Quorum sensing, virulence and secondary metabolite production in plant soft-rotting bacteria

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Quorum sensing describes the ability of bacteria to sense their population density and respond by modulating gene expression. In the plant soft-rotting bacteria, such as *Erwinia*, an arsenal of plant cell wall-degrading enzymes is produced in a cell density-dependent manner, which causes maceration of plant tissue. However, quorum sensing is central not only to controlling the production of such destructive enzymes, but also to the control of a number of other virulence determinants and secondary metabolites. *Erwinia* synthesizes both N-acylhomoserine lactone (AHL) and autoinducer-2 types of quorum sensing signal, which both play a role in regulating gene expression in the phytopathogen. We review the models for AHL-based regulation of carbapenem antibiotic production in *Erwinia*. We also discuss the importance of quorum sensing in the production and secretion of virulence determinants by *Erwinia*, and its interplay with other regulatory systems.

**Keywords:** *Erwinia*; quorum sensing; N-acylhomoserine lactone; carbapenem antibiotic; virulence; phytopathogenic

1. QUORUM SENSING IN PLANT SOFT-ROT BACTERIA

Over the past 15 years or so, it has become clear that bacteria are able to communicate with each other to coordinately regulate gene expression according to population density, in a process which has been termed ‘quorum sensing’ (reviewed in Whitehead et al. 2001). Depending on the bacterial strain, various bacterial processes can come under the control of the quorum sensing regulon, particularly systems involved in the production of secondary metabolites and those involved in virulence and symbiosis (Sebaihia 1999; Whitehead 1999; Corbett et al. 2005; Pemberton et al. 2005).

Quorum sensing has been well studied in some Gram-negative phytopathogens, including *Erwinia* species. *Erwinia carotovora* ssp. *carotovora* (Ecc) and the closely related *E. carotovora* ssp. *atroseptica* (Eca) are the causative agents of soft-rot disease in a range of host plants, including carrot and potato. *Eca* in particular is responsible for ‘blackleg’ infections in potato plants. Virulence depends on the production of an arsenal of plant cell wall-degrading enzymes (PCDWEs; including pectinases and cellulases) and many of these virulence determinants, and others, have been found to be under quorum sensing control. Quorum sensing signalling in *Erwinia* uses N-acylhomoserine lactone (AHL)-based systems (table 1; Jones et al. 1993; Pirhonen et al. 1993) as well as systems that depend on autoinducer-2 (AI-2; Coulthurst et al. 2006). It should be noted that the AHL signalling molecules and quorum sensing systems used can vary between different strains, even within the erwiniae (table 1).

Regulation of production of the secondary metabolite, 1-carbapen-2-em-3 carboxylic acid (carbapenem or Car), by *Ecc* strain ATCC 39048 remains one of the best-characterized AHL-mediated quorum sensing-regulated systems and the first section of this review will summarize the current regulatory model. Subsequent sections will focus on AI-2-based quorum sensing signalling and the role of quorum sensing in the virulence of *Erwinia* and related species.

2. QUORUM SENSING REGulates CARBAPENEM PRODUCTION IN *E. CAROTOVORA*

*Erwinia carotovora* ssp. *carotovora* strain ATCC 39048 first produces detectable levels of the β-lactam antibiotic, Car (figure 1), during the transition between late log and stationary phases of growth in laboratory culture (Bainton et al. 1992a,b). The Car biosynthetic and autoresistance functions of this strain are encoded by the *carABCDEFGH* gene cluster (figure 1; McGowan et al. 1996, 1997). The enzymes encoded by *carA*, *carB* and *carC* are essential for the production of Car (McGowan et al. 1997; reviewed in Coulthurst et al. 2005). CarB catalyses the first committed step in Car biosynthesis, the formation of carboxymethylproline, from precursors that are thought to be derived from acetate and glutamate (Sleeman & Schofield 2004). CarD and CarE may be involved in the supply of these precursors, but the *carD* and *carE* genes are not essential; disruption of either genes results in the reduction, but not the complete abolition, of Car production (McGowan et al. 1997).
<table>
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<td>EamI, EamR</td>
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<td>Venturi <em>et al.</em> (2004)</td>
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<td>EC153</td>
<td>3-oxo-C8-HSL, 3-oxo-C6-HSL</td>
<td>ExpI, ExpR</td>
<td>exoenzyme production</td>
<td>Chatterjee <em>et al.</em> (2005)</td>
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<td>Ecc 71</td>
<td>3-oxo-C6-HSL</td>
<td>ExpI (also known as AhlI, HslI), ExpR</td>
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<td><em>Pantoea stewartii</em></td>
<td>3-oxo-C6-HSL</td>
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<td>ATCC 39006</td>
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<td>SmaI, SmaR</td>
<td>prodigiosin and virulence factor production</td>
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*a In addition to the major AHL, 3-oxo-C6-HSL, the presence of 3-oxo-C8-HSL and C6-HSL has been described in these *Erwinia* strains (Toth *et al.* 2004).
CarA is the β-lactam synthetase, acting on the product of the CarB-catalysed reaction, (2S,5S)-carboxymethylproline, to close the β-lactam ring (Miller et al. 2002a, 2003; Gerratana et al. 2003). Finally, CarC catalyses an epimerization and desaturation reaction, forming the active carbapenem antibiotic (Li et al. 2000). In addition, ATCC 39048 possesses an intrinsic antibiotic resistance mechanism, encoded by carF and carG, to counter the action of the antibiotic it produces (McGowan et al. 1997). However, the function of the carH gene product remains unknown (McGowan et al. 1997).

The car gene cluster is shown in figure 1. The eight car genes are organized as an operon, with a quorum sensing-dependent promoter driving production of a single transcript, initiating 52 bp upstream from the translational start of carA (McGowan et al. 2005). However, there is also a weak quorum sensing-independent promoter within carD, which means that the cells constitutively express CarE–H at low cell densities and are therefore resistant to Car produced by other bacteria (McGowan et al. 2005). This quorum sensing-independent expression of the resistance genes also means that the Car resistance mechanism is in place before the quorum sensing-dependent production of the Car antibiotic.

Quorum sensing depends on a diffusible signalling molecule, N-(3-oxohexanoyl)-l-homoserine lactone (3-oxo-C6-HSL), produced by the CarI synthase. 3-Oxo-C6-HSL binds to and activates the response regulator CarR, which is encoded immediately upstream of the carA gene. CarR/3-oxo-C6-HSL binds to the carA promoter and activates transcription of carA–H. Car production is also controlled by the hor gene product. Various environmental factors such as temperature, pH of the culture medium, carbon source and anaerobiosis also influence expression of carA–H. Pointed arrows or (+) symbols indicate a positive effect, while blunted arrows indicate a negative effect. The (?) symbols indicate a possible effect.

Early work showed that Car production in Ecc could be induced prematurely (i.e. at lower cell densities) when grown in the presence of filter-sterilized spent culture supernatants from stationary-phase Ecc cultures (Bainton et al. 1992a, b). This suggested the accumulation of a diffusible signal in stationary-phase cultures that could activate Car production. It was subsequently shown that a single gene product was responsible for the production of this signalling molecule. The gene encoded a homologue of the previously identified LuxI protein from Vibrio fischeri and was named carl (Swift et al. 1993). CarI produces the AHL signal, N-3-(oxohexanoyl)-l-homoserine lactone (3-oxo-C6-HSL; figure 1 and table 1). Purified synthetic 3-oxo-C6-HSL was shown to induce
premature Car production in ATCC 39048 (Bainton et al. 1992a,b) and to alleviate the Car− phenotype of a carI mutant (Jones et al. 1993).

AHL-based quorum sensing systems rely on the presence of a transcriptional regulator, a member of the LuxR family of proteins, that detects the AHL signal and responds by bringing about a change in the transcriptional regulation. The LuxR homologue involved in the control of Car production in Erwinia is called CarR, and is encoded by the carR gene, located immediately upstream of carA (figure 1; McGowan et al. 1995). The carR gene is a separate transcriptional unit from the carA–H operon, initiating 108 bp upstream from its translational start site (McGowan et al. 2005). The carI gene is located elsewhere in the genome and is adjacent to a convergently transcribed gene, expR, that encodes a second LuxR homologue in Erwinia (Swift et al. 1993; Rivet 1998). Finally, there is a gene encoding a third LuxR homologue in the genome, VirR, which is an important regulator of virulence factors in Erwinia (see §12; Burr et al. 2006). Disruption of carI and/or carR leads to the abolition of Car production and transcription from the quorum-sensing-dependent carA promoter (McGowan et al. 2005). However, inactivation of either expR or virR has no obvious influence on Car production, either in the presence or absence of a functional carI gene. Therefore, it appears that CarR is the only LuxR homologue in ATCC 39048 that is involved in the regulation of Car production.

It is possible to bypass the dependence of Car production on 3-oxo-C6-HSL in a carI mutant by supplying carR in trans on a plasmid (McGowan et al. 1995). Therefore, CarR is capable of activating the carA promoter in a ligand-independent fashion when present at high non-physiological concentrations. However, carR transcription is tightly controlled via a CarR- and 3-oxo-C6-HSL-dependent autoregulatory mechanism (shown using a carR::lacZ reporter with the provision of a functional carR in trans on a plasmid), and therefore the concentration of CarR within the cell is limited (McGowan et al. 2005).

3. THE MODEL FOR QUORUM SENSING CONTROL OF CAR PRODUCTION IN ERWINIA

The model for quorum sensing-dependent regulation of antibiotic production in Erwinia is shown in figure 2. 3-Oxo-C6-HSL has been shown to interact directly with the CarR protein and increase its DNA-binding affinity for the carR–A intergenic region (Welch et al. 2000). Upon binding of CarR/3-oxo-C6-HSL to the carA promoter, carA–H transcription is activated, leading to Car production. Thus, under normal conditions, Car production is not activated by CarR until the concentration of 3-oxo-C6-HSL reaches a high enough level, which occurs during the transition from late log to stationary phase of growth. As 3-oxo-C6-HSL levels decline during the stationary phase of growth, we presume that 3-oxo-C6-HSL begins to dissociate from

Figure 2. Models for quorum sensing-dependent control of Car production in (a,b) Erwinia carotovora ssp. carotovora strain ATCC 39048 and (c,d) Serratia sp. strain ATCC 39006. At low cell densities, carA–H are not expressed in both Erwinia and Serratia (a,c); in Erwinia, this is because transcription of carA–H is not activated (a). However, in Serratia, the SmaR repressor adds an extra layer of control by preventing transcription of carA–H in the absence of quorum sensing signal (c). At high cell densities in Erwinia, the 3-oxo-C6-HSL quorum sensing signal binds and activates CarR, which promotes transcription and hence activates Car production (b). In Serratia, Car production is derepressed at high cell densities due to the AHL quorum sensing signal which inactivates the SmaR repressor. The Serratia CarR protein is then able to activate carA–H transcription in a ligand-independent manner (d).
CarR, which can no longer bind and activate the carR and carA promoters, and thus Car production is switched off.

4. THE Hor TRANSCRIPTIONAL REGULATOR
The quorum sensing system in Ecc is not the only genetic system responsible for the control of Car production. The hor gene product (figure 1) is a member of the SlyA family of transcriptional regulators, homologues of which are present in many bacterial species (Thomson et al. 1997). This family of proteins controls a diverse range of physiological functions, such as multidrug resistance, toxin production, secondary metabolite production and virulence, in both plant and animal pathogens. In Erwinia, Hor is an activator of Car and PCWDE production (Thomson et al., 1997). Analysis of carA transcriptional fusion strains has shown that Hor activates transcription from the carA promoter, although direct physical binding of Hor to the carA promoter has not been demonstrated (McGowan et al. 2005). Since Hor does not affect the transcription of either carR or carI, it appears to regulate Car biosynthesis via a quorum sensing-independent mechanism. However, the Car and exoenzyme phenotype of a hor mutant can be overridden (at least partially) by the provision of an excess of exogenously added 3-oxo-C6-HSL, suggesting that there may be some crosstalk between the quorum sensing- and the Hor-dependent pathways controlling Car production (McGowan et al. 2005).

5. ENVIRONMENTAL FACTORS INFLUENCING Car PRODUCTION
In addition to the cell density-dependent regulation described above, it is clear that Car production in Ecc also responds to a variety of other environmental cues, such as temperature, anaerobiosis, pH and carbon source availability (Byers et al. 2002; McGowan et al. 2005). Although less well characterized at the molecular genetic level, at least some of these inputs are integrated into the Car regulatory network via the quorum sensing system itself, as summarized in figure 1.

The chemical stability of AHL signalling molecules themselves is greatly influenced by pH (Byers et al. 2002; Yates et al. 2002). Under alkaline conditions, AHLs are rapidly hydrolysed, and hence ‘inactivated’, and are unable to function as quorum sensing signals. In a laboratory setting, unbuffered Erwinia culture medium becomes alkaline during the stationary phase of growth, owing to metabolism of peptides (L-broth). Under these alkaline conditions, the 3-oxo-C6-HSL signal is degraded and Car production is switched off during stationary phase. However, if the medium is buffered at a neutral pH, the quorum sensing signal is allowed to accumulate and the concentrations of 3-oxo-C6-HSL and Car detected in culture supernatants during stationary phase of growth remain constant (Byers et al. 2002). The sensitivity of AHLs to extremes of pH may be important during infection in planta, since one of the first plant responses to infection by Erwinia is alkalization of the plant infection site (Nachin & Barras 2000). This might aid the plant in resisting Erwinia infection by interfering with its quorum sensing system (Byers et al. 2002).

Another environmental factor that can influence Car production in Ecc is temperature (McGowan et al. 2005). The optimal temperature for both carA transcription and Car production in Ecc is 34°C. However, higher temperatures are inhibitory to antibiotic biosynthesis and Car production is completely abolished at growth temperatures of 37°C and above. Although carI transcription is apparently unaffected by an increase in temperature, the concentration of 3-oxo-C6-HSL detected in culture supernatants is reduced at 37°C, suggesting that a diminution of 3-oxo-C6-HSL may be responsible for the effect of temperature on Car production. The addition of exogenous 3-oxo-C6-HSL can partially restore Car production by Ecc grown at 37°C. However, in addition to the effect of temperature on 3-oxo-C6-HSL production, transcription of hor (and so production of the Hor transcriptional activator) is repressed at 37°C. Thus, the effect of growth temperature on Car production in Ecc is mediated by both Hor and the quorum sensing system (McGowan et al. 2005).

Car production by Erwinia is also influenced by the nature of the carbon source present in the growth medium (McGowan et al. 2005). In minimal medium containing sucrose as the sole carbon source, transcription of carA was approximately 50% higher than in minimal medium containing glucose; while growth in medium containing glycerol completely inhibited carA transcription. Transcription of carR and hor were unaffected by the nature of the carbon source, but carI transcription was found to be reduced by approximately 50% in minimal glycerol medium compared with minimal sucrose medium. This effect on carI transcription was also reflected in 3-oxo-C6-HSL levels detected in the culture supernatants (McGowan et al. 2005).

It is also known that oxygen availability affects Car production, which could be an important consideration during plant infection. Antibiotic cannot be detected in laboratory cultures grown under oxygen-limiting conditions. However, since Ecc cultured under these conditions is severely affected in growth rate, it is not possible to distinguish between the effect of a diminished growth rate and the effect of anaerobiosis on Car production (McGowan et al. 2005).

6. CRYPTIC CARBAPENEM GENE CLUSTERS ARE WIDESPREAD IN ERWINIA SPECIES
Upon identifying the carR–H locus in ATCC 39048, genetic screens were conducted to determine if the gene cluster was widely distributed among Erwinia species (Holden et al. 1998). The results of these screens showed that, while production of the AHL quorum sensing signalling molecule appeared to be universal in a diverse range of erwiniae (43 Ecc and 39 Eca isolates were tested), only a few Erwinia species produced Car under standard laboratory conditions (only two of the Ecc isolates tested; Holden et al. 1998). However, Southern blot analyses using probes to the Car biosynthetic genes revealed that the car cluster was present in a further seven Ecc strains, as well as two of the Eca strains tested. The lack of Car production could not
be attributed to a lack of the AHL quorum sensing signalling molecule and, furthermore, Car production could be activated in these strains by the provision of the carR gene from ATCC 39048 in trans on a plasmid (Holden et al. 1998). One of the strains containing a cryptic car cluster was Ecc strain SCRI193. This strain was found to have a complete carR–H gene locus but did not produce Car, even though it synthesized 3-oxo-C6-HSL. However, the lack of Car production could not be attributed to a defective CarR protein either, as, when supplied on a plasmid, the SCRI193 carR gene could activate Car production in this strain (Holden et al. 1998). There are a number of hypotheses that could explain this result. It is possible that transcription of carR is reduced in SCRI193 and thus, while CarR 193 is functional, its concentration within the cell is just too low. Alternatively, 3-oxo-C6-HSL and/or DNA binding by CarR193 may be weaker than the corresponding CarR protein from strain ATCC 39048. It is also possible that SCRI193 requires additional factors to produce Car that are not necessary for ATCC 39048 when grown under standard laboratory conditions.

Another potential explanation for the presence of cryptic car genes is that Erwinia species may be in the process of ‘degenerating’ the car clusters perhaps because it represents some unknown fitness cost to the species. If this explanation is correct, then it is interesting that the Car resistance functions are maintained in SCRI193. It might be ideal for some strains to be resistant to Car without the metabolic burden of producing the antibiotic. The real ecological value of antibiotic production by Erwinia is not known.

7. Car PRODUCTION IN SERRATIA SPECIES ATCC 39006
Serratia sp. ATCC 39006 (39006) is a taxonomically ill-defined strain of Serratia that produces pectate lyase and cellulase activity, like the erwinias, and also produces the red pigment, prodigiosin (Slater et al. 2003), like other Serratia species. Interestingly, this strain also possesses functional homologues of the Ecc car genes (including carR), which are also regulated by quorum sensing in 39006 (Cox et al. 1998; Slater et al. 2003; Fineran et al. 2005). However, the means by which quorum sensing-mediated control of Car production is achieved in 39006 shows striking differences when compared with the model proposed for the control of Car production in Ecc (figure 2).

In 39006, the luxR homologue is called smaI and is found in the genome adjacent to, and transcribed convergently with, a gene called smaR, which encodes a second LuxR homologue (Thomson et al. 2000;
SmaI is the AHL synthase responsible for the production of the major quorum sensing signal, N-butanoyl-l-homoserine lactone (C4-HSL) as well as a small amount of N-hexanoyl-l-homoserine lactone (C6-HSL; figure 3). When either smaI or carR are disrupted in 39006, Car production is switched off (Slater et al. 2003). A smaI mutation, but not a carR mutation, can be complemented by the addition of exogenous C4-HSL. These results confirm that smaI is required for Car production because AHL is required to activate Car production. It also confirms that CarR39006 is essential for Car production even in the presence of AHL.

When the other luxR homologue, smaR, is disrupted, Car production is unaffected (Slater et al. 2003). Surprisingly, however, a smaR mutation can phenotypically suppress a smaI mutation and switch Car production back on in the presence of a functional CarR39006 (Slater et al. 2003). This result suggests that SmaR is a repressor of the carA–H transcription in the absence of the AHL quorum sensing signal. This interpretation is supported by bandshift experiments demonstrating that purified SmaR protein can bind to the carA promoter region, but binding is reduced in the presence of C4-HSL (Slater et al. 2003). However, a smaR mutation cannot bypass a carR mutation (so that Car remains switched off), confirming that CarR39006 is essential for Car production in 39006. Finally, in a smaI, smaR and carR triple mutant, Car production is switched off. This confirms once again that CarR39006 is essential for Car production and, when combined with the data for the smaIR double mutant, shows that the CarR39006 protein is an apparently quorum sensing signal-independent activator of Car production, as proposed by Cox et al. (1998).

By analysing the above data, the current model for Car regulation in 39006 is as follows. At low cell density and AHL concentration, SmaR acts as a repressor by binding to the carA promoter and preventing the transcription of the carA cluster, as depicted in figure 2. At high cell density, the AHL concentration increases. The interaction of AHL with SmaR weakens the affinity of SmaR for the carA promoter and the SmaR repressor dissociates from the DNA. This allows CarR39006 to bind to the carA promoter independently of AHL and activate the carA–H transcription. As the AHL concentration declines, SmaR dissociates from AHL and binds to the carA promoter, switching off Car production.

It is interesting that the same gene cluster is regulated by AHL-based quorum sensing in two different organisms, ATCC 39048 and 39006, yet by two very different mechanisms. In ATCC 39048, Car production is positively regulated by a ligand-responsive activator, CarRelec, while in 39006, it is derepressed by the competing actions of the ligand-responsive repressor, SmaR, and the ligand-independent activator, CarR39006 (figure 2).

8. Car Production in Photorhabdus luminescens Strain TT01

A third entero-bacterial species known to possess a carA–H gene cluster and to produce the carbapenem antibiotic is Photorhabdus luminescens strain TT01 (Derzelle et al. 2002). TT01 is an insect pathogen and can also exist in a symbiotic relationship with nematode species. In strain TT01, the gene locus is referred to as cpmA–H and each gene in the cluster exhibits sequence similarity with the corresponding genes in the ATCC 39048 and Serratia 39006 carA–H gene clusters. The final three genes in strain TT01, cpmFGH, are also duplicated elsewhere in the chromosome and called cpmJKL, although it is not known if they are functionally expressed (Derzelle et al. 2002).

TT01 is not known to produce AHL signal molecules. Therefore, it is perhaps not surprising that there is no LuxR homologue encoded immediately upstream of the cpmA–H cluster. However, while it is difficult to measure Car production directly, owing to the many other antibiotics produced by TT01, there is evidence that Car production in this strain is repressed during the stationary phase of growth by a LuxS-type (AI-2-based) quorum sensing system (Derzelle et al. 2002). While there is no evidence that LuxS regulates Car production in ATCC 39048, there is evidence that a LuxS mutant of 39006 has reduced Car production suggesting that AI-2 has a positive input into Car production in this strain (Coulthurst et al. 2004, 2006). Photorhabdus luminescens strain TT01 serves as yet another example of how a single gene locus can be regulated very differently by quorum sensing in different genetic backgrounds.

These data suggest that bacterial regulation of secondary metabolites by quorum sensing may be versatile, such that bacterial gene clusters (presumably acquired by horizontal gene transfer) can come under the control of existing regulatory pathways.

9. THE ROLE OF QUORUM SENSING IN ERWINIA VIRULENCE

While the role of quorum sensing in the control of Car production has been well characterized, it is by no means the only quorum sensing-controlled phenotype in Erwinia (Sebaihia 1999; Whitehead 1999; Corbett et al. 2005; Pemberton et al. 2005; Burr et al. 2006). Many Erwinia genes whose products are involved in pathogenesis and virulence in plant hosts also lie within the quorum sensing regulon.

10. QUORUM SENSING AND PROTEIN SECRETION IN ERWINIA

The production of secreted proteins is key to virulence in the soft-rot erwinias. As reviewed by Toth et al. (2003) and Toth & Birch (2005), these secreted proteins include both those involved in ‘brute-force’ attack on the plant tissue, namely the battery of PCWDEs, and also those involved in more subtle interactions with the plant host, for example, type III effectors (such as DspE/A), Nip and Svx. Production of many of these known secreted virulence factors has been shown to be quorum sensing dependent (Toth et al. 2003; Corbett et al. 2005; Pemberton et al. 2005; Toth & Birch 2005; Burr et al. 2006).

Corbett et al. (2005) used the proteomic technique of two-dimensional difference gel electrophoresis (2D-DIGE; Unlu et al. 1997) to identify quorum
sensing-dependent proteins in the secretome of Eca SCRl 1043. This study identified three novel quorum sensing-dependent secreted proteins: Svx, a putative cellulase and a putative proteoglycan hydrolase, in addition to previously known quorum sensing-dependent secreted enzymes, e.g. PelC and CelV. Following its identification by proteomic analysis, Svx was subsequently shown to be required for full virulence in planta, demonstrating the usefulness of this approach for the identification of novel virulence factors. In addition, 2D-DIGE also revealed reduced levels of Pel, Cel and Prt enzymes in the secretome of the luxS mutant of Ecc ATn10 (Coulthurst et al. 2006). A recent proteomic study of Ech strain 3937 showed that secretion of multiple pectinases together with a rhamnogalacturonase and an esterase were induced by the addition of appropriate plant-derived inducing compounds (Kazemi-Pour et al. 2004). A novel protein, AvrL, homologous to Svx, was also identified, although its role, if any, in Ech virulence is unknown (Kazemi-Pour et al. 2004).

Although such proteomic analyses are proving valuable in identifying and comparing secreted proteins from wild-type Erwinia and defined mutants, such proteomic analyses only provide a ‘snapshot’ of the secretome. Growth under a variety of different conditions, e.g. in different inducing media, is expected to reveal many more secreted proteins and, of course, some key proteins may only be expressed in planta. Furthermore, some proteins cannot be seen on two-dimensional gels, often owing to low abundance and/or solubility (although other approaches, such as mass spectrometry-based, are expected to help address this issue). It is therefore anticipated that there are many more secreted proteins yet to be discovered, possibly with important roles in planta. With the increase in genomic sequence information, approaches such as proteomics and transcriptomics are an emerging and attractive way to gain a more global picture of the extent of the quorum sensing regulon in an organism. For example, in Pseudomonas aeruginosa, transcriptomic analyses have revealed that quorum sensing regulates, directly or indirectly, the expression of at least 300 genes, including many important for virulence (Schuster et al. 2003; Wagner et al. 2003). Moreover, a proteomic analysis in this organism has revealed that a significantly greater proportion of the proteome than the transcriptome seems to be affected by quorum sensing, implying that quorum sensing regulation is mediated at a post-transcriptional, as well as a transcriptional, level (Arevalo-Ferro et al. 2003).

Given that many important secreted virulence factors are produced by Erwinia, many in a quorum sensing-dependent fashion, it is also pertinent to consider how these secreted proteins are moved out of the bacterial cell and, in a few cases, into the plant cell. In both E. carotovora and Ech, proteases are secreted by the one-step type I secretion system, with the Ech Prt system (comprising the transporter PrtDEF and its substrates, proteases PrtABCg) being one of the model systems for this means of secretion (Delepelaire 2004). The majority of the key secreted virulence factors of E. carotovora and Ech, including multiple Pels, Peh, Cel and Svx, are secreted by a two-step type II secretion system known as Out (He et al. 1991; Reeves et al. 1993; Corbett et al. 2005). Type II secretion systems are complex assemblages, comprising 12–16 different proteins, which span the periplasm and translocate their substrate proteins from the periplasm to the exterior of the cell, following Sec (or Tat)-dependent export to the periplasm (Filloux 2004). Type II secretion is important for virulence in a variety of Gram-negative bacteria (Sandkvist 2001). Type III secretion systems (complex multienzyme structures that deliver ‘effector’ proteins directly into host cells) are important for virulence in plant and animal pathogens. In plant pathogens, type III systems are generally known as Hrp (‘hypersensitive response and pathogenicity’) systems and are involved in the establishment of infection in host plants and eliciting a defence response known as the hypersensitive reaction (HR) in non-host (resistant) plants (Alfano & Collmer 2004; He et al. 2004). Ecs, Eca and Ech all have hrp gene clusters, but the exact role of these secretion systems in virulence and the identity of all of their substrates remain to be fully clarified (Rantakari et al. 2001; Yang et al. 2002; Toth et al. 2003; Holeva et al. 2004). Several type III-dependent proteins have been identified in the soft-rotting erwinias: the harpin, HrpN, has been implicated in virulence and/or HR elicitation in Ecs, Eca and Ech, and the effector, DspE/A, has been implicated in pathogenicity in Eca (Mukherjee et al. 1997; Yang et al. 2002; Holeva et al. 2004). Interestingly, expression of HrpN is reported to be under AHL quorum sensing control in Ecs and Eca, but not in Ech (Mukherjee et al. 1997; Ham et al. 2004; Smadja et al. 2004).

There are a number of questions that are currently the subject of further investigation: is the secretion process itself also quorum sensing controlled? Alternatively, are the secretion systems expressed and ‘primed’ ready to secrete their substrates as soon as their expression is activated by quorum sensing, or are they simply able to secrete both quorum sensing-dependent and -independent substrates whenever required? Quorum sensing-dependent secretion systems have been reported in other bacteria, e.g. the Lip type I secretion system of S. liquefaciens and the Xcp type II secretion system of P aeruginosa (Chapon-Herve et al. 1997; Riedel et al. 2001).

11. MECHANISM OF QUORUM SENSING-MEDIATED CONTROL OF VIRULENCE FACTOR PRODUCTION

As illustrated by the cell density-dependent control of Car production in Erwinia and Serratia, members of the LuxR family of proteins play a key role in quorum sensing. Indeed, in the majority of the known cases, LuxR-type proteins are crucial for detecting the AHL signal produced by LuxI family members and modulating gene expression accordingly. It has been known for some time that the E. carotovora LuxI homologue, CarI (in Ecs) or its equivalent, ExpI (in Eca), is required for optimal virulence factor production (Jones et al. 1993; Pirhonen et al. 1993) and thus the involvement of a LuxR protein in regulating virulence factor production was postulated (McGowan & Salmond 1999;
revealed a potential role for ExpR 71 in controlling are also called luxIR genes. The corresponding Erwinia Ecc well-studied example of LuxR-regulated, AHL-dependent pathway eluded investigators. It is only in the light of details of this quorum sensing-dependent regulatory recent studies that the nature of this regulation is becoming clear.

As discussed, the regulation of carbapenem antibiotic production by CarR in Erwinia is a particularly well-studied example of LuxR-regulated, AHL-dependent gene expression. However, mutations in the Ecc carR gene have no effect on virulence factor production in this strain. Additionally, as previously discussed, like many so-called LuxIR systems, the Ecc carR gene has an adjacent overlapping luxR gene (expR) that encodes a second LuxR-type regulator (Swift et al. 1993). The same organization is seen in Ecc, with the expI and expR genes. The corresponding luxIR genes in Ecc strain 71 are also called expI and expR, and recent studies have revealed a potential role for ExpR71 in controlling virulence factor production (Chatterjee et al. 2005). In the absence of an AHL signal, ExpR71 stimulates the production of the RsmA protein (regulator of secondary metabolites), which in turn is known to negatively regulate the production of virulence factors (Chatterjee et al. 1995; Cui et al. 2005). In a recent study by Cui et al. (2005), ExpR71 was shown to bind in vitro to the rsmA promoter in the absence of AHL and this binding was abolished by the addition of the AHL signal, 3-oxo-C6-HSL. This correlates with the observation that RsmA levels in an expI mutant are higher than in an expI, expR double mutant. However, while virulence factor production in an expI mutant is increased by the expR mutation, it is not restored to the wild-type level seen in Ecc 71 cells expressing 3-oxo-C6-HSL (Cui et al. 2005). It seems then that some additional factor(s) must be contributing to the quorum sensing-dependent control of these genes in Ecc strain 71. A recent investigation by Welch et al. (2005), that investigated ligand binding by Ecc ATCC 39048 LuxR proteins, supports this notion. They studied binding using the ligands derived from the physiological 3-oxo-C6-HSL quorum sensing signal and the results show that, of the various ligands tested, 3-oxo-C6-HSL is the ligand most tightly bound by Ecc CarR and results in the greatest induction of carbapenem production. In contrast to this result, 3-oxo-C6-HSL is not the ligand most tightly bound by ExpR, but it is the ligand that most strongly induces production of PCWDEs. Thus, it is possible that the part of stimulating virulence factor production in response to 3-oxo-C6-HSL is performed (or supported) by another player.

12. THE ERWINIA VirR REGULATOR

Another LuxR protein has now been described in both Eca and Ecc, which is intimately involved in the control of virulence factor production in response to quorum sensing. Like Ecc71 ExpR, this additional LuxR protein (named VirR for virulence repressor) acts to negatively regulate a number of virulence products in the absence of its presumed ligand, 3-oxo-C6-HSL (Burr et al. 2006). The complement of factors regulated by VirR is extensive and includes PCWDEs such as Pel and Cel, as well as less well-characterized virulence factors such as the recently described Svx and Nip proteins (Corbett et al. 2005; Pemberton et al. 2005).

By homology with other LuxR family members, VirR possesses an N-terminal ‘autoinducer domain’ (used by other LuxR proteins to bind AHL ligands) and a C-terminal DNA-binding domain, characterized by the presence of a helix-turn-helix motif. The fact that VirR acts as a repressor only in the absence of the AHL signal explains why it has not previously been isolated in genetic screens looking for genes involved in the quorum sensing-dependent regulation of PCWDE production. Analysis of the Ecc 1043 genome sequence identified the gene encoding VirR in the genome of this strain and prompted further investigation of its role in quorum sensing (Bell et al. 2004). Critically, a virR mutation completely restores virulence factor production in an Ecc expI mutant to wild-type levels. This increase in expression is reflected in an increase in the level of virulence gene mRNA and, importantly, translates into a corresponding restoration of virulence in planta to wild-type levels. Thus, VirR has a central role in the quorum sensing regulatory network controlling Ecc virulence.

The simplest model for repression of virulence gene expression by VirR is that it binds directly to the promoter regions of those genes to prevent transcription. However, it is currently unknown whether the repression of transcription by VirR is direct, or indirect perhaps, as suggested in the case of ExpR71, via an additional factor. The mechanism by which VirR represses transcription of its target genes requires further study. Indeed, members of the LuxR family have been shown to vary in their response to the AHL signal. Some function only in the presence of the AHL signal (e.g. CarR Ecc Escherichia coli SdiA and Agrobacterium tumefaciens TraR; Qin et al. 2000; Welch et al. 2000; Yao et al. 2006; Van Houdt et al. 2006), while others are functional only in the absence of the signal (e.g. Pantoea stewartii EssR, Ecc ExpR71 and Serratia Smars90069 Minogue et al. 2002; Slater et al. 2003; Chatterjee et al. 2005). Other LuxR family members do not appear to respond to an AHL signal at all (e.g. Serratia CarR900066 Cox et al. 1998).

Like E. carotovora, the related soft-rotting phytopathogen, Erwinia chrysanthemi (Ech), also has the expI locus but, significantly, virR does not appear to be present in the genome of Ech (Burr et al. 2006; E. chrysanthemi sequencing project at http://www.tigr.org). This can be explained by the observation that in Ech, production of most PCWDEs (Pel), while being growth-phase dependent, is not significantly affected by an expI mutation (Nasser et al. 1998; Ham et al. 2004). Similarly, in agreement with this observation, the virulence of Ech strains is not affected by the expI mutation. Thus, it would not be necessary for this strain to possess the VirR protein and, presumably, cell density-dependent control of PCWDEs is achieved via an alternative mechanism.

That both VirR and ExpR are known to repress the expression of virulence factors in the absence of AHL suggests that, in E. carotovora, the expression of these factors is actively prevented when AHL levels are low, i.e. when the population of cells is low. It should be noted that, in an E. carotovora expI, virR double

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mutant, PCWDEs are still produced in a cell density-dependent manner (Burr et al. 2006). This suggests that, while VirR plays a key regulatory role, there are additional players in this complex regulatory network that are yet to be characterized.

13. INTEGRATION OF OTHER SIGNALS TO CONTROL ERWINIA VIRULENCE

It is important to note that quorum sensing is not the only means by which Erwinia virulence factors can be regulated and therefore the contribution of quorum sensing to gene regulation must be examined in context. A number of other regulators from a variety of erwiniae have been characterized, which integrate signals from other stimuli in order to regulate the expression of PCWDEs (reviewed in Brencic & Winans 2005).

14. THE Rsm REGULATORY NETWORK

The Rsm (regulator of secondary metabolites) system is proving to be a major, global gene regulatory system, homologues of which are widely distributed among Gram-negative bacteria. RsmA is a homologue of the global regulator, CsrA, from E. coli, which operates in the post-exponential phases of growth to negatively regulate a number of metabolic pathways, such as glycogen biosynthesis and gluconeogenesis. CsrA binds to selected mRNA transcripts in a region that overlaps the ribosome-binding site, blocking ribosome binding and promoting transcript degradation (Cui et al. 1995; Liu et al. 1995; Baker et al. 2002; Dubey et al. 2003). Recent SELEX (systematic evolution of ligands by exponential enrichment) studies have confirmed the importance of a conserved GGA motif for preferential CsrA binding in vitro (Dubey et al. 2005). However, CsrA does not degrade mRNA itself, so other factors (potentially nucleases) must be involved (Liu et al. 1995). CsrA has also been shown to act as a positive regulator of other genes by stabilizing these target mRNA transcripts. For example, CsrA activates flagellum biosynthesis and motility in E. coli (Wei et al. 2001). The regulator RsmA was originally identified in Ecc strain 71 (Chatterjee et al. 1995; Cui et al. 1995), but homologues of rsmA have been found in many different erwiniae, indicating its potential universal importance in the regulation of gene expression (Cui et al. 1995). Mutants of rsmA in Ecc strain 71 were found to be hypervirulent and overproduced PCWDEs. In addition, mutants also produced more 3-oxo-C6-HSL than the wild-type strain, suggesting a link between the Rsm system and quorum sensing. In support of this idea, the LuxR homologue, ExpR, from Ecc strain 71 has been shown to activate transcription of rsmA (Cui et al. 2005).

A second component of the Rsm system is the small untranslated RNA, rsmB (also known as crsB or aepH; Murata et al. 1994; Liu et al. 1998), whose predicted secondary structure contains multiple GGA repeats exposed in the loop regions of stem-loop structures. It was originally noted that the number of these repeats correlates with the number of RsmA molecules sequestered by rsmB (Liu et al. 1998) and, later, direct binding of RsmA to these repeats was demonstrated in the Pseudomonas fluorescens CHAO system (Valverde et al. 2004). Thus, rsmB enhances expression of genes encoding PCWDEs by sequestering RsmA and therefore antagonizing the action of RsmA on its mRNA targets (Liu et al. 1998). A similar method of regulation exists between CsrA and crsB in E. coli.

The third component of this Ecc system, RsmC (Cui et al. 1999), also known as HexY (Shih et al. 1999), was identified by two independent groups as a gene which, when mutated, caused elevated levels of exoenzyme production. RsmC negatively controls the expression of rsmB. It also has a positive effect on the expression of rsmA, although this might be mediated by rsmB rather than being a direct effect.

15. KdgR

It has long been known that full expression of the genes encoding PCWDEs in Erwinia requires the presence of plant cell wall breakdown products in the culture medium. The implication for erwiniae is that large quantities of PCWDEs are not made unless there is a suitable (plant) substrate nearby on which the enzymes can act. This requirement is mediated at a molecular level by the KdgR repressor, which acts as a global repressor of genes involved in pectin degradation. The kdgR gene can be found in the genome of Ecc and of multiple Ecc strains, as well as in the EcA strain for which the genome sequence has recently become available (SCRI 1043; Hugouvieux-Cotte-Pattat et al. 1996; Liu et al. 1999; Bell et al. 2004). KdgR is a DNA-binding transcriptional repressor that binds to defined KdgR boxes located within the promoter regions of its target genes (EcH reviewed in Barras et al. 1994; Ecc in Liu et al. 1999). These targets include genes encoding enzymes involved in pectin catabolism, such as pelI and pehI in Ecc (Liu et al. 1999). KdgR has also been shown to bind to the promoter region of the gene encoding the small regulatory RNA, rsmB (Liu et al. 1999). The DNA binding of KdgR is abolished by plant cell wall breakdown products such as KDG (2-keto-3-deoxyglucuronate), DKG (2,5-diketo-3-deoxyglucuronate) and DKG (2,5-diketo-3-deoxyglucuronate). Thus, these products induce expression of PCWDE-encoding genes by a depression mechanism.

16. OTHER REGULATORS

An additional kdgR homologue, rexZ (regulator of exoenzymes), exists in the genomes of both Ecc and EcA (Thomson et al. 1999; Bell et al. 2004). However, unlike other KdgR homologues, RexZ activates the production of PCWDEs (Thomson et al. 1999). RexZ clearly has a role distinct from that played by KdgR, but its true function requires clarification. Various other regulators of PCWDE production are also known, although their functions have not been well characterized. AepA (activator of extracellular proteins) has been shown to act as a transcriptional activator of pel and affects levels of protease and cellulase production in the cell (Liu et al. 1993; reviewed in detail by Barras et al. 1994). The Hor regulator, previously described, coordinately activates PCWDE and Car production in Ecc ATCC 39048 (Thomson et al. 1997). Recently, hor was identified as one of the two genes required for infection of Drosophila by a strain of Erwinia (Ecc 15;
Bassett et al. (2003), suggesting that Hor may have additional roles distinct from those involved in the regulation of Car and PCWDE production. The Erwinia stationary-phase sigma factor, RpoS, has been shown to be important in virulence, since rpoS mutants are less able to infect tobacco plants and exhibit impaired growth (Andersson et al. 1999). Interestingly, rpoS mutants show elevated production of PCDWEs, an effect that is mediated, at least in part, by a reduction in the levels of the rsmA transcript (Mukherjee et al. 1998; Andersson et al. 1999). Therefore, although PCWDEs are clearly important virulence determinants, there must be additional factors that are required for successful infection of a plant host. The HexA global regulator (Harris et al. 1998) also appears to influence PCWDE production by affecting both RpoS (RpoS levels are elevated in a hexA mutant; Mukherjee et al. 2000) and rsmB transcription (which is reduced in a hexA mutant; Mukherjee et al. 2000). Finally, the ExpA/ExpS (GacA/GacS) two-component system also influences the Rsm system (Eriksson et al. 1998). In a gacA mutant, rsmB transcription is reduced, while RsmA levels remain unaffected, thus resulting in a reduction in the production of PCWDEs (Cui et al. 2001).

Clearly, the regulation of virulence factor production in the erwiniae is extremely complex to allow the bacteria to effect rapid and precise changes in the expression profile of PCWDEs and other virulence determinants, in response to changes in environmental conditions. Additionally, this intricate system of interconnecting gene regulatory networks has obvious implications for the design of laboratory experiments to study the role of quorum sensing in Erwinia.

17. luxS/autoinducer-2-mediated quorum sensing in E. carotovora

Another type of quorum sensing system, distinct from AHL-mediated quorum sensing, has also been described in Gram-negative bacteria: the AI-2 signalling system. This system has been reviewed recently by Vendeville et al. (2005). The AI-2 quorum sensing system was first discovered in the marine bacterium Vibrio harveyi, where it is one of at least three parallel quorum sensing systems that converge to regulate bioluminescence and several other phenotypes including type III secretion (Bassler et al. 1994; Henke & Bassler 2004a,b). As reviewed by Federle & Bassler (2003), Henke & Bassler (2004c) and Vendeville et al. (2005), at high cell densities, AI-2 is bound by the periplasmic protein LuxP, which interacts with the hybrid sensor kinase, LuxQ. This initiates a dephosphorylation cascade resulting in the dephosphorylation of the phosphotransfer protein, LuxU, and thus inactivation of the response regulator, LuxO. Inactivation of LuxO alleviates small RNA-mediated repression of the master regulator, LuxR_Vh. The other quorum sensing signals feed into the phosphotransfer relay at LuxU via dedicated sensor kinases.

Production of AI-2 is dependent on the luxS gene (Surette et al. 1999). The observation that many species of bacteria, both Gram-positive and Gram-negative, produce AI-2-like activity and/or possess a luxS homologue led to the suggestion that AI-2 might be a universal (non-species specific) and/or interspecies signalling molecule (Schauer & Bassler 2001; Sun et al. 2004). Inactivation of luxS in a variety of bacterial species has led to a range of phenotypes, from very little impact through to altered production of virulence determinants and decreased virulence (reviewed in Vendeville et al. 2005). For example, phenotypes of luxS mutants have been reported to include decreased expression of type III secretion and motility genes in E. coli, decreased production of secreted virulence factors in Streptococcus pyogenes and Porphyromonas gingivalis, impaired biofilm formation in Streptococcus mutans, and reduced virulence in Neisseria meningitidis and Streptococcus pneumoniae (Sperandio et al. 1999, 2001; Lyon et al. 2001; Burgess et al. 2002; Winzer et al. 2002a; Stroher et al. 2003; Wen & Burne 2004). Hence, LuxS appears to have an important role in virulence-associated phenotypes, and therefore potentially in virulence, in many bacterial species.

However, the elucidation of the biosynthetic pathway for AI-2 revealed that LuxS has a metabolic role in the S-adenosyl methionine (SAM) utilization pathway. The product of SAM-dependent methyltransferase reactions, S-adenosyl homocysteine, is detoxified by the nucleosidase Pfs to give S-ribosyl homocysteine (SRH), and then LuxS acts on SRH, yielding homocysteine, which can be recycled to methionine, and AI-2 (Schauer et al. 2001; Winzer et al. 2002b). The immediate product of LuxS, 4,5-dihydroxypentanedione (DPD), spontaneously forms multiple, interconvertible, cyclized derivatives that exhibit AI-2 activity. As revealed by the crystal structures of the cognate receptors, the structure of AI-2 detected by V. harveyi is a furanosyl borate diester, whereas in Salmonella typhimurium, it is a hydrated furanone (Chen et al. 2002; Miller et al. 2004). Indeed, these findings indicate that ‘AI-2’ is actually a collective term for multiple chemical species (Vendeville et al. 2005).

Since LuxS has a metabolic role, it is currently unclear how many of the phenotypes described for luxS mutants are actually the result of a signalling defect due to the absence of AI-2, and how many are simply the result of a metabolic defect due to the loss of methionine recycling. In some cases, either conditioned medium from a LuxS+AI-2+ strain or, rarely, in vitro-synthesized AI-2 have been used to complement luxS mutant phenotypes or show an impact on gene expression, implying an extracellular signalling role for luxS (Vendeville et al. 2005). For example, conditioned medium from a LuxS+/AI-2+ strain has been used to complement the toxin production defect of a Clostridium perfringens luxS mutant and the reduced prodigiosin production of a Serratia marcescens luxS mutant, while Borrelia burgdorferi shows altered expression of several proteins in response to synthetic DPD (Ohtani et al. 2002; Coulthurst et al. 2004; Babb et al. 2005).

Quorum sensing signal transduction systems similar to that of V. harveyi have been found in other Vibrio spp., detecting AI-2 and, in some cases, AHLs and/or a third signal, CAI-1. These quorum sensing systems have been implicated in virulence and biofilm formation in V. cholerae, colonization in V. fischeri and virulence in V. vulnificus (Miller et al. 2002b; Kim et al. 2003;
Hammer & Bassler 2004; Lupp & Ruby 2004). In *S. typhimurium* and *E. coli*, a system for the ABC-transporter-mediated uptake and subsequent phosphorylation and degradation of AI-2, the Lsr system, has been described (Taga et al. 2001, 2003; Xavier & Bassler 2005). However, whether this uptake of AI-2 is for the purpose of signal detection/termination or simply for metabolite scavenging of AI-2 is currently unclear.

The potential role of luxS/AI-2 has recently been examined in *E. carotovora*, the first such report in a plant pathogen (Coulthurst et al. 2006). Both *Ecc ATTn10* (a derivative of ATCC 39048) and *Eca SCR1* 1043 produce luxS-dependent extracellular AI-2 activity. In each strain, a defined luxS mutant was constructed and the phenotypes of the resulting mutant characterized. In *Ecc ATTn10*, a proteomic analysis revealed that a defined set of intracellular proteins were altered in abundance in the luxS mutant compared with the wild-type, including several potentially involved in virulence. For example, both PelX (periplasmic exopolygalacturonate lyase) and TogB (component of the Tog[\(\text{MNAB}\)] transporter), involved in the generation and uptake, respectively, of pectin oligomers during pectin catabolism, were found to be reduced in the luxS mutant. In contrast, a similar analysis of the *Eca SCR1* 1043 luxS mutant revealed a negligible impact on the intracellular proteome. Analysis of the secreted proteome of *Ecc ATTn10* found that levels of key secreted enzymes, Pel, Cel and Prt, were reduced in the luxS mutant. Similarly, the levels of Pel, Cel and Prt activities were reduced in the luxS mutant of *Eca SCR1* 1043. As has been observed in the luxS mutants of other bacteria, the motility of the luxS mutant of *Eca SCR1* 1043 was found to be reduced when compared with the wild-type.

Emphasizing the strain-dependent nature of the impact of luxS inactivation, the virulence of the luxS mutant of *Ecc ATTn10* was found to be reduced compared with that of the wild-type in *vitro*, whereas no statistically significant decrease in virulence was observed in the luxS mutant of *Eca SCR1* 1043, at least in the models used (Coulthurst et al. 2006). Strain dependence in the impact of luxS has also been noted in *Serratia* spp. (Coulthurst et al. 2004). In addition, no link was detected between this potential AI-2 quorum sensing system and the AHL quorum sensing system in *Erwinia*. Although the molecular mechanism(s) of luxS regulation in *Erwinia* remain to be determined, it appears that luxS plays a strain-dependent role in virulence in this organism.

18. QUORUM SENSING IN RELATIVES OF THE SOFT-ROT ERWINIAS

(a) *Pantoea stewartii*

*Pantoea stewartii* ssp. stewartii (*P. stewartii*, formerly known as *Erwinia stewartii*) is the causative agent of Stewart’s wilt and leaf blight in maize and sweetcorn. Symptoms result from the production by *P. stewartii* of large amounts of exopolysaccharide (EPS), which block the plant xylem vessels (Von Bodman et al. 1998, 2003a,b). The synthesis and translocation of EPS is encoded by the *cps* gene cluster and EPS-deficient mutants are avirulent (Minogue et al. 2005). Production of EPS is cell density dependent and under the control of the EsaI/EsaR AHL quorum sensing system. EsaI, a LuxI homologue, produces 3-oxo-C6-HSL, and EsaR, a LuxR homologue, provides the cognate 3-oxo-C6-HSL receptor. Mutation of *esaI* eliminates EPS synthesis and virulence (Von Bodman et al. 1998). EsaI was the first LuxI family protein for which a crystal structure was reported, identifying critical residues for catalysis and substrate specificity (Watson et al. 2002).

EsaR was the first LuxR homologue identified as being a negative regulator, rather than an activator, of gene expression (Von Bodman et al. 1998). Genetic studies revealed that EsaR represses production of EPS in the absence of 3-oxo-C6-HSL (at low cell densities) and that 3-oxo-C6-HSL relieves this repression, allowing expression of EPS. Mutation of *esaR* leads to growth-phase-independent overexpression of EPS, in either a wild-type or an *esaI* background (Von Bodman et al. 1998). Interestingly, both the *esaR* and the *esaIR* mutants display impaired virulence in * planta*, implying that quorum sensing-mediated repression of EPS production in the early stages of an infection is crucial to its success, perhaps by allowing initial attachment of the bacterium to the host (Von Bodman et al. 1998, 2003a). EsaR represses expression of *esaR* in the absence of 3-oxo-C6-HSL by binding to a lux box overlapping its own promoter, with binding of 3-oxo-C6-HSL relieving this repression. However, EsaR does not regulate the expression of *esaI* (Minogue et al. 2002). EsaR-mediated repression of *cps* expression has recently been shown to be mediated via direct transcriptional repression of rcsA. RcsA is an essential transcriptional coactivator (together with the response regulator RcsB) of the *cps* genes (Minogue et al. 2005). The authors suggest that, since EsaR can act as a transcriptional activator in the absence of AHL (if its binding site is appropriately positioned with reference to the promoter sequences), EsaR may also positively regulate other genes at low AHL concentrations (Von Bodman et al. 2003b; Minogue et al. 2005).

(b) *Erwinia amylovora*

*Erwinia amylovora* (*Ea*) causes the necrotic disease fire blight in apple, pear and related plant species, with key virulence traits being production of EPS and type III secretion of Hrp proteins into host cells. The existence of AHL quorum sensing systems in *Ea* has only recently been reported. Venturi et al. (2004) describe the production of a single AHL, most probably 3-oxo-C6-HSL, by several Italian strains of *Ea*; for one strain, production of an AHL was observed in * planta*. Molina et al. (2005) describe the detection of AHL activity in culture supernatant of a Swiss strain of *Ea*. In this strain, overexpression of the *AiiA* lactonase eliminated detectable AHL activity and considerably reduced EPS production, oxidative stress tolerance and virulence on apple leaves, indicating that AHL is likely to play an important role in *Ea* virulence (Molina et al. 2005). Both reports describe the detection and partial sequencing of pairs of convergent luxI and luxR homologues, named *eamI/eamR*. However, we await analysis of *eamI/eamR* mutants in order to investigate
the quorum sensing mechanisms and definition of the quorum sensing regulon in this species.

(c) Serratia sp. 39006
As described previously, the atypical Serratia, species 39006, produces extracellular pectate lyase and cellulase activity, suggesting that, like the erwinias, it may be capable of soft rotting in its natural environment. The quorum sensing-mediated control of Car production in this strain has already been discussed, but production of both Pel and Cel as well as the red pigment, prodigiosin, is also under quorum sensing control in 39006 (Slater et al. 2003). The LuxR homologue, SmaR, has been shown to repress the expression of the prodigiosin biosynthetic genes (pigA–O) and exoenzyme production (as well as Car as described above) in the absence of AHL (i.e. at low cell density). This repression is relieved in the presence of AHL (i.e. at high cell density).

The quorum sensing system of 39006 is integrated into a complex regulatory network controlling production of Car and prodigiosin in response to a variety of environmental inputs (Slater et al. 2003; Fineran et al. 2005). Regulatory components acting above, below and independently of the quorum sensing system have been detected. Expression of smaI (and also of pigA–O independently of smaI) is upregulated under conditions of phosphate limitation, via the Pst phosphate transport system (Slater et al. 2003). The smaIR system controls the expression of several regulators, rap, pigR and pigQ (predicted to encode a SlyA-family transcriptional regulator, an adenylate cyclase and a GacA-family response regulator, respectively), which themselves control expression of pigA–O in response to unknown signals (Fineran et al. 2005). A novel pleiotropic regulator, pigP, activates or represses the expression of multiple prodigiosin regulators, some of which are also regulated by smaIR (Fineran et al. 2005). Hence, multiple cues, including quorum sensing/cell density, can be integrated to finely tune the production of secondary metabolites in response to environmental signals.

19. WHY QUORUM SENSE?
One of the fundamental questions in the field of quorum sensing is that of why bacteria regulate gene expression according to population density. There are, of course, many possibilities for the role of quorum sensing and this topic has been hotly debated.

In Erwinia, there are several potential advantages that might be conferred by placing production of the carbapenem antibiotic under quorum sensing control. Car production in Ecc is an energetically demanding process for each cell in the population. As discussed above, it requires the expression of the Car biosynthetic and resistance genes, together with the precursors for Car production (glutamate semi-aldehyde and malonyl-CoA) and a supply of ATP to drive Car biosynthesis. Therefore, it makes sense that the bacteria should control antibiotic synthesis, so that it is only produced when it could provide a beneficial effect. It has been hypothesized that Car production may be involved in eliminating competing bacteria from the site of infection in planta. If these competing bacteria produce an AHL signal to which Erwinia can respond, Car production by Erwinia would be switched on and any Car-sensitive bacteria would be eliminated. It is probable that Ecc might encounter other AHL-producing organisms occupying a similar environmental niche. 3-Oxo-C6-HSL production has been demonstrated in a variety of Erwinia strains, including strains that do not make the carbapenem antibiotic (table 1). In addition, it has been shown (both in vitro and in vivo) that CarR_Ecc is capable of responding to a variety of AHL signals, not just 3-oxo-C6-HSL, further widening the scope for cross-activation of Car production in Ecc by exogenously provided AHLS (Welch et al. 2000, 2005). The presence of other bacteria may also help to increase the local 3-oxo-C6-HSL concentration (in the absence of further AHL synthesis), which could serve to activate Car production by Ecc.

As discussed, the quorum sensing system in Erwinia also prevents the production of secreted proteins, such as PCWDEs, at low cell densities. One suggested rationale for this is that Erwinia might be better able to prevent detection by the plant host defence systems if it delays secretion of virulence factors until a suitable population density has been achieved at which it can overcome the host by brute-force (induction of those genes). In support of this idea, Måe et al. (2001) reported that resistance to infection by wild-type Ecc was improved by the presence of AHL produced in a tobacco host harbouring the Ecc expl gene. In contrast, however, Toth et al. reported that potato plants engineered to express an AHL synthase (YenI from Yersinia enterocolitica) were in fact more susceptible to disease (as measured by the ‘disease score’ of Toth et al. (1999)) caused by wild-type Erwinia strains, even when inoculated with numbers as low as 10^2 cells (Toth et al. 2004). It is important to note that the conclusions of Måe et al. (2001) are drawn from the observation that a reduction was seen in the fraction of plants showing disease. However, no difference in disease progression was observed in cases where an infection had been established. Therefore, although quorum sensing is clearly important in Erwinia virulence and infection, its actual role may have more subtleties than previously thought, and thus is an area that requires further clarification.
accumulation of AHL may serve to signal to the cells that they are in a plant host environment and not, for example, in the less-confined environments of the soil. Alternatively, for organisms that are able to form biofilms, the AHL signal may allow the population of bacteria to distinguish between growth in planktonic culture and growth in a biofilm, and allow the population to modulate gene expression accordingly.

A related suggestion is simply that Erwinia is responding to a limitation of nutrients, which is the inevitable consequence of population increase in a finite space. In this case, on sensing a population increase, expression of genes that result in the liberation of additional nutrients from a plant host would allow further growth. In this way, Erwinia might be able to put off expression of PCWDEs for as long as possible, thus preventing the premature death of its host.

Antibiotics are effective against competing organisms only if they are produced and secreted in large enough amounts, such that the local concentration of antibiotic is greater than the minimum inhibitory concentration required for cell death. If Car were produced under circumstances when 3-oxo-C6-HSL concentrations are low, owing to extensive diffusion of the signalling molecule away from the producer cells, Car would also be subject to similar diffusion and so would not reach the concentrations required for it to be effective as an antibiotic. However, by linking Car production to the detection of high 3-oxo-C6-HSL concentrations, it is ensured that Car is only made when diffusion is limited sufficiently to allow antibiotic concentrations to reach high enough levels to be effective against competitor organisms. Finally, it is always possible that 3-oxo-C6-HSL and Car production may have other, as yet unidentified, advantages to Erwinia.

Of course, the reality is likely to involve aspects of each of these models, but the fact remains that, in Erwinia, virulence is responsive to quorum sensing, a fact that has made it the subject of intense study as a potential target of therapeutics but also reinforce the importance of AHLs in plant defense.

21. CONCLUDING COMMENTS
Quorum sensing plays an important role in the regulation of production of secondary metabolites and virulence factors in Erwinia and other soft-rotting bacteria. However, quorum sensing is just one component of an extremely complicated regulatory hierarchy that allows bacteria to titrate and respond to external signals. The challenge now is to determine what position quorum sensing occupies in the global regulatory hierarchy and to elucidate its true physiological and evolutionary function.

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