JOURNAL OF BACTERIOLOGY, Jan. 2011, p. 399–410 Vol. 193, No. 2
0021-9193/11/$12.00 doi:10.1128/JB.01079-10


# MexT Regulates the Type III Secretion System through MexS and PtrC in *Pseudomonas aeruginosa*▽

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Received 10 September 2010/Accepted 2 November 2010

**The type III secretion system (T3SS) is the most important virulence factor in *Pseudomonas aeruginosa*, and its expression level varies in different isolates. We studied the molecular basis for such differences in two laboratory strains, PAK and PAO1. A chromosomal clone library from the high-T3SS-producer strain PAK was introduced into the low-producer strain PAO1, and we found that a *mexS* gene from PAK confers high T3SS expression in the PAO1 background. Further tests demonstrated that both *mexS* and its neighboring *mexT* gene are required for the repression of the T3SS in PAO1, while the PAK genome encodes a defective MexS, accounting for the derepression of the T3SS in PAK and the dominant negative effect when it is introduced into PAO1. MexS is a probable oxidoreductase whose expression is dependent on MexT, a LysR-type transcriptional regulator. Various genetic data support the idea that MexS modulates the transcriptional regulator function of MexT. In searching for the MexT-dependent repressor of the T3SS, a small gene product of PA2486 (*ptrC*) was found effective in suppressing the T3SS upon overexpression. However, deletion of *ptrC* in the PAO1 background did not result in derepression of the T3SS, indicating the presence of another repressor for the T3SS. Interestingly, overexpression of functional *mexS* alone was sufficient to repress T3SS even in the absence of MexT, suggesting that MexS is another mediator of MexT-dependent T3SS repression. Overexpression of *mexS* alone had no effect on the well-known MexT-dependent genes, including those encoding MexEF efflux pump, elastase, and pyocyanin, indicating alternative regulatory mechanisms. A model has been proposed for the MexS/MexT-mediated regulation of the T3SS, the MexEF efflux pump, and the production of elastase and pyocyanin.**

*Pseudomonas aeruginosa* is a ubiquitous Gram-negative bacterium, capable of causing acute and chronic infections in individuals with cystic fibrosis and immunocompromised patients such as AIDS patients, cancer patients, and burn victims (9, 18, 29, 42, 43). Its pathogenicity, to a large extent, is attributable to its ability to secrete a broad arsenal of virulence determinants, including effector proteins secreted through a type III secretion system (T3SS) (3, 8, 59). *P. aeruginosa* is also well known to possess high intrinsic antibiotic resistance and is capable of developing multidrug resistance, posing serious therapeutic problems (21).

The T3SS is an important virulence factor in *P. aeruginosa*, through which effector proteins are directly injected into the cytosols of eukaryotic host cells, evading host defense by inducing apoptosis in macrophages, polymorphonuclear phagocytes, and epithelial cells (10, 23, 25). The *P. aeruginosa* T3SS comprises 43 coordinately regulated genes organized in 10 transcriptional units, encoding the needle complex, translocation apparatus, regulatory proteins, effector proteins, and their chaperones (52, 56). To date, four effector molecules have been characterized in *P. aeruginosa*: ExoS and ExoT share similar structures, both with an ADP-ribosyltransferase activity and a GTPase-activating protein activity, while ExoY and ExoU display adenylate cyclase and phospholipase activities, respectively (17, 37, 45, 57).

In *P. aeruginosa*, the expression of type III secretion-related genes is coordinately regulated by a transcriptional activator, ExsA, in response to varieties of environmental signals, such as low calcium and direct contact with eukaryotic host cells. ExsA is an AraC-family transcriptional regulator, which recognizes and binds to two adjacent highly conserved consensus sequence sites on the promoter region to activate the expression of T3SS genes, including the *exsA* gene itself (5, 14). Besides ExsA, three other proteins, "anti-activator" ExsD, "anti-anti-activator" ExsC, and secretable repressor ExsE, are involved in the "partner switching" mechanism to couple transcription with secretion in the T3SS (6, 12, 14, 31, 35, 51). Under non-inducing conditions, ExsE binds to ExsC and resides inside the bacterial cytosol and ExsD binds to the N terminus of ExsA, inactivating its transcriptional activator function (12, 31, 35, 46, 50, 51). While under the inducing condition, ExsE is secreted by T3SS and frees ExsC to sequester its lower-binding-affinity target ExsD, liberating ExsA to activate transcription of T3SS-related genes. Recently, this regulatory model has been confirmed *in vitro* with purified components (7, 34).

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▽ Published ahead of print on 12 November 2010.

Besides the regulators involved in the partner-switching model, a large number of additional genes have been reported to affect the expression of the T3SS, although the regulatory mechanisms are not well understood. Those genes can be divided into two types, the regulator-related genes and the metabolism-related genes. The former includes a membrane-associated adenylcyclase (CyaB) and a cyclic AMP binding cyclic AMP receptor protein (CRP) homolog called Vfr (53), an Rhl quorum-sensing system (4, 22), stationary-phase sigma factor RpoS and its transcriptional activator PsrA (22, 41), and a hybrid sensor kinase/response regulator (RtsM/RetS) (19). The latter includes *aceA* and *aceB*, encoding the subunits of pyruvate dehydrogenase (11); *trpA* and *kynA*, encoding tryptophan synthase and tryptophan dioxygenase (40); *truA*, encoding pseudouridinase enzyme (1); and *mgtE*, a magnesium transporter gene (2).

The expression of T3SS genes is also coordinately regulated by small proteins, called miniproteins, under various stress conditions (20, 55). For instance, we have previously demonstrated that a *ptrA* gene, highly inducible during infection of the burned mouse model, encodes a small protein which suppresses the T3SS by directly bounding to the ExsA and thus acts as an anti-ExsA factor (20). Expression of this gene is specifically induced by a high-copper signal through a CopR/S two-component regulatory system; therefore, PtrA coordinates the T3SS and the copper stress response (20, 32). We have also shown that another gene, *ptrB*, encoding a small protein with a prokaryotic DskA/TraR $C_4$-type zinc-finger motif, inhibits the expression of the T3SS. This gene is upregulated under the stress of DNA damage through the PrtR/PrtN pathway and thus coordinates the T3SS and the SOS response (55). It was thus predicted that T3SS is coordinately repressed by a particular miniprotein under each stress condition (20, 55).

MexEF-OprN is one of the well-characterized resistance-nodulation-division (RND) multidrug efflux systems (27). The MexEF-OprN efflux system is quiescent in so-called wild-type strains under routine laboratory conditions, while it is highly expressed in the *nfxC*-type strain, which displays resistance to fluoroquinolones, chloramphenicol, and trimethoprim (13, 16, 27). The expression of *mexEF-oprN* was shown to be positively regulated by their immediate upstream gene product, MexT, a LysR-family transcriptional regulator (26, 27). Although the transcriptional levels of *mexT* in *nfxC*-type mutants are comparable to those of the wild type, introduction of a plasmid-carried *mexT* gene is sufficient to confer an *nfxC*-type multidrug resistance phenotype on the susceptible wild-type strain (26, 27).

In the present study, we investigated the molecular mechanism by which two commonly used laboratory strains, PAK and PAO1, display drastic differences in T3SS gene expression levels under type III inducing conditions. We demonstrate that both functional MexS and MexT are required to repress the T3SS while activating the MexEF-OprN efflux pump. Strain PAO1 carries functional copies of both genes while PAK carries nonfunctional *mexS*. MexT represses the T3SS through two parallel pathways, involving *ptrC* and *mexS*, respectively. However, MexT-dependent regulation of MexEF efflux pump, elastase, and pyocyanin does not go through the same pathway. A model has been proposed to explain MexS/MexT-dependent regulation of all these virulence factors.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** Bacterial strains and plasmids used in this study are listed in Table 1. Both *Escherichia coli* and *Pseudomonas aeruginosa* strains were grown in L broth (LB) medium. Whenever needed, the following antibiotics were used at the indicated concentrations (μg/ml) for *E. coli*: ampicillin (100), kanamycin (50), tetracycline (10), gentamicin (10), chloramphenicol (34), spectinomycin (25), and streptomycin (25). For *P. aeruginosa*, the following antibiotics at the indicated concentrations (μg/ml) were used: carbenicillin (150), tetracycline (75), gentamicin (100), spectinomycin (200), and streptomycin (200).

For the *mexT*-complementing clone, a 1.5-kb *mexT*-containing fragment with its own promoter was PCR amplified using PAO1 genomic DNA as a template (primers listed in Table 2), digested with BamHI and EcoRI, and then ligated into a shuttle vector, pUCP21, which was digested with the same restriction enzymes, resulting in pYX0908. A *mexS* gene-complementing plasmid, pYAN0693, was also constructed using a similar method. For the *PA2486* gene-overexpressing construct pYX1004, a 330-bp PA2486 coding sequence was PCR amplified from PAO1 genomic DNA, digested with EcoRI and HindIII, and then ligated into pUCP20, where *PA2486* is under the control of the *lac* promoter on the vector.

For the *PA2486* gene deletion construct, a 933-bp fragment immediately upstream of the *PA2486* start codon and an 829-bp fragment immediately downstream of the *PA2486* stop codon were PCR amplified and digested with HindIII-XbaI and XbaI-EcoRI, respectively. The two fragments were then ligated into pEX18Tc plasmid, which was digested with EcoRI and HindIII, resulting in pYX1022, in which the whole *PA2486* open reading frame had been deleted. Similarly, a *mexS* deletion construct, pYAN0649, was generated. Gene disruption constructs for *mexS*, *mexT*, and *mexE* were generated in pEX18 vectors by insertion of either a Ω fragment or a gentamicin resistance cassette, resulting in pYAN0683, pYAN0699, and pYAN0670, respectively. Gene knockouts in PAO1 or PAK were generated by conjugal transfer of these plasmids followed by selection for single crossovers and double crossovers as described previously (39).

For the *PA2486-lacZ* reporter construct pYX1038, a 933-bp sequence containing the putative *PA2486* promoter region was PCR amplified, digested with EcoRI and BamHI, and then ligated into the same sites on pDN19*lac*Ω. Similarly, promoters of *mexS* and *mexE* have also been cloned in front of the promoterless *lacZ* gene in pDN19*lac*Ω, resulting in pYX1037 and pYAN0655, respectively.

**Preparation of PAK chromosome DNA clone library.** Chromosome DNA from PAK was isolated and digested with Sau3A, recovering 3- to 5-kb DNA fragments from an 0.8% agarose gel, and ligated into a shuttle vector, pUCP19, which was digested with BamHI and treated with shrimp alkaline phosphatase. The ligation mixture was transformed into *E. coli* XL-1, resulting in approximately $2 \times 10^5$ independent transformants with DNA insertions, and saved as a PAK chromosomal DNA clone bank. Plasmids were isolated from the clone bank as a mixture and transformed into strain PAO1 harboring reporter plasmid *exoT-lacZ* (pHW0006) as an indicator for T3SS gene expression. The transformants were spread on L agar plates containing carbenicillin, spectinomycin, and streptomycin; X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; 20 μg/ml); and 2.5 mM EGTA to select for dark blue colonies after 24 to 48 h of incubation at 37°C.

**Elastase assay.** Fresh bacteria from overnight culture in an L agar plate were inoculated onto the surface of a 1.5% agar plate containing 0.4% elastin (Sigma) (24). The plates were incubated at 37°C for 40 h. Elastolysis zones, through digestion of the elastin substrate, were observed surrounding colonies that produce elastase (33).

**Other methods.** DNA manipulations were done according to the method of Sambrook et al. (36). Western blot analysis for the detection of secreted ExoS in culture supernatant was performed following a previously reported protocol (55). The β-galactosidase activity assay and the BacterioMatch two-hybrid assay were carried out as described previously (20, 54).

## RESULTS

**Variation in type III secretion activities between PAO1 and PAK.** *P. aeruginosa* strains PAO1 and PAK are widely used as laboratory model organisms, both harboring functional T3SS gene clusters. However, strain PAK secretes a much higher level of type III effector molecules than does PAO1 when grown in the presence of 5 mM EGTA (low calcium), a stan-

TABLE 1. Strains and plasmids used in this study

| Strain or plasmid | Description | Reference or source |
|---|---|---|
| Strains | | |
| *E. coli* | | |
| DH5α | F$^-$ ϕ80d*lacZ*ΔM15 *endA1 recA1 hsdR17*($r_K^-$ $m_K^+$) *supE44 thi-1 relA1* Δ(*lacZYA-argF*)*U169 gyrA96 deoR* | 36 |
| RS | BacterioMatch two-hybrid system reporter strain, Km$^r$ | Stratagene |
| S17-1 | RP4-2 Tc::Mu Km::Tn*7* Tp$^r$ Sm$^r$ Pro Res$^-$ Mod$^+$ | Stratagene |
| XL-1 | *E. coli* clone strain | Stratagene |
| *P. aeruginosa* | | |
| PAO1 | Wild-type *P. aeruginosa* strain | B. Iglewski |
| PAK | Wild-type *P. aeruginosa* strain | David Bradley |
| PAO1*mexS*::Ω | PAO1 with *mexS* gene disrupted by insertion with Ω, Sp$^r$ Sm$^r$ | This study |
| PAO1*mexT*::Gm | PAO1 with *mexT* gene disrupted by insertion with Gm cassette, Gm$^r$ | This study |
| PAO1Δ*ptrC* | PAO1 with the *PA2486* gene deletion | This study |
| PAO1*mexE*::Gm | PAO1 with the *mexE* gene disrupted by insertion with Gm cassette, Gm$^r$ | This study |
| PAKΔ*mexS* | PAK with the *mexS* gene deletion | This study |
| PAK*mexT*::Gm | PAK with *mexT* gene disrupted by insertion with Gm cassette, Gm$^r$ | This study |
| PAK*mexE*::Gm | PAK with *mexT* gene disrupted by insertion with Gm cassette, Gm$^r$ | This study |
| Plasmids | | |
| pCR2.1-TOPO | Cloning vector for the PCR products; Amp$^r$ Kan$^r$ | Invitrogen |
| pGEM-T Easy | Cloning vector for the PCR products; Amp$^r$ | Promega |
| pDN191acΩ | Promoterless *lacZ* fusion vector, Sp$^r$ Sm$^r$ Tc$^r$ | 49 |
| pUCP19 | Broad-host-range shuttle vector, Ap$^r$ | 38 |
| pUCP20 | Broad-host-range shuttle vector, Ap$^r$ | 38 |
| pUCP21 | Broad-host-range shuttle vector, Ap$^r$ | 38 |
| pEX18Gm | Gene replacement vector, Gm$^r$ *oriT*$^+$ *sacB*$^+$ | 39 |
| pEX18Tc | Gene replacement vector, Tc$^r$ *oriT*$^+$ *sacB*$^+$ | 39 |
| pEX18Amp | Gene replacement vector, Amp$^r$ *oriT*$^+$ *sacB*$^+$ | 39 |
| pYAN0650 | Remove EcoRI site from pEX18Amp; Amp$^r$ | This study |
| pYAN0648 | *mexE* and *mexT* genes cloned into pCR2.1-TOPO; Amp$^r$ Kan$^r$ | This study |
| pYAN0649 | Deletion of *mexS* gene on pEX18Gm, Gm$^r$ | This study |
| pYAN0666 | *mexT* gene cloned into pEX18Amp; Amp$^r$ | This study |
| pYAN0669 | *mexT* gene inserted by Gm in pEX18Amp; Amp$^r$ Gm$^r$ | This study |
| pYAN0667 | *mexE* gene cloned into pYAN0650; Amp$^r$ | This study |
| pYAN0670 | *mexE* gene inserted by Gm in pYAN0650; Amp$^r$ Gm$^r$ | This study |
| pYAN0657 | *mexS* and *mexT* with own promoters cloned into pCR2.1-TOPO; Amp$^r$ Kan$^r$ | This study |
| pYAN0682 | *mexS* and *mexT* with own promoters cloned into pEX18Gm; Gm$^r$ | This study |
| pYAN0683 | *mexS* was disrupted with Ω cassette in pYAN0682; Sp$^r$ Sm$^r$ Gm$^r$ | This study |
| pBT | Bait vector encoding full-length bacterial phage λ*c*I protein; Chl$^r$ | Stratagene |
| pTRG | Target vector plasmid encoding RNA polymerase alpha subunit protein; Tc$^r$ | Stratagene |
| pTRG-popN | *popN* gene in pTRG vector | 58 |
| pBT-*pcr1* | *pcr1* gene in pBT vector | 58 |
| pYAN0699 | *mexS* from PAK cloned into pTRG; Tc$^r$ | This study |
| pYAN0700 | *mexS* from PAO1 cloned into pTRG; Tc$^r$ | This study |
| pYAN0701 | *mexT* from PAO1 cloned into pBT; Chl$^r$ | This study |
| pHW0006 | *exoT-lacZ* fusion reporter in pDN19lacΩ, Sp$^r$ Sm$^r$ Tc$^r$ | 55 |
| pHW0032 | *exsA-lacZ* fusion reporter in pDN19lacΩ, Sp$^r$ Sm$^r$ Tc$^r$ | 20 |
| pHW0243 | *popN-lacZ* fusion reporter in pDN19lacΩ, Sp$^r$ Sm$^r$ Tc$^r$ | 20 |
| pHW0244 | *pscN-lacZ* fusion reporter in pDN19lacΩ, Sp$^r$ Sm$^r$ Tc$^r$ | 20 |
| pYAN0655 | *mexE-lacZ* fusion reporter in pDN19lacΩ, Sp$^r$ Sm$^r$ Tc$^r$ | This study |
| pYX0908 | PAO1 *mexT* gene with own promoter cloned to pUCP21, Amp$^r$ | This study |
| pYAN0693 | PAO1 *mexS* gene with own promoter cloned to pUCP19, Amp$^r$ | This study |
| pYX1004 | *PA2486* gene without the *mexT* binding site cloned to pUCP20, Amp$^r$ | This study |
| pYX1027 | *PA2486* gene ATG→ATC on pUCP20, Amp$^r$ | This study |
| pYX1029 | *PA2487* gene cloned into pUCP20, Amp$^r$ | This study |
| pYX1030 | *PA2485* gene cloned into pUCP20, Amp$^r$ | This study |
| pYX1022 | Deletion of *PA2486* gene on pEX18 Tc, Tc$^r$ | This study |
| pYX1037 | *mexS-lacZ* fusion reporter in pDN19lacΩ, Sp$^r$ Sm$^r$ Tc$^r$ | This study |
| pYX1038 | *PA2486-lacZ* fusion reporter in pDN19lacΩ, Sp$^r$ Sm$^r$ Tc$^r$ | This study |
| pYAN0652 | PAK *mexS* gene with own promoter on pUCP19 vector, Amp$^r$ | This study |
| pYAN0653 | PAK *mexT* gene with own promoter on pUCP19 vector, Amp$^r$ | This study |
| pYAN0659 | PAK *mexS* and *mexT* genes with own promoter on pUCP19 vector, Amp$^r$ | This study |
| Bank 1 to 30 | PAK chromosome DNA clones on pUCP19, Amp$^r$ | This study |
| pYAN0662 | Bank 15/NcoI/HindIII/T4 DNA polymerase self-ligation | This study |
| pYAN0668 | Bank 1/BglII/EcoRI plus 0.86-kb fragment from pYAN0654/BglII/EcoRI to make *mexT* intact in Bank 1 | This study |

TABLE 2. PCR primers used in this study

| Gene (purpose) | Amplicon size (bp) | Primer name | Primer sequence |
|---|---|---|---|
| *mexS* (cloning) | 1,376 | mexS1 | 5′-CGAATTCCGGCTGGCCGAGGAACAGTTTCTCTGC-3′ |
| | | mexS2 | 5′-CAAGCTTCTCTTTCGCTGCGGGTCGTCCTCAATC-3′ |
| *mexT* (cloning) | 1,512 | mexT1 | 5′-CGGGATCCCGCGGCATCAGCACCACGTCGCCATAG-3′ |
| | | mexT2 | 5′-GGAATTCTCGATTTTCCCGTTGCGACGCCTCGTG-3′ |
| *mexE* (promoter) | 540 | mexEp1 | 5′-CGGATCCTGCCTTGGGTGGTTTCCGGGGCCTTGC-3′ |
| | | mexEp2 | 5′-CGAATTCCTGGCCGGCACCGACATCATCGCCACC-3′ |
| *mexE* (mutation) | 3,117 | mexET1 | 5′-GCGCGGCATCAGCACCACGTCGCCATAG-3′ |
| | | mexET2 | 5′-GGAAGTTGGCGCGGACCACGACGGTCGG-3′ |
| *mexT* (mutation) | 3,117 | mexET1 | 5′-GCGCGGCATCAGCACCACGTCGCCATAG-3′ |
| | | mexET2 | 5′-GGAAGTTGGCGCGGACCACGACGGTCGG-3′ |
| *PA2486* (cloning) | 329 | PA2486-1 | 5′-GAATTCCCCTACTCGCCCGGTTCAGGCCAGC-3′ |
| | | PA2486-2 | 5′-AAGCTTTGGTGGCGGATTGGGAAAACGCCGC-3′ |
| *PA2487* (cloning) | 420 | PA2487-1 | 5′-GGAATTCGACTGCGGATGTAGCCTGGCGGCTC-3′ |
| | | PA2487-2 | 5′-GCTCTAGATACCTCCAGTGCCCGTGTGCCGCTC-3′ |
| *mexS* (two hybrid) | 475 | mexS2h-1 | 5′-TCATGGATCCCGGTCAACGATCTGTGGATCTGAA-3′ |
| | | mexS2h-2 | 5′-AGCTGGATCCATGTCCCGAGTGATCCGTTTTCAT-3′ |
| *mexT* (two hybrid) | 346 | mexT2h-1 | 5′-ATTAGGATCCATGAACCGAAACGACCTGCGCCGC-3′ |
| | | mexT2h-2 | 5′-AGCTGGATCC GGTTATGGACAGTGCTTTCGATGG-3′ |
| *PA2486* (deletion) (upstream) | 1,101 | PA2486du-1 | 5′-CCCAAGCTTCGGCCTCGACCGCCAGCAGGGAATC-3′ |
| | | PA2486du-2 | 5′-GCTCTAGAGGATGCCTCCTTGGGCTCATTTCTT-3′ |
| *PA2486* (deletion) (downstream) | 1,020 | PA2486dd-1 | 5′-GCTCTAGATACCTCCAGTGCCCGTGTGCCGCTC-3′ |
| | | PA2486dd-2 | 5′-GGAATTCGGATAGGGCAGGCGGGTCCGCTGGG-3′ |
| *PA2486* (mutated ATG) | 278 | PA2486mutatg | 5′-GGAATTCATGAGCCCAAGGAGGATCCCCATCCTCTCAG-3′ |
| | | PA2486mutatg-2 | 5′-AAGCTTTGGTGGCGGATTGGGAAAACGCCGC-3′ |
| *mexS* (promoter) | 591 | mexSp1 | 5′-GGAATTCGGTGGGCTCCATGCTGCGTCCGG-3′ |
| | | mexSp2 | 5′-CGGGATCCGTCAGAACCTGGCCCCGGAGCAG-3′ |
| *PA2486* (promoter) | 933 | PA2486p1 | 5′-CCCAAGCTTCGGCCTCGACCGCCAGCAGGGAATC-3′ |
| | | PA2486p2 | 5′-CGGGATCCGGATGCCTCCTTGGGCTCATTTCTT-3′ |

dard type III-inducing condition (Fig. 1A). Upon infection of HeLa cells at similar multiplicities of infection (MOIs), much higher levels of ExoS and ExoT injection by PAK than of injection by PAO1 were also noted (data not shown). Consistent with these, HeLa cells were rounded and lifted at much earlier times in PAK-infected cells than in those infected by PAO1 (data not shown).

We wondered whether the difference of T3SS expression

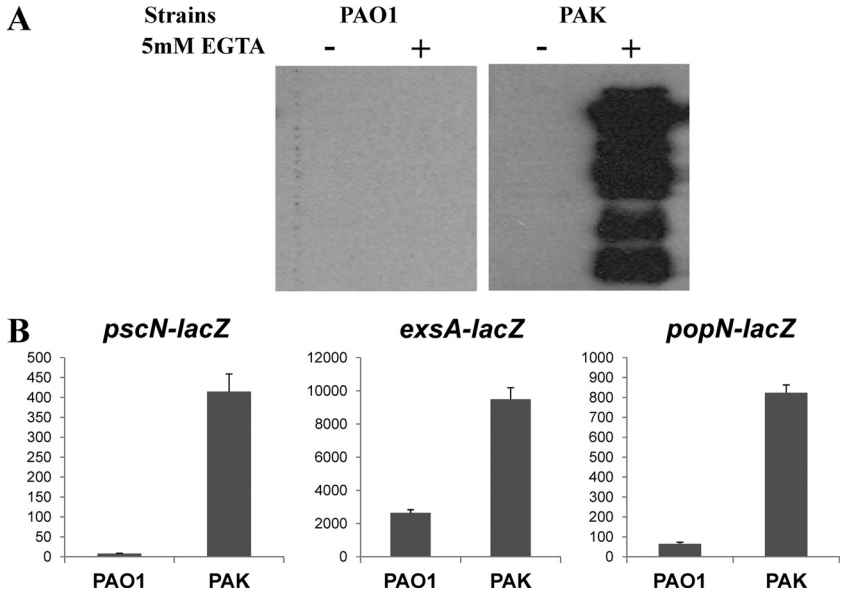


FIG. 1. Expression and secretion of the T3SS in laboratory strains of *P. aeruginosa* PAO1 and PAK. (A) Overnight cultures were diluted to 2% in LB with (+) or without (−) 5 mM EGTA and grown at 37°C for 3 h. Proteins secreted into supernatants were concentrated 20-fold with trichloroacetic acid precipitation, loaded onto SDS-PAGE gels with equivalent bacterial cell numbers, and immunoblotted with antibody against ExoS. (B) β-Galactosidase activities in PAO1 and PAK carrying a P*exsA-lacZ* (pHW0032), P*popN-lacZ* (pHW0243), or P*pscN-lacZ* (pHW0244) reporter gene. Overnight cultures were diluted to 2% in LB with 5 mM EGTA and grown at 37°C for 4 h before being assayed for β-galactosidase activities. Error bars indicate standard deviations of triplicate assays. Numbers on *y* axes of graphs are β-galactosidase activity units.

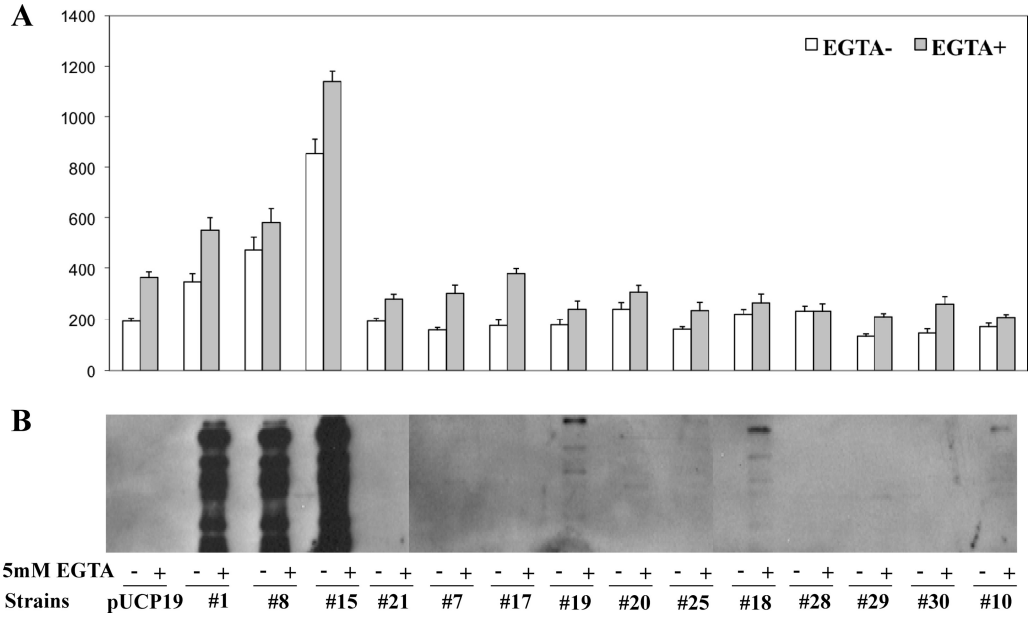


FIG. 2. Expression and secretion of ExoS in selected transformants of PAO1 carrying the PAK chromosome DNA clone bank or empty vector pUCP19. (A) Overnight cultures carrying the *exoT-lacZ* reporter gene were diluted to 2% in LB with (+) or without (−) 5 mM EGTA plus appropriate antibiotic and grown at 37°C for 4 h before being subjected to β-galactosidase assay. Error bars indicate standard deviations of triplicate assays. Numbers on the *y* axis are β-galactosidase activity units. (B) Overnight cultures were diluted to 2% in LB with (+) or without (−) 5 mM EGTA and grown at 37°C for 3 h. Proteins secreted into supernatants were concentrated 20-fold with trichloroacetic acid precipitation, loaded onto SDS-PAGE gels with equivalent bacterial cell numbers, and immunoblotted with antibody against ExoS.

between PAK and PAO1 is limited to the effector proteins or covers the whole type III secretion apparatus. To test this, *lacZ* reporter plasmids for T3SS master transcriptional activator *exsA* (pHW0032); the initial gene in the plug operon, *popN* (pHW0243); and the initial gene of the secretion machinery operon, *pscN* (pHW0244), were introduced into both PAK and PAO1 backgrounds. β-Galactosidase activity measurements under the type III-inducing condition indicated that the whole type III secretion system genes in PAO1 were expressed at much lower levels than were those in PAK (Fig. 1B), suggesting possible differences in the master regulator functions.

**MexS is the major cause of difference in the T3SS expression levels between PAK and PAO1.** The difference in T3SS expression levels between the two strains possibly means that either there is a transcriptional activator present in PAK which PAO1 lacks or there is an inhibitor expressed in PAO1 but not in PAK. To test the first possibility, a 3- to 5-kb PAK chromosome DNA clone library was constructed, containing $2 \times 10^5$ independent clones. The library plasmids were transformed into PAO1 harboring a reporter plasmid of *exoT-lacZ*, and the transformants were plated on L agar containing calcium chelator EGTA for type III induction, X-Gal for β-galactosidase activity, and proper antibiotics for plasmid selection. After 24 to 48 h of incubation, 26 dark blue colonies were identified out of a total $5 \times 10^5$ light blue colonies, indicating elevated expression of T3SS genes in those isolates.

Plasmids were isolated from the dark blue colonies and subjected to sequencing analysis. Based on the DNA fragment inserts, the 26 clones can be grouped into three categories. The first group included 12 clones, each of which contains a chromosome fragment covering genome positions from 2804400 to 2808500. The second group contained 4 constructs, all harboring the *PA1873* gene and its flanking regions. The third group had 10 constructs, each with a unique insert from various chromosomal regions.

To validate the screening results, selected isolates were grown under type III-inducing conditions and subjected to a β-galactosidase activity assay for the transcriptional level of *exoT-lacZ* and Western blot analysis for ExoS secretion. Three representatives from the first group, one from the second group, and all clones from the third group were tested. As shown in Fig. 2, both T3SS transcriptional activity and secretion level were increased significantly only in the first group of isolates, especially in the strain designated 15. The plasmid in isolate 15 contained four genes: *PA2489*, encoding a probable transcriptional regulator with 46% similarity to the putative transcriptional regulator in *Streptomyces coelicolor*; *PA2490*, encoding a conserved hypothetical protein of unknown function with 60% similarity to hypothetical protein YdbB in *Bacillus subtilis*; *PA2491*, encoding MexS protein, which is a probable quinone oxidoreductase; and *PA2492*, encoding MexT protein, which is a LysR-family transcriptional regulator.

To determine which of these four genes is responsible for the increased T3SS expression in PAO1, a series of deletion constructs were further generated and introduced into PAO1 or PAO1 carrying the *exoT-lacZ* reporter plasmid to test T3SS secretion and transcriptional levels, respectively. As shown in Figure 3, the ExoS secretion assay clearly showed that the *mexS* gene of PAK alone could transactivate T3SS secretion in PAO1. To our surprise, unlike isolate 15, β-galactosidase activity did not show a significant difference between PAO1 containing the *mexS* clone of PAK and an empty vector. A possible

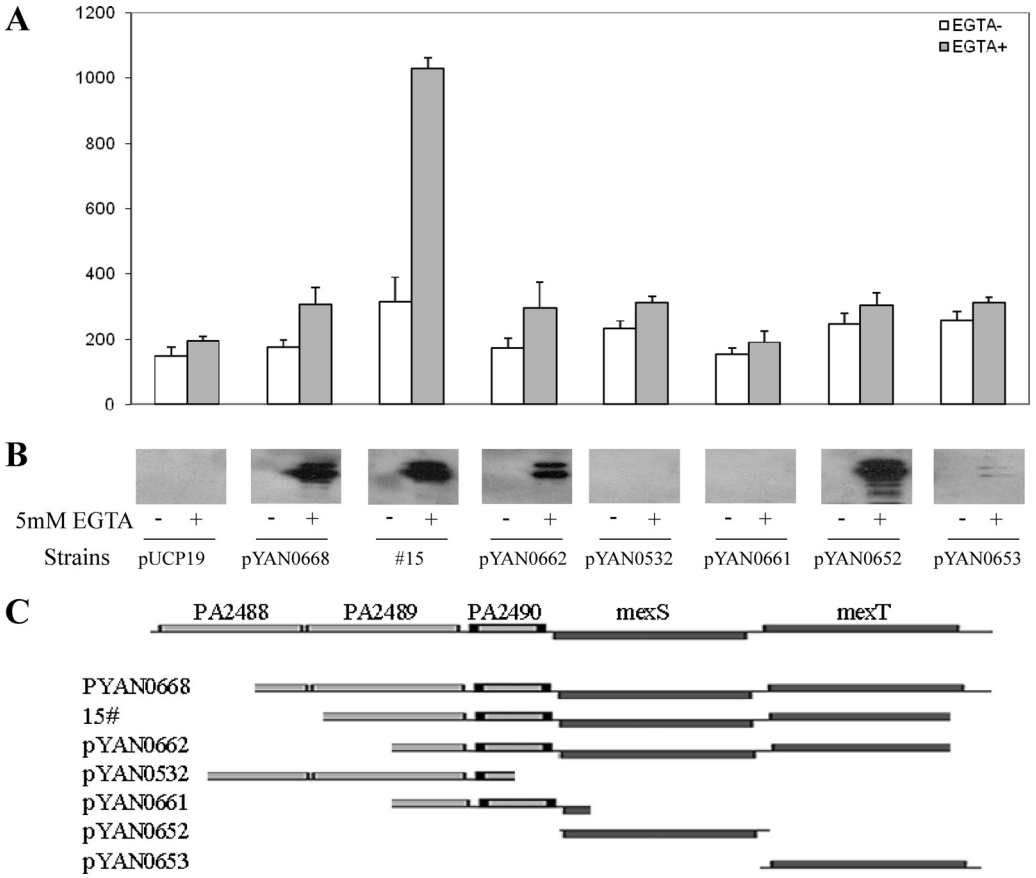


FIG. 3. Expression and secretion of ExoS in PAO1 carrying various DNA fragments from PAK. (A) Overnight cultures carrying various constructs and the *exoT-lacZ* reporter gene were diluted to 2% in LB with (+) or without (−) 5 mM EGTA plus appropriate antibiotics and grown at 37°C for 4 h before being subjected to β-galactosidase assay. Error bars indicate standard deviations of triplicate assays. Numbers on the *y* axis are β-galactosidase activity units. (B) Overnight cultures were diluted to 2% in LB with 5 mM EGTA (+) or not (−) plus appropriate antibiotics and grown at 37°C for 3 h. Proteins secreted into supernatants were concentrated 20-fold with trichloroacetic acid precipitation, loaded onto SDS-PAGE gels with equivalent bacterial cell numbers, and immunoblotted with antibody against ExoS. (C) Schematic gene map for the indicated plasmid constructs.

explanation for the difference is the sensitivity differences between the two assay methods.

**Both MexS and MexT are required for repression of the T3SS and activation of the MexEF efflux pump.** To confirm the role of MexS in the T3SS, we knocked out the *mexS* gene in the PAK background. Western blot analysis showed little change of ExoS secretion in the *mexS* mutant compared to that in its parental strain PAK (Fig. 4A). However, disruption of *mexS* in the PAO1 background resulted in a significant increase in ExoS secretion, reaching a level as high as that secreted by strain PAK (Fig. 4A). These data demonstrated that *mexS* in PAO1 functions as a T3SS repressor, whereas *mexS* in PAK does not have such a repressor function. Therefore, introduction of the nonfunctional *mexS* gene from PAK into PAO1 might have interfered with the function of endogenous MexS in PAO1, resulting in derepression of the T3SS genes.

It has previously been reported that when overexpressed on a plasmid, *mexT* can decrease the T3SS level in wild-type PAO1 (47). To test if *mexT* also contributes to the difference of T3SS expression between PAK and PAO1, the *mexT* genes were disrupted by insertion of a gentamicin resistance cassette in both strain backgrounds. Mutation of the *mexT* gene in PAK (PAK*mexT*::Gm) resulted in no difference in ExoS secretion compared to that by its parental strain PAK. However, a *mexT* mutant of PAO1 (PAO1*mexT*::Gm) resulted in much higher ExoS secretion than that of its parental strain PAO1, similar to its *mexS* mutant (Fig. 4A). These results indicated that both *mexS* and *mexT* contribute to the negative regulation of T3SS in PAO1, whereas *mexS* and *mexT* in PAK lost such repressor functions.

To confirm the above results further, the *mexS* and *mexT* genes from PAO1 were used to complement *mexS* and *mexT* mutants in PAO1 as well as PAK backgrounds. As evaluated by Western blotting for ExoS secretion, the *mexS* gene from PAO1 is able to inhibit type III secretion in *mexS* mutants of both PAK and PAO1 strains (Fig. 4B). However, the *mexT* gene of PAO1 can suppress the type III secretion only in PAO1*mexT*::Gm and is unable to suppress that of PAK*mexT*::Gm (Fig. 4C). These results together suggest that in strain PAK, *mexS* is defective while *mexT* remains functional.

The above results were further confirmed by testing the effect of MexS/MexT on one of its well-known downstream genes, namely, the *mexEF-oprN* operon for efflux pump. The

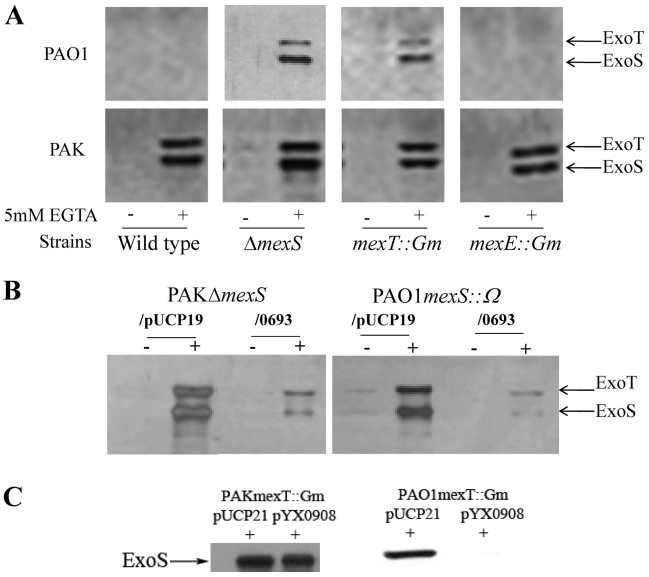


FIG. 4. T3SS secretion by mutant derivatives of PAK and PAO1. (A) Secretion of ExoS and ExoT by the indicated strains under type III-inducing and noninducing conditions. (B) Secretion of ExoS and ExoT in *mexS* mutants of PAO1 and PAK carrying *mexS*-complementing plasmid (pYAN0693) or empty vector (pUCP19). (C) Secretion of ExoS by *mexT* mutants of PAO1 and PAK carrying *mexT*-complementing plasmid or an empty vector (pUCP21). Overnight cultures were diluted to 2% in LB with (+) or without (−) 5 mM EGTA and grown at 37°C for 3 h.

expression of the *mexE-lacZ* reporter gene was high in PAO1 but low in the PAK background (Fig. 5A). Mutation of either *mexS* or *mexT* abolished the *mexE-lacZ* expression in PAO1, while similar mutations in PAK had no effect on the *mexE-lacZ* expression. Also, introduction of either a *mexS* or a *mexT* gene from PAK into PAO1 resulted in decreased expression of *mexE-lacZ* by 11-fold and 1/2-fold, respectively (Fig. 5B). These data further suggested that the nonfunctional MexS encoded by the PAK genome displays a dominant negative effect on functional MexS in PAO1, while *mexT* of PAK is partially functional.

Combining all of the above, it is clear that both functional MexS and MexT are required for not only the activation of *mexEF-oprN* expression but also the repression of T3SS. While the PAO1 genome encodes functional MexS/MexT, the PAK genome encodes totally nonfunctional MexS and partially functional MexT.

**Sequence analysis of *mexS* and *mexT* in PAK and PAO1.** To account for the functional differences of the *mexS*/*mexT* genes from PAK and PAO1 strains, both genes in the two strain backgrounds were subjected to DNA sequencing analysis. The *mexS* gene of our PAO1 strain is 100% identical to the genome-sequenced strain (www.pseudomonas.com) while *mexS* of PAK has five nucleotide differences, resulting in only a single-amino-acid change at position 249, from aspartic acid (D) in PAO1 to asparagines (N) in PAK (GenBank accession no. HQ433550). Since MexS of PAK is totally nonfunctional, the observed critical single-amino-acid change from D to N should account for the loss of function.

Compared to the genome-sequenced strain, the *mexT* genes from both PAK and PAO1 laboratory strains had 8-bp deletions at the 108th nucleotide position (CAGCCGGC). The MexT protein of the genome-sequenced strain includes a 347-amino-acid (aa)-long peptide, whereas the MexT proteins encoded by our PAK and PAO1 strain genomes are 304-amino-acid-long proteins, sharing high similarity to the LysR family of transcriptional factors. According to an earlier study, the same 8-bp deletion produced an active form of MexT protein (30). Besides the 8-bp deletion, the *mexT* gene of our PAO1 strain has two silent mutations compared to the *mexT* gene from the genome-sequenced PAO1 strain, whereas *mexT* of PAK carries 5 nucleotide changes relative to that of sequenced PAO1, resulting in only one amino acid change at position 31, from the basic amino acid lysine (K) in PAO1 to the acidic amino acid glutamic acid (E) in PAK (GenBank accession no. HQ433549). As MexT of PAK is partially defective, the drastic K-to-E amino acid change should account for the observed functional defect.

**MexT activates *mexS* expression while MexS modulates MexT activity.** The *mexS* and *mexT* genes are located next to each other but in head-to-head opposite directions, and both genes are required for not only the expression of the MexEF-OprN efflux pump but also for the repression of T3SS. Also, as shown in Fig. 6, expression of the *mexS-lacZ* reporter construct is much higher in PAO1 than in the PAO1*mexT*::Gm background, indicating that *mexS* expression is dependent on the transcriptional activator MexT. Therefore, the MexS/MexT pair of regulators behave very much like a bacterial two-component regulatory system (TCS), although they structurally do not fall into that group of regulators. Like the TCS, although the expression of *mexE-lacZ* in PAO1 requires both *mexS* and *mexT*, overexpression of *mexT* on a plasmid can increase *mexE-lacZ* expression in PAO1 (26), suggesting that MexS modulates MexT function and that high levels of MexT can overcome the requirement for MexS.

Modulation of MexT transcriptional activator function by MexS suggested a possible interaction between the two proteins. To test this, we performed a bacterial two-hybrid experiment. The *mexS* genes from both PAO1 and PAK were cloned into the pTRG vector, and the *mexT* gene from PAO1 was cloned into the pBT vector. The pBT empty vector and the pTRG-*mexS* clone pair were used as negative controls, while the *popN*-TRG and *pcr1*-BT pair, previously shown to interact strongly (58), were used as the positive control. Test results did show a positive interaction between the MexS and MexT proteins (Fig. 7). A weak interaction between MexS and MexT may indicate a transient interaction, such as enzymatic modification of MexT by the MexS. We were unable to observe physical interaction through a pulldown assay.

**MexT represses the T3SS through PA2486 and MexS.** A previous report said that overexpression of the MexEF-OprN efflux pump was associated with the impairment of the T3SS in *P. aeruginosa* (28). We asked if MexS and MexT repress the T3SS through the *mexEF-oprN* operon. The *mexE* gene was disrupted by insertion of a gentamicin resistance cassette in the middle of the *mexE* gene in both PAK and PAO1 chromosomes. Judging from the ExoS secretion patterns (Fig. 4A), *mexE* disruption has no effect on the T3SS in PAK or PAO1, indicating that the MexEF-OprN efflux pump itself is not directly involved in the regulation of the T3SS.

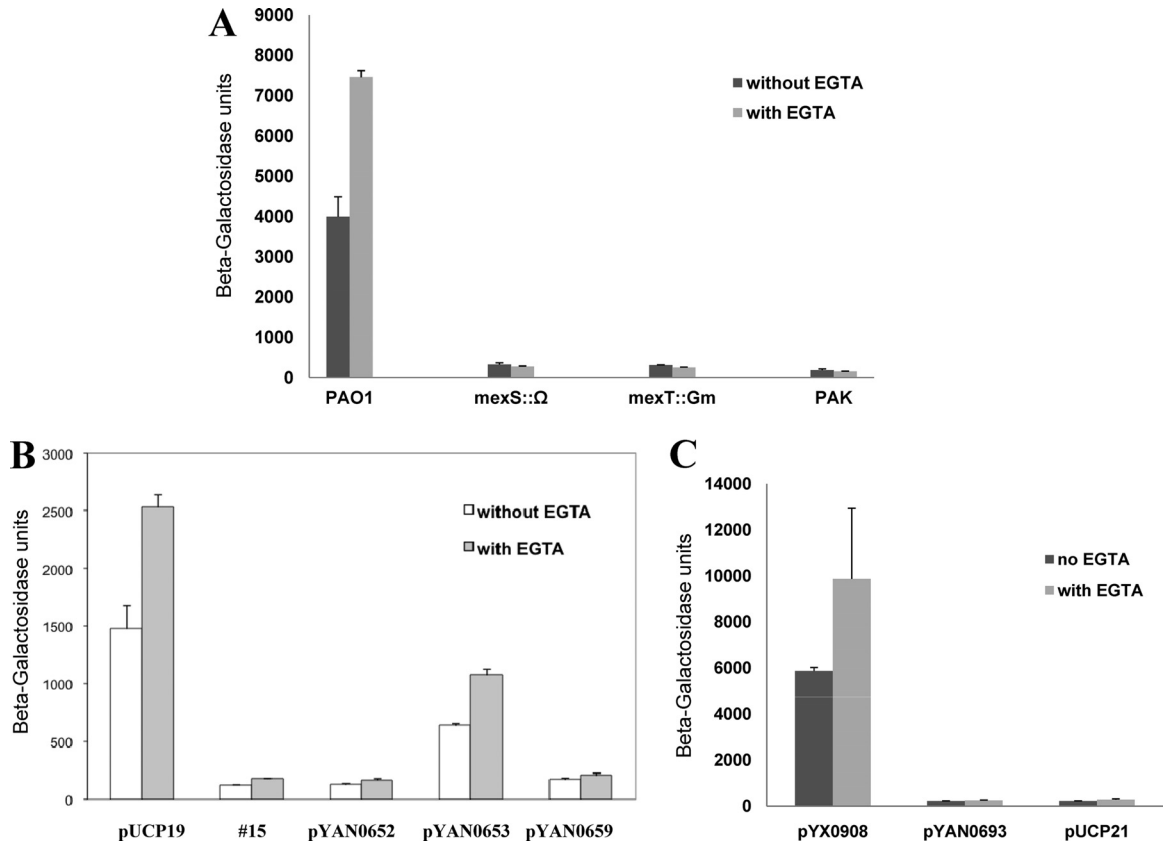


FIG. 5. Expression of *mexE-lacZ* fusion in various strain backgrounds. (A) Expression levels of *mexE*::*lacZ* fusion in PAO1, PAO1*mexS*::Ω, PAO1*mexT*::Gm, and PAK backgrounds. (B) Expression levels of *mexE*::*lacZ* fusion in PAO1 carrying indicated constructs. (C) Expression levels of *mexE*::*lacZ* fusion in PAO1*mexT*::Gm carrying *mexT*- and *mexS*-complementing plasmids or empty-vector control. Overnight cultures were diluted to 2% in LB with or without 5 mM EGTA and grown at 37°C for 4 h before being subjected to β-galactosidase activity assay. Error bars indicate standard deviations of triplicate assays.

MexT is a LysR-family regulator which activates many target genes by binding to their promoter regions. However, none of the T3SS gene promoters contain the putative MexT binding sequence; thus, MexT is unlikely to repress T3SS genes directly; rather, a MexT-dependent repressor might be involved. We have previously characterized two T3SS repressors under the control of stress response regulons (20, 55). According to the characteristics of those T3SS repressors, the MexT-dependent repressor should be a highly charged small peptide (<100 amino acids [aa]) with close physical proximity to the regulator MexT. Gene *PA2486* fits well into this criterion, as it is located close to *mexT*, contains a putative MexT binding site in its upstream regulatory region, and encodes a 64-aa-long highly charged peptide. First, its promoter was fused to a promoterless *lacZ* reporter gene and tested for expression in the wild type and the *mexT* mutant strain of PAO1. As shown in Fig. 6, expression of *PA2486* was indeed highly dependent on the presence of functional MexT. Next, its coding region was cloned under the *lac* promoter in pUCP20 and the resulting plasmid (pYX1004) was introduced into PAO1*mexT*::Gm. As the results in Fig. 8A show, forced expression of *PA2486* suppressed T3SS expression in PAO1*mexT*::Gm to the level in PAO1 without an adverse effect on the bacterial growth. Tests of similar-size open reading frames next to *PA2486*, namely, *PA2485* and *PA2487*, had no effect on the T3SS (Fig. 8A). Finally, to test if *PA2486* exhibits its repressor function as a small RNA or miniprotein, its ATG start codon was mutated to ATC in pYX1004. Upon introduction of the resulting plasmid (pYX1027) into PAO1*mexT*::Gm, suppression of ExoS secretion could no longer be observed (Fig. 8A), indicating that *PA2486* exerts its repressor function as a peptide. We name this gene *ptrC* for *P*seudomonas type III repressor gene C.

To further confirm its repressor function, the *ptrC* gene was deleted in the PAO1 background, where the type III secretion system was expected to increase. To our surprise, however, the ExoS secretion did not change in PAO1Δ*ptrC* compared to the wild-type strain PAO1, thus suggesting that PtrC might function as a T3SS repressor only under certain stress conditions, while under normal conditions there is an additional MexT-dependent repressor.

Since both *mexS* and *mexT* are required to repress the T3SS, and MexT positively regulates *mexS*, we wondered if MexS alone could suppress the T3SS, functioning in parallel with PtrC. To test this, the *mexS*-expressing plasmid (pYAN0693) was introduced into PAO1*mexT*::Gm and ExoS secretion was measured under type III-inducing conditions. As shown in Fig.

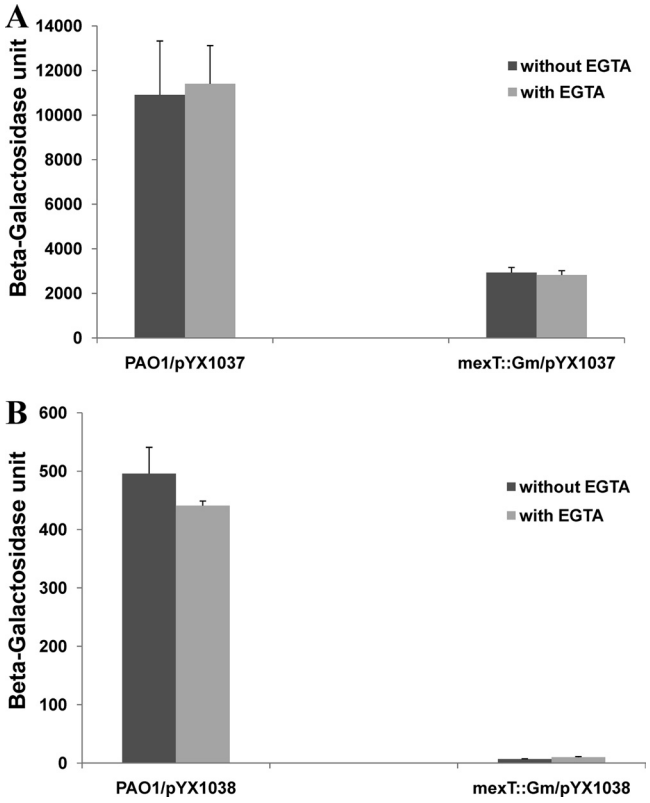


FIG. 6. Expression levels of *mexS*::*lacZ* and *PA2486*::*lacZ* fusions in PAO1 or PAO1*mexT*::Gm background. Overnight cultures were diluted to 2% in LB with or without 5 mM EGTA and grown at 37°C for 4 h before being subjected to β-galactosidase activity assay. Error bars indicate standard deviations of triplicate assays.

8B, the type III secretion system was repressed to the wild-type PAO1 level by the expression of *mexS* alone without the presence of functional *mexT*. On the other hand, introduction of a *mexT*-expressing plasmid (pYX0908) into PAO1*mexS*::Gm did

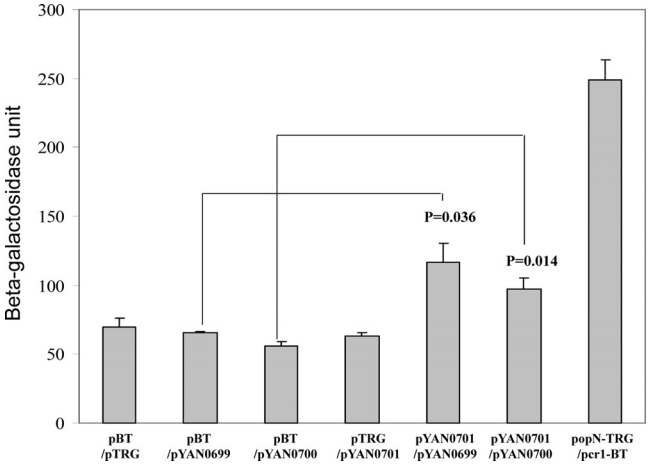


FIG. 7. Test of protein-protein interactions between MexS and MexT using the Bacterial-Match two-hybrid system. pBT, bait vector; pTRG, target vector; pYAN0699, *mexS* from PAK cloned into pTRG; pYAN0700, *mexS* from PAO1 cloned into pTRG; pYAN0701, *mexT* from PAO1 cloned into pBT. The interaction between *popN*-TRG and *pcr1*-BT was used as a positive control.

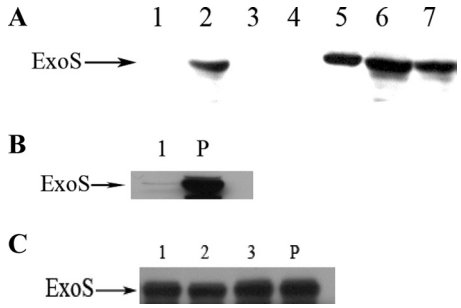


FIG. 8. Secretion of ExoS by the indicated strains. (A) ExoS secretion by PAO1*mexT*::Gm carrying different constructs derived from PAO1. Lane 1, PAO1 with pUCP20; lane 2, PAO1*mexT*::Gm with pUCP20; lane 3, PAO1*mexT*::Gm with *mexT*-complementing plasmid (pYX0908); lane 4, PAO1*mexT*::Gm with PA2486 overexpression plasmid (pYX1004); lane 5, PAO1*mexT*::Gm with PA2486 ATG→ATC overexpression plasmid (pYX1027); lane 6, PAO1*mexT*::Gm with PA2485 overexpression plasmid (pYX1030); lane 7, PAO1*mexT*::Gm with PA2487 overexpression plasmids (pYX1029). (B) ExoS secretion by PAO1*mexT*::Gm carrying *mexS*-complementing plasmid pYAN0693 (lane 1) or an empty vector (lane P). (C) ExoS secretion by PAO1*mexS*::Ω carrying *mexT*-complementing plasmid pYX0908. Lanes 1 to 3 are three parallel samples, and lane P is empty vector. Overnight cultures were diluted to 2% in LB with 5 mM EGTA plus appropriate antibiotics and grown at 37°C for 3 h. Proteins secreted into supernatants were concentrated with 15% trichloroacetic acid, loaded onto SDS-PAGE gels, and immunoblotted with antibody against ExoS.

not suppress the ExoS secretion (Fig. 8C). Clearly, MexS can inhibit the T3SS independently of MexT; thus, MexT represses the T3SS through two independent pathways, one involving MexS and the other involving PtrC, each possibly responding to different environmental signals.

**MexT-mediated regulation of the MexEF efflux pump, elastase, and pyocyanin does not go through MexS or PtrC.** It has been reported that MexT also plays a regulatory role in the production of elastase and pyocyanin (48). To examine if MexS is required, elastase and pyocyanin production was examined in both *mexS* and *mexT* mutant backgrounds. As shown in Fig. 9A and B, the *mexS* and *mexT* mutants of PAO1 shared the same phenotype of increased elastase and pyocyanin production compared to that in wild-type PAO1, indicating that MexS is also required. Since MexT represses the T3SS through MexS, we wanted to know if the MexT-mediated regulation of the MexEF efflux pump, elastase, and pyocyanin also goes through MexS. Examination of *mexE-lacZ* expression in the PAO1*mexT*::Gm strain containing a *mexS*-expressing plasmid, pYAN0693, indicated that MexT does not regulate *mexEF-oprN* through MexS (Fig. 5C). Similarly, overexpression of *mexS* in the PAO1*mexT*::Gm mutant did not change the pyocyanin or elastase levels (Fig. 9A and B), indicating that MexT-mediated repression of elastase and pyocyanin production does not go through MexS. Clearly, MexS/MexT-mediated regulation of the MexEF efflux pump, elastase, and pyocyanin goes through a unique regulatory pathway that is different from the ones repressing T3SS gene expression.

## DISCUSSION

MexT is a LysR-family transcriptional regulator that modulates many virulence phenotypes, including the T3SS of *P.*

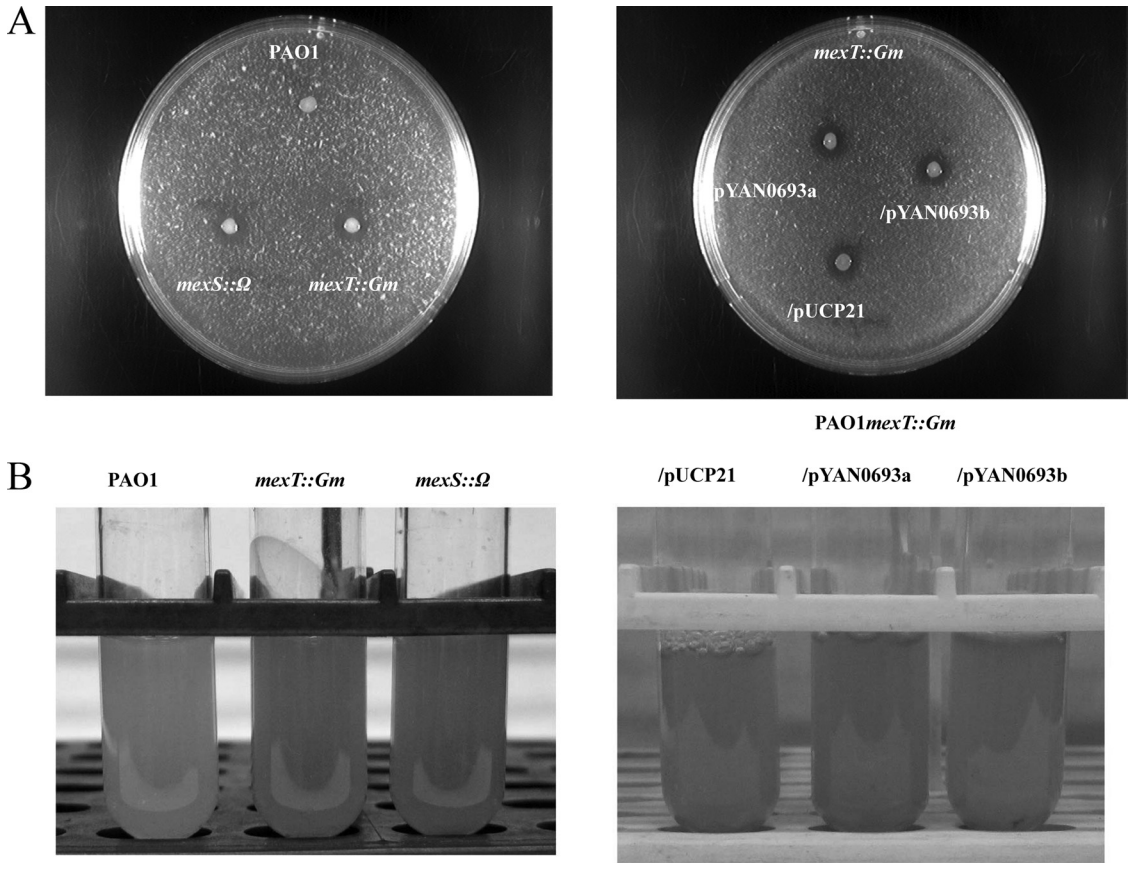


FIG. 9. Elastase and pyocyanin secretion assays. (A) Elastolytic activity of indicated strains on an elastin-agar plate incubated at 37°C for 40 h. (B) Pyocyanin production of indicated strains upon overnight growth at 37°C in LB medium.

*aeruginosa* (48). However, the precise mechanism by which MexT regulates the T3SS remains to be elucidated. In the present study, we have shown that MexT regulates T3SS through two independent pathways, involving PtrC in one and MexS in the other. *ptrC*, whose upstream regulatory region contains a putative MexT binding site, is expressed at much higher levels in wild-type PAO1 than in its *mexT* mutant derivative; however, such "normal"-level expression is not sufficient to suppress the T3SS gene expression, and a much higher level of PtrC expression may occur in response to certain environmental stimuli. PtrC includes a small hypothetical protein that comprises 64 amino acids, of which 21 are charged amino acids. This small protein is 100% identical in all of the sequenced *P. aeruginosa* strains. When overexpressed in the PAO1*mexT*::Gm mutant, PtrC can effectively repress the T3SS; however, when *ptrC* was knocked out in PAO1, the T3SS was not derepressed. These observations hinted at the presence of another MexT-controlled repressor functioning under normal growth conditions. Our experimental data indicated that MexS is the key molecule linking the elusive repressor to T3SS suppression.

We have previously shown that the T3SS is specifically repressed by miniproteins that are highly induced under various stress conditions. For example, the T3SS is specifically suppressed by PtrA, a 63-aa-long anti-ExsA peptide that is highly and specifically induced by the stress of high copper cation levels (20). Also, PtrB, a 66-aa-long highly charged peptide repressor, is highly induced under the stress of DNA damage and exhibits a T3SS-specific inhibitory function (55). Both PtrA and PtrB are miniproteins (<100 aa) with high proportions of charged amino acids; thus, it is reasonable to predict that PtrC is another repressor of the T3SS, responding to antibiotic stress. Recently, 12 genes with unknown functions in the PAO1 genome were reported to be highly induced by overexpression of *mexT*, independently of MexEF-OprN (47). Consistent with our finding, *ptrC* was one of them. According to the characteristics of the miniproteins, as mentioned above, four additional small proteins, namely, PA1970, PA3229, PA4354, and PA4881, were tested for possible repressor functions for the T3SS. When the four candidate genes were overexpressed in PAO1*mexT*::Gm, ExoS secretion was unaffected compared to that in PAO1*mexT*::Gm harboring an empty vector (data not shown), indicating that these four genes are not mediators connecting MexT and T3SS repression.

*mexS*, previously called *qrh*, is located immediately upstream of the *mexT* gene with an opposite transcriptional direction and encodes a possible quinone oxidoreductase, acting on NADH or NADPH with quinone or a similar compound as acceptor (http://v2.pseudomonas.com). This is the first report demonstrating the repressor function of MexS for the T3SS in *P. aeruginosa*, linking MexT to T3SS regulation. MexS does not have structural features of transcriptional activators; thus, it is

unlikely to regulate the T3SS genes through direct DNA binding. A single-amino-acid change at position 249 of MexS, from aspartic acid (D) to asparagine (N), resulted in a loss of function in PAK. It is interesting that the 249th amino acids are also N′s in sequenced genomes of PA7, PA14, and PA LesB58, indicating that these strains' genomes also encode nonfunctional MexS, in terms of MexEF-OprN activation and T3SS repression. Also, the 249th amino acid is located in the conserved region of the cofactor-binding domain of the quinone oxidoreductase and thus is likely to affect its enzymatic activity, linking to its regulatory role. Our bacterial two-hybrid assay results demonstrated physical interaction between MexS and MexT. Since MexS modulates the MexT activity through direct interaction, it is possible that MexS might also be able to interact with another MexT-like transcriptional factor which regulates T3SS gene expression. The identity of the MexS-interacting transcriptional factor as well as the nature of MexS-mediated modulation of MexT remains to be identified.

It has been reported that MexT can increase *mexEF-oprN* expression in wild-type PAO1 when introduced in *trans* on a multicopy plasmid (26). Also, using a clinical isolate of *P. aeruginosa* which displays a pan-aminoglycoside-resistant phenotype, Sobel et al. have previously shown that the *mexEF-orpN* efflux pump genes were actually repressed by MexS (44). However, in our study, both functional *mexS* and *mexT* are required to activate the *mexEF-oprN* operon. This major discrepancy may relate to the use of different strain backgrounds. Consistent with our findings, in the *nfxC*-type mutant derivatives of PAO1, expression of both *mexS* and *mexE* is elevated while the *mexT* transcription level is comparable to that in its parental strain PAO1 (26). Also, a transcriptome analysis of *P. aeruginosa* PAO1 after interaction with airway epithelial cells revealed substantial increases in the expression of both *mexEF-oprN* (12.9-fold) and *mexS* (13.3-fold) (15).

MexEF-OprN, one of the several efflux pumps in *P. aeruginosa*, is overexpressed in an *nfxC*-type mutant which confers resistance to quinolones, chloramphenicol, and trimethoprim. In the present study, the expression of *mexE* requires both functional MexS and MexT. However, previous studies indicated that overexpression of *mexT* alone can activate *mexE* without the presence of MexS. For instance, the *mexE* expression could be activated in *E. coli* by merely introducing a *mexT*-expressing plasmid (26), while introduction of a *mexT*-overexpressing plasmid into wild-type PAO1 is sufficient to confer an *nfxC*-type phenotype (26, 27). Therefore, MexT seems to be synthesized in a low-active form and becomes highly activated upon modification by MexS. When the amount of low-active MexT reaches a certain threshold level, MexT can override the need for the helper MexS. In this regard, the MexS/MexT function resembles the classical two-component regulatory system, although what environmental signals they sense and the nature of MexT modification by MexS are not known at present.

In the present study, both MexS and MexT are also shown to be essential for the suppression of pyocyanin and elastase production. However, unlike the repressor function for the T3SS, MexS is not the mediator connecting MexT and the production of pyocyanin or elastase. Combining all of the above, we propose a model for the *mexT* gene function (Fig. 10). Under laboratory culture conditions, MexS modulates MexT, which in turn activates *mexEF-oprN* expression while suppressing genes involved in the production of pyocyanin and elastase. MexT exerts a repressive function on the T3SS through activation of *mexS* expression, and MexS may interact with another LysR-type regulator to negatively control the T3SS. Under specific environmental conditions, MexT gets highly activated, leading to elevated *ptrC* expression, which in turn represses the T3SS. Considering the important roles that the T3SS plays, it is not surprising to see redundant regulatory pathways controlling its expression, involving PtrC and MexS, as they may represent regulatory mechanisms under two totally different environmental conditions, ensuring shutoff of the T3SS.

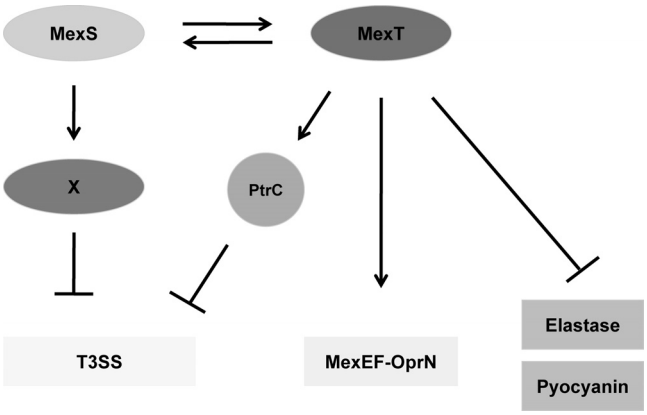


FIG. 10. Proposed model of MexS/MexT-mediated regulation of the type III secretion system (T3SS), the efflux pump (*mexEF-oprN*), and elastase and pyocyanin production. "X" denotes a LysR-family transcriptional regulator that is activated by MexS and represses the T3SS directly or indirectly through a downstream repressor.

## ACKNOWLEDGMENTS

This work was supported in part by funding from the UF Opportunity Fund (S.J.) and a grant (30970114) from the General Program of NSFC, People's Republic of China (H.Y.). Y.J. is the recipient of a scholarship from the Chinese Ministry of Education.

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