A novel host-responsive sensor mediates virulence and type III secretion during Pseudomonas aeruginosa–host cell interactions

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Sensitive sensory mechanisms are instrumental in affording Pseudomonas aeruginosa the capacity to establish diverse yet severe human infections, which can manifest themselves in long-term untreatable disease. The ability of P. aeruginosa to tightly regulate gene expression and virulence factor production, in response to activation of these sensory components, enables the pathogen to sustain infection despite the host immune response and aggressive antibiotic treatment. Although a number of factors are recognized as playing a role in early infection, very little is known regarding the sensors involved in this process. In this study, we identified P. aeruginosa PA3191 as a novel host-responsive sensor that plays a key role during P. aeruginosa–host interactions and is required for optimum colonization and dissemination in a mouse model of infection. We demonstrated that PA3191 contributed to modulation of the type III secretion system (T3SS) in response to host cells and T3SS-inducing conditions in vitro. PA3191 (designated GtrS) acted in concert with the response regulator GltR to regulate the OprB transport system and subsequently carbon metabolism. Through this signal transduction pathway, T3SS activation was mediated via the RsmAYZ regulatory cascade and involved the global anaerobic response regulator Anr.

INTRODUCTION

Pseudomonas aeruginosa is a highly virulent nosocomial pathogen and the source of a wide variety of acute infections in immunocompromised individuals, while also being the primary cause of morbidity and mortality in chronically infected cystic fibrosis (CF) patients (Navon-Venezia et al., 2005; Quinn, 1998; Siegel, 2008). Chronic infection by this pathogen is especially problematic, as it is rarely eradicated, even following aggressive antibiotic treatment. Several factors contribute to the recalcitrance of P. aeruginosa, including its ability to adapt and thrive in a wide variety of niches and to produce several virulence-associated factors, and its capacity to form biofilms (Mahajan-Miklos et al., 1999; Rahme et al., 1995; Spiers et al., 2000). P. aeruginosa is also intrinsically resistant to several antibiotics, further aided by its biofilm mode of growth. The change in growth conditions encountered during the transition from acute to chronic infection correlates with alterations in P. aeruginosa behaviour causing phenotypic changes, including increased antibiotic tolerance and a switch to the biofilm mode of growth. In addition, P. aeruginosa must contend with fluxes in exogenous signals encountered during the course of microbial–host interactions, such as foreign cell contact, diffusible signals, nutrients and what is now emerging as a variable oxygen gradient (Schobert & Jahn, 2010; Son et al., 2007).

While the ubiquitous nature of bacterial molecular sensors employed to perceive and react to environmental and nutrient signals is essential to P. aeruginosa survival during host interaction, few membrane sensors have been confirmed to play a role during infection (Goodman et al., 2009). Recently, RoxS has been shown to play a role in the polymorphonuclear leukocyte (PMN) transepithelial migration response during epithelial infection, although its signals have not yet been identified (Hurley et al., 2010). LadS and RetS are hybrid histidine kinase sensors that respond to unidentified environmental cues and are thought to trigger...
the transition from acute to chronic infection (Ventre et al., 2006). Both LadS and RetS work in concert with a third sensor kinase protein, GacS, to modulate the GacA response regulator. To date, GacA has been shown to act exclusively to control the expression of two small RNAs, rsmY and rsmZ, which in turn sequester and modulate the activity of RsmA, a global post-transcriptional regulator (Brencic et al., 2009; Humair et al., 2010). RsmA is central to the modulation of both cell-associated and secreted virulence determinants in P. aeruginosa (Brencic & Lory, 2009; Burrowes et al., 2006; Heurlier et al., 2004; Mulcahy et al., 2006; Pessi et al., 2001), and it plays a role during colonization and dissemination in a mouse model of infection (Mulcahy et al., 2008).

In P. aeruginosa, the anaerobic response is mediated through the anaerobic regulator Anr, a homologue of Escherichia coli FNR, which is known to be the general positive activator of the anaerobic response (Sawers, 1991; Trunk et al., 2010; Zimmermann et al., 1991). We recently reported an interplay between the Rsm regulatory system and the anaerobic respiratory pathway, whereby Anr directs NarL binding and repression of rsmY and rsmZ transcription (O’Callaghan et al., 2011). This results in RsmA-mediated activation of the type III secretion system (T3SS) under microaerobic conditions. The T3SS is a key virulence factor, which functions to inject potent toxins via a needle-like structure directly into the host cell cytosol (Cowell et al., 2005; Garrity-Ryan et al., 2000; Geiser et al., 2001). The T3SS has previously been associated with acute P. aeruginosa infection and biofilm growth, and is known to be tightly regulated by several interlinked regulatory circuits, culminating in the activation of the master T3SS regulator, exsA (Schulert et al., 2003; Roy-Burman et al., 2001; Manos et al., 2009; Waite & Curtis, 2009; Mulcahy et al., 2006; Yahr & Wolfgang, 2006; Brutinel et al., 2010; O’Callaghan et al., 2011).

Membrane sensors provide a critical control point at which intracellular signal transduction networks are primed and integrated to ensure the rapid and appropriate response to the varying environmental conditions. To address the deficit in our knowledge of the sensor proteins involved in the microbial–host interaction, we designed a proteomic screen to identify P. aeruginosa factors altered during bacterial–host interaction, with the objective of identifying a sensor(s) and defining the signal transduction circuitry associated with the adaptive response. We discovered that the novel sensor PA3191 was induced in response to the host cell, and in conjunction with its putative cognate response regulator was involved in virulence and type III secretion during P. aeruginosa–host cell interactions and in response to a number of environmental conditions. PA3191-mediated regulation of the T3SS involved the OprB carbohydrate uptake system, and the Anr–NarL and RsmAYZ regulatory circuits, and was necessary for PAO1 dissemination to the spleen in a mouse model of infection.

METHODS

Cell lines and bacterial strains. The 16HBE14o− S-1 non-CF human bronchial epithelial cell line (Rajan et al., 2000) was chosen as the principal in vitro infection model, as this cell line becomes fully differentiated and forms tight junctions when grown in monolayers on BSA/collagen/fibronectin-coated plastic. An alternative non-epithelial cell line, the Chinese Hamster Ovary (CHO) cell line, was chosen as an additional in vitro model. Cells were cultured and maintained in Minimum Essential Medium (Sigma) supplemented with 10% (v/v) FBS, 2 mM l-glutamine, 100 U penicillin ml⁻¹ and 100 μg streptomycin ml⁻¹, and in the case of 16HBE14o− S-1, 400 μg G–418 ml⁻¹ was also included. Both cell lines were incubated at 37 °C in a humidified 5% CO₂ atmosphere.

The P. aeruginosa strains used in this study are shown in Supplementary Table S1. PAO1 and associated mutants were routinely grown at 37 °C in the described cell culture medium without antibiotics (infection media), Luria–Bertani (LB) or tryptic soy broth (TSB). Gentamicin-resistant transposon insertion mutants were obtained from the PA14 Transposon Insertion Mutant Library (Jacobs et al., 2003; Liberati et al., 2006). Transposon type, insertion, location and orientation were confirmed for all mutants. Type III-inducing and -non-inducing media were prepared by addition of 5 mM EGTA and 5 mM CaCl₂ to TSB, respectively (Wolfgang et al., 2003). Anaerocult A (Merck) was used for the induction of a microaerophilic production of the production of a microaerophilic, as previously described (O’Callaghan et al., 2011). E. coli strains were routinely grown in LB media at 37 °C. Where appropriate, antibiotics were added to growth media at the following concentrations: tetracycline 50 μg ml⁻¹, kanamycin 100 μg ml⁻¹ and gentamicin 15 μg ml⁻¹, for P. aeruginosa; and ampicillin 50 μg ml⁻¹, gentamicin 20 μg ml⁻¹ and tetracycline 10 μg ml⁻¹ for E. coli.

Infection conditions. For infection studies, bacterial strains were cultured aerobically with shaking for 16–18 h in the infection media (cell culture media without antibiotics) at 37 °C, and then PBS-washed to remove extracellular components. Medium was removed from epithelial cell monolayers, followed by PBS washing to remove antibiotics. Bacterial densities were adjusted to fully infect confluent epithelial cell monolayers or epithelial cell-free controls at an m.o.i. of 50:1. Serial dilutions were plated onto LB agar to confirm the m.o.i. used.

2D proteomic analysis. Infections were carried out as described above. Following 1.5 h infection, extracellular and loosely adherent PAO1 was removed from co-culture by centrifugation (7000 r.p.m., 10 min, 4 °C). Protein isolation, 2D gel electrophoresis, in-gel digestion, peptide mass mapping, and peptide sequence analysis and protein identification, were carried out as previously described (Kiely et al., 2008; Nouvens et al., 2000) using 17 cm nonlinear pH 3–10 immobilized pH gradient (IPG) strips (Amersham Biosciences). Gels represent triplicate biological experiments. A twofold cut-off was considered significant in terms of deviation from the control.

Quantitative real-time PCR (qRT-PCR). For isolation of bacterial RNA, the infection conditions described above were carried out for the time stated. Bacterial samples cultured in infection media without epithelial cells were used as controls. Total bacterial RNA was subsequently extracted using the Qiagen RNaseqy Mini RNA extraction kit in accordance with the manufacturer’s guidelines. The RNA was treated with Ambion TURBO DNase at 37 °C for 1 h, and the RNA pellet was then resuspended in sterile diethylpyrocarbonate (DEPC)-treated water. For qRT-PCR analysis, RNA was isolated from three independent infection experiments. cDNA was synthesized using Promega AMV reverse transcriptase, RNasin (100 U µl⁻¹), dNTPs (10 mM) and random primers (0.5 µg µl⁻¹). Standards were generated by PCR, and qRT-PCR analysis of expression was carried out using the Qiagen QuantiFast SYBR Green PCR kit. qRT-PCR
signals were normalized to a constitutively expressed housekeeping gene, proC, for bacterial expression analysis (Savli et al., 2003).

Plasmid construction and transformation. The PCR primer sequences employed are listed in Supplementary Table S2 and are based on the PAO1 genome sequence (http://www.pseudomonas.com) (Stover et al., 2000). Unmarked, in-frame deletions of 1236, 649 and 2055 bp, spanning PA3191, gltR and both PA3191 and gltR in PAO1 and PA14, were carried out by allelic exchange (Hoang et al., 2000), generating the mutants ΔPA3191, ΔgltR and ΔPA3191ΔgltR, respectively. To generate complementation and overexpression constructs, fragments containing the genes of interest were subcloned into pBBR- MCS2 (Kovach et al., 1995) and mobilized by triparental mating into their respective mutants. The transfer of plasmids into the P. aeruginosa strains was confirmed by PCR.

The sequences of promoter regions for transcriptional reporter fusions provided by other laboratories were sequenced and confirmed to be identical to that of the PAO1 and PA14 sequences. Promoter regions used for the construction of transcriptional reporter fusions were amplified using primer pairs (Supplementary Table S2) and subcloned into pmini-CTX-lux (Becher & Schweizer, 2000). Plasmids derived from pmini-CTX-lux were mobilized into PAO1 and mutants by triparental mating. After integration into the attB site, vector backbone sequences were excised by Flp recombine as described elsewhere (Hoang et al., 1998). Each reporter construct was therefore present as a single, unmarked copy integrated at the chromosomal attB site.

Quantification of cell cytotoxicity by measuring lactate dehydrogenase (LDH) release. The release of LDH into culture supernatants was measured using the LDH Cytotoxicity Detection kit (Roche) according to the manufacturer's instructions. FBS was omitted from the infection medium, as inclusion of FBS resulted in high background levels of LDH activity. Infection of epithelial and CHO cells was carried out as described above, and allowed to proceed for the indicated time points post-infection. Bacteria were subject to serial dilutions and plating to confirm uniform growth between wild-type and mutant strains in the presence of epithelial cells.

Ingestion and adhesion assays. Ingestion assays were performed as described elsewhere (Burns et al., 1996), with modifications. Briefly, following a 1 h infection, gentamicin (1 mg ml⁻¹) and ceftazidime (2 mg ml⁻¹) were added for a further 2 h, after which the epithelial cells were washed in PBS and lysed using 0.1% Triton X-100, and the number of intracellular bacteria was enumerated by serial dilutions and plate counts. The number of adherent bacteria following 1 h co-culture was enumerated by gently washing extracellular bacteria from the epithelial cells, which were then lysed to provide a total intracellular and adherent count, from which the previously determined number of intracellular bacteria was subtracted.

Measurement of luciferase and calculation of transcription. Infection of epithelial cells was carried out as described above, with strains carrying the promoter::lux and empty::lux fusions. Control infections were set up under identical conditions in the absence of epithelial cells. At the indicated time points post-infection, relative luciferase counts (RLC) were compared between strains carrying the promoter::lux and empty::lux fusions using the IVIS 100 Imaging System (Xenogen Imaging Technologies). Bacteria were subjected to serial dilutions and plating to confirm uniform growth between wild-type and mutant strains in the absence and presence of epithelial cells.

Bacteria were cultured under microaerobic and aerobic growth conditions as described above, in low- and high-Ca⁺² TSB media. At OD₆₀₀ 0.5, RLC were compared between strains carrying the promoter::lux and empty::lux fusions using a Tecan GENios plate reader (Tecan Group). RLC were normalized to OD₀₀₀ readings.

In vitro competition experiments. Competitive growth experiments between PAO1 and ΔPA3191Gm or ΔPA3191ΔgltR and ΔPA3191Gm (as controls) were carried out in vitro in LB medium for up to 30 generations of growth (24 h). Strains were grown overnight and diluted to a starting OD₀₀₀ of 0.1. The starting ratios of PAO1 and ΔPA3191Gm or ΔPA3191ΔgltR and ΔPA3191Gm were confirmed by plate counts on LB and LB+ gentamicin (LBGm). Bacteria were subcultured every 8 h into fresh medium, and samples were taken every 4 h to enumerate c.f.u. on LB and LBGm selective agar.

Mouse model of acute pneumonia. Acute mouse infections were carried out as described elsewhere, with modifications (Comolli et al., 1999). Briefly, P. aeruginosa strains were grown in LB broth at 37 °C overnight with shaking, collected by centrifugation, washed and resuspended in PBS to 1 × 10⁷ c.f.u. 50 μl⁻¹. The exact number of bacteria was determined by plating serial dilutions of each inoculum on LB agar plates. Female BALB/c mice (6–8 weeks) were anaesthetized by intraperitoneal administration of ketamine hydrochloride (65 mg kg⁻¹) and xylazine (13 mg kg⁻¹). Anaesthetized animals were intranasally infected with 50 μl of culture, 25 μl per nostril, producing a final inoculum of 1 × 10⁷ c.f.u. per mouse. At 18 hours post infection (h.p.i.), mice were sacrificed to remove and homogenize the lungs, liver and spleen. Serial dilutions in PBS were plated onto LB to enumerate the recovered bacteria. The resultant c.f.u. were calculated and are presented as the level of bacterial infection in the whole lungs, liver or spleen. All animal experiments were approved by the animal ethics committees of University College Cork.

In silico analysis of the PA3191 protein. TMpred (http://www.ch.embnet.org/software/TMPRED_form.html) and Phobius (http://phobius.sbc.su.se/) transmembrane topology and signal peptide predictor software programs were employed to examine the putative sensor histidine kinase protein PA3191.

Statistical analysis. The statistical significance of data was analysed using Student’s t test or analysis of variance (ANOVA), where appropriate. All analysis was carried out using the SPSS and Excel software packages. A P value of 0.05 or less was considered significant.

RESULTS

P. aeruginosa PA3191, an uncharacterized sensor histidine kinase, is induced during infection of airway epithelial cells

We employed a strategy to study the response of P. aeruginosa to host cells during infection with the objective of identifying novel sensors involved in the adaptive response during infection. Whole-cell protein was isolated from P. aeruginosa cultured in the presence and absence of epithelial cells, and a series of 2D protein profiles were compared to screen for proteins key to P. aeruginosa–host interactions. Of the 10 PAO1 proteins found to be differentially expressed to a significant level following 1.5 h airway epithelial cell interaction (Table 1), the discovery of PA3191 was notable, as it signified an uncharacterized sensor histidine kinase protein induced in response to host cells. qRT-PCR analysis was performed and validated a 2.1-fold induction of PA3191 gene expression in response to epithelial cells (+2.1 ± 0.3). It also determined that induction occurred at the transcriptional rather than post-transcriptional level.
In the Pseudomonas Genome database, PA3191 is annotated as a probable two-component sensor with a type I export signal and a predicted cleavage point after residue 22 (http://www.pseudomonas.com). In silico analysis predicts PA3191 to be a classic sensor histidine kinase protein. The N terminal of PA3191 has two transmembrane helices, while the remainder of the PA3191 protein is located in the cytoplasm and contains HAMP, histidine kinase A and HATPase domains, typical of sensor histidine kinase proteins (http://www.pseudomonas.com; Lewenza et al., 2005). PA3191 is located downstream of gltR, encoding a glucose uptake response regulator (Sage et al., 1996). The in silico analysis and genomic organization of PA3191 and GltR suggest that they function as a classic two-component system (TCS), whereby phosphorylated GltR activates target gene expression in response to signals perceived by PA3191, although these have not yet been characterized as a cognate pair. Operon prediction models indicate that PA3191 and gltR are encoded in an operon with a glucokinase (glk) gene (http://www.pseudomonas.com), and this was subsequently confirmed by reverse transcription (data not shown). To examine the role of the putative PA3191–GltR TCS during infection, non-polar single and double mutants were constructed for use in this study.

Inactivation of PA3191 resulted in elevated ingestion and reduced cytotoxicity of eukaryotic cells

The observation that PA3191 expression was increased in response to epithelial cells prompted us to investigate the impact of PA3191 on P. aeruginosa virulence phenotypes and gene expression. Therefore, in vitro co-culture assays were set up to study the role of PA3191 and GltR in ingestion, adhesion and cytotoxicity of eukaryotic cells. Wild-type PAO1 and PA3191 and gltR single and double mutants were co-cultured with an airway epithelial cell line (HBE-S) and a CHO cell line. Significantly elevated ingestion of ΔPA3191 relative to PAO1 by epithelial cells was observed following 1 h of co-culture and 2 h of antibiotic exclusion (Fig. 1a). However, the observed difference in ingestion was not due to elevated adhesion, as there was no difference in the adherence of ΔPA3191 and PAO1 wild-type strains to epithelial cells (Fig. 1b). Similar ingestion and adhesion phenotypes were observed for ΔgltR and ΔPA3191ΔgltR (Fig. 1a, b). Cytotoxicity assays demonstrated reduced levels of epithelial cell LDH release following infection with ΔPA3191, ΔgltR or ΔPA3191ΔgltR compared with infection with PAO1 wild-type (Fig. 1c), indicating reduced cytotoxicity. All phenotypes were fully complementable when the relevant genes were introduced in trans into the respective mutants. Furthermore, we also sought to ascertain whether the role of PA3191 during infection was specific to the epithelial cell line used. These phenotypes were not cell line-specific, as ΔPA3191 also resulted in elevated P. aeruginosa ingestion and reduced cytotoxicity towards CHO cells (Supplementary Fig. S1a). Comparable results were obtained using P. aeruginosa PA14 mutants of PA3191 and gltR homologues (PA14Δ22960 and PA14Δ22940, respectively), verifying that the observed PA3191- and GltR-dependent phenotypes occurred in PA14–host cell interactions and were not PAO1-specific (Supplementary Fig. S1b). The consistency of ΔPA3191, ΔgltR and ΔPA3191ΔgltR mutants in demonstrating similar phenotypes during infection further implied that this putative sensor–regulator pair, PA3191–GltR, may function as a TCS controlling ingestion and cytotoxicity during infection of eukaryotic cells.

### Table 1. P. aeruginosa PAO1 proteins identified to be differentially expressed following 1.5 h infection of airway epithelial cells

Differential expression of proteins was analysed using Phoretix PG200 software (Nonlinear Dynamics), and proteins were identified by MALDI-TOF analysis. Locations of identified proteins in the 2D gels correlate with their predicted molecular mass and isoelectric point. A twofold cut-off was considered significant in terms of deviation from the control.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Description</th>
<th>Expression in PAO1 post-infection</th>
<th>Score [−10×log(P), P&lt;0.05]</th>
<th>Sequence coverage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA0300</td>
<td>SpuD, polyamine transport protein</td>
<td>−2.2</td>
<td>82</td>
<td>34</td>
</tr>
<tr>
<td>PA0575</td>
<td>Conserved hypothetical</td>
<td>−2.0</td>
<td>95</td>
<td>42</td>
</tr>
<tr>
<td>PA0668</td>
<td>TyrZ, tyrosyl tRNA synthetase 2</td>
<td>−1.9</td>
<td>81</td>
<td>34</td>
</tr>
<tr>
<td>PA1596</td>
<td>HtpG, heat-shock protein 90</td>
<td>+2.8</td>
<td>100</td>
<td>39</td>
</tr>
<tr>
<td>PA2396</td>
<td>PvdF, pyoverdine synthetase</td>
<td>−2.0</td>
<td>70</td>
<td>32</td>
</tr>
<tr>
<td>PA3191</td>
<td>Probable two-component sensor</td>
<td>+2.1</td>
<td>84</td>
<td>41</td>
</tr>
<tr>
<td>PA3244</td>
<td>MinD, cell division inhibition factor</td>
<td>Undetected-detected</td>
<td>88</td>
<td>37</td>
</tr>
<tr>
<td>PA3655</td>
<td>Tsf, stress response</td>
<td>+2.0</td>
<td>74</td>
<td>40</td>
</tr>
<tr>
<td>PA4238</td>
<td>RpoA, DNA-directed RNA polymerase alpha chain</td>
<td>Undetected-detected</td>
<td>52</td>
<td>52</td>
</tr>
<tr>
<td>PA4265</td>
<td>TufA, stress response</td>
<td>+2.0</td>
<td>87</td>
<td>39</td>
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</table>
et al., 2001; Hauser et al., 1998; Mulcahy et al., 2006). Therefore, we investigated whether PA3191–GltR played a role in modulating the expression of the T3SS. Promoter activities of the type III regulatory operon exsCEBA and the effector toxin exoS were assessed in ΔPA3191, ΔgltR and wild-type backgrounds in response to host cell contact, known to induce the T3SS. Interestingly, the T3SS appeared to be induced in the culture medium without cells but further induced in response to cells. Nevertheless, the promoter activity of exsC and exoS was significantly lower in ΔPA3191 than in PAO1 both in the media control and in the presence of epithelial cells (Fig. 2a, i, ii). A similar trend was observed for exoS expression in the gltR mutant in co-culture experiments (Fig. 2a, iii). Thus, PA3191 and GltR are essential for optimal activation of the PAO1 T3SS in response to host cell contact.

We then sought to determine whether PA3191–GltR controls T3SS activity in response to other T3SS-inducing conditions. exsCEBA and exoS promoter activity was compared between PAO1 and ΔPA3191/ΔgltR in response to low calcium (Ca^{2+}) and limiting oxygen (O_2) (O’Callaghan et al., 2011). As was observed in response to host cells, both ΔPA3191 and ΔgltR exsC and exoS promoter activity was significantly lower than that of PAO1 in response to low Ca^{2+} (Fig. 2b), confirming a role for PA3191 in the ExsC-dependent T3SS pathway (Dasgupta et al., 2006). Furthermore, ΔPA3191 exhibited reduced exsC and exoS promoter activity relative to PAO1 under limiting oxygen tension (Fig. 2b). Taken together these data indicate that the induction of the key virulence trait, T3SS, by P. aeruginosa in response to host cell contact and extracellular factors of physiological significance during infection is
mediated through the PA3191–GltR signal transduction system.

**PA3191 plays a role in initial colonization and dissemination in a mouse model of acute pneumonia**

The T3SS has been shown to be required for optimum survival and systemic spread in vivo (Vance et al., 2005). In order to determine whether PA3191 is necessary for optimal in vivo colonization and dissemination during *P. aeruginosa* infection, PAO1 and ΔPA3191 were inoculated in a mouse model of acute pneumonia. Initial in vitro competition experiments were carried out to determine whether loss of PA3191 influences the fitness of the mutant population relative to the parent strain. Cultures containing PAO1 and ΔPA3191Gm at a ratio of 1:1 or ΔPA3191 and ΔPA3191Gm at a ratio of 1:1 were assessed after 4, 8, 12 and 24 h (30 generations) growth in LB medium. No significant difference in the fitness of the mutant strains relative to each other or to PAO1 wild-type was observed under these conditions (data not shown).

PAO1 was capable of colonizing the lungs of infected mice, and the number of c.f.u. increased eightfold over the course of the 18 h infection (Fig. 3). Additionally, PAO1 was able to disseminate into the liver and the spleen. In contrast, ΔPA3191 exhibited significantly reduced colonization of the lungs and liver, whereby the bacterial load was 2 logs lower for both these organs in ΔPA3191-infected mice compared with PAO1-infected mice (Fig. 3). Strikingly, ΔPA3191 was not detected in the spleen. The complemented strain showed full restoration of colonization. Therefore, loss of PA3191 attenuated PAO1 infection in a mouse model of acute pneumonia, which suggests a role for this sensor in dissemination of the pathogen in vivo. These data led us to investigate and define associated molecular pathway(s) linking PA3191 and the T3SS in *P. aeruginosa*.

**PA3191–GltR mediates its effects on the T3SS through the OprB transport system**

While *P. aeruginosa* response regulators are reported to have several targets, to date the OprB transport system remains the only known target of GltR (Sage et al., 1996). OprB is an outer-membrane porin that is part of the high-affinity glucose transport pathway in *P. aeruginosa*, which is also active during anaerobic growth (Saravolac et al., 1991; Wylie & Worobec, 1994). While OprB is induced by and facilitates the transport of glucose, it has also been shown to facilitate the uptake of several carbohydrates, including fructose, mannitol and glycerol, and to be differentially regulated by salicylic acid, pH and temperature (Wylie & Worobec, 1995; Williams et al., 1994; Adewoye & Worobec, 1999). Therefore, we investigated whether PA3191 functions together with GltR to influence oprB promoter activity and in turn whether they mediated their effect on the T3SS through the OprB transport system in response to host cell interactions. As predicted, oprB promoter activity was repressed approximately threefold in ΔPA3191 and twofold in ΔgltR relative to PAO1 both in the absence and presence of epithelial cells (Fig. 4a), providing further evidence to support the hypothesis that PA3191 and GltR may form a TCS to modulate the OprB transport system. For this reason PA3191 was designated GtrS (glucose transport sensor).

We then investigated whether expression of OprB influenced T3SS activity. Using a PA14 oprB transposon mutant, available from the PA14 non-redundant library (Liberati et al., 1997), we performed bacterial competition experiments with PAO1, ΔPA3191 and ΔPA3191comp carrying a single copy of the (i) exsC::lux or (ii) exoS::lux transcriptional reporter, and (iii) PAO1, ΔgltR and ΔgltRcomp carrying a single copy of exoS::lux, was measured following 3 h bacterial infection of airway epithelial cell monolayers or tissue culture plastic (media control) with a starting m.o.i. of 50:1. (b) Luminescence produced by PAO1, ΔPA3191 and ΔPA3191comp carrying (i) exsC::lux and (ii) exoS::lux following aerobic and microaerobic growth in TSB, TSB+5 mM CaCl$_2$ or TSB+5 mM EGTA. Error bars, sds for triplicate experiments. Asterisks indicate $P\leq0.05$ for PAO1 versus the mutant strain for all conditions (Student’s t test).
et al., 2006), exoU promoter activity (a PA14 T3SS effector toxin) was repressed in the oprB transposon mutant relative to PA14 following 3 h airway epithelial cell interactions (Fig. 4b), suggesting that GtrS/GltR modulated the T3SS through the OprB carbohydrate uptake system.

**GtrS-GltR-OprB directs type III activation through Anr–NarL regulation of the RsmAYZ pathway**

The high-affinity glucose uptake pathway is active during anaerobic growth (Hunt & Phibbs, 1983), and here we provide evidence that GtrS is required for microaerobic induction of the T3SS. Furthermore, we recently showed that the T3SS is induced in response to micro- and anaerobic conditions via Anr–NarL regulation of the RsmAYZ network (O’Callaghan et al., 2011). Therefore, we investigated whether the molecular mechanism through which GtrS-GltR-OprB modulated the T3SS in response to host cells also involved the RsmAYZ pathway.

While the small non-coding RNAs (sRNAs) rsmY and rsmZ sequester and titrate RsmA to dictate the critical balance of the regulatory protein within the bacterial cell, RsmA in turn can also modulate sRNA production. Therefore, rsmA, rsmY and rsmZ promoter activity was examined in ΔPA3191, ΔgltR and oprB at 3 h.p.i. Significantly elevated rsmY and rsmZ promoter activity was evident in ΔPA3191, ΔgltR and oprB relative to their wild-type strains at 3 h.p.i. (Fig. 5a–c) and in the absence of epithelial cells (data not shown), while there was no difference in rsmA::lux expression in ΔPA3191 relative to PAO1 in the presence or absence of airway epithelial cells (Fig. 5a–c). Thus, it appears that GtrS-GltR-OprB may mediate their effect on the T3SS through perturbation of rsmY and rsmZ transcription, which in turn modulates RsmA activity. This was further supported by the finding that overexpression of rsmA in ΔPA3191 restored exoS promoter activity to wild-type levels (Fig. 5d).

**Fig. 4.** PA3191–GltR regulates the OprB uptake system to direct T3SS activation. (a) Luminescence produced by ΔPA3191 (black bars) and ΔgltR (white bars) relative to PAO1 carrying a single copy of the oprB::lux transcriptional reporter was measured over 3 h following bacterial infection of airway epithelial cell monolayers or tissue culture plastic (media control) with a starting m.o.i. of 50:1. (b) Luminescence produced by the oprB mutant relative to PA14 carrying a single copy of the exoU::lux transcriptional reporter was measured over 3 h following bacterial infection of airway epithelial cell monolayers or tissue culture plastic (media control) with a starting m.o.i. of 50:1. Error bars, sds for triplicate experiments. Asterisks indicate $P<0.05$ for PAO1 or PA14 versus the mutant strain (Student’s $t$ test).
Subsequently, we sought to determine whether the Anr-NarL regulatory pathway also played a role in GtrS/GltR-dependent T3SS activation in response to host cell contact. qRT-PCR analysis revealed that anr transcript levels were reduced 10-fold in ΔPA3191 (−10.0 ± 1.7) and eightfold in ΔgltR (−8.2 ± 2.3) compared with PAO1 at 1.5 h.p.i. Furthermore, overexpression of narL in ΔPA3191 restored exoS, rsmY and rsmZ promoter activity to wild-type levels (Fig. 5d). Together these data suggest that induction of the GtrS-GltR-OprB pathway induces the T3SS via Anr/NarL regulation of rsmY and rsmZ. Thus, we provide evidence linking the GtrS/GltR/OprB carbohydrate transport pathway, RsmA activity and the induction of the T3SS.

Interplay between central carbon metabolism and the T3SS

Several publications have reported the effect of mutating metabolic genes on virulence factor production and pathogenicity among a range of infective bacteria (An & Grewal, 2010; Alteri et al., 2009). Dacheux et al. (2002) reported reduced T3SS activity following mutagenesis of the metabolic gene pyruvate dehydrogenase (PDH), which functions further downstream of the OprB transport system.
to feed pyruvate into the tricarboxylic acid (TCA) cycle. Therefore, we sought to determine whether PDH activity influenced RsmAYZ and the T3SS. In agreement with previously published work, exoU promoter activity was repressed in the aceE (PDH) transposon mutant (Liberati et al., 2006) relative to PA14 following 3 h airway epithelial cell interactions (Fig. 6), suggesting that PDH enzyme activity is necessary for induction of the T3SS. Furthermore, loss of aceE resulted in elevated rsmYZ promoter activity in the presence of epithelial cells following a 3 h infection (Fig. 6). This observation correlates with the oprB mutagenesis data to reveal novel interplay and crosstalk between pathways of central carbon metabolism and Rsm regulatory circuitry with subsequent effects for the T3SS.

**RsmA forms a feedback loop to modulate the GtrS-GltR-OprB and OpbA transport systems**

We previously observed that GtrS protein expression is altered in the rsmA mutant PAZH13 strain (J. O’Callaghan, unpublished data). In addition, the *P. aeruginosa* PAK comparative transcriptomes suggest that RsmA positively influences genes associated with the OprB uptake pathway (Brenic & Lory, 2009). Therefore, we sought to determine whether *P. aeruginosa* PA01 RsmA forms a feedback loop to modulate these transport systems. qRT-PCR analysis revealed that a PA01 rsmA mutant had reduced gtrS (−3.9 ± 0.6), gltR (−3.8 ± 0.8) and oprB (−4.1 ± 0.3) transcript levels relative to the wild-type at 3 h.p.i., confirming that RsmA forms a feedback loop to modulate the GtrS-GltR-OprB system.

This study demonstrates for the first time to our knowledge that induction of the GtrS-GltR-OprB carbohydrate transport system, in response to host cell contact, low Ca²⁺ and low oxygen, modulates RsmA activity to induce the T3SS and to form a positive feedback loop, in turn modulating gtrS, gltR and oprB expression.

**DISCUSSION**

During early *P. aeruginosa*–host cell encounters, the capacity to perceive and transduce environmental signals to trigger the appropriate adaptive response greatly enhances the ability of the bacterium to survive and establish infection. *P. aeruginosa* is a highly adaptable opportunistic pathogen, and this is reflected in the fact that 8.4% of its genome is predicted to encode transcriptional regulators and environmental sensors (Stover et al., 2000). In this study, we identified *P. aeruginosa* PA3191 as a putative novel sensor induced in response to host cell contact and required for optimum colonization and dissemination in vivo in a mouse model of infection. While the PA3191 protein had been suggested to be a cognate sensor kinase for GltR based on its predicted protein structure and genomic proximity, there was no experimental evidence to confirm this. We have confirmed by RT-PCR that the PA3191–93 operon is transcribed as a single unit incorporating a glucokinase (gkl), in addition to the proposed sensor and response regulator. Our expression data demonstrating that PA3191 and GltR similarly modulated the induction of oprB, the only known target of GltR, and the T3SS, as well as the associated phenotypes, also support the hypothesis that PA3191–GltR acts as a TCS, and therefore we assigned the name GtrS (glucose transport sensor) to PA3191. While further assessment of the kinase activity and signal transduction mechanism between GtrS and GltR is warranted, it is clear that GtrS/GltR are required for the induction of the OprB uptake system and in turn for type III secretion during early *P. aeruginosa*–host interactions, and in response to the apparently distinct, low-Ca²⁺ and low-oxygen T3SS-inducing conditions. While it is not clear what are the specific signal(s) that GtrS may be sensing to trigger its activation of the response regulator GltR, the fact that the OprB carbohydrate uptake pathway is involved suggests that these ‘signals’ may be influenced by perturbation of central carbon metabolism. Several studies have suggested that the induction of the T3SS may be influenced by the metabolic state of the cell (Rietsch et al., 2004) or metabolic pathways such as that of intracellular cAMP, which dictates T3SS activity through CyaB and Vfr (Wolfgang et al., 2003; Wu et al., 2004). Rietsch & Mekalanos (2006) also suggested that the metabolite regulating the induction of the T3SS may be derived from acetyl-CoA and may involve the intracellular concentration of cAMP. Dacheux et al. (2002) demonstrated that specific mutations in genes encoding PDH, which catalyses the conversion of pyruvate to acetyl-CoA, prevent the induction of T3SS genes in response to calcium depletion. Here, we demonstrate that mutation of the
PDH gene *aceE* also prevented T3SS in PA14 in response to host cells, and further demonstrate that this effect is mediated through an increase in the expression of the sRNAs *rsmY* and *rsmZ*. Interestingly, a study by Takeuchi *et al.* (2009) demonstrated that a mutation in the pyruvate carboxylase genes *pycAB* in *Pseudomonas fluorescens* CHAO results in downregulation of the homologous sRNA *rsmy* and *rsmz*, whereas a mutation in the fumarase gene *fumA* leads to upregulation of *rsmXYZ*. In the present study we found that inactivation of *pycA* in *P. aeruginosa* PA14 also downregulated the expression of *rsmYZ*, and in turn there was a significantly enhanced induction of the T3SS in response to host cells (data not shown). Taken together the data support the hypothesis that product(s) of pyruvate metabolism, possibly involving acetyl-CoA, may play a central role in modulating the T3SS in *P. aeruginosa*, and provide further evidence that the resultant modulation of the T3SS is mediated through regulation of *rsmYZ* expression and subsequently RsmA activity.

Recently we demonstrated that low oxygen induces the *P. aeruginosa* T3SS through the Anr–NarL pathway, whereby NarL binds to *rsmY* and *rsmZ* and represses their expression (O’Callaghan *et al.*, 2011). This study suggests that GtrS/GltR-mediated induction of the T3SS in response to host cell contact may also involve Anr–NarL regulation of the RsmAYZ network. Anr is proposed to directly monitor available oxygen via ligation of an oxygen-sensitive [4Fe–4S] iron–sulfur cluster to its sensory domain in a similar manner to its *E. coli* homologue FNR (Crack *et al.*, 2004; Moore *et al.*, 2006). However, this study provides evidence that GtrS/GltR plays a host-responsive role in inducing anr transcription and consequently narL induction (Fig. 7). Further studies are warranted to determine whether this induction of anr transcription is a result of metabolic flux and to investigate a mechanism for this transcriptional regulation.

Fig. 7. Model linking the GtrS/GltR/OprB carbohydrate uptake system with RsmA regulation of the T3SS in *P. aeruginosa*. The GtrS/GltR TCS is activated to induce the OprB carbohydrate uptake system and subsequently the T3SS in response to host cells and environmental cues, including low oxygen and low Ca$^{2+}$. This GtrS/GltR/OprB system indirectly regulates the T3SS, possibly through intermediates of pyruvate metabolism, which act to increase anr transcription, leading to direct activation of the narL TCS. NarL subsequently inhibits *rsmYZ* transcription through direct binding of the respective promoter regions, allowing free RsmA to positively modulate the T3SS. RsmA is thought to directly modulate the T3SS through regulation of ExsA, the positive transcriptional regulator of the T3SS. RsmA also forms a feedback loop to modulate the transcription of the GtrS/GltR/OprB system. Arrowheads indicate positive regulation, blunt ends indicate negative regulation, solid lines indicate direct regulation, dashed lines indicate indirect regulation (modified from O’Callaghan *et al.*, 2011).

Nevertheless, the finding that RsmAYZ is part of this newly defined host-responsive pathway provides further evidence that RsmA is a global regulator of virulence and may act as a common point of convergence in response to diverse environmental triggers. Cross-talk between TCSs may represent a molecular mechanism through which the bacterial cell can integrate temporal and host-specific signals with exquisite precision to ensure an appropriate adaptive response to host infection. Indeed the effect of PA3191 mutation on PAO1 infection in the mouse model of acute pneumonia reflected data outlined in a previous study showing a similar role for RsmA *in vivo* (Mulcahy *et al.*, 2008). In both cases colonization of the lungs and dissemination to the liver by the mutant were significantly lower compared with wild-type, while dissemination to the spleen was limited for the *rsmA* mutant and negligible for...
ΔPA3191 (GtrS). This suggests that the observed GtrS phenotype is possibly due to modulation of RsmA activity.

In conclusion, we have identified PA3191 (designated GtrS) as a novel host-responsive sensor, which, in cooperation with GtrR and the OprB carbohydrate uptake pathway, was found to modulate T3SS activity via the RsmAYZ regulatory network. During infection bacteria encounter gradients in nutrient availability and composition. This is likely to modulate and/or integrate into central metabolic pathways. This work and that of others suggest that the RsmAYZ global regulatory network is fine-tuned to respond to these fluctuations. We propose that GtrS may provide the cell with the ability to coordinate the perception of altered environmental conditions or nutrients, and in turn mediate the appropriate cellular response.

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REFERENCES


GtrS mediates the Pseudomonas aeruginosa T3SS


