The PhoBR two-component system regulates antibiotic biosynthesis in Serratia in response to phosphate

Tamzin Gristwood², Peter C Fineran², Lee Everson¹, Neil R Williamson¹ and George P Salmond*²

Address: ¹Department of Biochemistry, University of Cambridge, Cambridge, CB2 1QW, UK and ²Department of Microbiology & Immunology, University of Otago, PO Box 56, Dunedin, New Zealand

Email: Tamzin Gristwood - tamzin.gristwood@otago.ac.nz; Peter C Fineran - peter.fineran@otago.ac.nz;
Lee Everson - le212@mole.bio.cam.ac.uk; Neil R Williamson - nrw26@mole.bio.cam.ac.uk; George P Salmond* - gpcs@mole.bio.cam.ac.uk

* Corresponding author

Abstract

Background: Secondary metabolism in Serratia sp. ATCC 39006 (Serratia 39006) is controlled via a complex network of regulators, including a LuxIR-type (SmaIR) quorum sensing (QS) system. Here we investigate the molecular mechanism by which phosphate limitation controls biosynthesis of two antibiotic secondary metabolites, prodigiosin and carbapenem, in Serratia 39006.

Results: We demonstrate that a mutation in the high affinity phosphate transporter pstSCAB-phoU, believed to mimic low phosphate conditions, causes upregulation of secondary metabolism and QS in Serratia 39006, via the PhoBR two-component system. Phosphate limitation also activated secondary metabolism and QS in Serratia 39006. In addition, a pstS mutation resulted in upregulation of rap. Rap, a putative SlyA/MarR-family transcriptional regulator, shares similarity with the global regulator RovA (regulator of virulence) from Yersina spp. and is an activator of secondary metabolism in Serratia 39006. We demonstrate that expression of rap, pigA-O (encoding the prodigiosin biosynthetic operon) and smal are controlled via PhoBR in Serratia 39006.

Conclusion: Phosphate limitation regulates secondary metabolism in Serratia 39006 via multiple inter-linked pathways, incorporating transcriptional control mediated by three important global regulators, PhoB, SmaR and Rap.

Background

Phosphate is an essential component of numerous biomolecules. Therefore, the control of intracellular phosphate concentrations is vital for bacterial survival. At least two major systems are involved in managing intracellular concentrations of inorganic orthophosphate (P_i), the preferred primary source of phosphate [1]. When P_i is abundant, the low affinity Pit transporter appears to be primarily responsible for P_i uptake [2-4]. When P_i becomes limited, the high affinity Pst transport system (PstSCAB-PhoU) is activated, and takes over as the predominant P_i uptake system [5-8].

In Escherichia coli and other Enterobacteriaceae, the cellular response to P_i availability is mediated via the PhoBR two-component system. Under conditions of P_i limitation, the sensor histidine kinase PhoR is autophosphorylated [9]. PhoR then activates its cognate response
regulator, PhoB [9], which in turn activates expression of multiple genes, termed the Pho regulon, via direct binding to a conserved Pho box sequence found overlapping - 35 regions in target gene promoters [10-12]. In E. coli, the Pho regulon is believed to consist of approximately 30 genes involved in the adaptation to survival under low P\textsubscript{i} conditions, including \textit{pstSCAB-phoU} and \textit{phoBR} [1]. Phosphate regulation is controlled via similar mechanisms in \textit{Bacillus subtilis} and \textit{Streptomyces} species, although the consensus Pho boxes are different in each system [13,14]. Mutations in the \textit{pstSCAB-phoU} operon result in constitutive activation of PhoR and hence, constitutive phosphorylation of PhoB [15,16]. Therefore, \textit{pst} mutants are proposed to mimic low P\textsubscript{i} conditions.

P\textsubscript{i} has been found to negatively regulate the biosynthesis of antibiotics and other secondary metabolites in multiple bacterial species (reviewed in [17]). However, the complex molecular mechanisms underlying the P\textsubscript{i} mediated regulation of secondary metabolism are not well characterised. In this study we investigate the role of the PhoBR two-component system, and P\textsubscript{i} availability, on the regulation of antibiotic production in the Gram-negative Enterobacteriaceae, \textit{Serratia} sp. ATCC 39006 (\textit{Serratia} 39006). \textit{Serratia} 39006 synthesises the red, tripyrrole antibiotic, prodigiosin (Pig; 2-methyl-3-pentyl-6-methoxy-prodigiosin) [18]. The natural physiological role of Pig in the producing organism may be as an antimicrobial agent [19]. In addition, Pig is of clinical interest due to the observed anticancer and immunosuppressive properties of this compound [20-22]. \textit{Serratia} 39006 also produces the \textit{\beta}-lactam antibiotic, carbapenem (Car; 1-carbapen-2-\textit{\epsilon}-em-3-carboxylic acid) [23,24]. Both the Pig and Car biosynthetic gene clusters have been characterised (\textit{pigA-O} and \textit{carA-H}, respectively) [25,26].

Production of secondary metabolites in \textit{Serratia} 39006 is controlled by a hierarchial network of regulators [27]. This includes a LuxIR-type quorum sensing (QS) system (SmaIR) [25,28,29], which allows gene expression to be regulated in response to cell density via the production and detection of low molecular weight signal molecules [30]. In \textit{Serratia} 39006, the N-acyl homoserine lactone (AHL) synthase Smal produces two signalling molecules, N-butanoyl-L-homoserine lactone (BHL) and N-hexanoyl-L-homoserine lactone (HHL), with BHL being the major product [25]. At low cell density, SmaR acts as a transcriptional repressor of target genes [28,29]. At high cell density, and hence high BHL/HHL levels, SmaR binds BHL/HHL, resulting in decreased DNA-binding affinity with a consequent alleviation of repression. QS controls secondary metabolism in \textit{Serratia} 39006 via at least four other regulatory genes (\textit{carR}, \textit{pigQ}, \textit{pigR} and \textit{rap}) [28,29]. The putative SlyA/MarR-family transcriptional regulator, Rap (regulator of antibiotic and pigment), is an activator of Pig and Car production in \textit{Serratia} 39006 [31]. Rap shares similarity with the global transcriptional regulator RovA (regulator of virulence) from \textit{Yersinia} spp. [32-34]. More than 20 additional genes have been shown to regulate secondary metabolism in \textit{Serratia} 39006, and these are predicted to be responding to additional environmental stimuli [19,27,35,36].

Previously, we demonstrated that, in \textit{Serratia} 39006, mutations within genes predicted to encode homologues of the \textit{E. coli} PstSCAB-PhoU phosphate transport system resulted in over-production of both Pig (10-fold) and Car (2-fold), at the level of transcription of the biosynthetic genes [29]. In this study we investigate further the molecular mechanism by which these effects are occurring. We demonstrate that secondary metabolism in \textit{Serratia} 39006 is upregulated in response to mutations in PstSCAB-PhoU or P\textsubscript{i} limitation, via the PhoBR two-component system. In addition, we provide evidence that expression of the \textit{smaI}, \textit{pigA} and \textit{rap} genes are activated via PhoBR in \textit{Serratia} 39006. Hence, we propose a model in which P\textsubscript{i} limitation increases secondary metabolism in \textit{Serratia} 39006 via multiple, inter-linked pathways, incorporating the global transcriptional regulators PhoB, SmaR and Rap.

**Results**

**Sequence analysis of the \textit{pstSCAB-phoU} operon in \textit{Serratia} 39006**

Previously, \textit{Serratia} 39006 mutants were identified which contained transposon insertions in regions sharing sequence similarity to the \textit{pstS} and \textit{pstA} genes from \textit{E. coli} [29]. DNA sequencing analysis of this region revealed that \textit{Serratia} 39006 possesses a complete \textit{pstSCAB-phoU} operon, the organisation of which is consistent with other enteric bacteria in which a \textit{pst} operon has been identified (Fig. 1A).

The \textit{Serratia} 39006 \textit{pstS} gene was predicted to encode a protein most similar to PstS from the enteric bacteria \textit{Erwinia carotovora} ssp. \textit{atroseptica} SCR1043 (\textit{Eca} 1043) (82\% identity/90\% similarity). The putative protein product encoded by \textit{pstC} shared 90\% identity and 95\% similarity with PstC of \textit{Eca} 1043. The \textit{pstA} gene is predicted to encode a protein most similar to PstA of \textit{Eca} 1043 (87\% identity/92\% similarity). The predicted protein encoded by \textit{pstB} was most similar to PstB of \textit{Eca} 1043 (88\% identity/91\% similarity). Finally, \textit{phoU} was predicted to encode a protein most similar to PhoU of \textit{Eca} 1043 (94\% identity/98\% similarity).

**Isolation and sequence analysis of \textit{phoBR} mutants of \textit{Serratia} 39006**

Mutations in the \textit{pstSCAB-phoU} operon are thought to mimic growth in limiting phosphate, and hence result in constitutive activation of the Pho regulon [15]. We previ-
Pig, Car and AHL production were increased in the pstS mutant [29]. A possible explanation for this effect is that pigA, carA and smaI are regulated via the *Serratia* 39006 Pho regulon.

Random transposon insertions in the phoBR operon were isolated based on their lack of hyperpigmentation when grown on Pi-limiting media. Growth on Pi-limiting media results in increased Pig production in the wild-type (WT; throughout this manuscript WT refers to the LacA parental strain) [29]. Potential phoBR mutants were then checked for their loss of alkaline phosphatase activity (*phoA*, encoding alkaline phosphatase, is a conserved Pho regulon gene [1,37]) and the sequence of the operon was determined, as described in Methods. The phoB gene was predicted to encode a 229 amino acid (aa) protein with highest similarity to PhoB from *Eca* 1043 (96% identity/98% similarity). The phoR gene was located 28 bp downstream of phoB, and was predicted to encode a 440 aa protein sharing the highest degree of similarity to *Eca* 1043 PhoR (87% identity/90% similarity).

**PhoB regulates expression of pstC in Serratia 39006**

In *E. coli*, the *pst* operon is activated via direct binding of PhoB to a conserved Pho box upstream of *pstS* [10-12]. As *Serratia* 39006 is a member of the Enterobacteriaceae, we identified potential Pho boxes based on the *E. coli* consensus sequence. A potential Pho box was identified within the *pstS* promoter region of *Serratia* 39006, centred 122 bp upstream of the *pstS* start codon (Fig. 1B). This suggested that, as could be expected based on regulation of the *pst*-SCAB-phoU genes in other bacteria, the *pstSCAB-phoU* genes in *Serratia* 39006 may be regulated by PhoB. A putative Pho box was also identified upstream of *phoB* (Fig 1B), centred 68 bp upstream of the *phoB* start codon, suggesting that *phoBR* may be auto-regulated via the putative Pho box.

**β-Glucuronidase activity produced from a chromosomal *pstC::uidA* transcriptional fusion**

β-Glucuronidase activity produced from a chromosomal *pstC::uidA* transcriptional fusion was measured in the presence or absence of a secondary mutation in *phoB*. The *pstC::uidA* fusion strain does not contain a functional Pst transporter and is therefore believed to mimic low phosphate conditions. These data showed that, in the presence of functional PhoB, *pstC* was expressed constitutively throughout growth (Fig. 1C). Expression was dramatically reduced following inactivation of *phoB*, indicating that PhoB activates expression of the *pst* operon in *Serratia* 39006 (Fig. 1C).

**Insertions within phoBR abolish upregulation of secondary metabolism and QS in the pstS mutant**

It was hypothesised that the upregulation of Pig, Car and QS in a *Serratia* 39006 *pst* mutant was mediated via the...
PhoBR two-component system. Assessment of Pig, Car and QS phenotypes in \( \text{pstS} \), \( \text{phoB} \) and \( \text{pstS} \), \( \text{phoR} \) double mutants confirmed that \( \text{phoB} \) and \( \text{phoR} \) were responsible for the upregulation of secondary metabolism in a \( \text{pstS} \) mutant background. The \( \text{pstS} \) mutant was increased for Pig (9-fold), Car (2-fold) and AHL (2.5-fold) production compared with the WT (Fig. 2). However, the \( \text{pstS}, \text{phoB} \) and \( \text{pstS}, \text{phoR} \) double mutants were restored to WT levels for Pig, Car and AHL production in LB (Fig. 2). Single \( \text{phoB} \) or \( \text{phoR} \) mutations had no effect on Pig, Car or AHL production (Fig. 2). As it has been previously shown that upregulation of Car in response to a \( \text{pst} \) mutation is mediated via the upregulation of QS [29], we focused on the effects on \( \text{pigA} \) and \( \text{smaI} \) expression for the remainder of this study.

**Insertions within phoBR abolish transcriptional upregulation of pigA and smaI in the \( \text{pstS} \) mutant**

Phenotypic analysis showed that PhoBR are required for the upregulation of secondary metabolism and QS in response to mutation of the \( \text{pstSCAB-phoU} \) operon (described above). To confirm that these effects are exerted at the transcriptional level, primer extension analysis was used to assess levels of the \( \text{pigA} \) and \( \text{smaI} \) transcripts throughout growth in WT, \( \text{pstS} \) mutant and \( \text{pstS}, \text{phoB} \) double mutant strains. The abundance of \( \text{pigA} \) mRNA in the \( \text{pstS}, \text{phoB} \) double mutant was restored to levels similar to those displayed in WT \( \text{Serratia} \) 39006 (Fig. 3A). A chromosomal \( \text{pigA::lacZ} \) transcriptional fusion was used to confirm this result; a 3-fold increase in \( \text{pigA} \) transcription was observed in a \( \text{pstS} \) mutant, this was restored to WT levels following a secondary mutation in \( \text{phoB} \) or \( \text{phoR} \) (Fig. 3B). The upregulation of \( \text{smaI} \) transcription in the \( \text{pstS} \) mutant was also abolished by a \( \text{phoB} \) mutation (Fig. 3C). This is consistent with the hypothesis that PhoB, either directly or indirectly, activates expression of \( \text{pigA} \) and \( \text{smaI} \) in response to constitutive phosphorylation by PhoR as a result of the \( \text{pstS} \) insertion.

**Insertions within \( \text{pstSCAB-phoU} \) result in increased transcription of rap**

A complex network of regulators controls secondary metabolism in \( \text{Serratia} \) 39006 [27]. Therefore, it was possible that the effects on Pig and AHL production, in response to a \( \text{pst} \) mutation, were mediated via one or more of these regulators. To test if the effect on \( \text{smaI} \) and \( \text{pigA} \) transcription was mediated through any of the known secondary metabolite regulators, the expression of chromosomal \( \text{lacZ} \) transcriptional fusions in \( \text{pigP}, \text{pigQ}, \text{pigR}, \text{pigS}, \text{pigT}, \text{pigV}, \text{pigW}, \text{pigX}, \text{pigZ}, \text{rap} \) and \( \text{carR} \) was assessed throughout growth in the presence or absence of a \( \text{pstS::mini-Tn}5\text{Sm/Sp} \) insertion (data not shown). No effect was seen on any of the regulatory genes except for \( \text{rap} \). The expression of \( \text{rap} \) was increased by 1.4-fold in the \( \text{pstS} \) mutant (Fig. 4A). Rap is a putative SlyA/MarR-family transcriptional regulator. As expression of \( \text{rap} \) is known to be regulated by QS [28], the effect of a \( \text{pstC} \) mutation on expression of a \( \text{rap::lacZ} \) transcriptional fusion was assessed in a \( \text{smaI} \) mutant background. A mutation within the \( \text{pstSCAB-phoU} \) operon was still able to activate \( \text{rap} \) transcription (1.5-fold increase), in the absence of functional \( \text{smaI} \), indicating that this effect is via both QS-dependent and -independent pathways (Fig. 4B).

**Figure 2**

The effects of a \( \text{pstS} \) mutation on secondary metabolism and QS are occurring via PhoBR. (A) Pig, (B) Car and (C) AHL production were measured from WT, \( \text{pstS} \) mutant (ROP2), \( \text{phoR} \) mutant (BR1), \( \text{phoB} \) mutant (BR9), \( \text{pstS}, \text{phoR} \) double mutant (PCF60) and \( \text{pstS}, \text{phoB} \) double mutant (PCF59) cells. Production was assayed from cells grown to early stationary phase in LB.
PhoB activates expression from the pigA and rap promoters in an E. coli system

To investigate the control of the pigA, rap and smal promoters in more detail, an E. coli plasmid-based system was used (described in Methods). β-Galactosidase activity was measured from E. coli strains carrying the pigA, rap or smal promoters, inserted upstream of a promoterless lacZ gene (encoded by vectors pTA15, pTA14 or pTG27, respectively) in the presence or absence of Serratia 39006 PhoB, encoded by plasmid pTA74. Transcription from the pigA and rap promoters increased in the presence of pTA74, indicating that these genes may be activated by PhoB (Fig. 5). Unfortunately, the level of expression from the smal promoter was negligible in this system (data not shown). Therefore, it was not possible to determine whether PhoB

Figure 3
A pstS mutation effects transcription of pigA and smal via PhoBR. Primer extension analysis was used to measure the level of (A) pigA or (C) smal transcripts in WT, pstS mutant (ROP2), or pstS, phoB (RBR9) double mutant strains throughout growth in LB. (B) β-Galactosidase activity was measured from a chromosomal pigA::lacZ fusion in an otherwise WT background (NW60), or in pstS (PCF76), phoR (PCF75), phoB (PCF74), pstS, phoR double (PCF78) or pstS, phoB double (PCF77) mutant backgrounds. Activity was assayed from cells grown to early stationary phase in LB.

Figure 4
Expression of rap is activated following mutation of the pstSCAB operon. β-Galactosidase activity was assayed throughout growth from a chromosomal rap::lacZ fusion in (A) an otherwise WT background (RAPL;diamonds and open bars) or a pstS mutant background (PCF45; squares and solid bars), or (B) a smal (ISRL;diamons and open bars) or pstC, smal (TG71; squares and solid bars) mutant background. In both graphs, bars represent β-galactosidase assays and dashed lines represent bacterial growth.
was modulating transcription from the smal promoter. In the *E. coli* system, the degree of activation from both the pigA and rap promoters in the presence of PhoB is comparable with the levels of activation observed using chromosomal pigA::lacZ and rap::lacZ transcriptional fusions as a result of pstS/pstC mutation in *Serratia* 39006 (Fig. 3B & Fig. 4). Putative weak Pho boxes were identified within the promoter regions of pigA and smal, overlapping the predicted -35 sequences and centred 28 bp and 34 bp, respectively, upstream of the transcriptional start sites, which were previously mapped by primer extension [29] (Fig. 1B). A putative weak Pho box was also identified within the rap promoter, centred 148 bp upstream of the rap start codon (Fig. 1B). The presence of putative Pho boxes suggest that PhoB may directly activate expression of pigA, smal and rap, although this has not yet been shown experimentally. In the *E. coli* reporter assays described, it is possible that *Serratia* 39006 PhoB may show activity in the absence of the cognate *Serratia* 39006 histidine kinase, PhoR, due to cross-regulation by non-cognate *E. coli* histidine kinases, or as a result of low level activity of the unphosphorylated *Serratia* 39006 PhoB.

### Figure 5

In *E. coli*, *Serratia* 39006 PhoB can activate expression from the pigA and rap promoters. β-Galactosidase activity was measured from *E. coli* cells grown in LB carrying plasmid pTA15 or pTA14 (containing the pigA or rap promoters respectively cloned upstream of a promoterless lacZ gene) and either an empty vector control (pQE-80L) (solid bar) or pTA74, encoding PhoB (unfilled bar).

#### Pₚ regulates secondary metabolism and QS in *Serratia* 39006

In other species, PhoBR upregulates expression of multiple genes when the cell is starved for Pᵢ. As Pᵢ has been shown to control secondary metabolism in multiple species [17], we investigated whether secondary metabolism and QS in *Serratia* 39006 were also modified by Pᵢ limitation. Growth of *Serratia* 39006 in phosphate-limiting medium (PL medium) without the addition of 5 mM KH₂PO₄ resulted in an increase in Pig (6-fold) and AHL (2-fold) production (Fig. 6A & 6B), reminiscent of the effects of pstS mutations. β-Galactosidase activity from strains containing chromosomal pigA::lacZ, smal::lacZ and rap::lacZ fusions grown in PL medium without the addition of 5 mM KH₂PO₄ was also assessed. Pᵢ limitation resulted in increased transcription of pigA (2-fold) and smal (5-fold) compared with Pᵢ-replete conditions (Fig. 7A & 7B), although there was not a clear increase in rap transcription (Fig. 7C). These experiments demonstrate that low Pᵢ, like pstSCAB-phoU mutations, controls the transcription of pigA and smal to up-regulate secondary metabolism and QS. However, in each instance, the fold increase in response to Pᵢ limitation is lower (by approxi-
Pi limitation affects secondary metabolism and QS. (A) Pig and (B) AHL production in WT cells were measured throughout growth in phosphate-limiting medium with (squares) or without (triangles) the addition of 5 mM KH$_2$PO$_4$. In all graphs, solid lines represent Pig or AHL assays and dashed lines represent bacterial growth.

The effect of Pi limitation on pigA, smal and rap transcription. β-Galactosidase activity was measured from a chromosomal (A) pigA::lacZ (MCP2L), (B) smal::lacZ (LC13) or (C) rap::lacZ (RAPL) strain throughout growth in phosphate-limiting medium with (squares) or without (triangles) the addition of 5 mM KH$_2$PO$_4$. In all graphs, solid lines represent β-galactosidase assays and dashed lines represent bacterial growth.
approximately 35%) than that observed in a pst mutant. As the increase in rap transcription in a pst mutant is below 2-fold, a lesser change, in response to P_i limitation, may be below the level of detection.

We predicted that a pstS mutation would be epistatic to the effects of P_i on secondary metabolism and QS. In a pstS mutant, P_i limitation did not result in an increase in maximal Pig production (Fig. 8A), although slightly premature production of Pig was observed (data not shown). In addition, P_i limitation resulted in only a small (1.3-fold) increase in AHL production in a pstS mutant (Fig. 8B). Taken together, the data suggest that in Serratia 39006, as in other bacteria, mutation of pstS mimics the effect of P_i-limiting media. However, other mechanisms also appear to play a role, facilitating the small increase in AHL production observed in response to P_i limitation despite the absence of a functional PstSCAB-PhoU system.

Discussion

There are multiple studies identifying environmental factors that effect Pig production in Serratia spp., including the effects of salt concentration, temperature, oxygen availability and multiple metal ion concentrations [27]. However, the molecular mechanism underlying most of these responses has not been elucidated. Here, we investigate the molecular mechanism by which P_i limitation affects secondary metabolism in the enteric bacteria Serratia 39006.

It was previously shown that a pstS mutation in Serratia 39006 resulted in the upregulation of QS and secondary metabolism [29]. Here, we demonstrate that these effects are occurring via the PhoBR two-component system, since a secondary mutation in phoBR abolished the effects of a pstS mutation. In addition, we confirm that QS and secondary metabolism are upregulated in response to P_i limitation, and that this is occurring primarily via the PstSCAB-PhoU transport system. We also demonstrate that expression of rap is upregulated in response to a pstS mutation. Rap is an activator of Pig and Car, and a repressor of surfactant production and swarming motility, in Serratia 39006 [19,29]. Rap shares similarity with the SlyA/MarR-family global transcription factor, RovA, which regulates genes required for host colonization in Yersinia spp. [32-34]. Therefore, our results indicate that three global transcriptional regulators, Rap, SmaR and PhoB, are involved in mediating the effects of P_i limitation on secondary metabolism in Serratia 39006.

A mutation of the pstSCAB-phoU genes resulted in a clear increase in Pig and AHL production, and a clear increase in pigA, smaI and rap transcription. However, following P_i limitation, the effects on secondary metabolism and gene

![Figure 8](http://www.biomedcentral.com/1471-2180/9/112)

**Figure 8**

**A pstS mutant is largely unresponsive to P_i limitation.** (A) Pig and (B) AHL production was measured from a pstS mutant (ROP2) grown to early stationary phase in phosphate-limiting medium with (open bars) or without (solid bars) the addition of 5 mM KH_2PO_4.
expression were less dramatic. The degree of activation of Pig and AHL production, andpigA transcription, was approximately 35% lower following Pi limitation than the levels of activation observed in apstS mutant. In addition, a clear increase in rap transcription was not observed following Pi limitation. It is possible that this reduced effect is due to the fact that a pstS mutant is constitutively mimicking extreme Pi limitation. However, when WT cells are transferred to phosphate limiting media, there may be phosphate carry over from the initial inoculum, and the cells may utilise existing intracellular phosphate stores, for example inorganic polyphosphate, before phosphate starvation occurs. As the increase in rap transcription in a pstS mutant is below 2-fold, we believe that a 35% reduction in activation, in response to Pi limitation, may be undetectable. An alternative explanation could be that rap is induced via PhoBR, but not in response to Pi limitation. Previously, PhoBR has been shown to activate expression of the asr (acid shock RNA) gene, but Pi limitation did not activate asr expression [38]. In addition, there is also evidence that PhoB can be activated by non-partner histidine kinases, in the absence of PhoR [39]. This has lead to the hypothesis that PhoBR may activate genes in response to a variety of environmental cues, in addition to Pi limitation [39].

It may not be entirely accurate to describe the effect of a pstS mutation, or Pi limitation, on QS as ‘upregulation’. For QS to function correctly, it is the absolute concentrations of the AHL signal molecule that is critical, not the amount per cell [30]. Due to the growth defect observed following a pstS mutation or Pi limitation, the amount of AHL per cell is increased, but the absolute value remains comparable to WT/Pi excess conditions. Therefore, it may be more accurate to state that the upregulation of smal transcription, following pstS mutation or Pi limitation, allows maintenance of QS regulon control despite the reduced growth rate. This idea is supported by the fact that although carR, pigQ, pigR and rap are all regulated by QS in Serratia 39006 [28,29], only rap transcription is upregulated in response to a pstS mutation. Our experiments indicate that, in response to a pstS mutation, rap is activated independently of QS, and that activation may be mediated via PhoB.

Activation of carA expression, following pstS mutation, was previously reported to be dependent on the upregulation of QS [29]. However, as Rap is also an activator of carA transcription [29], it is possible that Rap, rather than QS, is responsible for the activation of carA following a pstS mutation. We propose that a dual mechanism, involving (1) the alleviation of SmalR repression at lower cell density, via upregulation of smal, and (2) increased levels of Rap via PhoB mediated transcriptional activation, is responsible for the increase in carA expression followingpstS mutation. In the absence of AHL (and hence constitutive SmalR repression), carA transcription is essentially abolished [29] and hence, further activation by Rap, in response to a pstS mutation, might not be possible.

Based on our data, we propose a model by which Pi limitation results in upregulation of secondary metabolism via multiple inter-linked pathways (Fig. 9). In response to Pi limitation, or following mutation of thepstSCA genes, PhoB is activated by phosphorylation [9,15,16]. PhoB−P can then activate expression of genes involved in the Serratia phosphate response which includes smal, pigA and rap. Activation of pigA expression causes increased Pig production. Upregulation of smal allows appropriate derepression by Smal [28,29]. This allows activation of pigA, carA and rap transcription. Rap, which is activated via QS and the phosphate response, can then further activate carA and pigA transcription. This results in upregulation of both Car and Pig production via multiple pathways.

Multiple studies have linked Pi limitation to enhanced secondary metabolite production [17]. However, the complex molecular mechanisms underlying phosphate-mediated regulation have proven difficult to elucidate. Extensive studies in Streptomyces species have shown that PhoPR (PhoBR activates secondary metabolism in response to Pi limitation, including biosynthesis of undecylprodigiosin, a triptyrrrole closely related to Pig [40,41]. However, in Streptomyces, inactivation of PhoP or deletion ofphoPR also activates secondary metabolism [41]. In contrast, deletion ofphoB and/or phoR in Serratia 39006 had no impact on secondary metabolism, demonstrating clear differences between the regulatory mechanisms employed by these distantly related bacteria. Although the requirement for increased secondary metabolism under conditions of phosphate limitation is unclear, it has been proposed that enhanced secondary metabolism allows the production of compounds which may, for example, directly antagonise other microorganisms or act as signalling molecules, thereby providing producing organisms with a competitive advantage under nutrient deprived conditions [40,42,43].

**Conclusion**

In conclusion, we have established that via the global transcriptional regulators PhoB, SmalR and Rap, multiple inter-linked pathways are acting to upregulate secondary metabolism in Serratia 39006 under conditions of Pi limitation, highlighting the importance of Pig and Car production under these conditions.

**Methods**

**Bacterial strains, plasmids, phage and culture conditions**

Bacterial strains and plasmids are listed in Additional File 1[44-49]. Serratia sp. ATCC 39006 derivative strains were
grown at 30°C and E. coli strains were grown at 37°C in Luria broth (LB; 5 g l⁻¹ yeast extract, 10 g l⁻¹ bacto tryptone and 5 g l⁻¹ NaCl), minimal media (0.1% w/v (NH₄)₂SO₄, 0.41 mM MgSO₄, 0.2% w/v glucose, 40 mM K₂HPO₄, 14.7 mM KH₂PO₄, pH 6.9–7.1) or in phosphate limiting (PL) media (0.1% w/v (NH₄)₂SO₄, 0.41 mM MgSO₄, 0.2% w/v glucose, 0.1 M HEPES, pH 6.9–7.1 ± 5 mM KH₂PO₄) in shake flasks at 300 rpm, or on plates supplemented with 1.5% (w/v) agar (LBA). For the phoBR mutagenesis screen, Serratia 39006 was grown on PGM agar plates (5 g l⁻¹ bacto peptone, 1% v/v glycerol and 1.5% w/v agar). Bacterial growth (OD₆₀₀) was measured in a Unicam Heλios spectrophotometer at 600 nm. When required, media were supplemented with antibiotics at the following final concentrations; kanamycin 50 μg ml⁻¹ (Km), spectinomycin 50 μg ml⁻¹ (Sp), ampicillin 100 μg ml⁻¹ (Ap), and tetracycline 35 μg ml⁻¹ (Tc). The generalised transducing phage ϕOT8 was used for transduction of chromosomal mutations as described previously [25].

**DNA manipulations**

All molecular biology techniques, unless stated otherwise, were performed by standard methods [50]. Oligonucleotide primers were obtained from Sigma Genosys and are listed in Table 1. DNA sequencing was performed at the DNA Sequencing Facility, Department of Biochemistry, University of Cambridge, analysed using GCG (Genetics Computer Group, University of Wisconsin) and compared with GenBank DNA or non-redundant protein sequence databases using BLAST [51].

**Sequencing of the pstSCAB-phoU operon**

Preliminary sequence analysis indicated the mini-Tn5Sm/Sp insertions in strains ROP2 and KHC5 were in pstS and pstA respectively [29]. To determine the full sequence of pstS and its surrounding genes, a Serratia 39006 PstI sub-genomic library was created in pBluescript II KS+. One clone containing pstS was analysed further and was named pPST1. The pst region was sequenced via a ‘primer walking’ technique using primers PST1, PST2, PST3, PST4, PST5, PSTITN, PSTRN. To complete the pstSCAB-phoU operon, a 2.1 kbp region of pstSCA was PCR amplified with the primers NW244 and NW245, and then sequenced using primers NW244, NW245, NW246 and NW247. Random primed PCR was used to extend the phoU sequence obtained from primer walking of pPST1, as described previously [48]. Gene specific primer NW250 was used in two separate random primed PCR reactions, one with PF106, PF107, PF108 [48], and a second with NW225, NW226, NW227. The products generated were then amplified with the nested primer PF109 or NW251, respectively and the resulting PCR products sequenced with primer NW251.

**Transposon mutagenesis screen for phoBR mutants**

To isolate phoBR mutants, Serratia 39006 strain LacA was subjected to a random transposon mutagenesis by conju-
duced into non-Pi responsive mutants, and non-hyperpigmenting this screen). The regulatory element(s) common to these uncharacterised insertions had disrupted a repression effects. The possibility that insertion in phoBR had been disrupted was investigated further by measuring alkaline phosphatase activity, encoded by phoA, which is a well conserved member of enteric Pho regulons [1]. Mutants RBR1 and RBR9 did not produce elevated levels of alkaline phosphatase as observed in the pstS mutant (data not shown). Sequence analysis, described below, confirmed that the insertions in BR1 and BR9 were within phoR and phoB respectively.

### Sequencing of the phoBR operon
To determine the site of the transposon insertion in strain BR1, chromosomal DNA was digested with EcoRV and ligated into pBluescript II KS+. The ligation was used as template in a single-primer-site PCR using primers KML and KMR that anneal to the 5' and 3' ends of mini-Tn5. Sequencing of the resultant PCR products revealed that BR1 contained an insertion within a gene similar to phoR from E. coli. A further PCR using chromosomal DNA from the BR9 mutant with primers PHORL and PHORR (homologous to phoR 5' and 3' ends) and primers KML and KMR demonstrated that BR9 contained an insertion within a gene with similarity to phoB from E. coli. To further confirm the phoBR sequence, PCR products of phoB and phoR were generated with primer pairs PF154/PF155

### Table 1: Oligonucleotide primers used in this study

| Name     | 5'-3' sequence | Description                        | Restriction site |
|----------|----------------|------------------------------------|-----------------|
| HS34     | GCTGACTCATAAATATGCTACTG | primers for Km R gene of miniTn5 |  |
| HS36     | GCCGAAAATGCTCGGTCTGTTCTC | primers for Km R gene of miniTn5 |  |
| HS56     | GCTATATCCGACTCTTCC | carA, primer extension oligo |  |
| KML      | ACCGTAAAGTTACCAAGGAGTGGG | primer for Km R gene of miniTn5 |  |
| KMR      | CGGAGCTTGGTACCCACCTGC | primer for Km R gene of miniTn5 |  |
| NW225    | GACCAACAGCCTGACTGCTGCGN| primer for pTG27, PhoB expression construct | EcoRI |
| NW226    | GACCAACAGCCTGACTGCTGCGN | primer for pTG27, PhoB expression construct | EcoRI |
| NW227    | GACCAACAGCCTGACTGCTGCGN | primer for pTG27, PhoB expression construct | EcoRI |
| NW224    | CGTCTGCGACTCTATTGTTATG | primer for pTG27, PhoB expression construct | EcoRI |
| NW245    | GGTATACGAAAGCTGAAAGC | primer for pTA14, rap promoter construct | HindIII |
| NW246    | GCATCTGCGAGCATACAAGAG | primer for pTA14, rap promoter construct | HindIII |
| NW247    | GGGAGCATGCGAAGTACTGTCG | primer for pTA14, rap promoter construct | HindIII |
| NW250    | CATATTGCGATGCAATACAG | primer for pTA14, rap promoter construct | HindIII |
| NW251    | GTGACAGATTGAGTACTTGGT | primer for pTA14, rap promoter construct | HindIII |
| OTG124   | ATCAGAGAATCTAATTGGGAGTCTATTCCG | F primer for pTG27, small promoter construct | EcoRI |
| OTG125   | ATCCAGAAGCTTCTACATATACTGCGTCCTCC | R primer for pTG27, small promoter construct | HindIII |
| PF42     | GCATAAGCTTCCATCACTACTCC | R primer for pTA14, rap promoter construct | HindIII |
| PF43     | GATTAGTTGAGTAAATTGGGAGTACAG | primer for pTA74, PhoB expression construction | EcoRI |
| PHORL    | GCGTTAGTTTGGGAGATTTTC | primer for sequencing | EcoRI |
| PHORR    | CCATCTGCGAGCATACAAGAG | primer for sequencing | EcoRI |
| PST1     | CAGGTGCTGCGACTGTCGC | primer for sequencing | EcoRI |
| PST2     | GTCCAGGGTTGCTGAG | primer for sequencing | EcoRI |
| PST3     | CCAGGCTTACCAAGGACAGTAG | primer for sequencing | EcoRI |
| PST4     | CAGGCTTACAGGAGTTCAGG | primer for sequencing | EcoRI |
| PST5     | CGGAGGCGAAGCATGCAAGG | primer for sequencing | EcoRI |
| PSTSLN   | CACCAGGATAAAGGTAGTGGGAG | primer for sequencing | EcoRI |
| PSTSNR   | CTGCACGGTTTCCTTGCG | primer for sequencing | EcoRI |
| T3       | CGCGCATAAACCTTACATAAG | primer for sequencing | EcoRI |
| T7       | CGCGCATAAACCTTACATAAG | primer for sequencing | EcoRI |
and PF180/PF182 respectively and sequenced on both strands from independent products.

**Construction of a plasmid (pTA74) that expresses native PhoB**

A construct that enabled expression of native, untagged PhoB was created as outlined below. The phoB gene was amplified by PCR, using primers PF154 and PF155, which contain EcoRI and HindIII restriction sites, respectively. Additionally, primer PF154 contains a consensus ribosome-binding site (RBS, AGGAGGA). The PCR fragment of phoB was cloned into pQE-80L, previously digested with the enzymes EcoRI and HindIII. The resulting plasmid, pTA74, was confirmed by DNA sequencing. Expression of plasmid pTA74 in *E. coli* was induced with 1 mM IPTG.

**Construction of promoter::lacZ fusions and assay conditions**

Plasmid pTA15 was constructed as described previously [48]. The rap and smal promoter regions were cloned into the promoterless lacZ plasmid pRW50 [49] to give the plasmid constructs pTA14 and pTG27, respectively. Plasmid pTG27 was constructed by cloning an EcoRI/HindIII digested PCR product (generated using forward primer OTG124 and reverse primers OTG125) into EcoRI/HindIII digested pRW50. Plasmid pTA14 was constructed by cloning an EcoRI/HindIII digested PCR product (generated using forward primer PF43 and reverse primer PF42) into EcoRI/HindIII digested pRW50. All constructs were confirmed by DNA sequencing.

Promoter activity assays were performed in *E. coli* DH5α cells as described in [48]. Briefly, DH5α cells were transformed with the promoter::lacZ construct (pTA14, pTA15 or pTG27) and either pTA74 (encoding native PhoB) or the empty vector control, pQE-80L. The resulting strains were grown in LB containing Ap, Tc and 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). At late exponential phase, 1 ml samples were assayed for β-galactosidase activity.

**Prodigiosin, carbapenem, AHL, β-galactosidase, β-glucuronidase and alkaline phosphatase assays**

The assays for Pig and Car were performed as described previously [29]. Pig production was plotted as (ΔA534 ml⁻¹ OD₆₀₀⁻¹). Detection of AHLs was performed using the *Serratia* LIS bioassay described in [25]. β-Galactosidase activity was determined as described previously [28,29] and was represented as (ΔA₄₂₀ min⁻¹ ml⁻¹ OD₆₀₀⁻¹). β-Glucuronidase activity was determined as for β-galactosidase activity except that reactions were performed in GUS buffer (50 mM NaPO₄, 1 mM EDTA, 5 mM DTT, pH 7.0), using the substrate 4-nitrophenyl β-glucuronide (PNPG; 10 mM), and measured at A₄₀₅. β-Glucuronidase activity was represented as (ΔA₄₀₅ min⁻¹ ml⁻¹ OD₆₀₀⁻¹). Alkaline phosphatase activity was assayed as described previously [52]. Results presented are the mean ± the standard deviation of three independent experiments, unless stated otherwise.

**Primer Extension and RNA studies**

RNA was extracted from *Serratia* 39006 and primer extension analysis for the pigA and smal transcripts was performed as described previously [28,29]. All primer extension reactions were performed with 25 μg of total RNA and 0.2 pmol of the appropriate ³²P-labelled primer. Oligonucleotide primers HS34 and HS36 were used in primer extension reactions for pigA and smal respectively.

**Authors’ contributions**

TG drafted the manuscript, participated in design of the study and performed all experiments that are not credited to the additional authors, listed below. PF generated multiple strains (PCF# strains) and plasmids used in the study, participated in sequencing phoBR, participated in design of the study and critically reviewed the manuscript. LE isolated strains BR1 and BR9, performed primer extension analysis, participated in sequencing phoBR and pst-SCAB-phoU, and participated in design of the study. NW generated strain NW201 and NW202, measured pstC::uidA expression and participated in sequencing of pstSCAB-phoU. GS conceived of the study and participated in the design and coordination of the study.

**Additional material**

*Additional file 1*

Bacterial strains, phages and plasmids used in this study. A list of strains, phage and plasmids used in this study.

Click here for file [http://www.biomedcentral.com/content/supplementary/1471-2180-9-112-S1.doc](http://www.biomedcentral.com/content/supplementary/1471-2180-9-112-S1.doc)

**Acknowledgements**

We thank all members of the Salmond group for helpful discussions, I. Foulds for technical assistance and Corinna Richter for the identification of strain PCF58A9. This work was supported by the BBSRC, UK. TG and LE were supported by BBSRC scholarships.

**References**

1. Wanner BL: Phosphorous assimilation and control of the phosphate regulon. In *Escherichia coli and Salmonella: Cellular and Molecular Biology Volume 1*. Edited by: Neidhart RCI, Ingraham JL, Lin ECC, Low KB, Magasanik B, Reznikoff WS, Riley M, Schaechter M, Umbreher HE. American Society for Microbiology, Washington, DC; 1996:1357-1381.
2. Harris RM, Webb DC, Howitt SM, Cox GB: Characterization of PitA and PitB from *Escherichia coli*. *J Bacteriol* 2001, 183(17):5008-5014.
3. Rosenberg H, Gerdes RG, Chegwidden K: Two systems for the uptake of phosphate in *Escherichia coli*. *J Bacteriol* 1977, 131(2):505-511.
4. Rosenberg H, Gerdes RG, Harold FM: Energy coupling to the transport of inorganic phosphate in Escherichia coli K12. J. Bacteriol. 1987, 161(1):133-137.

5. Amemura M, Makino K, Shinagawa H, Kobayashi A, Nakata A: Nucleotide sequence of the genes involved in phosphate transport and regulation of the phosphate regulon in Escherichia coli. J. Mol. Biol. 1985, 184(2):241-250.

6. Surin BP, Rosenberg H, Cox GB: Phosphate-specific transport system of Escherichia coli: nucleotide sequence and gene-polyprotein relationships. J. Bacteriol. 1985, 161(1):189-198.

7. Webb DC, Rosenberg H, Cox GB: Mutational analysis of the Escherichia coli phosphate-specific transport system, a member of the traffic ATPase (or ABC) family of membrane transporters. A role for proline residues in transmembrane helices. J. Biol. Chem. 1992, 267(34):24661-24668.

8. Willinsky GR, Malamy MH: Characterization of two genetically separable inorganic phosphate transport systems in Escherichia coli. J. Bacteriol. 1990, 172(4):993-1002.

9. Yamada M, Makino K, Amemura M, Shinagawa H, Nakata A: Regulation of the phosphate regulon of Escherichia coli: analysis of mutant phoB and phoR genes causing different phenotypes. J. Bacteriol. 1989, 171(10):5601-5606.

10. Kimura S, Makino K, Shinagawa H, Amemura M, Nakata A: Regulation of the phosphate regulon of Escherichia coli: characterization of the promoter of the phoT gene. Mol. Gen. Genet. 1989, 215(3):374-380.

11. Makino K, Shinagawa H, Amemura M, Kimura S, Nakata A, Ishihama A: Regulation of the phosphate regulon of Escherichia coli. Activation of phoT transcription by PhoB protein in vitro. J. Mol. Biol. 1988, 203(1):85-95.

12. Steed PM, Wanner BL: Use of the recA polypeptide for allele replacement in Escherichia coli. J. Bacteriol. 1989, 171(10):6133-6138.

13. Hulett FM: The signal-transduction network for Pho regulation in Bacillus subtilis. Mol. Microbiol. 1996, 19(5):933-939.

14. Sola-Landa A, Rodriguez-Garcia A, Apel AK, Martin JF: Target genes and structure of the direct repeats in the DNA-binding sequences of the response regulator PhoP in Streptomyces coelicolor. Nucleic Acids Res. 2008, 36(4):1358-1368.

15. Nagel G, Lahrz A, Dersch P: Environmental control of invasin expression in Yersinia pseudotuberculosis is mediated by regulation of RovA, a transcriptional activator of the SlyA/Hor family. Mol. Microbiol. 2004, 51(6):1647-1660.

16. Wang Z, Choudhary A, Fineran PC, Gristwood T, Cox A, Salmond GP: Biosynthesis of the red antibiotic prodigiosin pigment in Serratia is under quorum sensing control. Mol. Microbiol. 2000, 36(3):539-556.

17. Williamson NR, Simonsen HT, Leeper FJ, Salmond GP: Biosynthesis of carboxaphenem antibiotic and prodigiosin pigment in Serratia is under quorum sensing control. Mol. Microbiol. 2005, 56(4):971-989.

18. Fineran PC, Slater H, Everson L, Hughes K, Salmond GP: The biosynthesis of tripyrrole and beta-lactam secondary metabolites in Serratia: integration of quorum sensing with multiple new regulatory components. J. Mol. Biol. 2006, 4(1):887-899.

19. Fineran PC, Slater H, Everson L, Hughes K, Salmond GP: Phosphate availability regulates biosynthesis of two antibiotics, prodigiosin and carboxaphenem, in Serratia via both quorum-sensing-dependent and -independent pathways. Mol. Microbiol. 2003, 47(2):303-320.

20. Van Houdt R, Givskov M, Michiels CW: Quorum sensing in Serratia. FEMS Microbiol. Rev. 2007, 31(4):407-424.

21. Thomson NR, Cox A, Bycroft BW, Stewart GS, Williams P, Salmond GP: The rap and hop proteins of Erwinia, Serratia and Yersinia: a novel subgroup in a growing superfamily of proteins regulating diverse physiological processes in bacterial pathogens. Mol. Microbiol. 1997, 26(3):531-544.

22. Cathelyn JS, Crosby SD, Lathem WW, Goldman WE, Miller VL: RovA, a global regulator of Yersinia pestis, specifically required for bubonic plague. Proc. Natl. Acad. Sci. USA 2006, 103(36):13514-13519.

23. Ellison DW, Lawrenz MB, Miller VL: Invasion and beyond: regulation of Yersinia virulence by RovA. Trends Microbiol. 2004, 12(6):296-300.

24. Nagel G, Lahrz A, Dersch P: Environmental control of invasin expression in Yersinia pseudotuberculosis is mediated by regulation of RovA, a transcriptional activator of the SlyA/Hor family. Mol. Microbiol. 2004, 51(6):1647-1660.

25. Simonsen HT, Leeper FJ, Salmond GP: Future Microbiol 2007, 2(6):605-618.

26. Mapleston RA, Stone MJ, Williams DH: The evolutionary role of secondary metabolites—a review. Gene 1992, 115(1):151-157.
43. Vining LC: Secondary metabolism, inventive evolution and biochemical diversity—a review. Gene 1992, 115(1–2):135-140.
44. Larsen RA, Wilson MM, Guss AM, Metcalf WW: Genetic analysis of pigment biosynthesis in Xanthobacter autotrophicus Py2 using a new, highly efficient transposon mutagenesis system that is functional in a wide variety of bacteria. Arch Microbiol 2002, 178(3):193-201.
45. Herrero A, Flores E: Transport of basic amino acids by the dinitrogen-fixing cyanobacterium Anabaena PCC 7120. J Biol Chem 1990, 265(7):3931-3935.
46. Bainton NJ, Stead P, Chhabra SR, Bycroft BW, Salmond GP, Stewart GS, Williams P: N-(3-oxohexanoyl)-L-homoserine lactone regulates carbapenem antibiotic production in Erwinia carotovora. Biochim J 1992, 288(Pt 3):997-1004.
47. de Lorenzo V, Herrero M, Jakubzik U, Timmis KN: Mini-Tn5 transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria. J Bacterial 1990, 172(11):6568-6572.
48. Fineran PC, Eversion L, Slater H, Salmond GP: A GntR family transcriptional regulator (PigT) controls gluconate-mediated repression and defines a new, independent pathway for regulation of the tripyrrole antibiotic, prodigiosin, in Serratia. Microbiology 2005, 151(Pt 12):3833-3845.
49. Lodge J, Fear J, Busby S, Gunasekaran P, Kamini NR: Broad host range plasmids carrying the Escherichia coli lactose and galactose operons. FEMS Microbiol Lett 1992, 74(2–3):271-276.
50. Sambrook J, Fritsch EF, Maniatis T: Molecular Cloning: a Laboratory Manual. 2nd edition. New York, NY: Cold Spring Harbour Laboratory Press; 1989.
51. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ: Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 1997, 25(17):3389-3402.
52. Brickman E, Beckwith J: Analysis of the regulation of Escherichia coli alkaline phosphatase synthesis using deletions and phi80 transducing phages. J Mol Biol 1975, 96(2):307-316.