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Virulence in *Pectobacterium atrosepticum* is regulated by a coincidence circuit involving quorum sensing and the stress alarmone, (p)ppGpp

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Summary

Pectobacterium atrosepticum **(Pca) is a Gram-negative phytopathogen which causes disease by secreting plant cell wall degrading exoenzymes (PCWDEs). Previous studies have shown that PCWDE production is regulated by (i) the *inter*cellular quorum sensing (QS) signal molecule, 3-oxo-hexanoyl-L-homoserine lactone (OHHL), and (ii) the *intra*cellular 'alarmone', (p)ppGpp, which reports on nutrient limitation. Here we show that these two signals form an integrated coincidence circuit which ensures that metabolically costly PCWDE synthesis does not occur unless the population is simultaneously quorate *and* nutrient limited. A (p)ppGpp null Δ*relA*Δ*spoT* mutant was defective in both OHHL and PCWDE production, and nutritional supplementation of wild type cultures (which suppresses (p)ppGpp production) also suppressed OHHL and PCWDE production. There was a substantial overlap in the transcriptome of a (p)ppGpp deficient *relA* mutant and of a QS defective *expI* (OHHL synthase) mutant, especially with regards to virulence-associated genes. Random transposon mutagenesis revealed that disruption of *rsmA* was sufficient to restore PCWDE production in the (p)ppGpp null strain. We found that the ratio of RsmA protein to its RNA antagonist, rsmB, was modulated independently by (p)ppGpp and QS. While QS predominantly controlled virulence by modulating RsmA levels, (p)ppGpp exerted regulation through the modulation of the RsmA antagonist, rsmB.**

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Introduction

Pectobacterium atrosepticum (hereafter, Pca) strain SCRI1043 is a plant pathogenic member of the Enterobacteriaceae which causes black leg and soft rot disease in potato (Bell *et al.*, 2004). The genome of Pca encodes a plethora of potent virulence factors including several protein secretion systems, phytotoxins and secreted plant cell wall degrading exoenzymes (PCWDEs) such as pectate lyases (Pels) and exoproteases (Prts) (Toth *et al.*, 2006). PCWDE production is tightly regulated by an *N*-acyl-homoserine lactone (AHL) dependent quorum sensing (QS) system (Smith *et al.*, 2006; Barnard *et al.*, 2007). The primary AHL produced by Pca is 3-oxo-hexanoyl-L-homoserine lactone (OHHL), synthesized by the LuxI homologue, ExpI (Bainton *et al.*, 1992a,b; Pirhonen *et al.*, 1993). OHHL is freely diffusible and accumulates in the culture milieu in proportion to the bacterial population cell density. At a threshold concentration, OHHL interacts with the LuxR homologue, VirR (also known as ExpR2). In the absence of OHHL, VirR binds to the promoter of *rsmA*, stimulating transcription of the gene. However, when OHHL binds to VirR, the complex dissociates from the *rsmA* promoter, thereby preventing *rsmA* expression (Burr *et al.*, 2006; Cui *et al.*, 2006; Sjöblom *et al.*, 2006; Monson *et al.*, 2013).

RsmA (also known as CsrA) is a small RNA-binding protein that represses PCWDE production in *Pectobacterium* species (Chatterjee *et al.*, 1995; Cui *et al.*, 1995; Mukherjee *et al.*, 1996). RsmA is able to bind to specific PCWDE transcripts and reduce their stability (Baker *et al.*, 2002; 2007; Chatterjee *et al.*, 2010). RsmA binding to mRNA is antagonised by the non-coding regulatory RNA, rsmB, which is able to sequester RsmA (Liu *et al.*, 1998; Romeo, 1998; Valverde *et al.*, 2004; Dubey *et al.*, 2005; Romeo *et al.*, 2013). Each rsmB molecule can bind multiple RsmA molecules, thereby very effectively lowering the

concentration of 'free' RsmA. As a consequence of this rsmB-dependent titration of RsmA, PCWDE expression increases. However, although the link between VirR-dependent *rsmA* expression and QS is now well established, and several regulatory inputs have been identified that can affect *rsmB* expression (Eriksson *et al*., 1998; Cui *et al*., 1999; 2001; 2008; Liu *et al*., 1999; Mukherjee *et al*., 2000) it is still unclear how the cell modulates the delicate balance between RsmA/rsmB levels to co-ordinate optimal virulence factor production.

The intracellular 'alarmone' (p)ppGpp has also been shown to regulate virulence in several species of bacteria (Pizarro-Cerdá and Tedin, 2004; Thompson *et al*., 2006; Potrykus and Cashel, 2008; Dalebroux *et al*., 2010; Dalebroux and Swanson, 2012). In *Escherichia coli* and other members of the Enterobacteriaceae, (p)ppGpp accumulates when the culture becomes nutritionally limited. The synthesis of pppGpp is catalysed by two proteins, RelA and SpoT (Potrykus and Cashel, 2008). RelA is the major source of pppGpp in the cell and responds to amino acid limitation. SpoT has weaker pppGpp synthetic ability and senses deprivation of other nutrient sources (e.g. nitrogen, sulphur and phosphorus-containing compounds). Unlike RelA, which can only synthesize pppGpp, SpoT can also hydrolyse (p)ppGpp. Consequently, and since (p)ppGpp is able to arrest cell growth by inhibiting the transcription of ribosomal RNA (Srivatsan and Wang, 2008) and initiation of DNA replication (Potrykus and Cashel, 2008) *spoT* mutants are non-viable (presumably because they accumulate growth-inhibitory levels of (p)ppGpp). The pppGpp synthesized by RelA and SpoT is processed by nucleotide phosphohydrolase to yield ppGpp. In *E. coli*, ppGpp is a more potent growth inhibitor than its precursor, pppGpp (Potrykus and Cashel, 2008).

We previously suggested that (p)ppGpp accumulation is a necessary pre-requisite for optimal virulence factor production in Pca, and proposed that the culture needs to be both quorate *and* nutrient-limited for optimal PCWDE production (Wang *et al*., 2007). In this work, we test this hypothesis further. We demonstrate that the QS and (p)ppGpp regulons overlap and are integrally linked through a common signalling pathway. We use a genetic approach to identify RsmA/rsmB as the central node in this regulatory circuit, and show that when *rsmA* is disrupted, exoenzyme production is restored to a (p)ppGpp^0 (Δ*relA*Δ*spoT*) mutant. Finally, we show that (p)ppGpp exerts its effects on virulence by promoting the expression of the RsmA antagonist, rsmB. A simple model is presented in which QS and (p)ppGpp inputs form a 'logic AND gate' enabling the cell to integrate signals reporting on the population cell density *and* nutritional status to control virulence in this important phytopathogen. This model resolves many of the outstanding issues associated with the timing of virulence factor production.

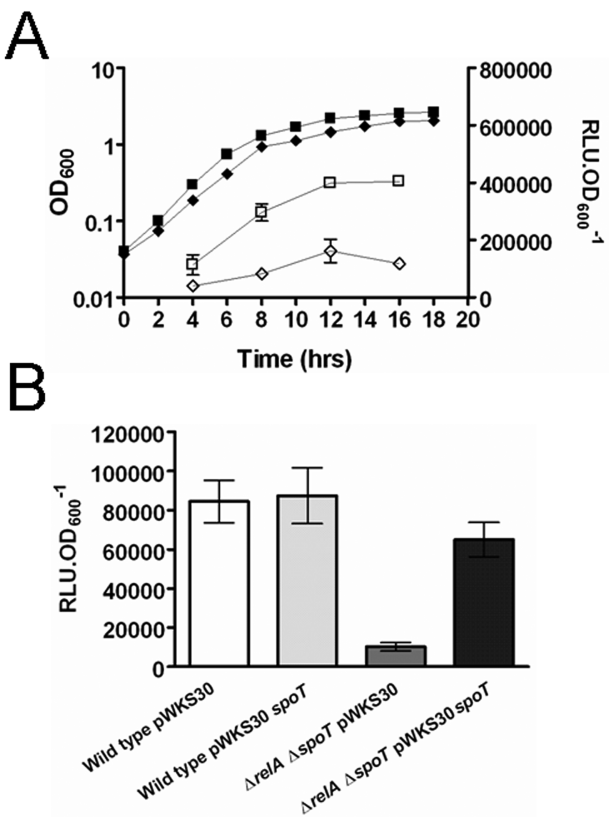

Fig. 1. OHHL production is greatly reduced in the Δ*relA*Δ*spoT* mutant.
A. Growth (filled symbols) and OHHL production (reported as OD_{600}-adjusted relative light units, open symbols) in LB-grown cultures of the wild type (squares) and SB1038 (Δ*relA*Δ*spoT*, diamonds).
B. *spoT* complementation of OHHL production. OHHL production was measured after 12 h growth in LB-grown cultures of the wild type (pWKS30) (white bars), wild type (pWKS30*spoT*) (light grey bars), SB1038 (pWKS30) (dark grey bars) and SB1038 (pWKS30*spoT*) (black bars). Error bars represent the standard deviation of three biological replicates.

Results

(p)ppGpp regulates PCWDE and OHHL production

A Δ*relA*Δ*spoT* double mutant (SB1038) was generated and confirmed to be (p)ppGpp^0 (Fig. S1A). As with *relA spoT* mutants of *E. coli*, SB1038 was auxotrophic. We determined that this was principally due to an inability to synthesize Ile, Leu, Met, Phe and Val. Consequently, SB1038 could not grow on minimal media unless supplemented with amino acids, although in amino acid-replete LB medium, SB1038 had a similar growth rate to the wild type (Fig. 1A). However, even when grown in LB, SB1038 showed greatly reduced OHHL production compared with the wild type (Fig. 1A). This defect could be complemented by introducing a plasmid expressing *spoT in trans* (Fig. 1B). We also found that secreted protease activity was lower in the Δ*relA*Δ*spoT* mutant rela-

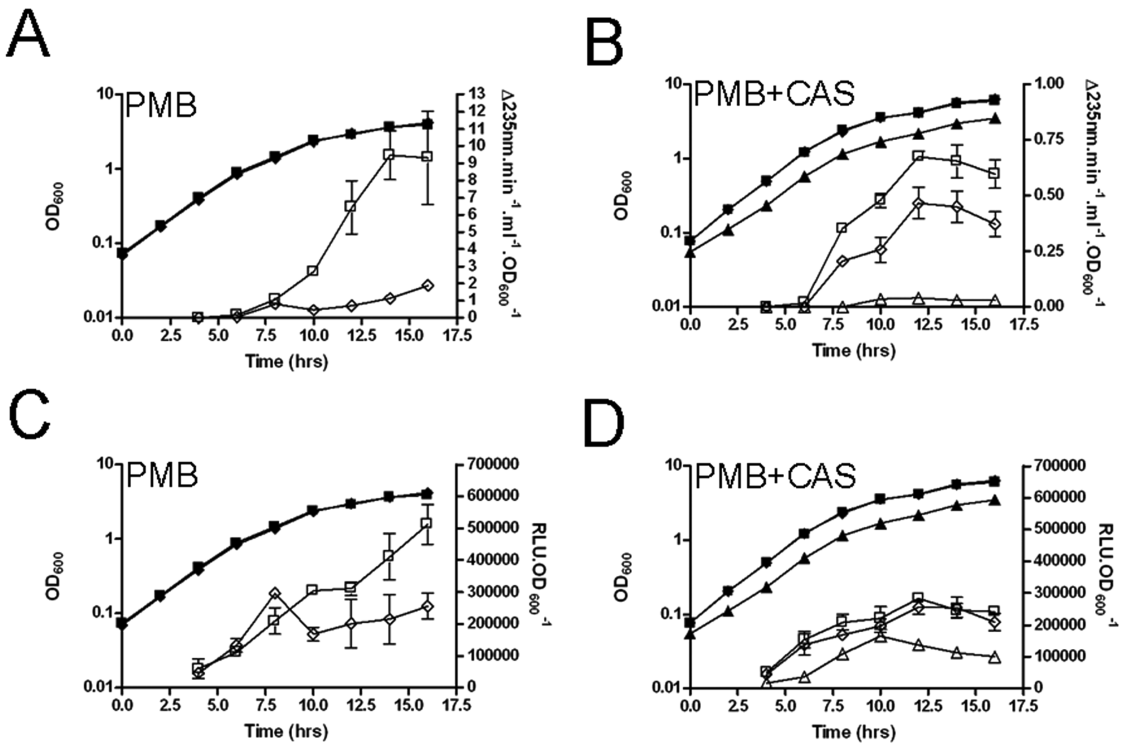

Fig. 2. Secreted Pel activity and OHHL production are lowered in the presence of amino acids in a manner that is dependent upon *relA*. Secreted Pel activity (panels A and B) and OHHL (panels C and D) were assayed in cultures grown in PMB (panels A, C) or PMB supplemented with 1% w/v casamino acids (panels B, D). Note the different vertical scales in panels A and B. Key to symbols: wild type (squares), SB1026 (Δ*relA*, diamonds), SB1038 (Δ*relA*Δ*spoT*, triangles), growth (filled symbols), Pel activity (open symbols in A, B), OHHL production (open symbols in C, D). Error bars represent the standard deviation of 3 biological replicates.

tive to the wild type or a Δ*relA* mutant (SB1026) (Fig. S1B) and again, this could be complemented by introducing the *spoT* gene *in trans* (Fig. S1C). However, the virulence defect in the Δ*relA*Δ*spoT* mutant was not solely due to the impact of (p)ppGpp on OHHL production; secreted protease production could not be restored in SB1038 by the addition of exogenous OHHL (Fig. S1D). This demonstrates that (p)ppGpp potentially regulates virulence in two ways: by decreasing OHHL synthesis on the one hand and *via* an OHHL-independent mechanism on the other.

relA and spoT regulate pectate lyase production in response to amino acids

To assess whether (p)ppGpp impacted on Pel production it was necessary to grow the bacteria in Pel minimal broth (PMB) (Shih *et al*., 1999); secreted Pel activity is negligible in rich media such as LB. PMB is limited for amino acids (Fig. S2A) and consistent with this, it was found that SB1038 had a severely reduced growth rate (doubling time 200 min) compared with the wild type (doubling time 122 min). However, addition of 1% w/v casamino acids to the PMB medium largely restored the growth of SB1038 (Fig. 2B). In contrast, a Δ*relA* mutant (SB1026) had an identical growth rate to the wild type in unsupplemented PMB (Fig. 2A) suggesting that SpoT-dependent (p)ppGpp synthesis was sufficient to maintain optimal growth in this medium. This notwithstanding, the Δ*relA* mutant displayed much lower secreted Pel activity compared with the wild type (Fig. 2A). Interestingly, the addition of casamino acids caused a large reduction in secreted Pel activity by the wild type, to levels close to that of the Δ*relA* mutant (Fig. 2B). This is consistent with the notion that casamino acids primarily inhibit Pel production by suppressing RelA-dependent (p)ppGpp synthesis. However, since the Δ*relA*Δ*spoT* mutant exhibited negligible Pel activity relative to the wild type or Δ*relA* strains (Fig. 2B), these data suggest that SpoT-derived (p)ppGpp also contributes towards PCWDE production.

relA and spoT regulate OHHL production in response to amino acids

The addition of casamino acids to PMB depressed OHHL production by wild type Pca to levels comparable with that of the Δ*relA* mutant (Fig. 2C and D). However, OHHL production by the Δ*relA*Δ*spoT* mutant was even lower (Fig. 2D). Taken together, these data suggest that the cell titrates nutrient availability *via* both RelA- and SpoT-

dependent (p)ppGpp production, and that this, in turn, can affect OHHL-dependent quorum sensing (although as noted earlier, this alone does not account for the profound virulence defect associated with the Δ*relA*Δ*spoT* mutant).

RelA and ExpI have overlapping virulence regulons

Microarray analysis was used to analyse the global gene expression profile of the wild type strain and of isogenic mutants containing deletions in the *relA*, *expI* or *rpoS* genes. We were particularly interested to see how mutations in these genes affected the expression of virulence determinants. Therefore, and to maximize PCWDE production (especially Pel induction) we grew the cultures in PMB. This is why the (p)ppGpp-deficient *relA* mutant was used instead of the (p)ppGpp null Δ*relA*Δ*spoT* mutant; recall that the latter exhibited a pronounced growth defect in PMB and that addition of casamino acids (which restores growth to the Δ*relA*Δ*spoT* mutant) concomitantly suppresses exoenzyme production in the wild-type comparator. The Δ*rpoS* mutant was included because RpoS is known to be an important regulator of the transition to stationary phase in *E. coli*, and its expression is strongly induced by (p)ppGpp (Battesti *et al*., 2011). All of the strains displayed similar growth rates in PMB medium (Fig. S2A and B). RNA was extracted from samples during exponential growth (6 h) and from the early stationary phase of growth (14 h), at which point the wild-type was expressing copious Pel activity and OHHL (Fig. S2B and C). The processed array data for each strain have been deposited in NCBI's Gene Expression Omnibus (Edgar *et al*., 2002) and are accessible through GEO Series Accession Number GSE50468 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE50468).

The Δ*relA* mutant displayed altered (i.e. twofold of greater) expression of just 5 genes (FDR = 0.05) during the exponential phase of growth (relative to the wild-type). Two of these were downregulated [*relA* and the adjacent downstream gene (*mazG*)] and three were upregulated, including two prophage genes located on a horizontally acquired island, HAI-17. However, the number of modulated transcripts increased to 358 (downregulated) and 930 (upregulated) by the stationary phase sampling point (Fig. S3). Transcripts in most classes of COG (Clusters of Orthologous Groups) were predominantly upregulated (relative to the wild-type) in the Δ*relA* mutant. Concordant with the central role played by (p)ppGpp in arresting cell growth, many of the upregulated transcripts encoded functions associated with cell division, cell envelope biogenesis, DNA replication and translation (Fig. 3A). In addition, genes encoding functions involved in translation and ribosomal structure were also strongly upregulated; an observation that is typical of the 'relaxed response' associated with *relA* mutants. The most highly upregulated genes in the Δ*relA* mutant (which were not modulated in the Δ*expI* mutant) included a divergent cluster of hypothetical genes (ECA2903-ECA2905) located in HAI13 (Bell *et al*., 2004) and surprisingly, several unlinked clusters of genes involved in branched chain amino acid metabolism (*ilvGMEDA*, *ilvIH*, *ilvBN* and *leuABCD*). This was unexpected since, based on the *E. coli* paradigm, amino acid biosynthesis should be generically downregulated in *relA* mutants. [Quantitative amino acid analysis of the spent culture supernatants after 6 h and 14 h growth did not reveal any major compositional differences between the *relA* mutant and wild-type, although the *relA* mutant seemed to secrete glycine into the medium during the stationary phase and used alanine and proline at a slower rate than the wild-type (Fig. S2A).] In addition, the *citW-citG* gene cluster (ECA2569–ECA2576, encoding enzymes involved in anaerobic citrate dissimilation) was strongly upregulated in the Δ*relA* mutant, as was the global regulator, *fis*.

Only two COGs displayed predominant downregulation in the Δ*relA* mutant; those relating to cell motility/protein secretion and virulence (discussed below). Consistent with the data in Fig. 2C showing that OHHL production was lower (cf. the wild-type) in the Δ*relA* mutant, levels of the *expI* transcript were also decreased by *c*. 50% in this strain. Moreover, with the exception of the achromobactin uptake system (*acr*/*cbrA-D* gene cluster), almost all iron uptake systems were downregulated in the *relA* mutant. [N.B. Iron uptake was even more restricted in the Δ*relA*Δ*spoT* mutant, where almost no secreted siderophore could be detected (Fig. S4 inset).] This was in stark contrast with the Δ*expI* mutant, where most of the iron uptake systems were upregulated (Fig. S4). In addition, a number of gene clusters showed pronounced downregulation in the *relA* mutant but not in the *expI* mutant. These included genes encoding functions involved in xylose/xylulose metabolism (*xylAB*, *xylFGH* operons), anaerobic formate metabolism and assimilation of hydrogen (the *hyp* and *hyb* operons). The most highly downregulated genes were ECA3950/ECA3951, encoding a putative Zn-dependent alcohol dehydrogenase and a LysE-type amino acid exporter respectively.

The Δ*expI* mutant displayed downregulation of 78 genes during the exponential phase of growth and 251 (mostly different) genes during the stationary phase. Notably, there was considerable overlap in the COG classes that were predominantly downregulated in the Δ*relA* mutant (motility/secretion and virulence) and also in the Δ*expI* mutant (Fig. 3A and B). In addition, the Δ*expI* mutant also displayed predominant downregulation of genes involved in secondary metabolism, signal transduction and lipid metabolism. These data are consistent with the microarray-based analyses of Liu *et al*. who examined the

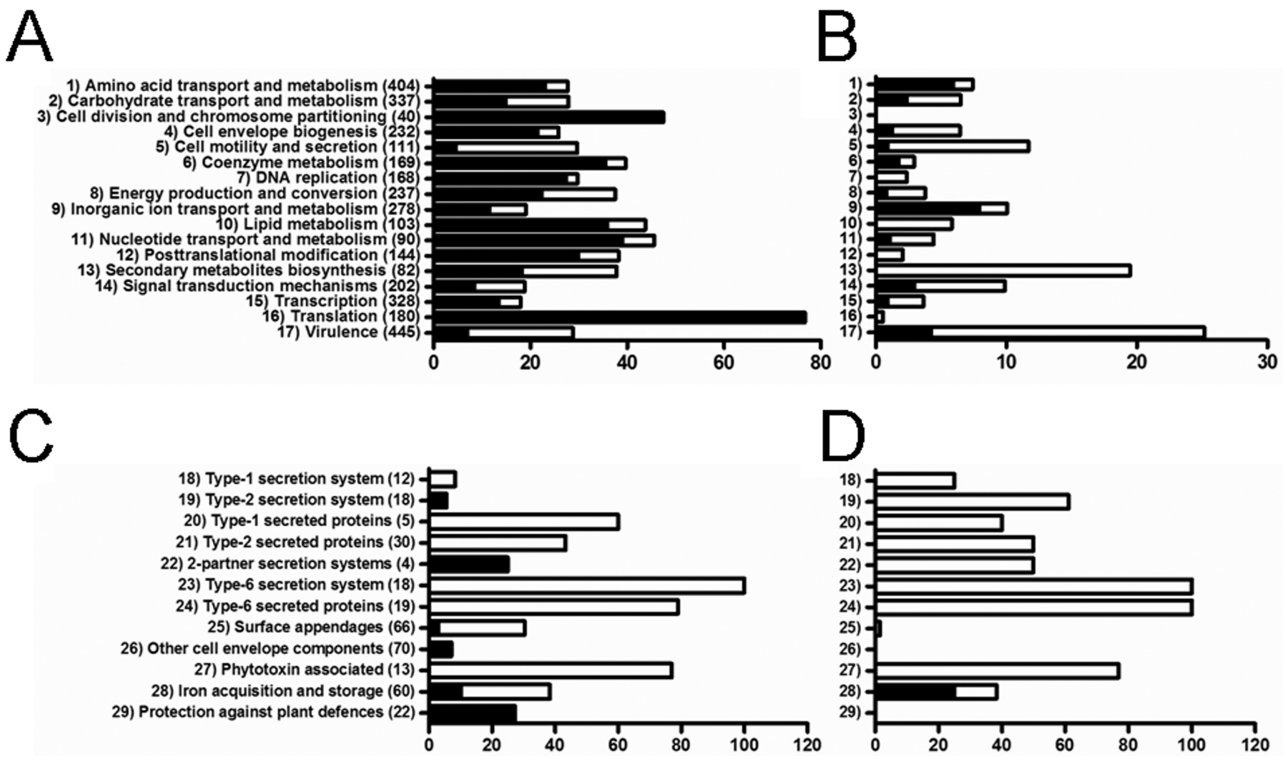

Fig. 3. Functional categories of *P. atrosepticum* genes that changed their expression profile in the Δ*relA* and Δ*expI* mutants during early stationary phase growth. Black bars and the white bars indicate the percentage of genes in each indicated functional category (COG) that were upregulated or downregulated respectively. The total number of genes within each COG category is indicated in brackets in panel A. Panels A and B show the percentage of genes within each functional category that were modulated in the Δ*relA* and Δ*expI* mutant respectively. Panels C and D show the virulence subcategories that were altered in the Δ*relA* and Δ*expI* mutant respectively. The microarray data used in these analyses have been deposited in NCBI's Gene Expression Omnibus (Edgar *et al*., 2002) and are accessible through GEO Series Accession Number GSE50468 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE50468). The list of genes included in each functional category was obtained from the Kyoto Encyclopedia of Genes and Genomes, KEGG (http://www.genome.jp/kegg/) or from the virulence categories cited previously (Bell *et al*., 2004; Liu *et al*., 2008).

gene expression profile of an *expI* mutant in tuber (Liu *et al*., 2008).

RpoS has been shown previously to regulate PCWDE production in *Pectobacterium carotovorum* subsp. *carotovorum* (Pcc) (Mukherjee *et al*., 1998). However, in our hands, the SCRI1043-derived Δ*rpoS* mutant displayed essentially wild-type protease activity (Fig. S1B and D) and Pel activity/OHHL production (Fig. S2B and C). Consistent with this, the only transcript that was significantly altered in the Δ*rpoS* mutant in either growth phase was that of the *rpoS* gene itself. This may suggest that (i) RpoS does not regulate gene expression during growth in PMB, (ii) the RpoS-dependency of PCWDE production is peculiar to Pcc or even to the specific Pcc strain investigated by Mukherjee *et al*. or (iii) the *rpoS* gene has accrued one or more mutations that make it non-functional in SCRI1043. If so, then sufficient compensatory mutations must have arisen to allow the strain to bypass this regulatory defect. In this regard, we note that the DNA sequence of the *rpoS* gene (and of the region *c*. 500 bp upstream of this gene) is identical in our laboratory lineage of SCRI1043 to the sequence of this region in the published genome. Since the DNA that was used for genome sequencing was from a strain that was frozen shortly after isolation from a Scottish potato plant in 1985 (Bell *et al*., 2004), it seems highly likely that this *rpoS* configuration is relevant in the field and is not a laboratory artefact.

Virulence, cell motility and protein secretion were COG categories that were predominantly downregulated in both the Δ*relA* and Δ*expI* mutants. The proportion of genes within individual virulence subcategories that were altered in each mutant is shown in Fig. 3C and D respectively. Genes encoding Type 2 secretion system substrates (e.g. Pels) were predominantly downregulated in both mutants, as were genes encoding the Type 1 secretion systems/substrates [which secrete the exoproteases (Salmond and Reeves, 1993)]. The Δ*relA* and Δ*expI* mutants also showed downregulation of almost all of the genes encoding the recently identified Type 6 secretion system (Liu *et al*., 2008) and of genes encoding the biosynthesis of coronofacic acid [known to contribute to Pca virulence in potato (Bell *et al*., 2004)]. Of the 251 genes that were

downregulated in the Δ*expI* mutant at the 14 h time-point, 56% were also downregulated in the Δ*relA* mutant (Fig. S3), and the majority of these were known virulence factors or were located on horizontally acquired islands (HAI) associated with pathogenicity (Bell *et al*., 2004).

Gene disruptions that restore Prt activity to the ΔrelAΔspoT mutant

Random transposon (Tn) mutagenesis was used to identify components of regulatory/effector pathways involved in the control of virulence downstream of (p)ppGpp. Our strategy was to identify Tn insertions in the Δ*relA*Δ*spoT* mutant that restored secreted Prt production. Approximately 12 500 Tn mutants were screened, yielding 15 Δ*relA*Δ*spoT* derivatives that were restored for exoprotease production (Fig. S5A). All of these mutants retained their auxotrophy phenotype, excluding the possibility that they arose due to spontaneous mutations in *rpoB* or *rpoC*. [Spontaneous *rpoB*/*rpoC* mutants are known to suppress the auxotrophy phenotype in *relA spoT* mutants (Murphy and Cashel, 2003).] Sequencing of the DNA flanking the Tn insertion sites revealed that these 'bypass' mutants were disrupted in 14 different genetic loci (Fig. S5B). Notably, three of the mutants contained Tn insertions within the known repressors of PCWDE production, *hexY* and *hexA* (Harris *et al*., 1998; Shih *et al*., 1999). However, except for mutant AE9 (see below), the remainder contained insertions at loci not previously known to impact on PCWDE production. We are currently investigating the possible function(s) of some of the encoded gene products in more detail.

Mutant AE9 was the most highly restored for Prt production and contained a Tn insertion in the promoter region of *rsmA*. Western blot analyses confirmed that this insertion abolished RsmA production (Fig. 4A). Despite numerous attempts, we failed to transduce the *rsmA* mutation into a 'clean' wild-type genetic background, suggesting that the *rsmA* gene may be conditionally essential in SCRI1043. This is consistent with previous work in the lab which failed to obtain an *rsmA* mutant in the wild-type background by allelic exchange (GPCS and MW, unpubl. results). Therefore, and to confirm that loss of *rsmA* expression was responsible for the restored protease production in AE9, *rsmA* was expressed *in trans* from a plasmid and was found to revert protease production to the low levels associated with the Δ*relA*Δ*spoT* parent strain (Fig. 4B). RsmA is an important negative regulator of virulence in *Pectobacterium* and *Dickeya* species (Chatterjee *et al*., 1995; Cui *et al*., 1995; 2006) and its homologue, CsrA (carbon storage regulator) is known to be regulated by RelA and SpoT in *E. coli* (Edwards *et al*., 2011; Romeo *et al*., 2013). Therefore, we decided to investigate the impact of (p)ppGpp on RsmA in Pca further.

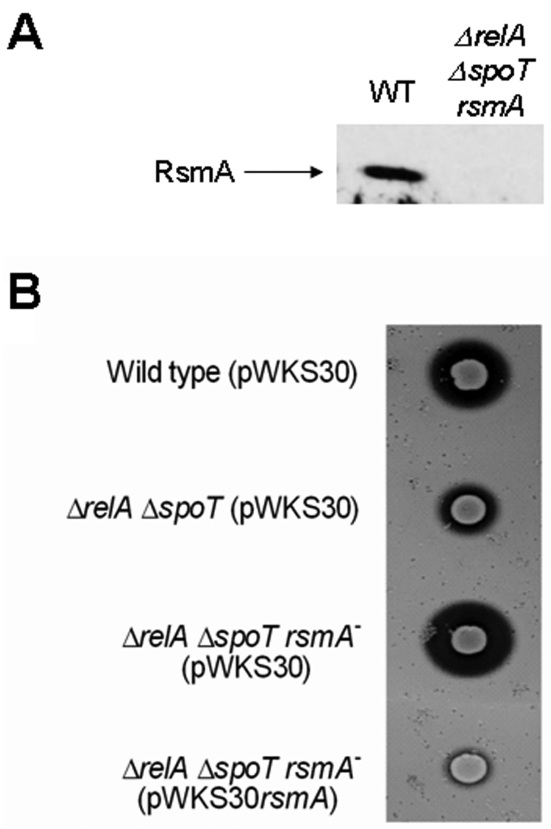

Fig. 4. Mutation of *rsmA* bypasses the protease defect in the (p)ppGpp null mutant.
A. Western blot analysis of cell extracts from wild-type and AE9 (Δ*relA* Δ*spoT rsmA*) mutant cultures (grown in PMB) probed with anti-RsmA antibodies. The WT was sampled after 12 h growth while the AE9 (which grew more slowly in minimal medium) was sampled after 24 h growth.
B. Complementation of exoprotease production in AE9 by plasmid-borne *rsmA*. The figure shows exoprotease production (visible as halos around the spotted colonies) by the indicated strains. pWKS30 is a low copy number vector. Note the impaired exoprotease production by the Δ*relA*Δ*spoT* mutant (SB1038) and that exoprotease production is fully restored in the corresponding Δ*relA*Δ*spoT rsmA* mutant (AE9). In contrast, expression of *rsmA in trans* from pWKS30 was sufficient to fully suppress exoprotease production by AE9.

(p)ppGpp and OHHL independently modulate the ratio of RsmA : rsmB

When grown in PMB medium (i.e. conditions in which virulence gene expression is activated) we observed marked differences between RsmA levels in the wild type/Δ*relA* mutant and the Δ*expI* mutant (Fig. 5A). [The ppGpp-deficient Δ*relA* mutant was examined because, as noted previously, the ppGpp null Δ*relA*Δ*spoT* double mutant displayed a pronounced growth defect in PMB.] In both the wild-type and the Δ*relA* mutant, RsmA levels showed similar patterns of growth phase-dependent change; RsmA concentrations were high in the exponential phase of growth and *c.* 2–2.5-fold lower during the stationary

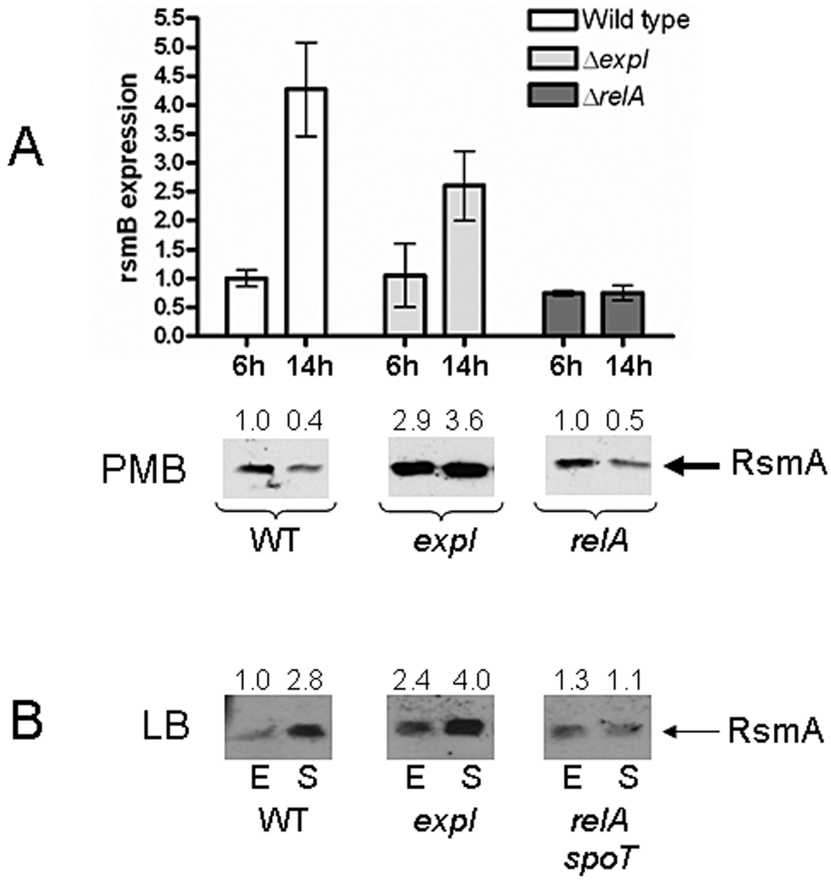

Fig. 5. RsmA and rsmB levels in the mutant strains.
A. Q-RT-PCR analysis of rsmB transcript levels in the wild type, Δ*expI* mutant and Δ*relA* mutant. RNA was extracted after 6 h or 14 h (as indicated) of growth in PMB and rsmB transcripts were measured by Q-RT-PCR. The reference gene used to normalize the Q-PCR signal was ECA0762. Key: wild type (white bars), Δ*expI* mutant (light grey bars) and Δ*relA* mutant (dark grey bars). RsmB transcript levels in the wild type at 6 h of growth were set at 1. Error bars represent the standard deviation from 3 biological replicates. Below each set of bars are the corresponding Western blots showing RsmA protein expression in each strain, as indicated. The relative RsmA band intensities were quantified and are indicated above each lane. RsmA expression in the wild type at 6 h of growth was set at 1.
B. The growth phase-dependency of RsmA expression is inverted in cultures grown in rich medium. Quantitative Western blotting was used to measure RsmA levels in cultures of the wild type, Δ*expI* mutant and Δ*relA*Δ*spoT* mutant grown in amino acid-replete LB medium.

phase (Fig. 5A). In contrast, RsmA levels in the Δ*expI* mutant were *c.* 3–4-fold higher (compared with the wild-type sampled after 6 h) at both growth phases (Fig. 5A). This is consistent with unrestrained stimulation of *rsmA* expression by VirR (also known as ExpR2) in the absence of OHHL (Cui *et al.*, 2006; Sjöblom *et al.*, 2006). However, additional factors must also regulate RsmA expression. For example, in amino acid-replete Luria Broth the wild-type RsmA expression profile was inverted, with nearly threefold higher RsmA expression during the stationary phase (Fig. 5B). This increased stationary phase expression of RsmA was abolished in the Δ*relA*Δ*spoT* mutant (which unlike PMB, grew well in LB) suggesting that under some conditions, (p)ppGpp can also stimulate RsmA expression, as noted by others (Edwards *et al.*, 2011).

RsmA activity is antagonized by the untranslated RNA, rsmB. RsmA is a very highly conserved protein that is thought to bind to a specific sequence motif (CAGGAXG) on rsmB, a motif that is present in multiple copies. In Pca, rsmB contains 8 dispersed perfect CAGGAXG motifs and a further 3 CAGGAXG motifs containing a single mismatch among the conserved bases (Fig. S6A). Additional CAGGAXG motifs are also present with a larger number of mismatches relative to the consensus. Therefore, in principle, rsmB can bind 8–11 functional units of RsmA with high affinity and further RsmA molecules with lower affinity. No other RsmA-antagonistic RNAs are known in Pca. Unfortunately, probes for *rsmB* were not included on the microarray. Therefore, we used two complimentary approaches [Q-RT-PCR (Fig. 5A) and Northern blotting (Fig. S6B)] to independently monitor *rsmB* transcript levels in virulence-inducing growth conditions. In the wild-type, the RsmA : rsmB ratio was high during exponential phase growth but became inverted upon entry into stationary phase. This was due to a 2.5-fold decline in RsmA protein levels coupled with a marked increase in rsmB levels, as assessed using both Q-RT-PCR and Northern blotting. This was not the case in the Δ*expI* mutant; upon entry into the stationary phase, RsmA levels remained high and but rsmB levels rose only slightly [as assessed by Q-RT-PCR (Fig. 5A)] or even became marginally depressed [as assessed by Northern blotting (Fig. S6B)]. Presumably, these changes in *rsmB* expression were insufficient to fully titrate the large amount of 'free' RsmA present in the Δ*expI* mutant. However, a more profound change was seen in the Δ*relA* mutant. As with the wild-type, RsmA levels declined *c.* twofold following entry into the stationary phase, but this was not accompanied by any increase in rsmB levels (indeed, in the Northern analysis, rsmB was barely detectable in the stationary phase samples). It therefore seems

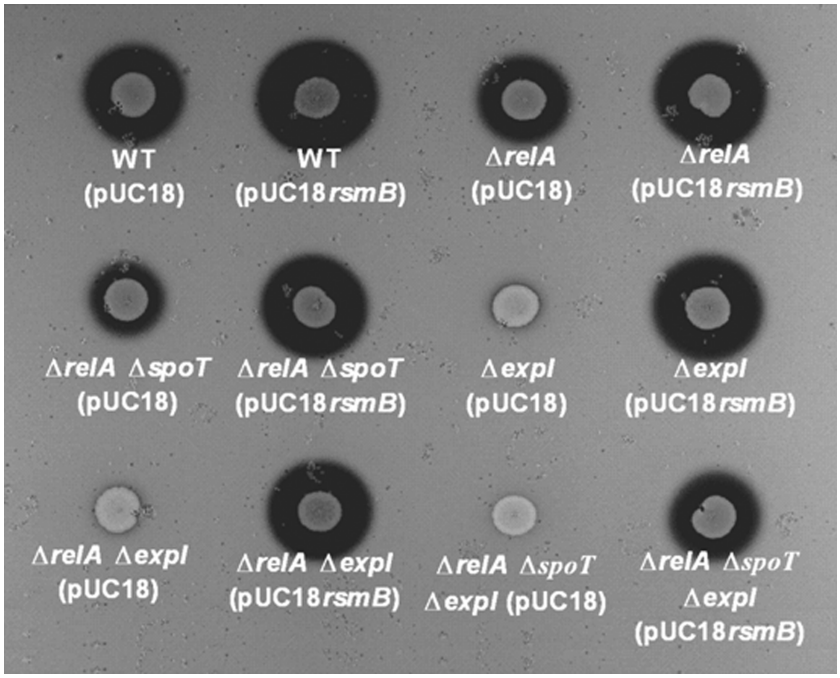

Fig. 6. Inhibition of RsmA by rsmB restores exoprotease production to mutants defective in (p)ppGpp production and QS. Exoprotease production by wild type, the Δ*relA* mutant, the Δ*expI* mutant, the Δ*relA*Δ*spoT* mutant, the Δ*relA*Δ*expI* mutant and the Δ*relA*Δ*spoT*Δ*expI* mutant, each containing either pUC18*rsmB* or pUC18 alone, as indicated.

that (p)ppGpp impinges on production of PCWDE primarily by regulating *rsmB* transcription.

(p)ppGpp and OHHL-dependent modulation of the RsmA : rsmB ratio leads to altered secretion of PCWDE

Our data suggest that the virulence defect associated with (p)ppGpp deficiency arises due to the inability of the Δ*relA* and Δ*relA*Δ*spoT* strains to induce *rsmB* expression. To test this possibility, we wanted to see whether expression of *rsmB in trans* from its own promoter on the high copy number plasmid, pUC18 (Yanisch-Perron *et al*., 1985) would restore protease production in the Δ*relA*, Δ*relA*Δ*spoT*, Δ*expI* and Δ*relA*Δ*spoT*Δ*expI* mutants. Strikingly, in all cases, it did (Fig. 6). Furthermore, expression of *rsmB in trans* from the medium copy plasmid, pACYC177 (Rose, 1988) led to increased Pel production in liquid culture by the wild type, presumably due to inhibition of RsmA activity (Fig. S7A). Similarly, expression of *rsmB in trans* also increased Pel production by the Δ*relA* and Δ*expI* mutants to levels close to that of the wild type/empty vector (Fig. S7B). This demonstrates that inhibition of RsmA activity by rsmB is sufficient to enable robust Pel production, even in the absence of RelA-derived (p)ppGpp or ExpI-derived OHHL.

Discussion

It has been known for many years that quorum sensing is a major regulator of virulence in *Pectobacterium* sp. (Jones *et al*., 1993; Pirhonen *et al*., 1993; reviewed by Smith *et al*., 2006; Barnard *et al*., 2007). It is now clear that (p)ppGpp is also an important regulator of virulence in Pca (Wang *et al*., 2007) and like QS, is a promising therapeutic target to prevent infections (Galloway *et al*., 2011). However, as far as we are aware, no previous workers have demonstrated a link between QS and (p)ppGpp signalling. In the current study, we show that these two major regulatory pathways are integrally linked and that (p)ppGpp and OHHL are *simultaneously* required for optimal PCWDE production i.e. the bacterial population must be nutrient limited and quorate in order to express virulence genes. If either signal is absent, PCWDE production is impaired. We further show that OHHL and (p)ppGpp effect this outcome because they independently regulate the level of 'free' RsmA, which appears to be the central node in a 'logic AND-gate' to control virulence (Fig. 7). While QS acts to decrease the synthesis of RsmA, (p)ppGpp is required to stimulate synthesis of the RsmA antagonist, rsmB. Indeed, rsmB-mediated antagonism of RsmA was sufficient to restore PCWDE production to Δ*expI* or Δ*relA*Δ*spoT* mutants (Fig. 6 and Fig. S7).

In the light of our results, we propose that the cell employs a 'belt-and-braces' mechanism for virulence control to ensure that metabolically costly exoenzyme secretion does not occur – even if the population is quorate – unless the culture is also nutrient-limited. This strategy makes good evolutionary sense; after all, why redirect valuable resources towards the biosynthesis and secretion of exoenzymes if the population is not nutrient-limited? In essence, by ensuring that *rsmB* expression is controlled by (p)ppGpp, the 'brakes' are only removed from

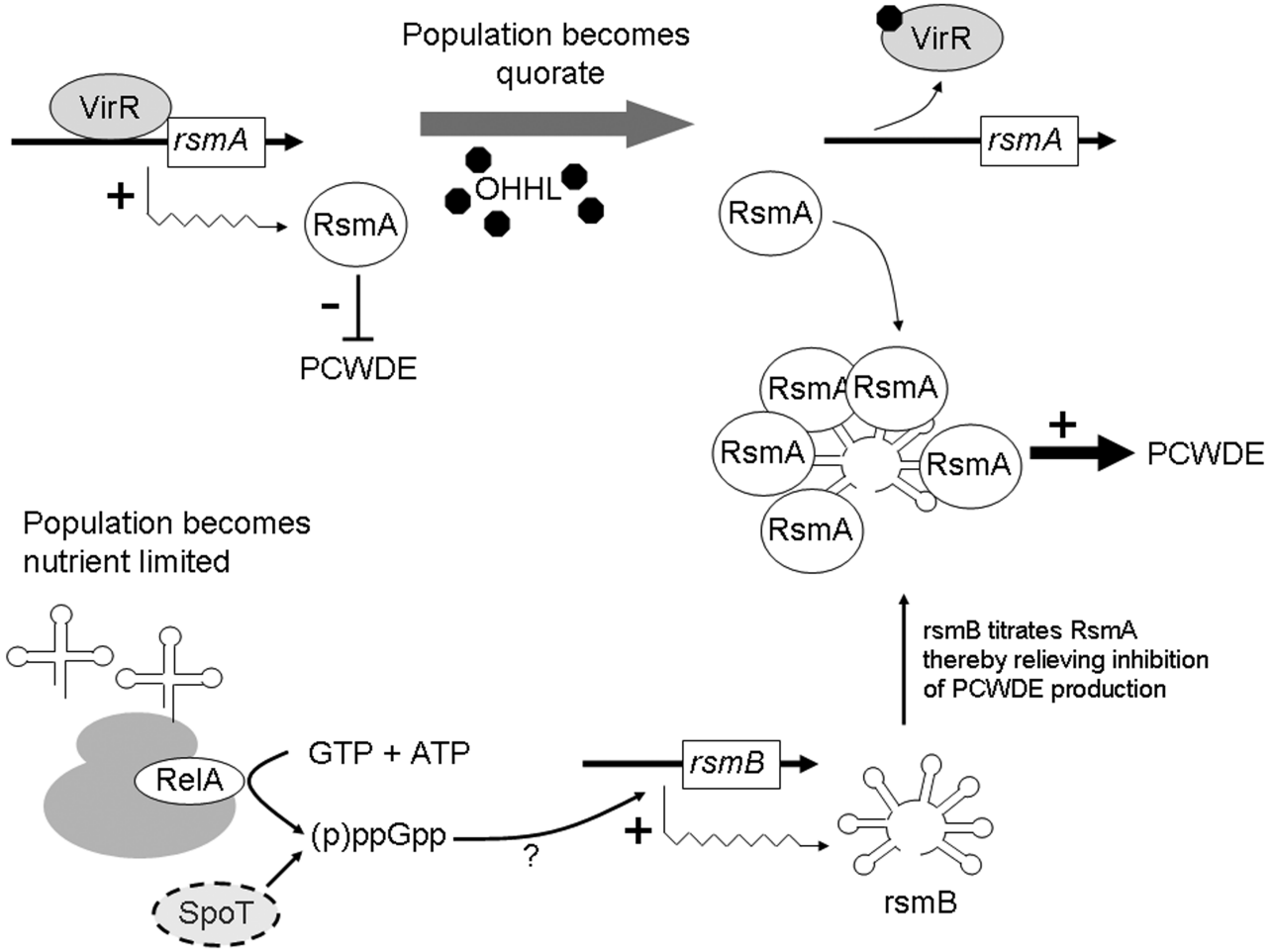

Fig. 7. Proposed model of how OHHL and (p)ppGpp control of virulence. **Upper arm.** At low cell densities (i.e. in the pre-quorate period) the LuxR homologue, VirR, activates transcription of *rsmA*. The RsmA protein targets PCWDE-encoding transcripts for degradation, thereby suppressing virulence. However, when the cell density is high, OHHL binds to VirR causing it to undergo a conformational change. In the ligand-bound conformation, the affinity of VirR for the *rsmA* promoter is diminished, and *rsmA* transcription decreases (Cui *et al*., 2006; Sjöblom *et al*., 2006). This is why RsmA levels are constitutively high in the *expI* mutant (Fig. 5A). **Lower arm.** RelA and SpoT synthesize (p)ppGpp when the culture becomes nutrient limited. (p)ppGpp directly or indirectly activates *rsmB* transcription. The rsmB binds and sequesters RsmA, thereby de-repressing PCWDE production. In the absence of rsmB production (i.e. in a *relA* or *relA spoT* mutant) PCWDE synthesis remains suppressed by RsmA, even in the presence of excess OHHL (Fig. S1D). The reciprocal effects of QS and (p)ppGpp on the RsmA/rsmB node would be expected to lead to a highly cooperative switch that is sensitive to both the physiological state of the cells and their population cell density.

exoenzyme production once the population has depleted more easily assimilated nutrients from the environment. Our model accounts for a number of previously unexplained observations. For example, the low steady-state levels of (p)ppGpp in exponentially growing cells explains why PCWDE production cannot be prematurely induced through the provision of exogenous OHHL to wild-type cultures early on in the growth curve. Moreover, the rapid accumulation of (p)ppGpp upon exiting exponential growth explains why QS-dependent virulence factor production generally peaks at this point. An intriguing corollary of these features is that the timing and magnitude of virulence factor production would be expected to vary depending upon the composition of the growth medium (which, in turn, will influence the steady-state levels of (p)ppGpp, RsmA and rsmB in the cell).

We noted that OHHL levels are depressed in the absence of (p)ppGpp (Fig. 2). Although this depression was clearly not responsible for the main virulence defect associated with the Δ*relA*Δ*spoT* mutant, it may nevertheless be an important evolutionary refinement. For example, if the absence of (p)ppGpp leads to lower OHHL production, then the converse should be true when (p)ppGpp accumulates. Indeed, van Delden and colleagues have shown that overexpression of *relA* in *P. aeruginosa* prematurely advances production of AHLs

(van Delden *et al*., 2001). This increased AHL production due to (p)ppGpp would be expected to further suppress *rsmA* gene expression and thereby amplify the impact of rsmB.

The effects of (p)ppGpp and QS were clearly separable, in spite of the fact that QS was influenced by (p)ppGpp. For example, the virulence phenotypes associated with the (p)ppGpp null mutant could not be bypassed by adding exogenous OHHL. This suggests that in spite of the complexity introduced by interactions between the two signalling pathways, the two arms (QS/RsmA- and (p)ppGpp/rsmB-dependent) of the model shown in Fig. 7 are essentially distinct. This does not preclude a degree of cross-talk between the two systems though. Indeed, in the case of *E. coli*, Edwards *et al*. recently showed that ppGpp accumulation was enhanced in a *csrA* mutant (CsrA is the *E. coli* homologue of RsmA) because CsrA represses the expression of *relA* (Edwards *et al*., 2011). If this were also the case in Pca, it would mean that ppGpp levels would be dictated (at least, to some extent) by QS, since *rsmA* expression is primarily regulated by the latter. However, we found that *relA* mRNA levels were essentially unaffected in our microarray datasets, even in the *expI* mutant (where RsmA is constitutively overexpressed). This does not preclude RsmA controlling RelA expression post-translationally as previously reported in *E. coli* (Edwards *et al*., 2011); it is formally possible that *relA* mRNA levels can remain unchanged while the rate of RelA translation is impeded by RsmA binding to the mRNA transcript. We note that like RelA and SpoT, the CsrA/csrB system also reports on nutritional status: CsrA negatively regulates anabolic pathways such as glycogen synthesis and gluconeogenesis, and promotes carbon skeleton catabolism. Consequently, a regulatory strategy involving (p)ppGpp and the Rsm system may have a common evolutionary rationale. Viewed from this perspective, the production of PCWDE is simply a specialized starvation response – a scavenging mechanism that comes into play when other resources become scarce.

A key issue that we are currently investigating further is how (p)ppGpp might regulate *rsmB* expression. Several possibilities suggest themselves. Based on the *E. coli* paradigm, (p)ppGpp may activate *rpoS* transcription (or stabilize the RpoS protein) and it is this that redirects the RNA polymerase (RNAP) to transcribe *rsmB*. However, this seems very unlikely given that an *rpoS* mutant of Pca strain 1043 had an identical gene expression profile to the wild-type. Alternatively, in the presence of ppGpp and some other alternative σ factor, RNAP may target the *rsmB* promoter directly. In this regard, we note that the region between the −10 hexamer and the +1 site (known as the discriminator region) in the *rsmB* promoter sequence is AT-rich, consistent with this being a (p)ppGpp-activated gene. A third possibility is that ppGpp indirectly promotes *rsmB* expression. If so, there is a good chance that some of the genes identified in our transposon 'bypass' screen may be involved. Indeed, we are currently testing these mutants further to see whether any of them are affected in *rsmB* expression. One obvious candidate in this regard is the *hexA* gene. HexA is a LysR-type transcriptional regulator that is known to repress *rsmB* expression (Mukherjee *et al*., 2000). There are two contiguous copies of *hexA* in the Pca genome (denoted *hexA1* and *hexA2*) and both are *c*. twofold upregulated in the Δ*relA* mutant during the stationary phase (GEO Series Accession Number GSE50468) suggesting that indeed, (p)ppGpp normally represses *hexA* expression. However, the *hexA* genes were even more strongly induced in the *expI* mutant, which is inconsistent with HexA acting on *rsmB* independently of (p)ppGpp.

A second gene identified in our transposon screen was *hexY* (also known as *rsmC*), a major repressor of exoenzyme production in Pca (Shih *et al*., 1999). It has been reported that in Pcc strain Ecc71, HexY controls *rsmB* transcription by binding to- and sequestering the $FlhD_4C_2$ protein complex (Chatterjee *et al*., 2009). The HexY-bound $FlhD_4C_2$ complex can no longer activate transcription of *fliA* (the flagellum-specific sigma factor, σ^{28}), *hexA* or *gacA* (another global regulator of virulence in Pca) (Cui *et al*., 2008; Chatterjee *et al*., 2009). Importantly, and consistent with our finding that Tn insertions in *hexY* can bypass the defect in virulence bought about by loss of (p)ppGpp, Cui *et al*. found that the relative levels of rsmB are increased in an Ecc71 *hexY* mutant (Cui *et al*., 1999). Alternatively, (p)ppGpp may regulate *rsmB* transcription by influencing GacS (also known as BarA, ExpS or RpfA) and GacA (also known as UvrY, SirA or ExpA) signalling (Heeb and Haas, 2001). GacA and GacS have been shown to activate transcription of *rsmB* in Pcc (Cui *et al*., 2001). (p)ppGpp regulation of *rsmB* transcription *via* the GacA/GacS system would be analogous that described for *csrB* expression *via* BarA and UvrY reported in *E. coli* (Edwards *et al*., 2011). As activators of virulence, *gacS* and *gacA* mutants would not have been isolated in our transposon screen; hence their role in (p)ppGpp dependent control of *rsmB* transcription remains a possibility.

As none of the bypass mutants (except the *rsmA* mutant, AE9) led to full restoration of protease production, we hypothesise that the regulation of *rsmB* expression is likely to be multifactorial, with many gene products contributing incrementally (and differentially, depending on growth conditions). This notwithstanding, all of the bypass mutants identified in this study are, by definition, repressors of PCWDE production and we cannot exclude the possibility that there also exist (p)ppGpp-dependent *activators* of *rsmB* expression. Current efforts are aimed at investigating these possibilities.

Table 1. Strains and plasmids used in this study.

	Description	Reference
Strain		
SCRI1043	Wild type *Pectobacterium atrosepticum* isolate	Bell *et al.* (2004)
SB1026	Δ*relA::cat* derivative of SCRI1043	This study
SB1031	Δ*expI::kan* derivative of SCRI1043	This study
SB1038	Δ*relA::cat*, Δ*spoT* derivative of SCRI1043	This study
SB1046	Δ*rpoS::aadA1* derivative of SCRI1043	This study
SB1026/31	Δ*relA::cat*, Δ*expI::kan* derivative of SCRI1043	This study
AE1	*eca0137::*miniTn5 Sm/Sp derivative of SB1038	This study
AE3	*eca3624::*miniTn5 Sm/Sp derivative of SB1038	This study
AE5	*eca4456::*miniTn5 Sm/Sp derivative of SB1038	This study
AE6	*eca4381::*miniTn5 Sm/Sp derivative of SB1038	This study
AE7	*eca3460::*miniTn5 Sm/Sp derivative of SB1038	This study
AE8	*eca1385::*miniTn5 Sm/Sp derivative of SB1038	This study
AE9	*rsmA::*miniTn5 Sm/Sp derivative of SB1038	This study
AE10	*eca0610::*miniTn5 Sm/Sp derivative of SB1038	This study
AE11	*eca1172::*miniTn5 Sm/Sp derivative of SB1038	This study
AE15	*hexY::*miniTn5 Sm/Sp derivative of SB1038	This study
AE16	*eca0400-0401::*miniTn5 Sm/Sp derivative of SB1038	This study
AE17	*eca0177::*miniTn5 Sm/Sp derivative of SB1038	This study
AE18	*hexY::*miniTn5 Sm/Sp derivative of SB1038	This study
AE19	*hexA2::*miniTn5 Sm/Sp derivative of SB1038	This study
AE22	*eca0577::*miniTn5 Sm/Sp derivative of SB1038	This study
Plasmids		
pUC18	High copy number cloning vector (Amp^R)	Yanisch-Perron *et al.* (1985)
pUC18-*rsmB*	pUC18 expressing *rsmB*	This study
pWKS30	Low copy number cloning vector (Amp^R)	Wang and Kushner (1991)
pSB1009	pWKS30 expressing *spoT*	This study
pWKS30-*rsmA*	pWKS30 expressing *rsmA*	This study

Experimental procedures

Bacterial strains and growth conditions

Strains used in this study are listed in Table 1. Unless otherwise stated, *P. atrosepticum* and *E. coli* strains were grown in Luria Broth (LB; 10 g l^{-1} tryptone, 5 g l^{-1} yeast extract and 5 g l^{-1} NaCl) at 30°C or 37°C respectively. Where necessary, antibiotics were used to maintain plasmids at the following concentrations, carbenicillin 50 μg ml^{-1} and tetracycline 10 μg ml^{-1}. When assaying for Pel activity, *P. atrosepticum* was grown in PMB [1 g l^{-1} yeast extract, 1 g l^{-1} $(NH_4)_2SO_4$, 1 mM $MgSO_4$, 0.5% v/v glycerol, 5 g l^{-1} polygalacturonic acid, 7 g l^{-1} K_2HPO_4 and 2 g l^{-1} KH_2PO_4 (pH 7.0)] and grown with vigorous aeration at 25°C. When indicated, the PMB was supplemented with 10 g l^{-1} vitamin-free casein acid hydrolysate.

Construction of Pectobacterium atrosepticum *strains*

(i) SB1038 (ΔrelA::cat ΔspoT). The Δ*relA*::cat *P. atrosepticum* strain SB1026 was constructed by marker exchange as described previously (Wang *et al.*, 2007). To construct the Δ*relA*::cat Δ*spoT* double mutant (SB1038) the *spoT* locus was PCR amplified (Phusion, NEB) from *P. atrosepticum* SCRI1043 genomic DNA with primers SBP3 and SBP6 and digested with SpeI (a complete list of the oligonucleotide primers used in this study is provided in Table S1). The resulting PCR product was gel-purified and cloned into the SpeI/SmaI sites of pBluescript SKII+ to yield plasmid pSB1001. This plasmid was digested with PmlI and BstZ171 to generate an in-frame deletion in the *spoT* reading frame. The resulting DNA fragment was self-ligated to generate pSB1012, which was then cloned into the XmaI/SpeI sites of pKNG101 (Kaniga *et al.*, 1991) to yield pSB1024. To generate the Δ*spoT* mutation in SB1026, a triparental mating procedure was used (Grinter, 1983). Briefly, pSB1024 was transferred from *E. coli* CC118λpir into SB1026 (Δ*relA*::cat) using *E. coli* HH26 (pNJ5000) as a helper. *P. atrosepticum* co-integrants were isolated by plating the mating mixture onto minimal medium supplemented with 50 μg ml^{-1} streptomycin. A single colony was picked, grown overnight in LB and then spread on an LB plate supplemented with 10% w/v sucrose. Surviving $strep^S$ colonies were PCR-screened for the Δ*spoT* mutation, which was verified by sequencing across the boundaries of the deletion. A verified colony was selected and denoted SB1038.

(ii) SB1031 (ΔexpI::kan). Lambda red mutagenesis (Datsenko and Wanner, 2000) with primers SBP23 and SBP24 was used to introduce the kan^R cassette from plasmid pKD4 into the *expI* ORF. To generate a more useful generic construct for allelic exchange, the chromosomal Δ*expI*::kan and its flanking DNA was PCR-amplified using primers SBP25 and SBP28. The PCR fragment was cloned into the XmaI/SpeI sites of pKNG101 and the construct was maintained in *E. coli* CC118λ*pir*. Marker exchange was then carried out as described above to generate strains SB1031 (Δ*expI*::kan), SB1026/31 (Δ*relA*::cat Δ*expI*::kan) and SB1038/31 (Δ*relA*::cat Δ*spoT* Δ*expI*::kan).

(iii) SB1046 (ΔrpoS::Sm/Sp). The sequence 5′ of *rpoS* was PCR-amplified from *P. atrosepticum* genomic DNA using primers SBP9 and SBP42 to generate PCR product 1 and the sequence 3′ of *rpoS* was PCR-amplified using SBP12 and SBP44 to generate PCR product 2. The spec/strep^R cassette from plasmid pCL1920 (Lerner and Inouye, 1990) was PCR-amplified using primers SBP43 and SBP45. PCR product 1 was digested with BamHI and BsaI, PCR product 2 was digested with SpeI and BsaI and PCR product 3 was digested with BsaI. Following a cleanup step, a four-way ligation was used to clone all 3 PCR products into the BamHI/XbaI sites of pBluescript SKII+. The resulting construct (pSB1008) possessed the spec/strep^R cassette from pCL1920 flanked by the upstream and downstream regions of the *rpoS* locus. Plasmid pSB1008 was digested with BamHI/NotI and the Δ*rpoS*::spec/strep^R fragment was subcloned into the same sites on pKNG101 to yield pSB1016. Marker exchange was carried out as described above to yield strain SB1046 (Δ*rpoS*:: spec/strep^R).

Construction of plasmids

Plasmids are listed in Table 1. Plasmid pWKS30*spoT* was constructed by PCR-amplifying *spoT* from *P. atrosepticum* genomic DNA using primers SBP89 and SBP90. The resulting amplicon was cloned into the BamHI/XbaI sites of pWKS30 (Wang and Kushner, 1991). Plasmid pUC18*rsmB* was constructed by PCR-amplifying *rsmB* and its 5′ upstream DNA using primers SBP287 and SBP288. The resulting amplicon was cloned into the BamHI/HindIII sites of pUC18 (Yanisch-Perron *et al*., 1985). Plasmid pACYC177*rsmB* was constructed by digesting pUC18*rsmB* with HindIII/SmaI and ligating the resulting fragment to HindIII/BstZ171-cleaved pACYC177 (Rose, 1988). Plasmid pWKS30*rsmA* was constructed by PCR-amplifying *rsmA* and its flanking DNA from *P. atrosepticum* genomic DNA using primers SBP121 and SBP122. The PCR product was digested with MfeI and the fragment containing *rsmA* and its promoter was cloned into the SmaI/EcoRI sites of pWKS30. All plasmids were introduced into *E. coli* and *P. atrosepticum* strains by electroporation.

Transposon mutagenesis

Overnight cultures of SB1038 and SM10λ*pir* (pUT-miniTn5 spec/strep) (de Lorenzo *et al*., 1990) were spotted on top of each other on LB plates and incubated overnight at 30°C. The colonies were resuspended in LB and plated onto caseinase assay plates (13 g l^{-1} Difco™ Nutrient Broth, 16 g l^{-1} agar and 10 g l^{-1} skimmed milk) supplemented with 50 μg ml^{-1} streptomycin and 12.5 μg ml^{-1} chloramphenicol. The plates were incubated for 48 h at 25°C. Transposon mutants with increased exoprotease activity were identified as having enlarged halos relative to the SB1038 progenitor. Southern blots were used to confirm that each mutant only contained one transposon insertion and the sites were identified using a random primed PCR sequencing method described previously (Jacobs *et al*., 2003).

HPLC analysis of (p)ppGpp

Pectobacterium atrosepticum strains were grown in 50 ml of LB to an OD_{600} ~ 0.7 and then pelleted at 3220 *g* for 10 min. The cell pellet was immediately resuspended in 50 ml of minimal media and incubated with shaking at 25°C for 10 min. Following this, the cells were pelleted and the pellet was immediately resuspended in 5 ml of 1 M formic acid. After 1 h on ice, cellular debris was sedimented and the supernatant was filter-sterilised [0.22 μm pore size (Millipore)]. The filtrate was freeze dried and resuspended in 200 μl of distilled water. The nucleotide-enriched samples were analysed by HPLC as described previously (Strauch *et al*., 1991) and quantified by comparison of the peak heights with a ppGpp standard.

Protease plate assays

Protease assays were performed as described previously (Pemberton *et al*., 2005). Briefly, the plates contained gelatin, which is hydrolysed by secreted proteases. The zones of hydrolysed protein are revealed after incubation of the plates (typically at 25°C for 2 days) by flooding each plate with saturated ammonium sulphate solution. The zones of hydrolysed protein remain clear against an opaque background of non-hydrolysed protein.

Luciferase bioassay of OHHL

Serial dilutions of *P. atrosepticum* culture supernatants were incubated with the *E. coli* sensor strain, JM109 (pSB401) as described previously (Winson *et al*., 1998). After 2 h incubation at 37°C the luminescence was measured with an Anthos Lucy 1 Luminometer. OHHL levels are reported in the form of relative light units.

Pectate lyase liquid culture assays

Pectate lyase activity was assayed as described previously (Starr *et al*., 1977). The rates of polygalacturonic acid breakdown were measured spectrophotometrically at 37°C and are expressed as ΔA_{235nm} ml^{-1} min^{-1} $OD_{600}{}^{-1}$.

Amino acid analysis of culture supernatants

This was carried out using quantitative chromatographic resolution of ninhydrin derivatives of amino acids in the indicated culture supernatants. The analysis was done by the Protein and Nucleic Acid Analysis facility in the Dept Biochemistry (Cambridge, UK).

Microarray analysis

RNA was extracted from three independent cultures of each strain [the wild-type and SB1026 (Δ*relA*), SB1031 (Δ*expI*), and SB1046 (Δ*rpoS*)] that had been grown with good aeration in PMB. Samples were harvested for RNA extraction after 6 h or 14 h growth, as indicated. RNA was extracted using the Qiagen RNeasy midiprep kit (Qiagen) as per the manufacturer's instructions for Gram-negative bacterial samples. Cy3-labelled cDNA was generated with reverse transcriptase (AffinityScript Multiple Temperature Reverse Transcriptase, Agilent) and Cy3-labelled dCTP (GE Healthcare Life Sci-

ences). The labelled cDNA was hybridized to custom 4 × 72k microarray slides (NimbleGen) as per the manufacturer's instructions and scanned using a Genepix 4000B dual laser scanner (Axon). Raw intensity values were obtained using NimbleScan. GeneSpring™ (Silicon Genetics) was used to normalize the data to that of the wild type values and to apply a parametric filter using ANOVA and the Benjamini and Hochberg multiple testing correction to adjust individual *P*-values using a false discovery rate (FDR) of 0.05.

RT-PCR

Superscript™ II Reverse Transcriptase (Invitrogen) was used to generate cDNA from *P. atrosepticum* RNA using random hexamer primers (Fermentas) as per the manufacturer's instructions. RT-PCR was performed using gene specific primers (Table S1) and Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen) according to the manufacturer's instructions. C_t values obtained for *rsmB* transcripts were normalised relative to the wild type 6 h values. The *rsmB* expression data were quantified relative to that of the *eca0762* transcript, which showed unaltered expression in the wild type, Δ*relA* mutant and Δ*expI* mutant in the microarray data (GEO Series Accession Number GSE50468).

Western blotting

Aliquots of culture were removed from cultures grown in the indicated medium and the cells were pelleted (4000 *g*, 5 min). The cell pellets were resuspended to the same final OD_{600} in Tris-NaCl lysis buffer (0.1 M Tris-HCl, 0.1 M NaCl, pH 6.8). We have found that this method yielded equivalent protein concentrations across different samples. After lysing the cells in SDS sample buffer, whole-cell lysates (20 µl) were separated on 12% SDS-polyacrylamide gels, transferred to polyvinylidene fluoride (PVDF) membrane and analysed by Western blotting. The primary anti-RsmA antibody was preabsorbed against an acetone powder extract of the *rsmA* mutant (AE9) before use. The anti-RsmA antibody was used at 1:10 000 dilution. ECL peroxidase labelled goat anti-rabbit antibodies (Sigma) were used as secondary antibodies (1:10 000 dilution). Blots were developed using Immobilon™ Western Chemiluminescent HRP Substrate (Millipore). All Western analyses were repeated on independent cultures at least three times. Representative results are shown. The Western blots were quantified using ImageJ software.

Northern blotting

Northern Blots were performed largely as described Majdalani *et al*. (1998) with the following modifications. RNA samples (3 µg) were heated in glyoxal sample buffer (Lonza) to 65°C for 15 min and separated on a 1.5% agarose gel for 120 min at 100 V. The RNA was transferred to Nylon positively charged membrane (Roche) overnight by capillary action. RNA was cross-linked with a UV cross-linker (3 UV) for 3 min on each side. The blots were washed in 2× SSC buffer for 5 min. Pre-hybridization and hybridization steps were carried out using Church's Buffer (0.5 M $NaPO_4$ pH 7.2, 7% SDS, 1 mM EDTA) for 1 h at 42°C. ^{32}P-labelled probes were added during the hybridization step. Hybridization steps were carried out at 42°C for the 5S rRNA probe and 43.5°C for the rsmB probe. Oligonucleotide probe oREM450 (5′-CGACATTCCTAATCGGCTCTCGG-3′) was used to detect rsmB and oREM443 (5′-CTACGGCGTTTCACTTCTGAG TTC-3′) was used to detect 5S rRNA. Signals were visualized using a Typhoon Phosphoimager (GE Healthcare) and quantified using ImageJ. Blots were stripped of bound probes by treatment with 1% SDS for 15 min at 85°C or until no bound probe was detected.

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