain further insights, a classical genetic approach for mapping biosynthetic pathways in bacteria was taken, i.e. the selection of mutants unable to make the final product of the pathway but which overproduce a particular intermediate. Using the strategy outlined diagrammatically in Fig. 5, the carbapenem-producing *Erwinia* strain was subjected to a programme of chemical and transposon mutagenesis to obtain blocked mutants unable to synthesize the antibiotic. These mutants were then analysed for the accumulation of potential precursors. A large number of *Erwinia* mutants with a carbapenem non-producing phenotype were obtained and from cross-feeding experiments they could be assigned to one of two classes (Bainton et al 1992a, b). Class 1 mutants secreted a low molecular mass, diffusible factor which restored carbapenem synthesis in class 2 mutants but not vice-versa. This diffusible substance was also found to be present in β-lactamase-treated (to inactivate endogenous carbapenem) supernatants of the parent *Erwinia* strain. The factor was subsequently isolated and purified from the culture supernatant of a class 1 mutant. Spectroscopic analysis of the compound led to the surprising discovery that it was not a biosynthetic intermediate on the carbapenem pathway but the *V. fischeri* pheromone OHHL. Both D- and L-isomers were synthesized and subsequent circular dichroism comparisons established that the natural product had the L-configuration (Bainton et al 1992b).

Substrate specificity for the pheromone-binding protein (LuxR) of *V. fischeri* appears to be high in that any structural modification resulted in a significant decrease in activity, although alterations to the 3-oxohexanoyl side chain were better tolerated than changes in the lactone ring (Eberhard et al 1986). To ascertain which structural features were important for the induction of carbapenem in *Erwinia*, a range of OHHL analogues was synthesized, differing in chirality, with side chain, ring size or ring heteroatom modifications (Chhabra et al 1993). Table 1 shows the structures of OHHL and the analogues synthesized, together with their relative biological activities. The homoserine lactone nucleus itself showed no activity indicating that the 3-oxohexanoyl side chain was required for induction of carbapenem biosynthesis. Similarly, chirality of the homoserine lactone nucleus would appear to be critical since the D-isomer of OHHL possessed a relative activity of 10% compared with the natural product (Table 1, compare 1 with 5). Analogues in

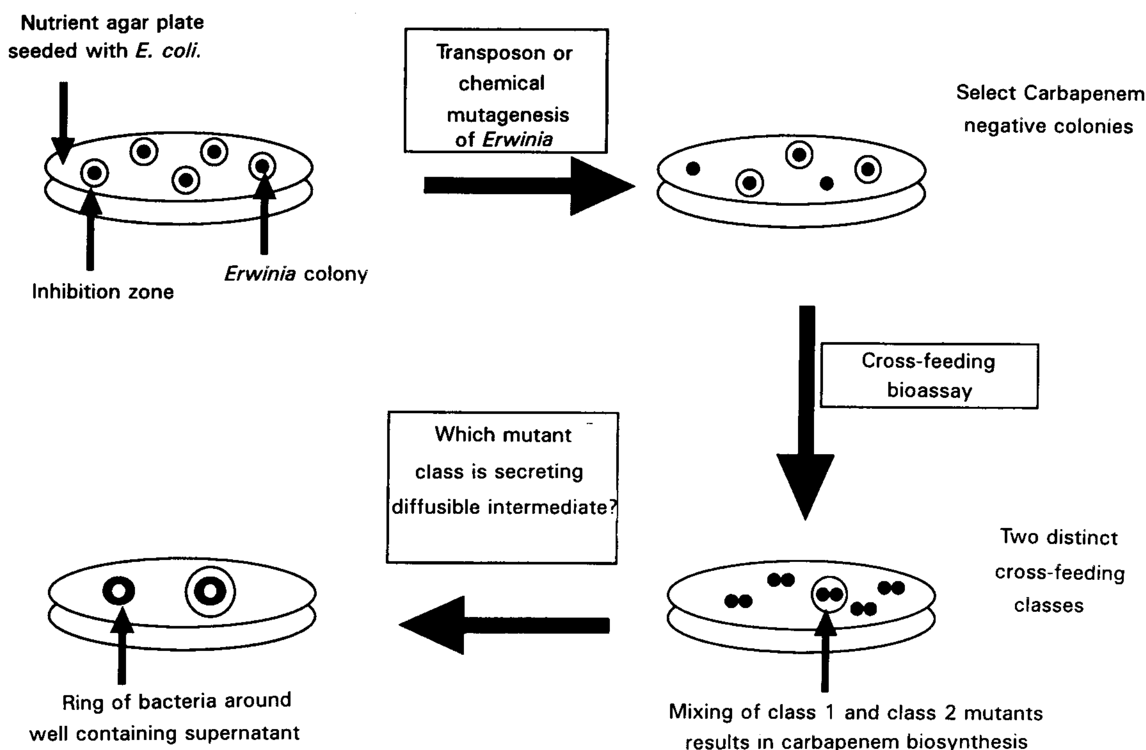


FIG. 5. Strategy for the selection of *E. carotovora* mutants defective in the synthesis of 1-carbapen-2-em-3-carboxylic acid and the subsequent identification of mutants secreting putative diffusible intermediates. Having identified two classes of mutants, the class 1 mutants were shown to secrete a diffusible compound by placing cell-free supernatants from a class 1 or a class 2 mutant in a well cut in the agar and then surrounding the well with a ring of either a class 1 or a class 2 mutant. Inhibition zones were only observed around wells containing supernatants from class 1 mutants surrounded by class 2 mutant cells indicating that the diffusible intermediate was being secreted by the class 1 mutant.

which the terminal region of the side chain were varied in both the length and branching of the hydrocarbon chain all showed decreased activity compared within the natural product. An increase by one methylene unit (Table 1, compound 2) decreased activity by 50%, whilst a two-methylene unit extension (Table 1, compound 3) reduced activity to 10%. Shortening the chain length by one methylene unit decreased activity some tenfold (Table 1, compound 8) and an even greater fall in activity is observed with the branched isopropyl unit (Table 1, compound 7). The introduction of a double bond into the side chain of OHHL (Table 1, compounds 9, 17 and 19) results in almost complete loss of activity possibly due to the rigidity introduced into the molecule. The β-keto carbonyl functionality also appears to be essential for induction of carbapenem since its loss (Table 1, compound 12) reduces the relative activity to only 0·3%. Replacement of the ring oxygen with sulphur or nitrogen (Table 1, compounds 4, 15) substantially reduced activity to 10 and 0·15%, respectively, whilst enlargement of the ring by one methylene unit completely abolished activity. Interestingly, the pheromone *N*-(3-hydroxybutyryl)homoserine lactone (Table 1, compound 21) which autoregulates bioluminescence in *V. harveyi* (Cao & Meighen 1989) was inactive as an inducer of carbapenem in *Erwinia*. These data indicate a tight substrate specificity for the *Erwinia* autoinducer-binding protein, analogous to the *V. fischeri* LuxR protein.

The induction of bioluminescence in *V. fischeri* follows a dose-response curve with a threshold of about 1 ng mL^{-1} and an optimum of 10 ng mL^{-1} of OHHL (Meighen 1991; Eberhard et al 1981). In a class 2 *Erwinia* mutant, the threshold and optimum concentrations of OHHL for induction of carbapenem biosynthesis were higher at approximately 0·1 and 1·0 μg mL^{-1}, respectively (Bainton et al 1992b). Furthermore, experiments in which carbapenem and OHHL production were monitored throughout growth, suggested that, like bioluminescence, antibiotic biosynthesis, was cell-density dependent (Williams et al 1992). Additional confirmation of this cell-density dependency was obtained by the addition of excess OHHL (5 μg mL^{-1}) at the time of first inoculation of the bacterial culture. This resulted in the early induction of carbapenem biosynthesis, a finding in accordance with the premature induction of bioluminescence in *V. fischeri* using conditioned media or exogenous OHHL (Nealson et al 1970).

Diverse Gram-negative Bacteria Employ OHHL as a Signalling Molecule

Although seen as a fascinating example of gene expression control, the OHHL-mediated autoinduction cascade had been associated only with a few esoteric luminescent marine bacteria. Our unexpected discovery that carbapenem biosynthesis in a terrestrial bacterium was regulated by the same molecule used by *V. fischeri* to control light emission, suggested that it was highly likely that other bacteria employed this elegant intercellular communication device. At the University of Nottingham, a research group in the Department of Applied Biochemistry and Food Science

Table 1. Structures of analogues of *N*-(3-oxohexanoyl)-L-homoserine lactone.

H, $(CH_2)_n$, X, R-HN, O

Compound	R	n	X	Relative† activity	Compound	R	n	X	Relative activity
1		1	O	100	12		1	O	0.3
2		1	O	50	13*		1	O	0.3
3		1	O	10	14		1	O	0.25
4		1	S	10	15		1	N	0.15
5 (D isomer)		1	O	10	16		1	O	0.15
6		1	O	2	17		1	O	0.1
7		1	O	2	18		1	O	0.05
8		1	O	2	19		1	O	zero
9		1	O	1	20		1	O	zero
10*		1	O	1	21		1	O	zero
11		1	O	0.5	22		2	O	zero

* Chirality at 3-hydroxy may be reversed. † This refers to the threshold concentration of the analogues which triggers the carbapenem production expressed relative to OHHL.

directed by Gordon Stewart, has been exploring the potential applications of bacterial bioluminescence as a reporter of bacterial injury and recovery and for the rapid detection of bacterial pathogens in food and pharmaceuticals. World-wide, there are few laboratories with expertise in the molecular genetics of bacterial bioluminescence and so this remarkable coincidence offered an immediate opportunity to explore the possible ubiquity of OHHL synthesis in diverse bacterial genera. An immediate and essential requirement was the availability of a sensitive bioassay for the detection of low levels of OHHL. This was achieved by constructing a recombinant derivative of the *lux* system for expression in *E. coli* in which the *luxR* gene and *lux* promoter regions were fused to the *luxA* and *luxB* genes encoding the luciferase. The resultant sensor plasmid (pSB237) enables *E. coli* to bioluminesce only in response to externally added OHHL and a long chain fatty aldehyde. *E. coli* [pSB237] is capable of sensing the pheromone at concentrations as low as 80 pg mL^{-1} and reporting its presence by emitting light (Bainton et al 1992a; Swift et al 1993). Using this assay, spent culture supernatants of over 40 different Gram-positive and Gram-negative bacteria were screened for pheromone-like activity. Table 2 lists the bacteria which were capable of inducing bioluminescence in *E. coli* [pSB237]. For *Pseudomonas aeruginosa*, *Serratia marcescens*, *Erwinia herbicola* and *Erwinia carotovora* (Bainton et al 1992a) as well as *Enterobacter agglomer-*

Table 2. Diversity of bacteria inducing bioluminescence in *E. coli* [pSB237]. Cell-free supernatants from each bacterium were added to *E. coli* [pSB237] in micro-titre wells and bioluminescence monitored using a photon video camera after exposure to the long chain fatty aldehyde decanal.

Erwinia carotovora	*Serratia marcescens*	*Pseudomonas aeruginosa*
Erwinia herbicola	*Citrobacter freundii*	*Pseudomonas fluorescens*
Aeromonas hydrophila	*Serratia liquefaciens*	*Pseudomonas putida*
Hafnia alvei	*Rhanella aquatilis*	*Klebsiella pneumoniae*
Proteus vulgaris	*Enterobacter agglomerans*	*Chromobacter violaceum*
Yersinia enterocolitica	*Aeromonas salmonicida*	*Agrobacterium tumefaciens*
Listeria monocytogenes	*Streptococcus pyogenes*	*Bacillus pumilis*
Streptococcus mutans	*Enterococcus faecalis*	*Micrococcus luteus*

ans and *Yersinia enterocolitica*, the inducing compound was isolated and confirmed as OHHL by infrared, mass spectrometric and NMR spectroscopic analysis (Bainton et al 1992a). High-level activity was also observed for *Klebsiella pneumoniae*, *Aeromonas hydrophila*, *Citrobacter freundii*, *Proteus mirabilis*, *Hafnia alvei* and *Rhanella aquatilis*. Low-level inducer activity was also noted across a range of Gram-negative and Gram-positive genera. This low-level activity may be due to the presence of either very low concentrations of OHHL or pheromones structurally related to OHHL. While culture supernatants are a useful first indication that these bacteria can synthesize OHHL, these supernatants frequently contain substances inhibitory to *E. coli* which can mask bioluminescence induction. By introducing an OHHL sensor plasmid directly into bacteria which exhibit autoinducer activity, it is possible to sense OHHL activity in-vivo. In particular, it is then possible to investigate the relationship between growth phase and OHHL production. Electroporation of the recombinant sensor plasmid, pSB315 (which is comparable with pSB237, but contains a different origin of replication and antibiotic resistance marker) into *E. carotovora*, *E. agglomerans*, *S. marcescens*, *H. alvei* and *R. aquatilis* enabled us to obtain bioluminescent transformants for each organism (Swift et al 1993). OHHL-mediated induction of bioluminescence in *V. fischeri* is characteristically cell density-dependent reflecting the requirement to accumulate to a threshold concentration of OHHL before triggering the autoinduction cascade. If a specific cell density is required to trigger an OHHL cascade in these bioluminescent transformants, then bioluminescence must increase suddenly and substantially and at a rate which exceeds the logarithmic increase in bacterial cell number. For each of *E. carotovora*, *E. agglomerans*, *S. marcescens*, *H. alvei* and *R. aquatilis*, this was the result obtained and is illustrated for *E. carotovora* [pSB315] by Fig. 6. Thus, cell density-dependent synthesis of OHHL is common to many different species of Gram-negative bacteria.

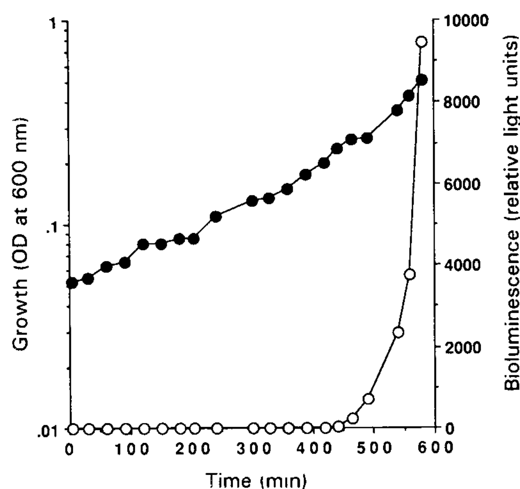


FIG. 6. Cell density-dependent induction of bioluminescence in *E. carotovora* containing the OHHL sensor plasmid pSB315. Bioluminescence (○ measured as relative light units using a Turner luminometer) and growth (● measured as OD_{600}) were determined simultaneously at 30-min intervals throughout the growth cycle.

Molecular Cloning and Characterization of Genes Analogous to *luxI*

If OHHL synthesis in bacteria such as *E. carotovora* is analogous to *V. fischeri*, then a protein equivalent to the 22 kDa LuxI protein should be present and transcribed from a gene analogous to *luxI* (Meighen 1991). In *V. fischeri* the *luxI* gene is located immediately upstream from *luxC* and is part of the same transcriptional unit (Fig. 3). It appears to be involved in the production of OHHL since luminescence can be restored to *luxI*$^-$-mutants of *V. fischeri* by the addition of exogenous OHHL (Engebrecht & Silverman 1984; Meighen 1991). It has therefore been suggested that the LuxI protein functions as an autoinducer synthetase, the substrates for which are derived from amino acid and fatty acid metabolism respectively (Eberhard et al 1981, 1991). These have been tentatively identified as *S*-adenosylmethionine and 3-oxohexanyl coenzyme A, although the experiments reported employed cell-free extracts and direct evidence for the function of the *luxI* gene product is still awaited.

Our strategy for cloning *luxI* analogues from OHHL-producing bacteria was based on the availability of *E. coli* [pSB315] which lacks the *luxI* gene so that the induction of bioluminescence is dependent upon the addition of exogenous OHHL. Chromosomal gene libraries were first prepared from an OHHL producer in a plasmid compatible with pSB315. This plasmid was then introduced into *E. coli* [pSB315]. The successful cloning of a *luxI* homologue in-trans with pSB315 would lead to the in-vitro production of OHHL and consequently bioluminescence. Once identified, bioluminescent colonies can be picked out and the recombinant plasmid they contained subjected to further analysis. Using this strategy, *luxI* homologues have been cloned from *Erwinia*, *Enterobacter*, *Serratia*, *Hafnia* and *Rhanella* (Swift et al 1993). Interestingly, the absence of hybridization both between these *luxI* homologues and with the cloned *V. fischeri luxI* provided no evidence of homology at the DNA

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LasI MIVQIGRREE FDKKLLGEMH K--LRAQVFK ERKGWDVSVI DEMEIDGYDA
CarI MLE-IFDVNH TLLSETKSEE LFTLRKETFK DRLNWAVQCT DGMEFDQYDN
ExpI MLE-IFDVSY TLLSEKKSEE LFTLRKETFK DRLNWAVKCI NGMEFDQYDD
EagI MLE-IFDVSY NDLTERRSED LYKLRKITFK DRLDWAVNCS NDMEFDEFDN
LuxI MTIMIKKSDF LAIPSEEYKG ILSLRYQVFK QRLEWDLVVE NNLESDEYDN
         ●                    **   **  ** *      ●  ● ● ***

LasI LSPYYMLIQE DTPEAQVFGC WRILDTTGPY MLKNTFPELL HGKEAPCSPH
CarI NNTTYLFGIK DNTV--ICSL -RFIETKYPN MITGTFFPYF KEINIP-EGN
ExpI DNATYLFGVE GDQV--ICSS -RLIETKYPN MITGTFFPYF EKIDIP-EGK
EagI SGTRYMLGIY DNQL--VCSV -RFIDLRLPN MITHTFQHLF GDVKLP-EGD
LuxI SNAEYIYACD DTEN--VSGC WRLLPTTGDY MLKSVFPELL GQQSAPKDPN
      *  *      ●     ●     ●         ●    ●  **      ●

LasI IWELSRFAI- NSGQKGSLG- FSDCTLEAMR ALARYSLQND IQTLVTVTTV
CarI YLESSRFFVD KSRAKDILGN EYPISSMLFL SMINYSKDKG YDGIYTIVSH
ExpI YIESSRFFVD KARSKTILGN SYPVSTMFFL ATVNYSKSKG YDGVYTIVSH
EagI YIDSSRFFVD KNRAKALLGS RYPISYVLFL SMINYARHHG HTGIYTIVSR
LuxI IVELSRFAVG KNSSK-INNS ASEITMKLFE AIYKHAVSQG ITEYVTVTST
       * *** ●  *   *         ●   **  ●        *      *  *

LasI GVE--KMMIR AGLDVSRFGP HLKIGIER-- -AVALRIELN AKT-QIALYG
CarI PMLTILKRSG WGIRVVEQGL SEKEERVY-- ----LVFLPV DDENQEALAR
ExpI PMLTILKRSG WKISIVEQGM SEKHERVY-- ----LLFLPV DNESQDVLVR
EagI AMLTIAKRSG WEIEVIKEGF VSENEPIY-- ----LLRLPI DCHNQHLLAK
LuxI AIERFLKR-- --IKVPCHRI GDKEIHVLGD TKSVVLSMPI N-E-QFKKAV
          ***     * *        *   *         *  *    * *   *

LasI GVLVEQRLAV S
CarI RINRSGTFMS NELKQWPLRV PAAIAQA
ExpI RINHNQEFVE SKLREWPLSF EPMTEPV G
EagI RIRDQSESNI AALCQCPMSL TVTPEQV
LuxI LN
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FIG. 7. Comparison of the amino-acid sequences of the protein CarI, ExpI and EagI compared with LuxI. * Represents at least three amino acids conserved with LuxI.

level. However, DNA sequencing of the *luxI* homologues from *E. carotovora* (*carI*) and *E. agglomerans* (*eagI*) indicated that in both cases, the translated protein sequence had partial homology (about 25%) with LuxI (Fig. 7). The region containing LuxI amino acid residues 24–50 is, however, especially well conserved with 13 out of 28 residues being conserved or identical. Despite this relative lack of amino-acid sequence homology, introduction of *luxI*, *carI* or *eagI* into the class 2 carbapenem negative *Erwinia* mutant, restored antibiotic production indicating that the gene products must be functionally equivalent (Swift et al 1993). Furthermore, although EagI is 23 amino-acid residues longer than LuxI, removal by deletion of the DNA encoding the last 18 amino acids does not impair biochemical activity as determined by OHHL production (Swift et al 1993).

A Role for OHHL in the Pathogenesis of Infection

Amongst Gram-negative bacteria, bioluminescence and β-lactam biosynthesis are rather unusual traits. Many different Gram-negative bacteria which do not emit light nor synthesize carbapenems nevertheless produce OHHL and possess genes analogous to *luxI*. These data suggest that different bacteria employ the pheromone to control quite different sets of genes. Amongst the OHHL producers identified to date, are several organisms capable of infecting man, animals and plants. 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 co-ordinately regulated virulence determinants in bacteria (Mekalanos 1992). Such information raises the possibility that OHHL may also be involved in co-ordinating the control of virulence. 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. The ability of a population of bacteria to co-ordinate their attack on the host may therefore be a crucial component in the development of infection.

An indication of a role for OHHL in the pathogenesis of human infection has come from studies of the opportunistic pathogen *P. aeruginosa*. This environmental bacterium can infect almost any body site given the right predisposing conditions. Individuals at greatest risk of *P. aeruginosa* infection include those with burn wounds and cystic fibrosis. *P. aeruginosa* secretes many extracellular toxic factors including an exotoxin which inhibits protein synthesis in mammalian cells and various tissue-damaging exoenzymes including an alkaline protease and an elastase. This latter enzyme, which appears necessary for the maximal virulence of *P. aeruginosa*, is a metalloprotease and the product of the *lasB* gene. Elastase synthesis is controlled by a gene termed *lasR*, the product of which (LasR) shares significant amino acid sequence homology with LuxR (Gambello & Iglewski 1991). When coupled with previous descriptions of the growth phase dependency of elastase production and our discovery that *P. aeruginosa* produces OHHL, there is strong circumstantial evidence for the involvement of OHHL or

structurally related compounds in controlling synthesis of this important virulence determinant.

To define a role for OHHL in controlling elastase expression we employed a similar strategy to that used in our studies of carbapenem biosynthesis in *Erwinia*, i.e. *P. aeruginosa* mutants were selected by chemical mutagenesis which were no longer able to make elastase (Jones et al 1993). Following the addition of exogenous OHHL, we were able to divide these *P. aeruginosa* mutants into two classes, those which responded to OHHL by synthesizing elastase and those which did not. Confirmation that the OHHL responsive mutants were indeed synthesizing the 33 kDa LasB protein was obtained by sodium dodecyl sulphate-polyacrylamide gel electrophoresis of the protein purified from spent culture supernatants of an OHHL-responsive mutant grown in the presence of OHHL (Jones et al 1993). These OHHL-responders are therefore likely to have mutations in the *P. aeruginosa* gene analogous to the *V. fischeri luxI* gene. This gene, termed *lasI*, has recently been cloned and sequenced (Passador et al 1993) and shown to be 34·6% identical and 55·9% similar to *luxI*. Passador et al (1993) also reported that *lasI* was involved in the synthesis of a small diffusible molecule, termed *P. aeruginosa* autoinducer (PAI), the structure of which was not reported but given our data (Jones et al 1993) this molecule is presumably OHHL or a closely related analogue. Apart from elastase synthesis, the *lasR* gene product has also been shown to be involved in the regulation of exotoxin A and alkaline protease (Gambello et al 1993). This information strongly suggests that bacterial intercellular communication facilitates co-ordination of the expression of multiple virulence determinants thus influencing the outcome of *Pseudomonas* infections. For other human pathogens such as *Y. enterocolitica* and *K. pneumoniae*, although we have established that they produce OHHL and have cloned the corresponding *lux*I homologue (unpublished data), the virulence determinants regulated by the pheromone have not yet been identified.

A further example of OHHL-mediated control of virulence has however come from studies of *E. carotovora* which is an important plant pathogen. In common with *P. aeruginosa*, this broad host range phytopathogen secretes multiple exoenzymes. These include cellulases, pectinases and proteases which degrade plant cell walls resulting in the extensive tissue maceration characteristic of soft-rot diseases. In *Erwinia*, synthesis of these exoenzymes is tightly controlled and mutants affected in the co-ordinate production of these exoenzymes exhibit significantly reduced plant virulence (Jones et al 1993; Pirhonen et al 1993). Since we have shown that OHHL controls carbapenem synthesis in *E. carotovora*, we also examined whether this pheromone might play a role in the regulation of exoenzyme synthesis (Jones et al 1993). *E. carotovora* SCRI193 is a naturally occurring virulent strain which does not make carbapenems. This strain does, however, cross-feed the class 2 mutants of the carbapenem-producing *Erwinia* strain described earlier indicating that SCRI193 does make OHHL. Further studies of the class 2 mutants revealed that they were not only unable to synthesize carbapenem but also the exoenzymes. Addition of exogenous OHHL restored antibiotic production as well as synthesis of pectinase, cellulase and protease. Furthermore, these OHHL negative mutants were unable to produce the characteristic tissue necrosis indicative of exoenzyme-mediated tissue damage when inoculated into potato tubers unless co-injected with exogenous OHHL or transformed with a recombinant plasmid carrying either the *V. fischeri luxI* or the *Erwinia luxI* homologue *carI* (Jones et al 1993; Swift et al 1993). Similar results were obtained by Pirhonen et al (1993) who described the cloning of a *luxI* homologue designated *expI* from *Erwinia*, the translated amino-acid sequence of which is highly homologous with that of CarI (Fig. 7).

Many isolates of the soil bacterium *Agrobacterium tumefaciens* contain a plasmid known as the Ti-plasmid. When such bacteria infect wounds in plants they stimulate, at the site of infection, the formation of a tumour, known as crown gall. This is because part of the Ti-plasmid, the T-DNA, enters and transforms the plant cells leading to uncontrolled growth and formation of a crown gall. The T-DNA also directs the plant cells to produce opines, unusual amino acids which can be metabolized for use as an energy source by the bacterium but not by the plant. These opines also induce *A. tumefaciens* strains that are donors of Ti-plasmids to synthesize a diffusible conjugation factor. This factor stimulates the bacteria to conjugate so that the Ti-plasmids can be spread throughout the population. Zhang et al (1993) have recently elucidated the structure of this conjugation factor which turns out to be an analogue of OHHL with an extension of the side chain by two methylene units, i.e. *N*-(3-oxo-octanoyl)-L-homoserine lactone (Fig. 1, IV). *A. tumefaciens* culture supernatants were also reported to contain other *N*-acyl-L-homoserine lactones including *N*-hexanoyl-L-homoserine lactone (Fig. 1, V), although a functional role for those compounds is not as yet apparent. In addition an *A. tumefaciens* gene designated *traR*, the translated product of which is a homologue of LuxR, has been described and the *lux* autoinducer OHHL shown to have some activity in this system (Piper et al 1993).

Compromising Bacterial Communication Skills—Therapeutic Possibilities

Bacterial pathogens possess distinct genetic properties which confer a significantly greater capacity to compete with other (commensal) bacteria, to gain a foothold within a susceptible host, to multiply in host tissues and to avoid host defences. Pathogens have therefore evolved complex regulatory pathways which control virulence-factor expression indicating the crucial importance of being able to maximize survival with the minimum expenditure of metabolic resources when adapting to new and potentially hostile environments. The ability to switch off exogenously, virulence gene expression, may therefore offer a novel strategy for the treatment or prevention of infection. The recent discovery that many Gram-negative bacteria employ pheromones to co-ordinate the control of virulence gene expression enables us to envisage such an opportunity. In particular, interference with transmission of the molecular message by an antagonist which competes for the pheromone binding site of the transcriptional activator protein (the LuxR homologue), thereby switching off virulence gene expression so attenuating the pathogen, is an attractive strategy. In this context it is perhaps worth noting that too high a concentration of

OHHL in both *V. fischeri* and *E. carotovora* switches off bioluminescence and carbapenem biosynthesis, respectively (Eberhard et al 1986; Bainton et al 1992a). Several analogues of OHHL have also been shown to inhibit the action of OHHL in *V. fischeri* (Eberhard et al 1986). The relative ease with which analogues of OHHL can be synthesized should make it experimentally feasible to evaluate the influence of an OHHL antagonist on the course of infection. Furthermore the observation that *A. tumefaciens* employs an OHHL analogue to promote conjugation and the spread of Ti-plasmids throughout the bacterial population is unlikely to be a unique example. It is probable that other bacteria employ pheromone-based self-sensing mechanisms to promote conjugation and the spread of perhaps not only plasmids carrying virulence determinants but also those conferring multiple antibiotic resistance. Pheromone antagonists might therefore also have a role in preventing the spread of antibiotic resistance thus prolonging the useful life of many antimicrobials—including the carbapenems.

Acknowledgements
I would like to thank all my colleagues and in particular Barrie Bycroft, Gordon Stewart, Ram Chhabra, Nigel Bainton, Michael Winson, Pan Chan, Philip Hill, Simon Swift, Catherine Rees at Nottingham and George Salmond (Department of Biological Sciences, Warwick University) who have been involved in the development of our understanding of pheromone-mediated gene-expression control. The work described has been funded by grants from the Biotechnology Directorate of the Science and Engineering Research Council and by Amersham International which are gratefully acknowledged.

References

Bainton, N. J., Bycroft, B. W., Chhabra, S. R., Stead, P., Gledhill, L., Hill, P. J., Rees, C. E. D., Winson, M. K., Salmond, G. P. C., Stewart, G. S. A. B., Williams, P. (1992a) A general role for the *lux* autoinducer in bacterial cell signalling: control of antibiotic biosynthesis in *Erwinia*. Gene 116: 87–91

Bainton, N. J., Stead, P., Chhabra, S. R., Bycroft, B. W., Salmond, G. P. C., Stewart, G. S. A. B., Williams, P. (1992b) *N*-(3-Oxohexanoyl)-L-homoserine lactone regulates carbapenem antibiotic production in *Erwinia carotovora*. Biochem. J. 288: 997–1004

Boyle, R. (1668) Experiments concerning the relation between light and air in shining wood and fish. Philos. Trans. R. Soc. London 2: 581–600

Bycroft, B. W., Maslen, C., Box, S. J., Brown, A., Tyler, J. W. (1988) The biosynthetic implications of acetate and glutamate incorporation into (3*R*,5*R*)-carbapenem-3-carboxylic acid and (5*R*)-carbapen-2-em-3-carboxylic acid by *Serratia* sp. J. Antibiotics 41: 1231–1241

Cao, J. G., Meighen, E. A. (1989) Purification and structural identification of an autoinducer for the luminescence system of *Vibrio harveyi*. J. Biol. Chem. 264: 21670–21676

Chhabra, S. R., Stead, P., Bainton, N. J., Salmond, G. P. C., Stewart, G. S. A. B., Williams, P., Bycroft, B. W. (1993) Autoregulation of carbapenem biosynthesis in *Erwinia carotovora* by analogues of *N*-(3-oxohexanoyl)-L-homoserine lactone. J. Antibiotics 46: 441–454

Dorman, C., Ni Bhriain, N. (1992) Global regulation of gene expression during environmental adaptation: implications for bacterial pathogens. In: Hormaeche, C. E., Penn, C. W., Smyth, C. J. (eds) Molecular Biology of Bacterial Infection (Society for General Microbiology Symposium volume 49). Cambridge University Press, pp 193–230

Eberhard, A., Burlingame, A. L., Kenyon, G. L., Nealson, K. H., Oppenheimer, N. J. (1981) Structural identification of autoinducer of *Photobacterium fischeri* luciferase. Biochemistry 20: 2444–2449

Eberhard, A., Widrig, C. A., Mcbath, P., Schineller, J. B. (1986) Analogs of the autoinducer of bioluminescence in *Vibrio fischeri*. Arch. Microbiol. 146: 35–40

Eberhard, A., Lonngin, T., Widrig, C. A., Stranick, S. J. (1991) Synthesis of the *lux* gene autoinducer is positively regulated. Arch. Microbiol. 155: 294–297

Engebrecht, J., Silverman, M. (1984) Identification of genes and gene products necessary for bacterial bioluminescence. Proc. Natl. Acad. Sci. USA 81: 4154–4158

Gambello, M. J., Iglewski, B. H. (1991) Cloning and characterisation of the *Pseudomonas aeruginosa lasR* gene, a transcriptional activator of elastase J. Bacteriol. 173: 3000–3009

Gambello, M. J., Kaye, S., Iglewski, B. H. (1993) LasR of *Pseudomonas aeruginosa* is a transcriptional activator of the alkaline protease gene (*apr*) and an enhancer of exotoxin A expression. Infect. Immun. 61: 1180–1184

Greenberg, E. P., Hastings, J. W., Ulitzur, S. (1979) Induction of luciferase synthesis in *Beneckea harveyi* by other marine bacteria. Arch. Microbiol. 120: 87–91

Jones, S., Yu, B., Bainton, N. J., Birdsall, M., Cox, A. J. R., Bycroft, B. W., Golby, P., Chhabra, S. R., Reeves, P. J., Stephens, S., Winson, M. K., Salmond, G. P. C., Stewart, G. S. A. B., Williams, P. (1993) The *lux* autoinducer regulates the production of exoenzyme virulence determinants in *Erwinia carotovora* and *Pseudomonas aeruginosa*. EMBO J. 12: 2477–2482

Kaiser, D., Losick, R. (1993) How and why bacteria talk to each other. Cell 73: 873–885

Meighen, E. A. (1991) Molecular biology of bacterial bioluminescence. Microbiol. Rev. 55: 123–142

Mekalanos, J. J. (1992) Environmental signals controlling expression of virulence determinants in bacteria. J. Bacteriol. 174: 1–7

Nealson, K. H., Platt, T., Hastings, J. W. (1970) Cellular control of the synthesis and activity of the bacterial bioluminescence system. J. Bacteriol. 104: 313–322

Passador, L., Cook, J. M., Gambello, M. J., Rust, L., Iglewski, B. H. (1993) Expression of *Pseudomonas aeruginosa* virulence genes requires cell-to-cell communication. Science 260: 1127–1130

Piper, K. R., Beck von Bodman, S., Farrand, S. K. (1993) Conjugation factor of *Agrobacterium tumefaciens* regulates Ti plasmid transfer by autoinduction. Nature 362: 448–450

Pirhonen, M., Flego, D., Heikinheimo, R., Palva, E. T. (1993) A small diffusible signal molecule is responsible for the global control of virulence and exoenzyme production in the plant pathogen *Erwinia carotovora*. EMBO J. 12: 2467–2476

Silverman, M., Martin, M., Engebrecht, J. (1989) Regulation of luminescence in marine bacteria. In: Hopwood, D. A., Chater, K. F. (eds) Genetics of Bacterial Diversity. Academic Press, London, pp 71–85

Stephens, K. (1986) Pheromones among the procaryotes. Critical Reviews in Microbiology 13: 309–334

Stewart, G. S. A. B., Williams, P. (1992) *lux* genes and the applications of bacterial bioluminescence. J. Gen. Microbiol. 138: 1289–1300

Swift, S., Winson, M. K., Chan, P. F., Bainton, N. J., Birdsall, M., Reeves, P. J., Rees, C. E. D., Chhabra, S. R., Hill, P. J., Throup, J. P., Bycroft, B. W., Salmond, G. P. C., Williams, P., Stewart, G. S. A. B. (1993) A novel strategy for the isolation of *luxI* homologues: evidence for the widespread distribution of a LuxR:LuxI superfamily in enteric bacteria. Mol. Microbiol. 10: 511–520

Williams, P., Bainton, N. J., Swift, S., Chhabra, S. R., Winson, M. K., Stewart, G. S. A. B., Salmond, G. P. C., Bycroft, B. W. (1992) Small molecule-mediated density-dependent control of gene expression in prokaryotes: bioluminescence and the biosynthesis of carbapenem antibiotics. FEMS Microbiol. Lett. 100: 161–168

Zhang, L., Murphy, P.J., Kerr, A., Tate, M. E. (1993) *Agrobacterium* conjugation and gene regulation by *N*-acyl-L-homoserine lactones. Nature 362: 446–448