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Metabolic regulation of type III secretion gene expression in *Pseudomonas aeruginosa*

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Summary

Type III secretion-mediated cytotoxicity is one of the key virulence mechanisms of the opportunistic pathogen *Pseudomonas aeruginosa*. Prior data from several laboratories have established that metabolism is a key factor in the regulation of type III secretion gene expression in *P. aeruginosa*. Here we use a fluorescence-activated cell sorter (FACS)-based approach to investigate expression of type III secretion genes at a single-cell level. The data demonstrate that the metabolic state regulates the percentage of cells that are able to induce type III secretion gene expression under inducing conditions. We also present evidence that this regulation is the result of an effect of the growth conditions on the ability of *P. aeruginosa* to assemble a functional type III secretion apparatus. Preliminary data suggest that the metabolite that controls type III secretion gene expression is derived from acetyl-CoA and that this regulation may, in part, be mediated by changes in the intracellular concentration of cyclic-AMP.

Introduction

Pseudomonas aeruginosa is a common cause of ventilator-associated pneumonia and wound infections in patients that are immunocompromised due to an underlying condition, such as cancer patients (Velasco *et al*., 1997; Carratala *et al*., 1998), the elderly and burn patients (Holder *et al*., 2001a; Pirnay *et al*., 2003). One of the major virulence factors of *P. aeruginosa* is the type III secretion system, which is used to directly deliver toxins into the cytoplasm of infected host cells (Yahr *et al*., 1996; Olson *et al*., 1997). Epidemiological studies of *P. aeruginosa* isolates from patients have shown that the presence of a functional type III secretion system is associated with a higher incidence of systemic spread and poor clinical outcome (Roy-Burman *et al*., 2001; Hauser *et al*., 2002; Garau and Gomez, 2003).

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To date, four type III secreted toxins (effectors) have been described in *P. aeruginosa*. Exoenzyme S (ExoS) and exoenzyme T (ExoT) are bi-functional proteins with an amino-terminal GAP domain and a carboxy-terminal ADP-ribosylating domain (Knight *et al*., 1995; Goehring *et al*., 1999; Pederson *et al*., 1999; Barbieri, 2000; Krall *et al*., 2000). The GAP domains of ExoS and ExoT are active towards small rho-like GTPases and effect disassembly of the actin cytoskeleton and cell rounding of infected host cells. The ADP-ribosylating activities of ExoS and ExoT have differing substrate specificity. ExoS targets small ras-like proteins, whereas ExoT appears to be specific for CrkI and CrkII, two signalling proteins involved in triggering phagocytosis (McGuffie *et al*., 1998; Sun and Barbieri, 2003). The two remaining effector proteins, exoenzyme Y (ExoY) and exoenzyme U (ExoU), have adenylate-cyclase and phospholipase activity respectively (Yahr *et al*., 1998; Sato *et al*., 2003). Curiously, most strains of *P. aeruginosa* only have the gene for either ExoS or ExoU, but not both (Feltman *et al*., 2001).

The expression of type III secreted toxins is induced on cell contact, and can be induced *in vitro* by the removal of calcium from the medium (Yahr *et al*., 1996; Frank, 1997; Vallis *et al*., 1999). This ability to induce effector expression under low-calcium conditions has also been linked to a more severe disease outcome.

Cell contact, or low-calcium conditions, appears to trigger induction of the system by way of a regulatory cascade that results in activation of ExsA, the main transcriptional regulator of the type III secretion regulon (Frank and Iglewski, 1991). This induction depends on the presence of a functional type III secretion system. Mutations that inactivate the type III secretion machinery result in an inability to induce effector expression under low-calcium conditions (Frank and Iglewski, 1991).

The activity of ExsA is controlled by interaction with another protein, ExsD. When ExsD is bound to ExsA, transcription of the type III secretion regulon is turned off (McCaw *et al*., 2002). Overexpression of ExsD therefore results in the inability to induce effector expression under low-calcium conditions, whereas deletion of *exsD* results

in a constitutive expression phenotype. The ExsD–ExsA interaction, in turn is controlled by ExsC. ExsC is a small protein that can bind tightly to ExsD and thereby relieve the inactivation of ExsA by ExsD (Dasgupta *et al*., 2004). ExsC appears to be similar to a chaperone of a type III secreted protein and its activity is in turn controlled by a recently discovered, secreted protein, ExsE (Rietsch *et al*., 2005; Urbanowski *et al*., 2005). Upon triggering of the type III secretion machinery, ExsE is exported, freeing ExsC to interact with ExsD, which in turn liberates ExsA and results in induction of the regulon.

Expression of the type III secretion genes is subject to regulation by at least two global regulatory pathways. Cyclic AMP and the *Pseudomonas* catabolite repression homologue, Vfr, are required for expression of the type III secretion regulon (Wolfgang *et al*., 2003). Second, the GacA/GacS two-component regulatory system can repress expression of the type III secretion genes by modulating the activity of the regulator RsmA. The activity of the GacA/GacS pathway is, in turn, controlled by the hybrid two-component regulator RetS (Goodman *et al*., 2004; Laskowski *et al*., 2004; Zolfaghar *et al*., 2005). The molecular basis of neither regulation is, as yet, clear.

The third factor controlling exoenzyme expression is metabolic in nature, and much less well understood. Media composition, in particular salt concentration, contributes to the ability to induce ExoS expression (Hornef *et al*., 2000). Mutations in the genes for pyruvate dehydrogenase, *aceA* and *aceB,* were reported to result in the inability to induce *exsA* expression under low-calcium conditions (Dacheux *et al*., 2002). Similarly, plasmid insertions in *gltR*, the regulator of the glucose transporter, resulted in the inability to induce the type III secretion regulon (Wolfgang *et al*., 2003). Finally, overexpression of the histidine transporter and degradation genes abolished the ability to induce ExoS expression and rendered the bacteria non-cytotoxic (Rietsch *et al*., 2004). The latter phenotype depended on the presence of histidine in the medium and the ability to take up and metabolize histidine. Furthermore, the phenotype was suppressed by a transposon insertion in *cbrA*. The CbrAB two-component regulatory system is involved in sensing metabolic imbalance and adjusting gene-expression of catabolic operons accordingly (Nishijyo *et al*., 2001). Taken together these results suggest that metabolism can in fact exert a significant level of control over the ability to induce the type III secretion regulon.

We have used a fluorescence-activated cell sorter (FACS)-based assay to study the induction of ExoS expression to further define how metabolism controls the type III secretion regulon. Our results demonstrate that growth under metabolically unfavourable conditions in fact results in a type III secretion negative phenotype, suggesting that metabolism controls either assembly or activity of the type III secretion machinery. This finding allows us to integrate metabolic control into the model for regulation of ExsA activity and induction of the type III secretion regulon.

Results

When measuring induction of gene expression using a conventional genetic reporter system, such as the *lacZ* gene, any change in expression is, by definition, a change in the expression of a population of cells. This type of method does not distinguish between changes in regulation that affect the entire population of cells uniformly, and regulation that only affects a subpopulation of cells. Using a FACS to monitor single cell gene expression of a green fluorescent protein (GFP) reporter inserted in the *exoS* locus of *P. aeruginosa*, we discovered that *exoS* is only induced in a subpopulation of cells under inducing conditions. In L-broth, roughly 45% of cells induce expression of the reporter upon addition of the chelator nitrilo-triacetic acid (NTA) (Fig. 1A). Furthermore, adding increasing amounts of chelator only serves to increase the percentage of cells that induce (Fig. S1A). Induction depends on the presence of a functional type III secretion system, because deletion of the structural genes *pscC* or *pscJ* results in an inability to induce GFP expression (Fig. 1B and data not shown). This mode of induction differs from induction of the *lac* or toluic acid promoters in *P. aeruginosa* (data not shown and Fig. S1B). In those cases, addition of increasing levels of inducer gradually raises expression of the entire population of cells rather than inducing expression in only a fraction of the population.

Induction of exoenzyme expression by addition of a chelator does not work in minimal glucose media (Fig. 1C). Addition of caseamino acids was able to restore induction of type III secretion genes in minimal glucose, suggesting that the inability to induce by addition of chelator was due to a change in growth conditions (data not shown). Interestingly, addition of just glutamate was able to partially restore the ability of *P. aeruginosa* to induce expression of *exoS* in minimal glucose media upon removal of calcium (Fig. 1D).

It had been previously reported that salt concentration strongly influences the intracellular glutamate concentration and also influences *exoS* induction, so we decided to determine if osmolarity can influence the percentage of cells that induce *exoS* expression. Indeed only about 4% of cells induce *exoS* expression in L-broth with 5 mM NaCl (Fig. 2A), whereas 90% induce in 200 mM NaCl (Fig. 2B). Increasing the concentration of salt in the medium correlated with an increase in the percentage of cells that were able to induce *exoS* expression upon removal of calcium from the medium (Fig. 2C). Salt could be replaced by

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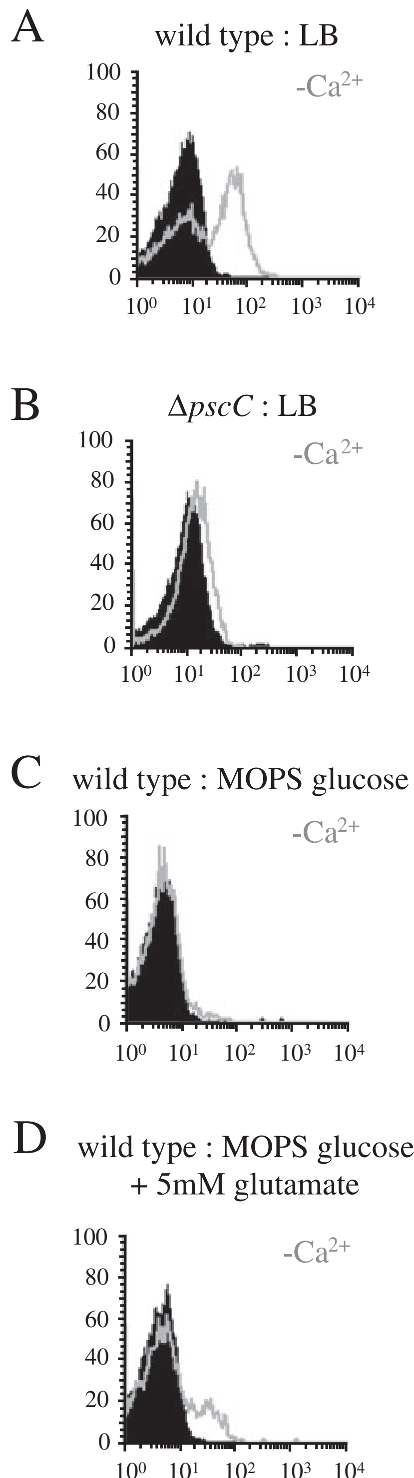

Fig. 1. Induction of *exoS* expression monitored by FACS analysis using a GFP reporter. *P. aeruginosa* PAK (A, C and D) or PAK Δ*pscC* (B) harbouring the *exoS*::GFP-*lacZ* reporter were grown in L-broth (A, B) or MOPS minimal media supplemented with 0.2% glucose (C) or 0.2% glucose and 5 mM glutamate (D). Relative fluorescence of individual bacteria is plotted on the *x*-axis. The *y*-axis denotes the number of bacteria of a given fluorescence intensity (10 000 bacteria were analysed). The grey overlay indicates the distribution for cultures in which calcium had been chelated by the addition of NTA to the media.

sucrose in these experiments, suggesting that *exoS* expression is in fact controlled by osmolarity (Fig. 2D). We also performed shift experiments in which the *exoS* reporter strain was grown in Luria–Bertani (LB) with 5 mM or 200 mM NaCl and shifted to media of the opposite osmolarity. Bacteria that had been grown in low-osmolarity LB were able to quickly adapt to the high osmolarity phenotype of being permissive for induction, whereas bacteria that had been grown in high-osmolarity media and then shifted to low-osmolarity LB were still able to induce *exoS* expression to a significant extent, especially if the shift occurred at the time of induction (Fig. S2). The simplest interpretation of these data is that being permissive for induction of *exoS* expression is associated with a cellular component that is not rapidly turned over, suggesting that it is not simply the result of a shift in pool size of a given metabolite.

While osmolarity controls the percentage of cells that induce *exoS* expression upon removal of calcium from the medium, osmolarity also affects the basal level of *exoS* expression in calcium-replete media. The basal level of *exoS* expression varies about fivefold between low- and high-osmolarity LB, as assayed using a *lacZ* reporter (Fig. 2E).

Induction of exoS *in regulatory mutants under low- and high-salt conditions*

To determine how this osmotic regulation integrates with the activity of the known regulators of the type III secretion regulon, we combined the reporter with deletions in *exsD*, *exsE*, *pcrV* and *popN*. ExsD is an anti-activator that binds ExsA and prevents induction of the type III secretion regulon. As mentioned in the *Introduction*, deletion of *exsD* results in constitutive expression of the type III secretion regulon. ExsE is a recently discovered negative regulator that is secreted via the type III secretion machinery upon triggering of secretion. ExsE binds ExsC and prevents activation of ExsA under conditions in which the secretion apparatus is closed. PcrV is also involved in the low-calcium response. PcrV is involved in inserting the translocase into the target cell membrane (Goure *et al*., 2004). It is likely to be surface exposed because antibodies directed against PcrV are commonly found in patient sera. Antibodies directed against PcrV are protective in an animal model and reduce cytotoxicity in an *in vitro* infection model (Sawa *et al*., 1999; Holder *et al*., 2001b; Moss *et al*., 2001; Frank *et al*., 2002; Faure *et al*., 2003). Like *exsD* mutants, deletion mutants lacking *pcrV* express the type III secretion regulon constitutively. *popN* mutants are deregulated for secretion and also express the type III secretion regulon constitutively (Sundin *et al*., 2004). PopN, like PcrV, is itself exported via the type III secretion apparatus and has been proposed to impose a block on

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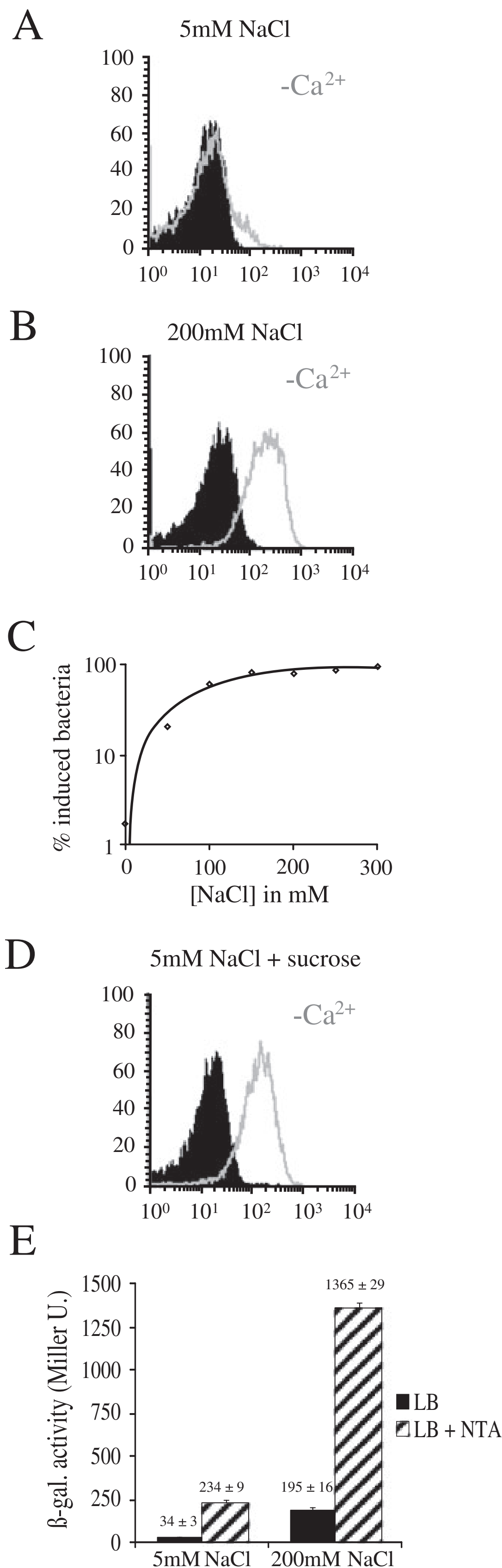

Fig. 2. Osmolarity controls the ability to induce *exoS* expression. Strain PAK harbouring the *exoS*::GFP-*lacZ* reporter was grown in LB containing the indicated concentration of NaCl (A, B) or 5 mM NaCl and 271 mM sucrose (D). The grey overlay indicates the distribution for cultures in which calcium had been chelated by the addition of NTA to the media. Panel C, a graph plotting the percentage of cells that induce *exoS* expression after removal of calcium from the media (*y*-axis) when grown in LB with the indicated NaCl concentration (*x*-axis). Panel E depicts levels of *exoS* expression as measured using the *lacZ* reporter in LB with 5 mM or 200 mM NaCl in the absence or presence of chelator (NTA). The actual activities are noted above each column of the graph.

secretion under repressing conditions. Unlike PcrV, PopN does not appear to be important for translocation of effectors into targeted host cells because a *popN* mutant is cytotoxic (Sundin *et al.*, 2004).

We grew *exsD*, *exsE*, *pcrG* and *pcrV* mutants of PAK harbouring the *exoS*-GFP reporter under low-salt and high-salt conditions and assayed *exoS* transcription under calcium-depleted and calcium-replete conditions. The *exsD* and *exsE* mutants displayed high levels of *exoS* transcription under all conditions tested (Fig. 3). This is in marked contrast to the *popN* and *pcrV* mutants, which only displayed high levels of transcription in the high-salt media (Fig. 3).

While *exsD*, *exsE*, *popN* and *pcrV* mutants have been described to constitutively express exoenzymes, they differ in one key respect. In *exsD* and *exsE* mutants, secretion is still low-calcium-dependent, whereas in *popN* and *pcrV* mutants, it is deregulated (McCaw *et al.*, 2002; Sundin *et al.*, 2002; 2004; Rietsch *et al.*, 2005; Urbanowski *et al.*, 2005). The constitutive induction phenotype of the *popN* and *pcrV* mutants could therefore be the result of constitutive secretion of ExsE and should therefore be type III secretion-dependent.

To determine if the deregulated expression of the type III secretion genes in the *popN* and *pcrV* mutants is type III-dependent, we combined the *exsD*, *exsE*, *popN* and *pcrV* mutations with a deletion in *pscC*, which encodes the outer membrane secretin of the type III secretion system. Indeed, expression of *exoS* continued unabated in the *exsD pscC* and *exsE pscC* mutants, but the *popN pscC* and the *pcrV pscC* double mutants, were unable to induce *exoS* expression, even under low-calcium conditions (Fig. 4). Taken together, these data suggest that the defect in *exoS* induction in low-osmolarity media behaves like a type III secretion defect and could therefore be the result of a defect in assembly of the type III secretion machinery. Interestingly, closer observation of the relative fluorescence of the *exsD* and *exsE* mutants harbouring the *exoS*-GFP reporter at low and high-osmolarity suggests that the basal level of transcription still varies with osmolarity and is therefore subject to a second, unrelated mechanism of regulation (Fig. 3).

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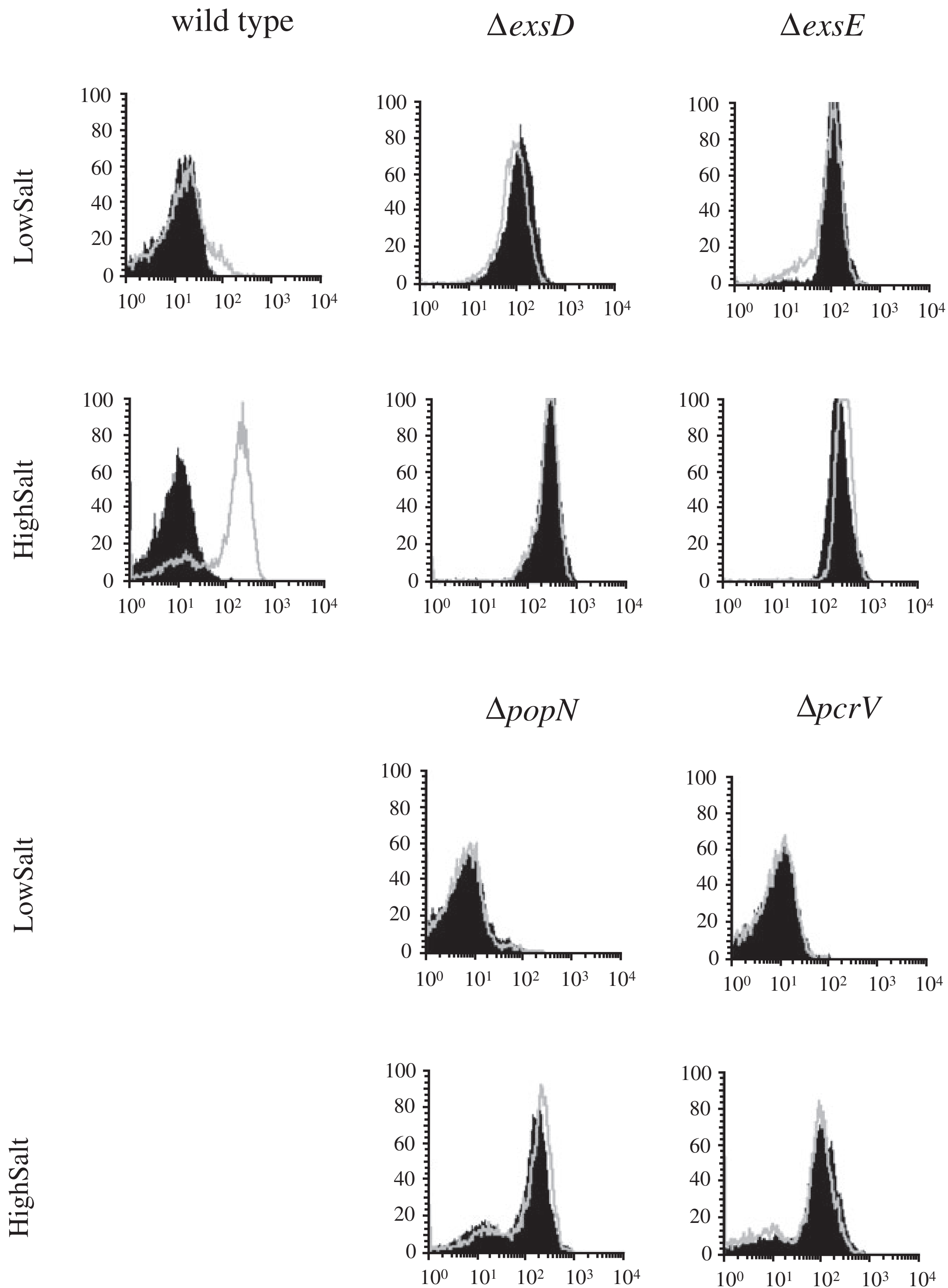

Fig. 3. Effect of regulatory mutations on induction in high- and low-osmolarity media. Indicated mutants of strain PAK were grown in LB with low (5 mM) or high (200 mM) salt and assayed, by FACS, for *exoS* expression in the presence (black) or absence (grey overlay) of calcium using a chromosomal *exoS*-GFP reporter.

PcrV is not surface-displayed in P. aeruginosa *grown in low-osmolarity media*

To test directly if the type III secretion machinery is assembled properly in bacteria grown in low-osmolarity media, we tested if PcrV is displayed on the surface of these bacteria. As PcrV is itself secreted via the type III secretion machinery (Yahr *et al*., 1997), its surface localization

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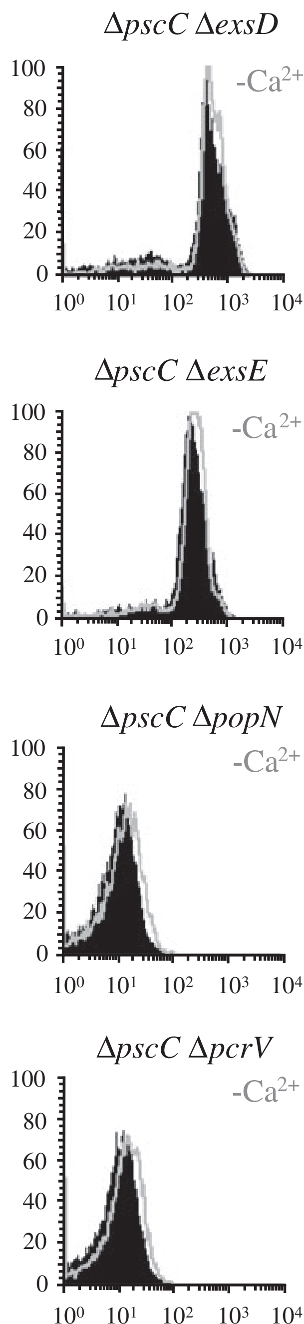

Fig. 4. *exoS* expression in various regulatory mutations in the context of a defective type III secretion apparatus. Derivatives of strain PAK bearing the indicated deletion mutations were assayed, by FACS, for *exoS* expression in LB with 200 mM NaCl in the presence (black) or absence (grey overlay) of calcium.

should serve as a convenient indicator of the ability of the bacteria to assemble a functional type III secretion apparatus. We fixed bacteria grown in low- or high-osmolarity media before staining them with an anti-PcrV antiserum and subjecting them to immunofluorescence microscopy. PcrV is clearly displayed on the surface of bacteria grown in high-osmolarity media (Fig. 5A). This is not the case for bacteria grown under low-osmolarity conditions (Fig. 5A). The control strains, a *pscC* mutant lacking the secretin of the type III secretion machinery, and a *pcrV* mutant strain were both negative for the PcrV signal (Fig. 5A). Control staining of the outer membrane porin OprH demonstrated that growth under low-osmolarity conditions did not interfere with the immunofluorescence staining procedure. To obtain a more quantitative analysis of this result, the stained cells were analysed for their fluorescent signal by FACS. These data confirm the microscopy data (Fig. 5B). In high-osmolarity media about 40% of cells are positive for the PcrV signal, whereas in low-osmolarity media, less than 1% of the cells stained positive. The controls were also both negative. Western blot analysis demonstrated that PcrV is produced under both conditions, although the level of expression was about fivefold lower in low-osmolarity media than in high-osmolarity media (Fig. S3A), which is consistent with the drop in the basal level of *exoS* expression we observed. Furthermore, PcrV is not aberrantly released into the supernatant under low-calcium conditions but remains associated with the cells (Fig. S3B).

One could argue that the inability to assemble the type III secretion machinery under low-osmolarity conditions is the result of the decrease in expression of the structural genes. Perhaps a critical threshold of expression is required to be able to assemble the machinery, and the decrease in transcription directly leads to the type III negative phenotype. To test this possibility we examined the ability of an *exsE* mutant, which expresses the type III secretion genes constitutively and to a high level, even in low-osmolarity media, to display PcrV on the surface of the bacterial cell. Even in the *exsE* mutant, however, the bacteria were not able to assemble a functional type III secretion machinery (Fig. 5B). This result suggests that the phenotype is the result of a defect in assembly or function of the machinery, or a post-transcriptional defect in expression, rather than a problem with the basal transcription level of the structural genes.

Tracing the nature of the metabolic cue

To begin to understand the nature of the metabolic cue that results in the type III secretion negative phenotype, we decided to use the FACS-based assay to monitor the effect of metabolic mutations on expression of *exoS*.

Pseudomonas aeruginosa is unable to induce *exoS* expression when grown in minimal medium with glucose as sole carbon source. Expression can be restored by adding back amino acids, for example, in the form of tryptone. We decided to use minimal medium with 0.1% tryptone and glutamate as a semi-defined condition to investigate this phenotype genetically. In this medium,

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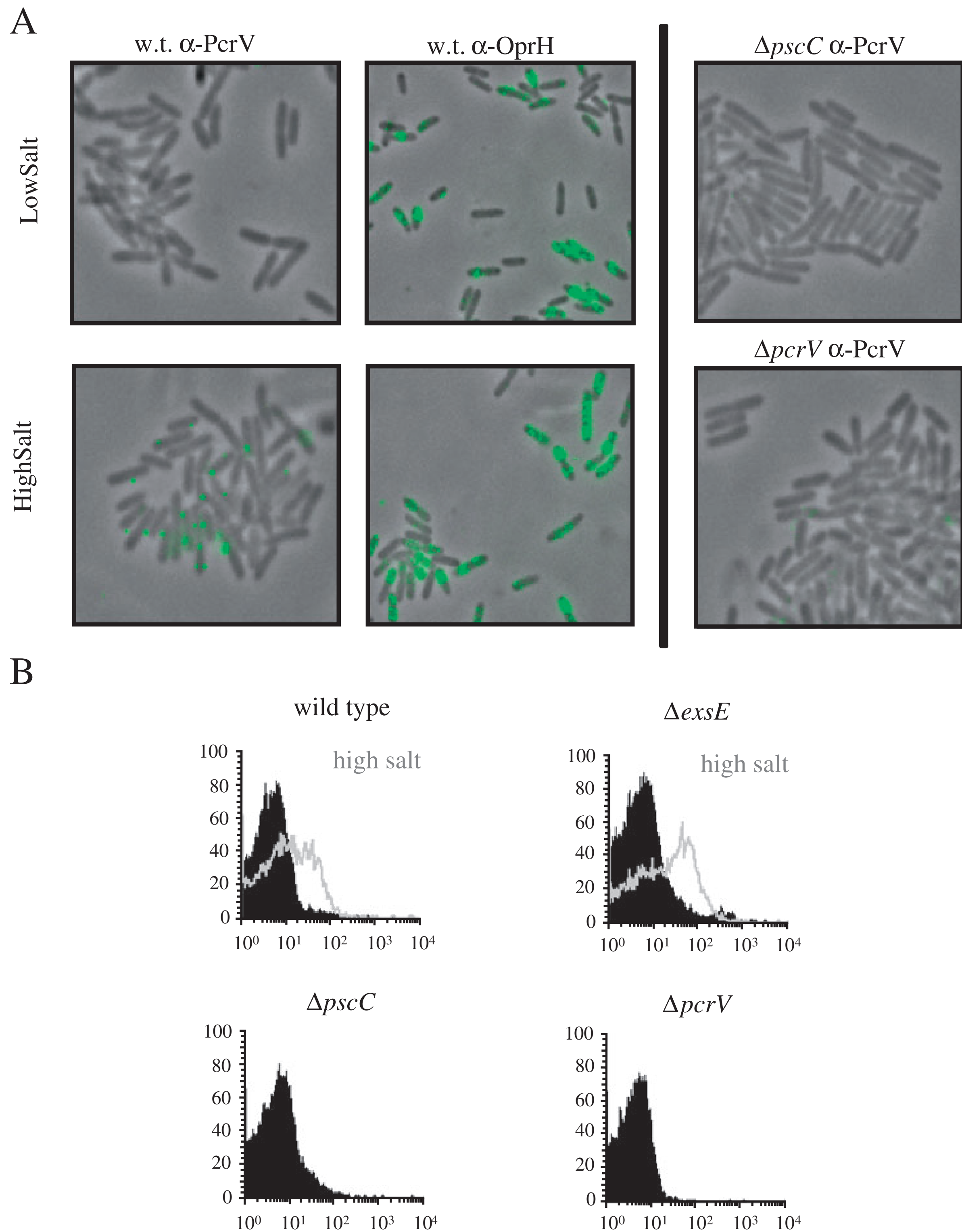

Fig. 5. Assembly of the type III secretion machinery in low- and high-salt media.
A. Bacteria grown in LB with 5 mM or 200 mM NaCl were stained with the indicated primary antibody (anti-PcrV or anti-OprH antiserum) and detected using a AlexaFluor 488 coupled secondary antibody. Phase contrast and fluorescence images of representative fields were overlayed. The *pcrV* and *pscC* mutants were only grown in LB with 200 mM NaCl.
B. FACS analysis of antibody stained cells. All strains were stained with the PcrV and AlexaFluor-488 coupled secondary antibody as described above and analysed by FACS. The wild-type and *exsE* mutant strain were grown in LB with 5 mM NaCl (black) or 200 mM NaCl (grey). The *pcrV* and *pscC* mutant strains were only grown in LB with 200 mM NaCl.

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about 35% of the cells induce *exoS* expression when calcium is removed from the medium, allowing for mutations that can shift the equilibrium towards either induction or repression. Based on the published metabolic conditions that affect expression, as well as preliminary data that suggested that adding back glutamate was able to partially restore induction (Fig. 1D), we decided to focus on mutations that impair the tricarboxylic acid cycle. Mutations in a large number of metabolic genes were analysed. The results of mutations that best define the metabolic pathway involved in the regulation are illustrated in Fig. 6. Deletion of *aceA*, one of the structural subunits of pyruvate dehydrogenase, was able to prevent induction in our media, whereas deletion of *gltA* (the main citrate synthetase) and *prpC* (citrates synthetase II), was able to enhance induction. Interestingly, the *aceA* mutation had no effect on induction in rich media in our hands (data not shown), somewhat contradicting previous data (Dacheux *et al*., 2002). However, subtle differences in media conditions or strains could account for this discrepancy. Taken together, these data suggest that acetyl-CoA, or a derivative thereof exerts metabolic control over *exoS* expression. Osmotic regulation and regulation seen when comparing minimal versus rich media are related and likely to be facets of the same regulatory phenomena, because the *gltA* and *gltA prpC* mutations also partially suppressed the inability of *P. aeruginosa* to induce *exoS* expression under low-osmolarity conditions (Table 1).

The nature of the metabolite or structural change in the cell (e.g. change in composition of the cell wall) that regulates the type III secretion machinery assembly is as yet unclear. Mutations in *ackA* and *pta* do not affect regulation, suggesting that acetylphosphate, which has been linked to regulation of SPI1 gene expression in *Salmonella* (Lawhon *et al*., 2002), is not a factor (data not shown). Similarly, no striking changes were observed in phospholipid head-group or fatty-acid side-chain composition (data not shown). While there are still other metabolites that derive from acetyl-CoA, future research will be aimed at understanding the nature of the assembly defect, which may shed light on the metabolite underlying the regulation.

While trying to express GFP under the control of the *Escherichia coli lac* promoter, in low- and high-osmolarity media we discovered that expression of GFP in *P. aeruginosa* was strongly regulated by the osmolarity of the media (data not shown). This defect could be partially suppressed by replacing the wild-type *lac* promoter with the cAMP-independent *lac*UV5 promoter, suggesting that changes in osmolarity result in changes in cAMP levels in *P. aeruginosa.* As cAMP regulates *exoS* expression, we measured cAMP levels in bacteria grown in low- and high-osmolarity media. Growth in high-osmolarity media resulted in higher levels of cAMP than growth in low-

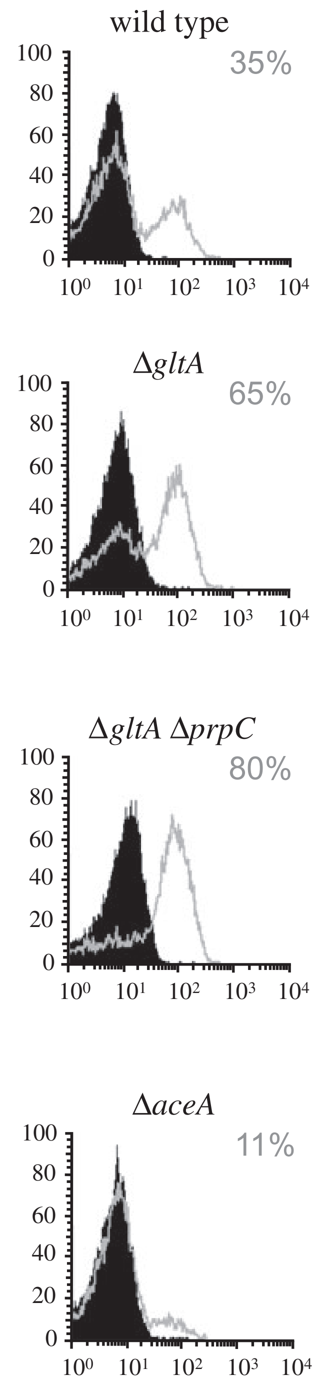

Fig. 6. Metabolic mutations control *exoS* induction. Wild-type *P. aeruginosa* as well as the indicated mutants were grown in MOPS minimal media supplemented with 0.1% tryptone and 20 mM glutamate. *exoS* expression was induced by removal of calcium from the media and analysed by FACS in the presence (black) or absence (grey overlay) of calcium (*aceE*, pyruvate dehydrogenase subunit E1, *gltA*, citrate synthetase 1, *prpC*, citrate synthetase 2). The percentage of cells that induce *exoS* expression in the absence of calcium is indicated in the upper right-hand corner of each graph.

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Table 1. Intracellular cAMP concentrations in low- and high-osmolarity media.

Strain	[NaCl] in media	Intracellular [cAMP] in μM	% of cells that induce *exoS* expression upon removal of calcium	Basal level of *exoS* expression[a]
PAK	5 mM	1.2 ± 0.4	4%	48 ± 6
PAK	200 mM	5.1 ± 0.8	70%	214 ± 5
PAK Δ*gltA* Δ*prpC*	5 mM	2.7 ± 1.0	15%	56 ± 3
PAK/pP35-*cyaB*	5 mM	3.3 ± 0.8	23%	nd

a. β-Galactosidase activity in Miller Units.
nd, not determined.

osmolarity media (Table 1), suggesting that changes in intracellular cAMP concentration may be responsible, at least in part, for the inability to induce expression in low-osmolarity LB. Consistent with this, overexpression of the adenylate cyclase CyaB was able to partially restore the ability to induce *exoS* expression in low-osmolarity media (Table 1). Interestingly, the Δ*gltA* Δ*prpC* double mutant, which also partially restored the low-calcium induction of *exoS* expression in low-osmolarity media, resulted in elevated levels of cAMP. Compared with wild type, however, the Δ*gltA* Δ*prpC* double mutation did not significantly elevate the basal level of *exoS* transcription (Table 1), suggesting that the two modes of regulation are distinct.

Discussion

A series of papers have established that the regulation of the type III secretion genes in *P. aeruginosa* is intimately tied to the metabolic state of the cell. Pyruvate dehydrogenase mutants have been described as being non-cytotoxic and unable to induce type III secretion gene expression. Osmolarity has been described as influencing *exoS* expression. Aberrant overexpression of a histidine transporter and subsequent uptake and metabolism of histidine also renders cells non-cytotoxic. Using a FACS-based assay to study induction of *exoS* at the single-cell level, we were able to determine that metabolically unfavourable conditions change the percentage of the population that can induce *exoS* expression, as well as the absolute level of expression per cell. We furthermore present data that the decrease in the percentage of cells that induce *exoS* expression is in fact due to a defect affecting either assembly or activity of the machinery. This defect causes a type III secretion negative phenotype, which in turn prevents induction by removal of calcium from the media.

Metabolism plays an important role in regulation of virulence factors in a wide variety of pathogens. In *Salmonella*, expression of the SPI1 type III secretion system depends on the alarmone ppGpp (Pizarro-Cerda and Tedin, 2004) and requires high salt and anaerobic growth (Lee and Falkow, 1990). Short chain fatty acids may also affect invasion gene expression in *Salmonella typhimurium* by altering levels of acetyl phosphate (Lawhon *et al.*, 2002). In *Listeria*, pyruvate dehydrogenase is required for growth in macrophage (O'Riordan *et al.*, 2003). Expression of cholera toxin in classical strains of *Vibrio cholerae* can be induced by growth at 30°C in media with a decreased pH (Waldor and Mekalanos, 1996), whereas induction in the El Tor biotype can be induce *in vitro* by growth under AKI conditions (Iwanaga *et al.*, 1986), which involve a fixed regimen of anaerobic, followed by aerobic growth, suggesting that there is a physiological cue that governs expression of this key virulence factor. Even with many examples to draw upon, for the most part, the mechanism by which metabolism exerts its control is unclear.

By using a combination of β-galactosidase assay and FACS analysis, we were able to determine that, in *P. aeruginosa*, growth under metabolically unfavourable conditions affects expression of the type III secretion genes in two ways. It reduces the basal level of *exoS* expression and decreases the percentage of cells that induce *exoS* expression upon removal of calcium from the medium. The two phenomena can be separated genetically, because in the *exsD* or *exsE* mutants, *exoS* expression is induced in all cells, even under metabolically unfavourable conditions, but the absolute level of expression per cell is reduced.

The block imposed on *exoS* expression by growth in metabolically unfavourable conditions behaves, genetically, like a type III secretion mutant. Mutant strains lacking *exsD* or *exsE* do not require an intact type III secretion system for constitutive expression of *exoS*, whereas mutants that do require an intact type III secretion system, *pcrV* and *popN*, are metabolically regulated. The decrease in the percentage of cells that induce *exoS* expression upon calcium removal correlates with an inability to display PcrV on the surface demonstrating that assembly or function of the type III secretion machinery is impaired. Constitutive expression of the type III secretion regulon in an *exsE* mutant was not able to restore the ability of the bacteria to assemble a functional type III secretion apparatus in low-osmolarity LB. The immunofluorescence data presented in this paper clearly demonstrate that *P. aeruginosa* assembles a functional type III secretion apparatus even under calcium-replete condi-

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tions. Furthermore, the data suggest that *P. aeruginosa,* on average, only assembles one or two type III secretion complexes per cell and that these secretion machineries are not necessarily located directly at the pole of the cell. Experiments using GFP labelled components of the apparatus will be needed to confirm and extend these observations.

While it is interesting to speculate about the role of osmolarity in regulation of this important virulence factor, it should be noted that changes in osmolarity alone are not sufficient to signal whether or not an environment is metabolically favourable. Simply increasing the salt concentration in minimal glucose media was not able to restore the ability of *P. aeruginosa* to induce *exoS* expression, while addition of glutamate did partially restore the ability to induce, suggesting that osmolarity, which was our primary means of controlling the metabolic 'on' and 'off' condition in this paper, was in fact merely a convenient way of skewing metabolism in a way that allows or prevents *exoS* induction upon removal of calcium from the media. The original thought in using osmolarity to manipulate the type III regulon stemmed from the observation that addition of glutamate to minimal glucose media was able to partially restore *exoS* induction. Increases in osmolarity result in significant increases in intracellular glutamate, and presumably alter flux through the citric acid cycle. Using the FACS assay, we therefore probed the citric acid cycle using metabolic mutation in order to triangulate the metabolite that controls *exoS* expression. Our efforts were met with partial success, because we were able to demonstrate that mutations in the citrate synthetases were able to increase the percentage of cells that induce *exoS* expression in a semi-defined media. Mutations in pyruvate dehydrogenase, on the other hand, reduced the percentage of cells that were able to induce *exoS* upon removal of calcium from the media. These experiments suggest that the critical metabolite regulating the type III regulon is acetyl-CoA or derived from acetyl-CoA. Deletion of both the gene for acetate kinase and that encoding phospho-transacetylase, *ackA* and *pta* respectively, did not affect the ability of the bacteria to induce *exoS* expression, suggesting that acetyl-phosphate is not, in fact, the controlling metabolite. Similarly, there were no overwhelming changes in the composition of the headgroups or side-chains of the phospholipids in the inner and outer membrane of *P. aeruginosa* grown under our test conditions. There are other metabolites that branch from acetyl-CoA. The *gltA prpC* double mutant was able to partially restore the ability of bacteria to induce *exoS* expression, but not elevate the basal level of expression, suggesting that these mutations restore the ability of the bacteria to assemble a functional type III secretion apparatus. As the type III secretion apparatus is a complex machinery that spans both the inner and the outer membrane, as well as the peptidoglycan layer of the cell, it seems reasonable to propose that the inability to induce *exoS* expression under metabolically unfavourable conditions is the result of a change in the envelope that prevents either assembly or proper function of the apparatus. Experiments are currently underway to better understand how the machinery is assembled. With regard to metabolic regulation, a better understanding of how this complex apparatus is assembled should lead to insights that will help characterize the assembly defect under metabolically unfavourable conditions and further focus our efforts to understand this remarkable phenomenon.

There are several global regulatory pathways that impact the expression of the type III secretion genes. The *P. aeruginosa* CRP homologue, Vfr, and the adenylate cyclases CyaA and CyaB are critically important for the expression of the type III secretion regulon. Interestingly, we were able to demonstrate that changes in osmolarity also alter the intracellular concentration of cAMP, and that artificially increasing the level of cAMP in the cell by over-expression of an adenylate cyclase was able to partially restore the ability to induce *exoS* expression, even in low-osmolarity media. As the *gltA prpC* double null mutation also resulted in elevated levels of cAMP, compared with the parental strain, it suggests that the cAMP pathway regulates, among other things, a gene or genes that are important for the proper assembly of the type III secretion machinery. Another way of interpreting the latter result is that the metabolic signal that derives form acetyl-CoA controls the activity of one or both adenylate cyclases. The second global regulatory pathway, the GacA/GacS system, however, does not appear to be relevant in this context of metabolic regulation, because expression of *exoS* in a *gacS* mutant is still media-dependent.

The fact that metabolism can severely impact the expression and assembly of the type III secretion machinery of *P. aeruginosa* is of considerable interest. It suggests that, if the controlling metabolic pathway is fully elucidated, it will provide a new target for antimicrobial development, with several potential benefits. As the controlling factor is a metabolite, it may be possible to use it as a lead compound for inhibitor development. Second, if the metabolite is an essential component of the cell, developing inhibitors of its synthesis may result in a bactericidal compound which, even if present at subinhibitory concentrations, can still impact the assembly of a key virulence factor.

We have presented evidence that metabolism controls expression of the type III secretion regulon in *P. aeruginosa* at both the level of basal transcription, as well as the assembly of the apparatus. These observations suggest that *P. aeruginosa* undergoes physiological changes when growing in the host, which directly influence the ability of

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Table 2. Strains and plasmids.

Strain	Relevant genotype	Reference/source
RP631	*P. aeruginosa* strain PAK, wild-type	Takeya and Amako (1966)
RP1407	PAK Δ*pcrV*	Rietsch *et al.* (2005)
RP1370	PAK Δ*pscC*	Rietsch *et al.* (2005)
RP955	PAK Δ*exoS*:: GFP-*lacZ*	Rietsch *et al.* (2004)
RP1224	PAK Δ*exoS*:: GFP-*lacZ* Δ*exsD*	Rietsch *et al.* (2005)
RP1389	PAK Δ*exoS*:: GFP-*lacZ* Δ*exsE*	Rietsch *et al.* (2005)
RP1221	PAK Δ*exoS*:: GFP-*lacZ* Δ*pscC*	Rietsch *et al.* (2005)
RP1192	PAK Δ*exoS*:: GFP-*lacZ* Δ*pcrV*	This study
RP1509	PAK Δ*exoS*:: GFP-*lacZ* Δ*popN*	This study
RP1302	PAK Δ*exoS*:: GFP-*lacZ* Δ*exsD* Δ*pscC*	This study
RP1438	PAK Δ*exoS*:: GFP-*lacZ* Δ*exsE* Δ*pscC*	Rietsch *et al.* (2005)
RP1305	PAK Δ*exoS*:: GFP-*lacZ* Δ*pcrV* Δ*pscC*	This study
RP1303	PAK Δ*exoS*:: GFP-*lacZ* Δ*popN* Δ*pscC*	This study
RP1150	PAK Δ*exoS*:: GFP-*lacZ* Δ*aceA*	This study
RP987	PAK Δ*exoS*:: GFP-*lacZ* Δ*gltA*	This study
RP1152	PAK Δ*exoS*:: GFP-*lacZ* Δ*gltA* Δ*prpC*	This study

Plasmid	Description	Reference/source
pPSV35	Shuttle vector with gentamicin-resistance gene (*aacC1*), PA origin, *lacI^q*, and the *lac*UV5 promoter and MCS of pUC18	Rietsch *et al.* (2005)
pP35-cyaB	cyaB gene cloned into pPSV35	This study

the bacterium to express key virulence factors and open exciting new possibilities for therapeutic intervention.

Experimental procedures

Bacterial strains and media

All *P. aeruginosa* strains described in this study are listed in Table 2. *E. coli* DH5α λ*pir* and SM10 λ*pir* were used, respectively, for cloning and conjugative transfer of plasmids into *P. aeruginosa* and were derived from laboratory stocks. Bacteria were routinely grown in L-broth (10 g tryptone, 5 g yeast extract, 5 g NaCl per litre). For experiments in which the effect of osmolarity was taken into account, bacteria were grown in LB (containing 10 g tryptone and 5 g yeast extract per litre) supplemented with 10 mM $MgCl_2$ and either 5 mM or 200 mM NaCl. MOPS minimal media were prepared as described (Neidhardt *et al.*, 1974).

Plasmids and strain construction

Deletion mutations were constructed using plasmid pEXG2 (Rietsch *et al.*, 2005). In-frame deletions were constructed by first generating two PCR products that carried the flanking regions for the deletion site. The two products were then spliced together in a second PCR reaction. The internal primers for the two PCR products defined the site of the deletion, as well as complementary 24 bp sequences (5′-TTCAGCAT GCTTGCGGCTCGAGTT-3′ and 5′-AACTCGAGCCGCAAG CATGCTGAA-3′ respectively), that allowed the two initial PCR products to be spliced together to generate the in-frame deletion. All primer sequences are available upon request.

β-Galactosidase assays

Cells were permeabilized with chloroform/SDS, and β-galactosidase activity was assayed as described previously (Miller, 1992).

FACS analysis

Overnight cultures of strains were diluted 1:200 into the appropriate fresh media and grown for 2 h at 37°C. At this point, the cultures were split into two and diluted into an equal volume of prewarmed media with or without NTA (final concentration 10 mM). Bacteria were then incubated with aeration for another 3 h at 37°C and subsequently chilled on ice for at least 10 min. The chilled bacteria were diluted into PBS and assayed for fluorescence using a FACScalibur flow cytometer (Becton Dickinson).

Immunofluorescence microscopy

Bacterial cells were stained based on a previously described protocol (Watarai *et al*., 2001). Overnight cultures of each strain were diluted 1:200 and grown in the appropriate media until mid-log. At this point 0.5 ml of culture were centrifuged, the supernatant was discarded and the bacteria were resuspended in a 2% solution of paraformaldehyde in KPO4 buffer (150 mM, pH 7.4) and incubated on ice for 30 min. The bacteria were subsequently pelleted, washed twice with PBS containing 2% goat serum (PBS-G) and resuspended in 40 μl of PBS-G. After 30 min of incubation at 37°C, the primary antibody was added at a final dilution of 1:250 (by addition of 10 μl of a 1:50 dilution in PBS-G) and the cells were incubated another 45 min at 37°C. At this point, 1 ml of PBS-G was added to each sample and the cells were again pelleted, washed once with 1 ml of PBS-G and resuspended in 50 μl of PBS-G containing a 1:500 dilution of a Alexa-fluor 488 conjugated goat-anti-rabbit secondary antibody (Molecular Probes). After 45 min of incubation at 37°C, in the dark, 1 ml of PBS-G was added to each sample and the cells were

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pelleted, washed twice with PBS-G, and resuspended in 30 μl PBS. Ten microlitres of each sample was spread evenly on a polylysine-coated microscopy slide, dried, covered with a drop of VectaShield mounting medium (Vector Laboratories) and a coverslip.

cAMP measurements

Bacteria were diluted 1:200 from overnight cultures and grown to mid-log in the appropriate medium at 37°C. The cultures were then chilled on ice for 5–10 min and 1 ml was removed and placed into a microcentrifuge tube. The bacteria were pelleted and washed twice with sterile, ice-cold saline (0.9% NaCl), then resuspended in 200 μl of ice-cold ddH_2O. One hundred microlitres of the suspension were used to determine the OD_{600}, the other half was extracted with HCl (0.1 N final concentration) for 15 min on ice. The extract was cleared by pelleting the cells. The supernatant was then transferred into a fresh microcentrifuge tube and dried in a SpeedVac. The amount of cAMP in the extract was determined using a commercial kit (Cayman) and the intracellular concentration was extrapolated, based on published values (D'Souza-Ault *et al*., 1993), by using the OD_{600} to determine the volume of the free intracellular water of the extracted bacteria. Changes in free intracellular water due to changes in osmolarity were taken into account.

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Supplementary material

The following supplementary material is available for this article online:

Fig. S1. FACS analysis of a plasmid-based *exoS* promoter (A) or toluic acid promoter (B) driving GFP expression in *P. aeruginosa* strain PAK. The concentration of inducer NTA (A) or toluic acid (B) is given in mM in the right hand corner of each plot.

Fig. S2. Shift experiments: PAK Δ*exoS*::GFP-*lacZ* (RP955) was grown overnight in LB with either 5 mM NaCl (low) or 200 mM NaCl (high), diluted 1:200 into fresh media and grown at 37°C with aeration. After 2 h, the culture was split and diluted 1:1 into LB with or without 10 mM NTA. Cultures were shifted between media of different osmolarity by pelleting cells in a microcentrifuge, washing them once with the medium the cells were being shifted to and resuspending them in the new medium at the indicated times (T0, at the time of diluting the overnight, or T2 after 2 h of incubation, at the time of the shift to media with or without NTA). After 3 h of incubation the cultures were chilled on ice and GFP fluorescence was assayed by FACS analysis [black, calcium replete; grey, in the presence of chelator (NTA)].

Fig. S3. PcrV production and localization in low- and high-osmolarity LB. (A) Western blot of cell lysates of PAK Δ*exoS*::GFP-*lacZ*(RP955) grown in LB with 5 mM NaCl (low) or 200 mM NaCl (high). Cells were grown to mid-log, pelleted and resuspended in SDS-sample buffer at an OD_{600} of 1. 10 μl of each lysate, and of a 1:4 and 1:16 dilution thereof, were separated by 10% SDS-PAGE and transferred to nitrocellulose. The blot was probed with a rabbit anti-PcrV antiserum and developed using a secondary antibody conjugated to HRP. After the first hybridization, the blot was stripped using a commercial stripping reagent (Pierce) and reprobed using a commercial mouse monoclonal-antibody directed against the α-subunit of RNA polymerase (Neoclone). (B) RP955 was grown in low- or high-osmolarity LB for 2 h at which point the culture was split and diluted 1:1 into LB of the same osmolarity with or without the chelator NTA. After 2 h of growth the cultures were chilled on ice and fractionated into cell pellet and supernatant fractions (supernatant proteins were precipitated using trichloroacetic acid at a final concentration of 10%). All fractions were subjected to SDS-PAGE, transferred to nitrocellulose and probed with an anti-PcrV antiserum.

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