

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 adenylate 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). This low-calcium regulation has also been observed in *Yersinia* sp., whose type III secretion machinery is highly homologous to that of *P. aeruginosa* (17, 27). The mechanism by which the extracellular calcium concentration is translated into a transcriptional signal remains enigmatic. 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*.

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MATERIALS AND METHODS

Bacterial strains and media. All of the *P. aeruginosa* strains described in this report are listed in Table 1. *Escherichia coli* DH5 α *lpir* and SM10 *lpir* were used,

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype or description	Reference or source
Strains		
PAO1	Wild type	16
PAK	Wild type	40
I.11E	PAO1 with TnTGL3 insertion upstream of PA5099 (Fig. 2)	This study
PAO1 $\Delta popB$	$\Delta popB$	This study
PAO1 $\Delta pilA$	$\Delta pilA::pP30\Delta$	This study
PAO1 $\Delta hutT$	$\Delta hutT$	This study
PAO1 $\Delta hutH$	$\Delta hutH$	This study
PAO1 $\Delta hutG$	$\Delta hutG$	This study
PAO1 $\Delta hutI$	$\Delta hutI$	This study
PAO1 $cbrA::TnFAC$	$cbrA::TnFAC$	This study
PAO1 $exoS::GL3$	$\Delta exoS::(GFP lacZ)$	This study
PAK $exoS::GL3$	$\Delta exoS::(GFP lacZ)$	This study
I.11E $\Delta hutT$	TnTGL3(I.11E) $\Delta hutT$	This study
I.11E $\Delta hutH$	TnTGL3(I.11E) $\Delta hutH$	This study
I.11E $\Delta hutI$	TnTGL3(I.11E) $\Delta hutI$	This study
I.11E $\Delta hutG$	TnTGL3(I.11E) $\Delta hutG$	This study
I.11E $hutU$	TnTGL3(I.11E) $hutU::pP30\Delta$	This study
I.11E $\Delta cbrA$	TnTGL3(I.11E) $\Delta cbrA$	This study
Plasmids		
pTGL3	Suicide vector bearing TnTGL3 (Fig. 1)	This study
pPSV18	Shuttle vector with pBR322 origin, PA origin, <i>lac</i> promoter, <i>bla</i> (ampicillin and carbenicillin resistance)	This study
pPSV32	Shuttle vector with pBR322 origin, PA origin, <i>lac</i> promoter, <i>lacI^q aacC1</i> (gentamicin resistance)	This study
pP18- <i>hutT</i> -PA5099	Chromosomal fragment carrying genes <i>hutT</i> , <i>hutH</i> , and PA5099 in pPSV18	This study
pP18- <i>hutT</i>	<i>hutT</i> in pPSV18	This study
pP18-PA5099	PA5099 in pPSV18	This study
pP32- <i>cbrB</i>	<i>cbrB</i> in pPSV32	This study

respectively, for cloning and conjugative 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 (PAori) from pUC181.1 (8). The *PstI* fragment from plasmid pUC181.1, carrying the PAori, was first cloned into the *BsmI* site of a pBR322 derivative, pBR-mob, with a short linker to introduce *SwaI* and *PacI* sites (generated by annealing primers 5'-ATTAATTAATTTAAATTTACCG-3') and 5'-GTAAATTTAAATTAATTAATTGCA-3'). The origin was then subcloned between the *NarI/MscI* sites of pBR322 as a *NarI/SwaI* fragment, generating plasmid pBR-PAori. pPSV18 was generated by combining the polylinker and most of the *bla* gene of pUC18 (pUC18 digested with *SapI*, blunted with T4 DNA polymerase, and subsequently digested with *AhdI*) with the *SfoI/AhdI* fragment of pBR-PAori, carrying the pBR322 origin and the PAori. Plasmid pPSV28 was derived from plasmid pPSV18 by replacing the *bla* gene with the *aacC1* (gentamicin resistance) gene, derived originally from plasmid pBSL182 (1). *aacC1* was amplified with primers gent5m (5'-AAAAAATATTACGCGTCAATTCTCGAATTGACATAA-3') and gent3mma (5'-AAAAGACTCCCGTCCAATTGACGCGTCGGCCGGGAAGCCGATCTC G-3') and cloned into pPSV18 as an *SspI/AhdI* fragment. Plasmid pPSV30 was generated by cloning the *EcoRI* fragment from plasmid pBSL237 (2), carrying the RP4 origin of transfer, into the *MfeI* site of pPSV28. Finally, plasmid pPSV32 was derived from plasmid pPSV30 by cloning the *lacI^q* gene, amplified with primers lacIq5 (5'-AAAAAAATATTAATGGAGCAAACCTTTCGCGGTA T-3') and lacI3 (5'-AAAAAAATATTCTCACATTAATTGCGTTGCGCTCA-3'), as an *SspI* fragment into pPSV30.

Transposon TnTGL3 was constructed by first replacing the Km^r cassette on pSCI23 (S. Chiang, unpublished data) with the *MluI* fragment carrying the tetracycline resistance gene from pBSL199 (1), thus generating pTnTet. In a second step, a cassette with the green fluorescent protein (GFP)-encoding gene and a translationally coupled copy of *lacZ* was generated by splicing together two PCR products (44). The GFP-encoding gene was amplified from pGFPmut3 (3)

with primers G5PST (5'-AAAAGTGCAGGAAGGAGATATACATATGAGTAAAG-3') and DGL3 (5'-AATCATGGTTCATTTGTATAGTTTCATCCATGC C-3'). The *lacZ* gene was amplified from the chromosome of *E. coli* strain MG1655 with primers DLG5 (5'-GAACTATACAAATGACCATGATTACGG ATTCAC-3') and L3PST (5'-GCAGACATGGCCTGCAGGGTTATTATTA TTTTGTAC-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 TnTGL3). 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 (*hutT*) to PA5099 with primers 5097 3' (5'-AAA AAAAGCTTCTCAGGCCGCGTTCGGTTCACACA-3') and perm5 (5'-G GCGCGCAATTCAGCGGAGTACAGCA-3') and cloning it into pPSV18 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*-5099 by digestion with *BamHI* and *HindIII*, followed by end filling with T4 DNA polymerase and religation. Plasmid pP32-*cbrB* was generated by amplifying the *cbrB* gene from the chromosome of PAO1 with primers *cbrB5* (5'-AAAAAGAATTCAGAGAGCTGAATACATGGCACATA TT-3') and *cbrB3* (5'-AAAAAAGCTTCCGCGGTAACAGGTTGCGGGTG TTA-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'-TTCAGCATGCTTGGCGGCTCGAGTT-3' and 5'-AACTC GAGCCGCAAGCATGCTGAA-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'-GGGGACA

AGTTTGTACAAAAAGCAGGCT-3'] and attB2 [5'-GGGGACCACTTTG TACAAGAAAGCTGGGT-3']. The actual cloning was performed with the Gateway cloning kit (Invitrogen) in accordance with the manufacturer's instructions.

The primers used to make deletion constructs were $\Delta hutT$ (5'-flanking PCR product, 5097/5-1 [5'-TTCAGCATGCTTGGCGCTCGAGTTCGCATCCCT CGGCTCTCCTT-3'] and 5097/5-2 [5'-GGGGACCACTTTGTACAAGAA AGCTGGGTCCATGCTCGGTTCCGGGCGCGTTCGAT-3']; 3'-flanking PCR product, 5097/3-1 [5'-GGGGACAAGTTTGTACAAAAAGCAGGCTC CAGCTCCGGCGCTGGTCTGGAAGGT-3'] and 5097/3-2 [5'-AACTCGA GCCCAAGCATGCTGAAGCCAGGGAGGGCTGTGACGGAACCGA-3']), $\Delta hutH$ (5'-flanking PCR product, 5098/5-1 [5'-TTCAGCATGCTTGGCG CTCGAGTTGCTCATCGAAGGCTCCTTGTTCGGT-3'] and 5098/5-2 [5'-G GGGACCACTTTGTACAAGAAAGCTGGGTTTCAGCCGCTGATGCTG CTCAGCGACAT-3']; 3'-flanking PCR product, 5098/3-1 [5'-GGGGACAAGTT TGTACAAAAAGCAGGCTGATCACCACGACATTGAGGA-3'] and n5098/ 3-2 [5'-AACTCGAGCCGAAGCATGCTGAACGGCTGTTGCCGAGTCTTT GATA-3']), and $\Delta hutG$ (5'-flanking PCR product, hutG5-1 [5'-TTCAGCATG CTTGGCGCTCGAGTACTCAGGACTTCCACGGTATACCT-3'] and hutG5-2 [5'-GGGGACCACTTTGTACAAGAAAGCTGGGTGCGAGGCCA GCGAGGAAGAAATGCT-3']; 3'-flanking PCR product, hutG3-1 [5'-GGGG ACAAGTTTGTACAAAAAGCAGGCTGGTGAAGCGCCGGGATAGT CTAAGT-3'] and hutG3-2 [5'-AACTCGAGCCGAAGCATGCTGAACATCC CGAACTGCTGGAAGCCTCCTCGCT-3']). A deletion in *exoS* was constructed as described previously (39), with primers ExoS 5' (5'-TACAAAA AAGCAGGCTGGCTCTGGTCTCGCTACTGGATCC-3') and ExoS SOE 3' (5'-ATCCTGTTCTTGGACCTCGCTGAGTTCGACTGGGAATCC AAACGCCACTGGCGCCTGGTG-3') for the 5'-flanking region and ExoS 3' (5'-TACAAGAAAGCTGGGTGAGCGCTTCGTCATCCTCAATCCG-3') and ExoS SOE 5' (5'-CACCAGGCCGCAAGTGGGCTTTGGAATTCCCA GTCGACTCAGGCGAGGTCCAGGAACAGGAT-3') for the 3'-flanking region.

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 pDONR Δ *exoS* (again made blunt with T4 DNA polymerase). The resulting plasmid was then recombined into deletion vector pEXG-mGW with the Gateway recombination system (Invitrogen), resulting in plasmid pEX Δ *exoS*::GL3.

Mutations in *pilA* and *hutU* were generated by insertional mutagenesis with pP30 Δ . pP30 Δ was derived from pPSV30 by digesting pPSV30 sequentially with *MscI* and *SmaI* and religation, thus removing the *lac* promoter and PAori of pPSV30. Internal fragments of *hutU* and *pilA* were cloned into this vector (primers hutUint5 [5'-AAAAACTGCGAGAACCCAGGGAACTGGTGGTTT A-3'] and hutUint3 [5'-AAAAACTGCGAGGCTTCGGCGGTGTAGCGCTG GAT-3'], as well as pilAint5 [5'-AAAAACTGCGAGGCTTTACCTTGATC GAACTGA-3'] and pilAint3-1 [5'-AAAAACTGCGAGAACATCGGATCCTG GGTAGATT-3'], respectively [the underlined sequences are restriction sites for cloning]), and the resulting plasmids, pP30 Δ *hutU*int and pP30 Δ *pilA*int, were moved into SM10 Δ *pir* and subsequently mated into *P. aeruginosa* PAO1 to generate the null mutants.

Fluorescence-activated cell sorting. The pool of transposon insertion mutants was grown in tryptic soy broth (Becton Dickinson) supplemented with 100 mM sodium glutamate and 1% glycerol. Low-calcium 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 (J. Budzik and G. A. O'Toole, 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 monocyte-derived cell line, were grown in RPMI 1640 tissue culture medium (Cell-Gro) 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 8×10^4 /well on the day

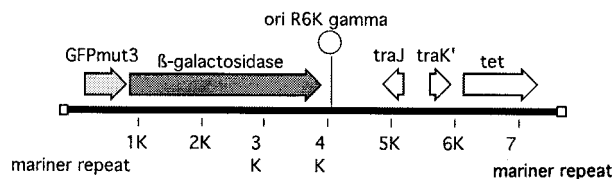


FIG. 1. Organization of promoter trap transposon TnTGL3. The transposon carries a promoterless version of GFPmut3, as well as a translationally coupled *lacZ* gene (indicated by the light and dark grey arrows, respectively).

prior to the experiment. On the day of the experiment, cells were infected at a multiplicity of infection of 50 (assuming $\sim 10^5$ 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 10^4 /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 (TnTGL3) 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-calcium 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-calcium 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

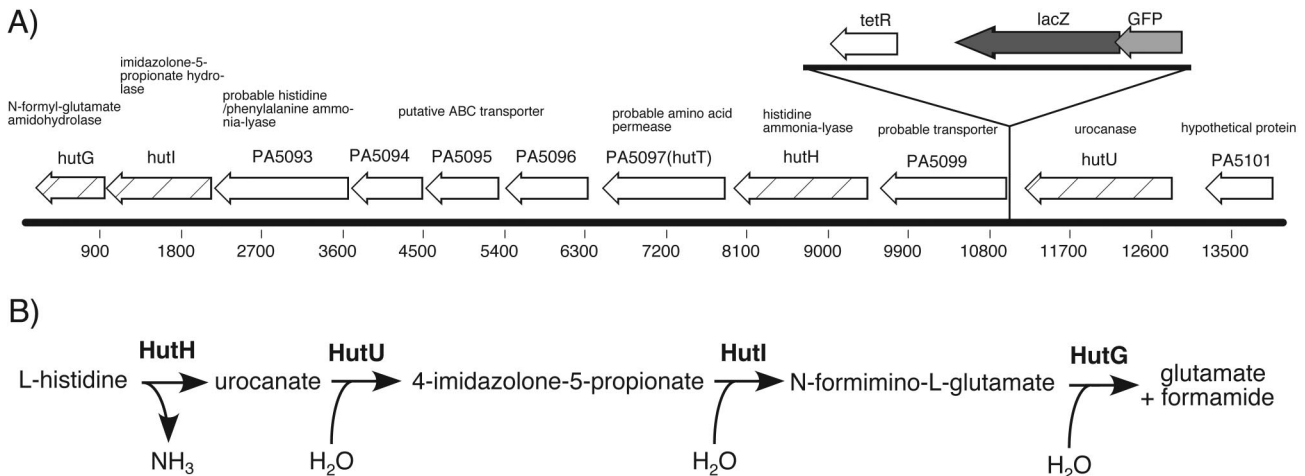


FIG. 2. Chromosomal location of the I.11E insertion mutant. (A) The transposon insertion in the cytotoxicity mutant I.11E is located 9 bp upstream of gene PA5099. Histidine utilization genes are indicated by the hatched arrows. (B) The histidine utilization pathway (<http://biocyc.org/>). The gene product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

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*,

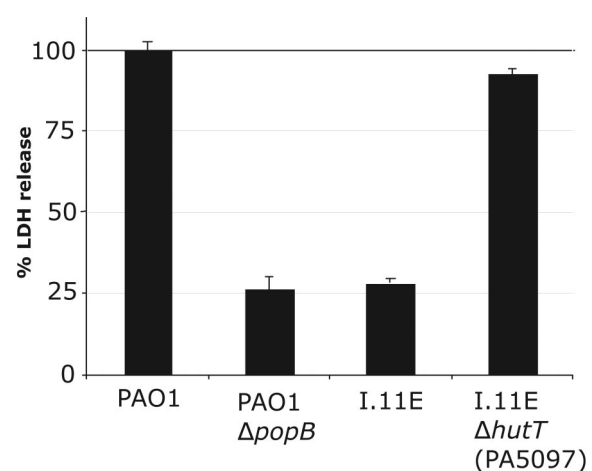


FIG. 3. Suppression of the I.11E phenotype by deletion of PA5097 (*hutT*). LDH release by RAW264.7 macrophages infected with *P. aeruginosa* PAO1, a mutant with an in-frame deletion of *popB*, the transposon insertion mutant I.11E, as well as a mutant of PAO1 combining the I.11E insertion with an in-frame deletion of PA5097 (*hutT*). The infections were carried out in complete RPMI tissue culture medium (which contains histidine) supplemented with glutamine and 10% FBS.

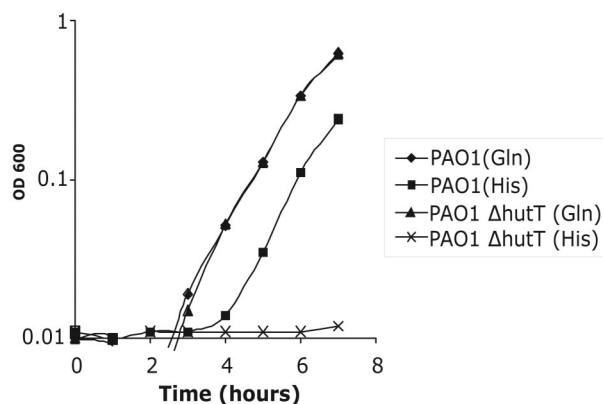


FIG. 4. Growth of PAO1 and PAO1 $\Delta hutT$ on glutamine or histidine as the sole carbon source. Either strain was grown overnight in MOPS minimal medium with glucose as the sole carbon source. On the following day, the bacteria were diluted into MOPS minimal medium with either 10 mM glutamine or 10 mM histidine as the sole carbon source and grown at 37°C with aeration. Growth was monitored by measuring the absorbance of the culture at 600 nm. OD, optical density.

leads 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

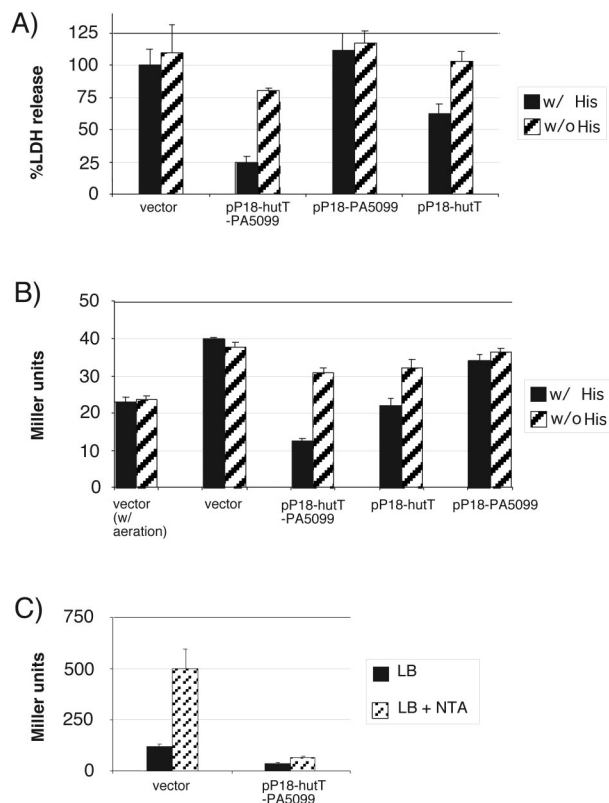


FIG. 5. Effect of overexpression of *hutT* and *hutH* on cytotoxicity and *exoS* expression. *hutT* (pP18-*hutT*), PA5099 (pP18-PA5099), or the chromosomal region spanning *hutT*, *hutH*, and PA5099 (pP18-*hutT*-PA5099) was expressed in multicopy in either wild-type PAO1 (A) or PAO1 $\Delta exoS::GL3$ (B). In panel A, cytotoxicity was assayed by monitoring LDH release by infected RAW264.7 macrophages. In panel B, *exoS* expression was monitored by β -galactosidase assay in cells grown with aeration (first two columns [w/aeration]) or under tissue culture conditions (no aeration, 5% CO₂). Both experiments were performed with RPMI medium supplemented with 10% FBS and glutamine and containing histidine (w/His) or lacking histidine (w/o His). In panel C, *exoS* expression was monitored in strain PAK $\Delta exoS::GL3$ carrying either plasmid pPSV18 (vector control) or pP18-*hutT*-PA5099. The bacteria were grown aerobically in LB medium to an optical density at 600 nm of approximately 0.3, at which point bacteria were diluted 1:1 into either fresh LB medium or LB medium containing the calcium chelator nitrilotriacetic acid (NTA; 10 mM final concentration) and incubated for another 3 h. Following this, the cultures were placed on ice and the β -galactosidase activity was determined.

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

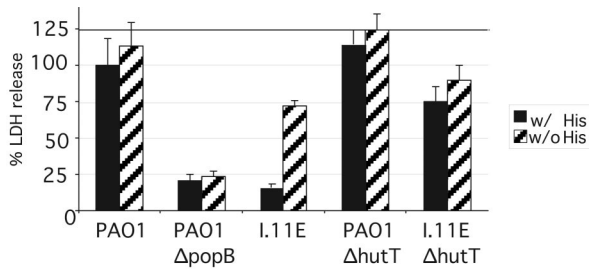


FIG. 6. Cytotoxicity of the I.11E insertion mutant in the presence (w/His) or absence (w/o His) of histidine. LDH release by RAW264.7 macrophages infected with *P. aeruginosa* PAO1, a *popB* deletion mutant, and the transposon insertion mutant I.11E, as well as mutants of PAO1 and I.11E lacking *hutT*, was measured. The infections were carried out with RPMI tissue culture medium supplemented with glutamine and 10% FBS and containing (w/His) or lacking (w/o His) histidine.

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 Tn*FAC* (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. 8A). 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 suppres-

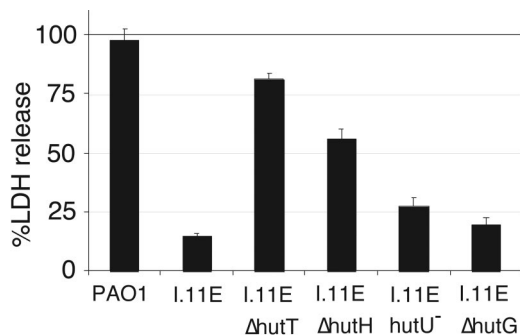


FIG. 7. Suppression of the I.11E phenotype by mutations in the histidine utilization pathway. LDH release by RAW264.7 macrophages infected with *P. aeruginosa* PAO1, the transposon insertion mutant I.11E, and mutants of PAO1 combining the I.11E insertion with mutations *hutT*, *hutH*, *hutU*, and *hutG*, was measured. The infections were carried out with RPMI tissue culture medium (complete) supplemented with glutamine and 10% FBS.

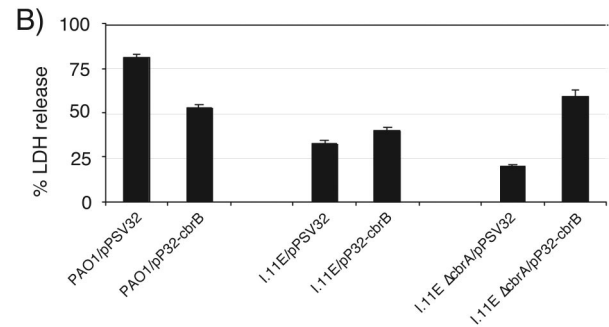
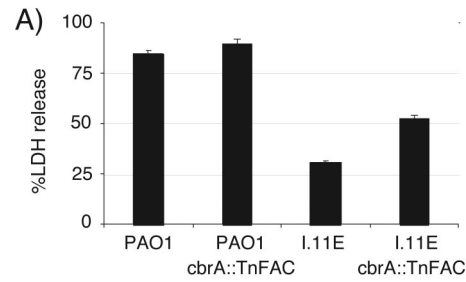


FIG. 8. Suppression of the I.11E phenotype by overexpression of *cbrB*. (A) LDH release by RAW264.7 macrophages infected with *P. aeruginosa* PAO1, transposon insertion mutant I.11E, or either strain harboring the *cbrA::TnFAC* insertion. (B) LDH release by RAW264.7 macrophages infected with PAO1, the I.11E insertion mutant, and the I.11E insertion mutant combined with a deletion in *cbrA* harboring either the vector (pPSV32) or a plasmid expressing *cbrB* (pP32-*cbrB*). The infections were carried out with RPMI tissue culture medium (complete) supplemented with glutamine and 10% FBS. In panel B, the medium was supplemented with 10 mM isopropyl- β -D-thiogalactopyranoside.

sion 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 Tn*FAC* 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 (*hutT*) 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 *cbrA*, which also results in overexpression of *cbrB*. The *cbrAB* two-component regulatory system was originally described as being involved in controlling a number of catabolic operons. Strains mutant for *cbrA* or *cbrB* 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 *cbrA::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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