Effect of Metabolic Imbalance on Expression of Type III Secretion Genes in Pseudomonas aeruginosa

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The type III secretion system is a dedicated machinery used by many pathogens to deliver toxins directly into the cytoplasm of a target cell. Expression and secretion of the type III effectors are triggered by cell contact. In Pseudomonas aeruginosa and Yersinia spp., expression can be triggered in vitro by removing calcium from the medium. The mechanism underlying either mode of regulation is unclear. Here we characterize a transposon insertion mutant of P. aeruginosa PAO1 that displays a marked defect in cytotoxicity. The insertion is located upstream of several genes involved in histidine utilization and impedes the ability of PAO1 to intoxicate eukaryotic cells effectively in a type III-dependent fashion. This inhibition depends on the presence of histidine in the medium and appears to depend on the excessive uptake and catabolism of histidine. The defect in cytotoxicity is mirrored by a decrease in exoS expression. Other parameters such as growth or piliation are unaffected. The cytotoxicity defect is partially complemented by an insertion mutation in cbrA that also causes overexpression of cbrB. The cbrAB two-component system has been implicated in sensing and responding to a carbon-nitrogen imbalance. Taken together, these results suggest that the metabolic state of the cell influences expression of the type III regulon.

Pseudomonas aeruginosa is an opportunistic pathogen that is a common cause of hospital-acquired infections. It generally infects immunocompromised patients, such as burn patients or cancer patients, in whom it causes wound infections, catheter infections, and ventilator-associated pneumonia (9, 30, 32). In cystic fibrosis patients, P. aeruginosa is the most common cause of severe, chronic lung infections, which ultimately lead to a decline in respiratory function and death (11).

P. aeruginosa possesses a large arsenal of virulence factors (11, 37). One virulence mechanism that has attracted significant attention in recent years is the ability to intoxicate eukaryotic cells by using a type III secretion system to inject toxins directly into the target cell (48). This mode of toxin delivery has also been termed contact-dependent secretion, since secretion of the effector molecules is triggered by cell contact (43). Four type III effector molecules (toxins) have been described in P. aeruginosa. Exoenzyme S (ExoS) and ExoT are highly homologous proteins that have a carboxy-terminal ADP-ribosylating motif and an amino-terminal GTPase-activating domain (10, 18, 20, 47). The GTPase-activating activity of these proteins targets small, Rho-like GTPases (19) and has been linked with cytoskeletal rearrangements and cell rounding in vitro (10, 18). The two other known effector molecules are ExoY and ExoU. ExoY is an adenylyl cyclase (50), and ExoU is a necrotizing toxin with phospholipase activity (5, 6, 13, 35).

Type III secretion has been linked to more severe disease progression in ventilator-associated pneumonia (12, 15, 34), and type III effectors are also associated with increased virulence in animal models of infection (6, 28, 29).

The regulation of the type III effector genes and secretion machinery appears to be coordinated (45). ExoS expression is induced by cell contact but can also be turned on in vitro by removing calcium from the medium (7, 49). This induction of effector genes under low-calcium conditions depends on the presence of an intact type III secretion machinery, suggesting that the machinery itself is involved in sensing the calcium signal (7, 25, 45). However, it was recently reported that the ability of P. aeruginosa strain CHA to respond to low-calcium conditions was abolished by transposon insertions in the aceAB genes, which encode pyruvate dehydrogenase (4). It was suggested that pyruvate dehydrogenase may act directly as a regulator of gene expression, but another interpretation is that there is some form of metabolic control over the transcription of the type III secretion genes.

In this paper, we report the isolation and characterization of a transposon insertion mutation in P. aeruginosa strain PAO1 that results in overexpression of histidine utilization genes and simultaneously abolishes the ability of the bacterium to induce type III-mediated cytotoxicity. The cytotoxicity defect can be partially suppressed by an insertion in cbrA. This gene encodes the sensor kinase of a two-component system reported to be involved in sensing and responding to a carbon-nitrogen imbalance (31). These findings suggest that the metabolic state of the cell is factored into the decision to induce expression of type III effectors by P. aeruginosa.

MATERIALS AND METHODS

Bacterial strains and media. All of the P. aeruginosa strains described in this report are listed in Table 1. Escherichia coli DH5α pir and SM10 kpir were used.
respectively, for cloning and conjugal transfer of plasmids into *P. aeruginosa* and were derived from laboratory stocks. Bacteria were grown in Luria-Bertani (LB) medium unless indicated otherwise. Exconjugants of *P. aeruginosa* were selected on Pseudomonas Isolation Agar (Becton Dickinson).

**Plasmids and strain constructions.** To generate plasmid pPSV18, the β-lactamase gene (∼bla) and lac promoter-polylinker region of pUC18 were combined with a pBR322 origin and the *Pseudomonas* origin of replication (PAAoI) from pUC183.1 (8). The *PstI* fragment from plasmid pUC183.1, carrying the PAoI, was first cloned into the *BomI* site of a pBR322 derivative, pBR-mob, with a short linker to introduce *SacI* and *PacI* sites (generated by annealing primers 5′-TAATTAATTTAAATTTACCG-3′ and 5′-TAATTAATTTAAATTTACCG-3′ respectively) that allowed the two initial 24-bp sequences (5′-AAAAAAGCTTCCGCGGTAACAGGTTGCAGGGTGACGCTGTA TAGGCTGTA-3′ and 5′-AAAAAAGCTTCCGCGGTAACAGGTTGCAGGGTGACGCTGTA TAGGCTGTA-3′) to be spliced together in a second PCR. The internal primers for generating two PCR products that carried the *bla* gene with the *SacI* and *PstI* recognition sequence were 5′-GAACTATACAAATGACCATGATTACGGACG-3′ (gentamicin resistance) and 3′-GGGGACA-5′ (pBR322 origin), respectively. These two PCR products were spliced together in a second PCR cloning system (45). In-frame deletions were constructed in this vector by generating two PCR products (44). The GFP-encoding gene was amplified with primers GFPf1 (5′-ACAGGTTGCAGGGTGACGCTGTA TAGGCTGTA-3′ and 5′-AAAAAAGCTTCCGCGGTAACAGGTTGCAGGGTGACGCTGTA TAGGCTGTA-3′) and GFPf2 (5′-AAAAAAGCTTCCGCGGTAACAGGTTGCAGGGTGACGCTGTA TAGGCTGTA-3′) and cloned into pPSV18 as an *SacI*-*PstI* fragment. Plasmid pPSV23 was derived from plasmid pPSV18 by digestion with *SacI* and *PstI*, and expression of GFP was confirmed by confocal microscopy.

**TABLE 1. Strains and plasmids used in this study**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype or description</th>
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<tr>
<td><strong>Plasmids</strong></td>
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<tr>
<td>pTGL3</td>
<td>Suicide vector bearing TnTGL3</td>
<td>This study</td>
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<tr>
<td>pPSV13</td>
<td>Shuttle vector with pBR322 origin, PA origin, lac promoter, bla (ampicillin and carbenicillin resistance)</td>
<td>This study</td>
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<tr>
<td>pPSV32</td>
<td>Shuttle vector with pBR322 origin, PA origin, lac promoter, lacP::aacC1 (gentamicin resistance)</td>
<td>This study</td>
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<tr>
<td>pP18-hutT-PA5099</td>
<td>Chromosomal fragment carrying genes hutT, hutH, and hutU in pPSV18</td>
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<td>pP18-hutT</td>
<td>hutT in pPSV18</td>
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<td>pP18-PA5099</td>
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<td>pP32-clbB</td>
<td>cbrA::TnFAC</td>
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with primers GSP5 (5′-AACACTGGCAAGAGGAGATACATATGAGTAAAGG-3′) and DGL3 (5′-AATCATGGCTATTGTTATAGATCATCAGCC-3′). The *lacZ* gene was amplified from the chromosome of *E. coli* strain MG1655 with primers DLS5 (5′-GAGAACATATACAACTCAGGACCATGATTACGGATTCTACT-3′) and L3PST (5′-GGCCGCGATCCAGCGATGACGCTGCGGTTGGTTATTTATTTGAGAGC-3′). These two PCR products were spliced together in a second PCR step and cloned into pTnTet as a *PstI* fragment, generating pTGL3 (harboring transposon TnGL3). The sequences of the GFP-encoding and *lacZ* genes in pTGL3 were confirmed by sequencing.

Plasmid pP18-hutT-PA5099 was generated by amplifying the chromosomal region carrying genes PA5097 (hutI) to PA5099 with primers 5097-3′ and 5097-5′, respectively, as an *EcoRI*/HindIII fragment. Plasmid pP18-hutT was derived from plasmid pP18-hutT-PA5099 by digestion with *EcoRI* and *EcoRV*, followed by end filling with T4 DNA polymerase and religation. Plasmid pP18-PA5099 was derived from plasmid pP18-hutT-PA5099 by digestion with BamHI and HindIII, followed by end filling with T4 DNA polymerase and religation. Plasmid pP32-clbB was generated by amplifying the *cbrB* gene from the chromosome of PAO1 with primers cbrB5 (5′-AAAAAGAACATCTCGAGAGCTGAATACATGCGCACTATA-3′) and cbrB3 (5′-AAAGAAGACATCTCGAGAGCTGAATACATGCGCACTATA-3′) and cloning it into pPSV18 as an *EcoRI*/HindIII fragment. Plasmid pP18-hutT-PA5099 was digested with *EcoRI* and *EcoRV*, followed by end filling with T4 DNA polymerase and religation. Plasmid pP18-PA5099 was derived from plasmid pP18-hutT-PA5099 by digestion with BamHI and HindIII, followed by end filling with T4 DNA polymerase and religation. Plasmid pP32-clbB was generated by amplifying the *cbrB* gene from the chromosome of PAO1 with primers cbrB5 (5′-AAAAAGAACATCTCGAGAGCTGAATACATGCGCACTATA-3′) and cbrB3 (5′-AAAGAAGACATCTCGAGAGCTGAATACATGCGCACTATA-3′) and cloning the product as an *EcoRI*/HindIII fragment into plasmid pPSV32.

**Deletion mutants of *P. aeruginosa* PAO1 were generated with a version of plasmid pEX18Gm (14) adapted for use with the Gateway recombinational cloning system (45). In-frame deletions were constructed in this vector by generating two PCR products that carried the flanking regions for the deletion site and splicing the two products together in a second PCR. The internal primers for the two PCR products defined the site of the deletion, as well as complementary 24-bp sequences (5′-TCTAGATCTGCGCGCTGAGTT-3′ and 5′-AAATTCGAGCCCGAACATCTGCTGAAAG-3′, respectively) that allowed the two initial PCR products to be spliced together to generate the in-frame deletion. The flanking primers contained the sequences necessary for subsequent recombinational cloning of the PCR product carrying the deletion (attB1 5′-GGGGAGC...-3′).
The exoS reporter was constructed by cloning the PstI fragment carrying the GFP-encoding and lacZ genes (blunted with T4 DNA polymerase) from pTGL3 into the EcoRI site of pDONR207 (again made blunt with T4 DNA polymerase). The resulting plasmid was then recombined into deletion vector pEXΔmGFP with the Gateway recombination system (Invitrogen), resulting in plasmid pEXΔmGFP-LacZ.

Mutations in pIL4 and hutU were generated by insertion mutagenesis with pPS60. pPS60 was derived from pPSV30 by digesting pPSV30 sequentially with MsiI and Smal and religation, thus removing the lac promoter and PAoI of pPSV30. Internal fragments of hutU and pIL4 were cloned into this vector (primers hutUn5′ [5′-AGATCTCAAGAAGACCGCTGACACTGCTAGGCGATTCGAGTCTT] and hutUn3′ [5′-GGGTACCAACTTATTTCAGAGAGATCGTATCCGGCTTCTT] ) and hutU3′, hutU5′ and pIL4′, pIL45′ (see supplementary sequences for restriction cloning), and the resulting plasmids, pPS50HutU and pPS60pIL41nt, were moved into SM1ΔΔs and subsequently mated into P. aeruginosa PA01 to generate the null mutants.

Fluorescence-activated cell sorting. The pool of transposition insertion mutants was grown in tryptone soy broth (Becton Dickinson) supplemented with 100 mM sodium glutamate and 1% glycerol. Low-calium conditions were achieved by addition of 1 mM EGTA, and calcium-replete conditions were achieved by adding 1 mM CaCl2. Fluorescent or nonfluorescent cells, depending on the stage in the enrichment cycle, were sorted with a FACScalibur flow cytometer (Becton Dickinson).

Phage transduction. Transduction was performed with a clear derivative of phage DMS3 (U. Budzik and G. A. O'Tool, unpublished data).

Cell lines and tissue culture conditions. A549 cells (CCL-185; ATCC), a human epithelial cell line, and RAW264.7 cells (IB-71; ATCC), a mouse macrophage cell line, were grown in RPMI 1640 tissue culture medium (CellGro) supplemented with 2 mM glutamine, 10 mM HEPES, and 10% fetal bovine serum (FBS) (RP10 medium). In experiments in which tissue culture medium without histidine was required (see Fig. 5 and 6), RPMI medium was generated in accordance with the published formulation (CellGro) but omitting histidine (RPMI-His). In those experiments, the data points of strains grown in the presence of histidine reflect results obtained with the same batch of RPMI-His to which histidine was added back to the concentration found in complete RPMI medium.

Cytotoxicity assays. Two types of assays were used to detect cytotoxicity. The first measures the ability of P. aeruginosa to cause rounding of epithelial cells. A549 cells were seeded in 24-well plates at approximately 8 × 104 cells/well on the day prior to the experiment. On the day of the experiment, cells were infected at a multiplicity of infection of 50 (assuming ~10 A549 cells/well) for 4 h. The infection was stopped by removing the medium and incubating the cells in a phosphate-buffered 4% paraformaldehyde solution. The extent of cell rounding was assessed by phase microscopy.

The second cytotoxicity assay exploits the ability of P. aeruginosa to cause necrosis in cultured RAW264.7 macrophages. The extent of necrosis was assayed by monitoring the release of the cytoplasmic enzyme lactate dehydrogenase (LDH). On the day of the experiment, RAW264.7 cells were scraped up, washed once with fresh RP10 medium, resuspended in fresh RP10 medium without the phenol red indicator dye (Invitrogen), and then dispensed into a 96-well plate (Corning) at 104 cells/well. The cells were infected with P. aeruginosa at a multiplicity of infection of 50. After 300 to 330 min of infection, the extent of LDH release was assayed with the CytoTox96 kit in accordance with the manufacturer’s (Promega) instructions.

β-Galactosidase assays. Cells were permeabilized with chloroform-sodium dodecyl sulfate, and β-galactosidase activity was assayed as described previously (26).

DNA microarray analysis. The expression profiles of mutant and wild-type PAO1 were determined with Affymetrix GeneChip microarrays. The wild-type and mutant were grown statically in a 5% CO2 atmosphere in 100 ml of RP10 medium. After 3 h of incubation, the cells were spun down, resuspended in Trizol reagent (GIBCO), and processed as described previously (45). The experiment was only performed once, and the results were not verified beyond the experiments indicated in this study (exoS expression and the effect of overexpression of the histidine degradation genes).

RESULTS

Isolation of a transposon insertion mutant that affects type III secretion-mediated cytotoxicity in P. aeruginosa PAO1. GFP has been used to identify differentially regulated genes (23, 24, 36, 41, 42). We used a modified version of this usually plasmid-based approach to identify differentially regulated genes in P. aeruginosa. Specifically, we designed a transposon (TnGL3) with a promoterless GFP-encoding gene and a translationally coupled lacZ gene for this purpose (Fig. 1).

At least one set of known virulence genes, encoding the type III secretion machinery and effectors, is regulated by low calcium. We therefore decided to use our system to identify additional genes that are induced under low-calcium conditions. A pool of approximately 58,000 transposon insertion mutants was grown in LB medium under low-calium conditions, and fluorescent bacteria were isolated by fluorescence-activated cell sorting. This pool of insertions was then grown in the presence of Ca2+ and sorted for nonfluorescent bacteria. After a second round of growth under low-calium conditions and sorting for fluorescent bacteria, the output pool was plated for single colonies. Of 190 colonies picked from this output pool, 77 (37%) responded to low calcium when tested individually. Sequencing of 36 mutants revealed 20 unique insertions, defining 11 genes or intergenic regions. This set of genes included an insertion four nucleotides upstream of pcrH, which encodes the promoter region of pcrH (Fig. 1). A6845 cells were seeded in 24-well plates at approximately 8 × 104 cells/well on the day prior to the experiment.
a proposed chaperone of the type III needle complex protein PopD. With the exception of two insertions (one in PA0388 and one just upstream of PA5099), this set matched a list of calcium-regulated genes previously identified by DNA microarray analysis (45).

*P. aeruginosa* causes rounding in epithelial cells and necrosis of macrophages in vitro. These activities depend on the presence of a functional type III secretion system. Since the type III effectors and secretion machinery genes are subject to induction under low-calcium conditions and we identified an insertion upstream of *pcrH*, we decided to subject the pool of low-calcium-regulated insertions to a secondary screen for cytotoxicity. One of these 77 mutants, I.11E, failed to induce rounding in A549 cells (data not shown) and displayed a significant defect in lysing RAW264.7 macrophages (Fig. 3).

A transposon insertion resulting in overexpression of the histidine utilization genes results in loss of cytotoxicity. The transposon insertion in mutant I.11E is located immediately upstream of gene PA5099, which encodes a putative transporter (Fig. 2). The mutant phenotype was not due to a secondary mutation, since crossing out the transposon insertion restored cytotoxicity to wild-type levels (data not shown). We also determined growth curves in LB medium and under tissue culture conditions and performed twitching motility assays to rule out a growth defect or loss of piliation as a cause for the loss of cytotoxicity. Neither growth nor piliation was affected in the mutant (data not shown).

DNA microarray analysis demonstrated that nine genes downstream of the transposon insertion were at least 15-fold overexpressed (data not shown). To determine if overexpression of one of the adjacent genes was responsible for the observed phenotype, deletions in adjacent genes were combined with the transposon insertion mutation, and cytotoxicity of the resultant strains was determined with the macrophage lysis assay. The ability of the bacteria to kill RAW264.7 macrophages was restored by deleting PA5097, a putative amino acid transporter gene (Fig. 3). Deletion of PA5097 in wild-type PAO1 had no effect on cytotoxicity (data not shown).

Since PA5097 is annotated as encoding a putative amino acid transporter and is situated among the histidine catabolism genes, we assayed the ability of a PA5097 deletion mutant to grow on each of the 20 amino acids as a sole carbon source. Of the amino acids that *P. aeruginosa* is able to utilize as a sole carbon source, the PA5097 deletion mutant only displayed a defect in utilizing histidine (Fig. 4). PA5097 therefore appears to encode the histidine transporter, and we have designated it *hutT*, in keeping with the nomenclature of the annotated histidine utilization genes. The unlinked ABC transporter *hisJQMP* is also annotated as a histidine transporter, on the basis of homology to the *E. coli* histidine transporter (38). However, since insertional inactivation of *hutT*, but not *hisM*,

![FIG. 2. Chromosomal location of the I.11E insertion mutant. (A) The transposon insertion in the cytotoxicity mutant I.11E is located 9 bp upstream of gene PA5099. Histidine utilization genes are indicated by the hatched arrows. (B) The histidine utilization pathway (http://biocyc.org/). The gene product involved in each individual step is in boldface.](http://iai.asm.org/)

![FIG. 3. Suppression of the I.11E phenotype by deletion of PA5097 (hutT)]. LDH release by RAW264.7 macrophages infected with *P. aeruginosa* PAO1, a mutant with an in-frame deletion of *popB*, the transposon insertion mutant I.11E, as well as a mutant of PAO1 combining the I.11E insertion with an in-frame deletion of PA5097 (hutT). The infections were carried out in complete RPMI tissue culture medium (which contains histidine) supplemented with glutamine and 10% FBS.](http://iai.asm.org/)
lead to histidine auxotrophy, the annotation of hisJQMP is likely to be incorrect (data not shown).

We next determined if overexpression of the histidine transporter by itself could replicate the mutant phenotype. To this end, a chromosomal fragment carrying genes PA5097 (hutT) to PA5099 was expressed from a multicopy plasmid in wild-type PAO1. Overexpression of this chromosomal fragment resulted in loss of cytotoxicity (Fig. 5A). Deletions of the plasmid that leave either PA5099 or hutT intact demonstrated that this defect depends on overexpression of hutT, not PA5099 (Fig. 5A). It is notable that the plasmid carrying only hutT did not have as severe an impact on cytotoxicity as the full-length construct. This difference is likely due to the effect of HutH, the histidine-ammonia lyase, which also contributes to the mutant phenotype (see below). The cytotoxicity defect was mirrored by a decrease in exoS transcription as measured by a GFP-lacZ reporter construct inserted into the chromosome at the exoS locus (Fig. 5B).

**hutT overexpression represses exoS transcription in strain PAK.** In the course of this study, we discovered that strain PAO1 does not induce expression of the type III effector genes under low-calcium conditions in vitro, and its overall level of type III effector expression is low compared to that of other *P. aeruginosa* strains. We therefore decided to determine if the effect of overexpressing the histidine catabolic genes could be extended to other strains of *P. aeruginosa*. Strain PAK induces expression of exoS under low-calcium conditions (45). We therefore introduced our chromosomal exoS reporter into PAK, as well as the plasmid bearing the hutT-PA5099 chromosomal fragment. Overexpression of the hutT-PA5099 fragment in PAK resulted in repression of the exoS reporter (Fig. 5C) and loss of cytotoxicity (data not shown).

**The defect in cytotoxicity is dependent on the presence of histidine in the medium and on an intact histidine degradation pathway.** To determine if the cytotoxicity defect in the I.11E insertion mutant was a nonspecific side effect of overexpressing the histidine transporter or actually related to its function, we examined the effect of exogenous histidine on the mutant’s cytotoxicity. The mutant phenotype was readily reversed by removing histidine from the tissue culture medium (Fig. 6), indicating that excessive uptake of histidine, rather than mere overexpression of the transporter, was responsible for the mutant phenotype. In fact, deletions in the histidine catabolic genes hutH, hutU, and hutG suppressed the mutant phenotype to various degrees, indicating that uptake and degradation of histidine are required for decreased cytotoxicity (Fig. 7). The poor suppression of the mutant phenotype by the mutations in later steps of the histidine utilization pathway (hutU, hutI, and hutG) may be due partly to the fact that combining these mutations with the I.11E insertion was deleterious for growth. This was especially true for hutI, which was
not included in Fig. 7, and, to a lesser degree, hutG (data not shown). Deletion of hutH, hutI, or hutG in the wild-type background had no effect on cytotoxicity (data not shown).

The histidine dependence of the mutant phenotype correlated with a histidine-dependent decrease in exoS expression in a strain overexpressing either hutT and hutH or just hutT (Fig. 5B).

The mutant phenotype is suppressed by a transposon insertion in cbrA. While constructing the hut deletion mutants in the I.11E background, we discovered that the original insertion mutant had a severe defect in growth on histidine as the sole carbon source and we used this phenotype to isolate suppressor mutants. The I.11E insertion mutant was subjected to a second round of transposon mutagenesis, this time with the mariner-based minitransposon TnFAC (46). From a pool of about 3,000 mutants, 3 were isolated that restored the ability of the I.11E mutant to grow efficiently on histidine as the sole carbon source. Of these, one partially restored cytotoxicity (Fig. 5A). The insertion in this mutant was located in cbrA, which encodes the sensor kinase of a two-component regulatory system (cbrAB) that has been implicated in sensing and responding to a carbon-nitrogen imbalance (31). The suppression phenotype is recapitulated by overexpressing cbrB in a mutant with an in-frame deletion in cbrA (Fig. 8B). Neither overexpression of cbrB alone nor deletion of cbrA alone affected cytotoxicity (Fig. 8B). A transductant in which the suppressing TnFAC insertion in cbrA was moved into wild-type PAO1 did not display significantly enhanced cytotoxicity. This suggests that cbrB is not simply an activator of type III secretion gene expression, but rather that the unique combination of deleting cbrA and overexpressing cbrB helps to, at least in part, alleviate the metabolic imbalance that caused the original insertion mutant to be noncytotoxic.

**DISCUSSION**

Here we describe the isolation of a transposon insertion mutant of *P. aeruginosa* that is unable to intoxicate eukaryotic cells in a type III secretion system-dependent manner. The insertion mutation results in overexpression of genes required for histidine utilization, and the concomitant cytotoxicity defect can be alleviated by removing histidine from the tissue culture medium. This suggests that the cytotoxicity defect results from excessive uptake of histidine, and not, for example, from aberrant overexpression of a variety of membrane proteins. Consistent with this observation is the fact that the defect in cytotoxicity can also be alleviated by combining the original insertion mutation with deletions in histidine degradation pathway genes, most notably hutT (the histidine transporter).
and hutH (the histidine-ammonia lyase, which catalyzes the first step in histidine degradation). The latter result indicates that both uptake and degradation of histidine are required for the loss of cytotoxicity.

Transcriptional profiling of the mutant indicated that, compared to that in the wild type, expression of the type III effectors and secretion machinery was reduced under the conditions of the cytotoxicity assays. We used a chromosomal exoS-lacZ reporter system to confirm this result. The defect in exoS expression is observed during overexpression of the histidine transporter (hutU) in a strain carrying a lacZ reporter gene in the exoS locus. This defect in exoS expression also depends on the presence of histidine in the medium.

The I.11E mutation causes PAO1 to grow extremely poorly on histidine as a sole carbon source. The reason for this appears somewhat puzzling, since none of the histidine catabolic genes are disrupted. If anything, they are overexpressed. One possible explanation is that overexpression of all of the histidine utilization genes except hutU (the gene for urocanase, which is located upstream of the insertion site [Fig. 2]) leads to a situation in which the relatively low level of urocanase creates a bottleneck in the catabolic pathway. As a result, it may be that ammonia levels rise because of the initial deamination reaction, while the assimilation of the carbon backbone proceeds more slowly, resulting in a metabolic imbalance that is deleterious for growth. This explanation fits conceptually with the results of the suppressor mutant screen. The defects in growth and cytotoxicity were partially suppressed by an insertion in cbreA, which also results in overexpression of cbreB. The cbreAB two-component regulatory system was originally described as being involved in controlling a number of catabolic operons. Strains mutant for cbreA or cbreB are incapable of growing on a variety of amino acids as the sole carbon source. This two-component regulatory system was postulated to sense and respond to a carbon-nitrogen imbalance because of the intriguing observation that a mutant is unable to grow on proline as the sole carbon and nitrogen source but can be complemented by addition of succinate to the medium. This complementation is abolished, however, by the further addition of ammonia to the medium (31). Since the cbreA::TnFAC insertion does not increase the cytotoxicity of the wild-type strain, it seems that this mutation helps counteract the metabolic imbalance incurred by the excessive uptake and catabolism of histidine.

A recent search for mutations that render P. aeruginosa strain CHA unable to induce its type III secretion machinery under low-calcium conditions identified a large number of insertions in the aceAB operon, which encodes the subunits of pyruvate dehydrogenase (4). The authors proposed that pyruvate dehydrogenase may act directly as a transcriptional activator. In view of our results, however, an alternative explanation is that these mutations affect the metabolic state of the cell, which, in turn, affects the expression of type III secretion genes.

Infection of a host represents a significant change in environment, and sensing its own metabolic state may be an expedient way to sense this transition and regulate virulence factor expression accordingly. Since several metabolic pathways funnel into and branch off of the tricarboxylic acid (TCA) cycle, its intermediates should be ideally suited to conveying the overall metabolic state of the cell. We propose that the I.11E and aceAB mutations affect the type III secretion system indirectly by modulating levels of TCA intermediates. Histidine is degraded to glutamate, which in turn is one step away from the TCA cycle intermediate α-ketoglutarate. Pyruvate dehydrogenase generates acetyl coenzyme A, which combines with oxaloacetate to form citrate, another TCA cycle intermediate. Current experiments are aimed at addressing this hypothesis directly, but there is clearly some supporting evidence from studies of other pathogenic bacteria. Glutamate is involved in the regulation of type III secretion genes in Yersinia sp. (33), and mutations in fatty acid biosynthesis genes in Salmonella sp. have also suggested that a change in metabolism may be factored into the expression of the type III secretion machinery on Salmonella pathogenicity island 1 (21, 22).

Finally, since we were able to recapitulate the mutant phenotype in strain PAK with a plasmid that overexpressed the histidine transporter and histidine-ammonia lyase, it appears that metabolic regulation of the type III secretion system is a general phenomenon. If so, it may be possible to target specific metabolic pathways to prevent induction of the type III secretion machinery and thus improve clinical outcomes.

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