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Short communication

MexT modulates virulence determinants in *Pseudomonas aeruginosa* independent of the MexEF-OprN efflux pump

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ABSTRACT

In the human pathogen *Pseudomonas aeruginosa*, the LysR-family regulator MexT modulates the induction of the tripartite MexEF-OprN resistance nodulation-division multi-drug efflux system resulting in increased resistance to diverse antibiotics. The MexEF-OprN system is normally quiescent in wild-type cells, but is highly induced in *nfxC*-type phenotypic mutants in a MexT dependent manner.

In addition to antibiotic resistance, induction of mexEF-oprN in *nfxC*-type mutants has been linked to reduced levels of homoserine lactone-dependent virulence traits, including pyocyanin, elastase, rhamnolipids and PQS and to reduced expression of type three secretion effector proteins. In this study, MexT is overexpressed in wild-type PAO1 and an isogenic mexEF deletion mutant to determine if MexT regulates diverse virulence phenotypes dependent or independent of MexEF-OprN. It is shown that MexT regulates type three secretion, pyocyanin production and early surface attachment independent of MexEF-OprN. In contrast, MexT modulation of the expression of the virulence genes rhlA, lasB and hcnB is dependent on MexEF-OprN, which apparently mediates these effects via efflux of cell-signaling intermediates. The data presented demonstrates that MexT may play a more global role in modulating *P. aeruginosa* virulence than previously reported and suggests that MexT regulates diverse targets that mediate phenotypic alterations independent of MexEF-OprN.

1. Introduction

Pseudomonas aeruginosa is an important opportunistic human pathogen and is the main cause of chronic lung disease in cystic fibrosis patients. The pathogenicity of *P. aeruginosa* is attributed to a plethora of virulence related phenotypes, which are modulated by a complex network of signaling and regulatory pathways. *P. aeruginosa* is also intrinsically resistant to several antibiotics and has the ability to acquire multi-drug resistance. This is partly attributable to the presence of several tripartite resistance nodulation-division (RND) multi-drug efflux systems .[1] MexEF-OprN is a unique multi-drug efflux pump, which is activated by a LysR-type transcriptional regulator, MexT [1,2]. The MexEF-OprN system is normally quiescent in wild-type cells under normal laboratory conditions, but is highly induced in *nfxC*-type phenotypic mutants, which exhibit increased resistance to chloramphenicol, trimethoprim and fluoroquinolones and susceptibility to certain β-lactam and aminoglycoside antibiotics [3,4]. Although the underlying mechanisms that generate *nfxC*-type mutants are not fully understood, and appear to be multifactorial [5–7], MexT is thought to be rendered active in these mutants with the resultant induction of the MexEF-OprN efflux pump. In addition to antibiotic resistance, induction of *mexEF-oprN* in *nfxC*-type mutants has been linked to reduced levels of homoserine lactone-dependent virulence traits, including pyocyanin, elastase, rhamnolipids and PQS [8] and to reduced expression of TTSS effector proteins [9]. It was suggested that MexEF-OprN mediates these effects via efflux of cell-signaling intermediates, which ultimately commits the cell to a state of reduced virulence [8]. However, it remains to be elucidated whether the pleiotropic effects of MexT on decreased virulence factor production are a direct result of MexEF-OprN induction or if MexT can mediate these effects through other regulatory mechanisms. In this study, we sought to determine if MexT played an independent role in regulating virulence in *P. aeruginosa* by using a defined genetic system overexpressing MexT in the wild-type PAO1 and its isogenic *mexEF* deletion mutant strains.

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2. Results and discussion

2.1. Interplay between MexT and nfxC-type phenotypes

In the wild-type PAO1 strain (Holloway collection) used in this study the *mexT* gene contains the 8 base pair deletion previously shown to render it active in *nfxC*-type mutants [5]. However, although low levels of *mexT* transcript were detected by semi-quantitative reverse transcription PCR (RT-PCR) when grown in routine LB growth conditions, *mexE* was not expressed (Fig. 1). This suggests that MexT may be inactive or the level of MexT protein may be insufficient for the activation of *mexE* expression under these conditions. Moreover, a *mexT* deletion mutant showed no phenotypic alterations compared to its isogenic wild-type. In contrast, in a *nfxC*-type mutant generated in this strain, by serial plating on high chloramphenicol concentrations [8], the transcriptional expression levels of *mexT* and *mexE* were highly increased in the same growth conditions (data not shown), with no sequence alterations in the open reading frames or upstream regulatory regions of *mexT* or *mexE*. These observations indicate that increasing *mexT* expression is a mechanism for generating an *nfxC*-type mutant under physiological conditions. Therefore, a plasmid overexpressing *mexT*, pME6032-*mexT*, was constructed and used to study the role of MexT in regulating virulence of *P. aeruginosa*.

A *mexEF* deletion mutant was constructed in the wild-type PAO1 to yield strain PAO1Δ*mexEF*. pME6032-*mexT* was introduced into PAO1 and this PAO1Δ*mexEF* mutant. No significant difference in growth rates was observed between the strains (data not shown). Introduction of pME6032-*mexT* into the wild-type PAO1 gave rise to a resistance profile typical of an *nfxC*-type mutant (Table 1). Deletion of *mexEF* alleviated the *nfxC*-type resistance phenotype observed upon over-expression of *mexT*, consistent with the resistance profile being dependent on the MexEF-OprN efflux system.

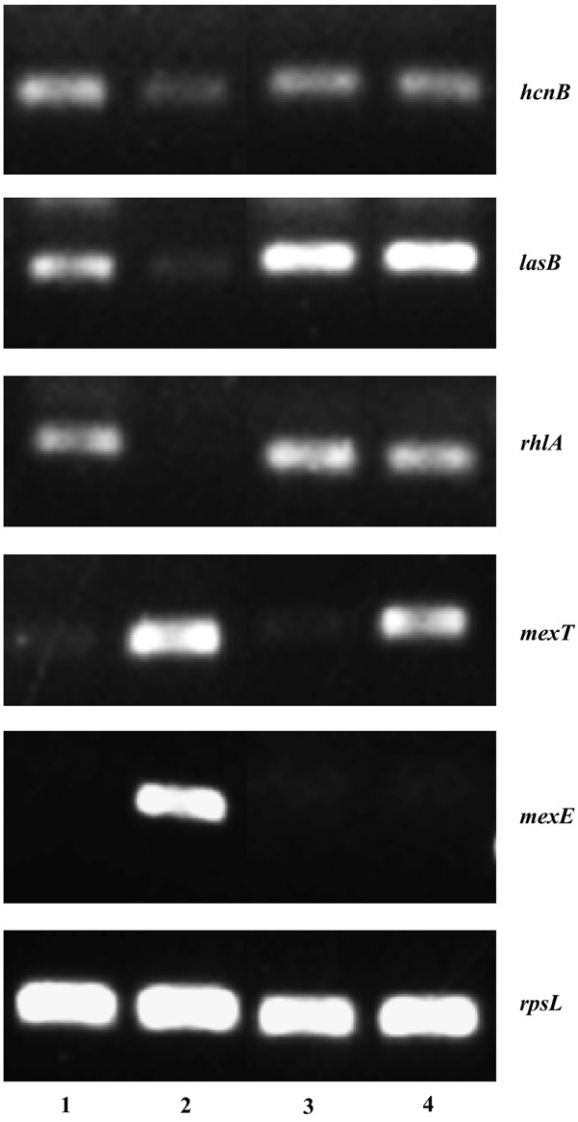

Fig. 1. Semi-quantitative RT-PCR of *hcnB*, *lasB* and *rhlA* expression in: lane 1, wild-type PAO1(pME6032); lane 2, wild-type PAO1(pME6032-*mexT*); lane 3, PAO1 △*mexEF* (pME6032); lane 4, PAO1 △*mexEF* (pME6032-mexT). Increased production of *mexT* by pME6032-*mexT* represses *hcnB*, *lasB* and *rhlA* expression but this is dependent on the presence of *mexEF*. *rpsL* was used as an RNA control.

2.2. The role of MexT in vitro virulence

Semi-quantitative RT-PCR was used to study the influence of MexT on the expression of virulence genes controlled by cell-to-cell signaling systems, previously associated with *nfxC*-type mutants [8]. Transcript levels of *rhlA*, *lasB* and *hcnB* encoding rhamnosyltransferase subunit, elastase and hydrogen cyanide synthase respectively, were assayed in PAO1 and PAO1Δ*mexEF* carrying either pME6032-*mexT* or pME6032. The transcript levels of the genes were considerably reduced in PAO1 strains, but there was no change in PAO1Δ*mexEF*, when *mexT* was overexpressed (Fig. 1). These results indicate that MexT regulates the expression of *rhlA*, *lasB* and *hcnB* in a MexEF-OprN-dependent manner. Reduced swarming and twitching motility was also observed in cells overexpressing MexT in a MexEF-OprN-dependent manner (data not shown).This could be explained by the fact that rhamnolipids are also decreased in cells overexpressing MexT in a MexEF-OprN-dependent matter, since rhamnolipids are known to modulate motility [8]. Our data confirms previous observations that the MexEF-OprN system plays a role in modulating cell signaling. However, further investigations revealed that MexT was capable of regulating certain virulence traits independent of MexEF-OprN expression.

2.3. MexT modulates type three secretion, pyocyanin production and early attachment independent of MexEF-OprN

Previously, increased expression of MexEF-OprN has been linked with reduced TTSS [9]. To determine the role of MexT in regulating the TTSS, an *exoS-lacZ* fusion was inserted at a neutral site in the chromosome of PAO1 and PAO1Δ*mexEF*. β-galactosidase activity was measured from each strain containing either pME6032-*mexT* or pME6032, when grown in TTSS-inducing or non-inducing conditions [10]. All strains exhibited comparable basal *exoS* expression under non-inducing conditions, however, under inducing conditions, there was a marked decrease in *exoS* expression in both strains when *mexT* was overexpressed (Fig. 2).

Table 1
Antibiotic resistance profiles.

Strain	Antibiotic MIC[a] (μg/ml)				
	CB	CM	TMP	NFX	KM
PAO1 (pME6032)	64	32	64	0.25	32
PAO1 (pME6032-*mexT*)	32	2048	1024	4	16
PAO1Δ*mexEF* (pME6032)	64	32	64	0.25	32
PAO1Δ*mexEF* (pME6032-*mexT*)	128	64	256	0.25	32

CB – carbenicillin, CM – chloramphenicol, TMP – trimethoprim, NFX – norfloxacin, Km – kanamycin.
[a] Minimum inhibitory concentration.

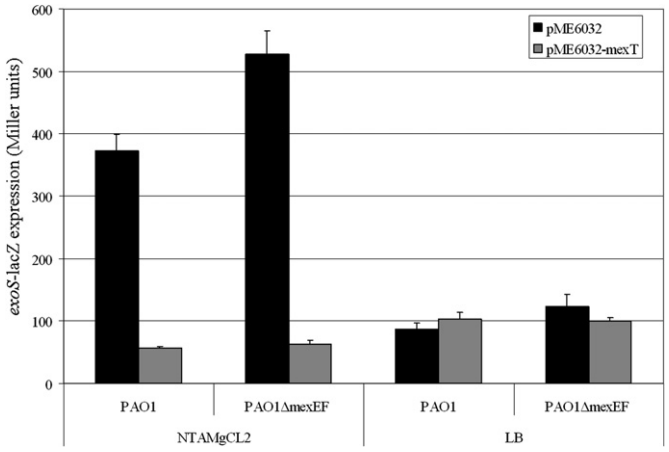

Fig. 2. β-galactosidase assays indicating *exoS* expression in TTSS-inducing (LB NTA $MgCl_2$) and non-inducing (LB) growth conditions. MexT exerts a negative effect on *exoS* expression independent of MexEF-OprN: MexT represses *exoS* expression in PAO1 and PAO1Δ*mexEF* under TTSS-inducing growth conditions.

Therefore, MexEF was not required for the modulation of *exoS* expression but overexpressing MexT prevented *exoS* induction under inducing conditions. Real-time RT-PCR was carried out to investigate if this MexT modulation of *exoS* was mediated through the TTSS regulators, *exsA*, *exsC* and *exsD* [11]. Overexpressing *mexT* resulted in a marked decrease in expression of all genes in both PAO1 and PAO1Δ*mexEF*: *exsA* expression was reduced 6 ± 3 and 7.5 ± 1.2-fold respectively; *exsC* expression was reduced 11.5 ± 2 and 11 ± 2.5-fold respectively and *exsD* was reduced 10 ± 5 and 13.5 ± 3-fold respectively. This data suggests that MexT represses the induction of the TTSS effector proteins through modulation of the expression of the transcriptional regulator ExsA, independent of the MexEF-OprN efflux pump.

The role of MexT in the regulation of the redox-active virulence factor, pyocyanin, independent of MexEF-OprN was also investigated (Fig. 3A). Previously, it has been reported that expression of MexEF-OprN in an *nfxC*-type mutant causes reduced production of pyocyanin [4]. Wild-type and PAO1Δ*mexEF*, containing either pME6032 or pME6032-*mexT*, were grown in glycerol–alanine media, previously shown to stimulate pyocyanin production [12]. Pyocyanin production was not significantly altered in PAO1Δ*mexEF* compared to PAO1 but was significantly reduced in both strains when *mexT* was overexpressed (Fig. 3A). This demonstrates that, in these growth conditions, MexT negatively regulated pyocyanin production, independent of MexEF-OprN.

Previously, it was reported that the early attachment to polystyrene substratum was impaired in a *mexS* (PA2491) mutant, which has been characterized as an *nfxC*-type mutant .[6,7] We investigated if MexT played an independent role in regulating this phenotype. PAO1 and PAO1Δ*mexEF*, containing either pME6032-*mexT* or pME6032, were incubated at 37 °C in 24-well polystyrene plates for 2 h and levels of attachment were quantified using crystal violet staining. The attachment was not significantly altered in PAO1Δ*mexEF* compared to PAO1 but was significantly reduced in both strains when *mexT* was overexpressed (Fig. 3B), demonstrating that MexT negatively regulated early attachment, independent of MexEF-OprN.

The data presented in this study demonstrate that the LysR transcriptional regulator MexT may play a more global role in modulating *P. aeruginosa* virulence than previously reported and that the MexEF-OprN efflux system may be just one of many MexT target phenotypes. The exact mechanism by which MexT modulates these diverse phenotypes remains to be determined but it is possible that MexT regulates quorum-sensing related phenotypes

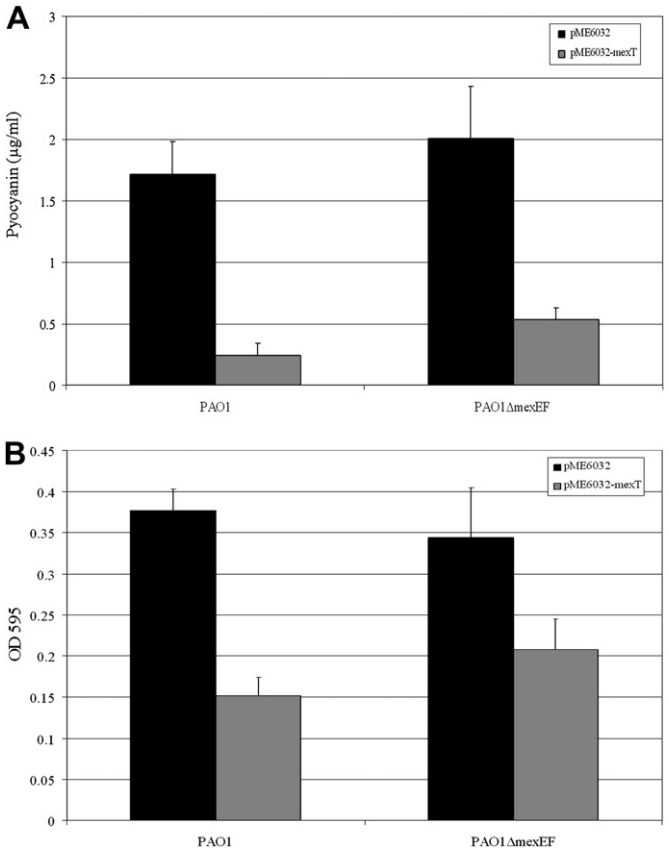

Fig. 3. MexT exerts negative effects on virulence phenotypes independent of MexEF-OprN. (A) Pyocyanin production in cell-free supernatants of stationary phase cultures of PAO1 and PAO1Δ*mexEF* grown in glycerol–alanine media was markedly reduced when pME6032-*mexT* was introduced; (B) Initial attachment to polystyrene substratum in M9-glucose was impaired on the introduction of pME6032-*mexT* to both PAO1 and PAO1Δ*mexEF*.

indirectly by modulating other changes in the cell through it's activation of *mexEF-oprN*. It is also possible that MexT directly regulates the expression of genes related to TTSS and phenazine production (Fig. 4). Recently, a LysR-type regulator YtxR has been shown to have a global regulatory role in *Yersinia enterocolitica*, including direct downregulation of the transcriptional expression of TTSS by overexpressed YtxR [13]. Further work is warranted to fully understand the global regulatory role of MexT in modulating virulence phenotypes in *P. aeruginosa* and to elucidate the exact mechanism(s) of this regulation.

3. Materials and methods

3.1. Bacterial growth

All *P. aeruginosa* and *Escherichia coli* strains were routinely grown at 37 °C in LB broth with aeration, unless otherwise stated. When required, antibiotics were added at the following concentrations ($\mu g\ ml^{-1}$): *E. coli*, tetracycline (Tc, 10) or gentamicin (Gm, 10); *P. aeruginosa* PAO1, gentamicin (Gm, 50), tetracycline (Tc, 50).

3.2. Generation of mexEF deletion mutant strain and construction of pME6032-mexT

In PAO1Δ*mexEF*, a 1.6-kb fragment was deleted in the *mexE-mexF* gene. The deletion includes from the 3rd codon of *mexE* to 118th codon of *mexF* and was obtained as follows. A 0.9-kb EcoRI–KpnI fragment, including the first 2 codons of *mexE*, which had

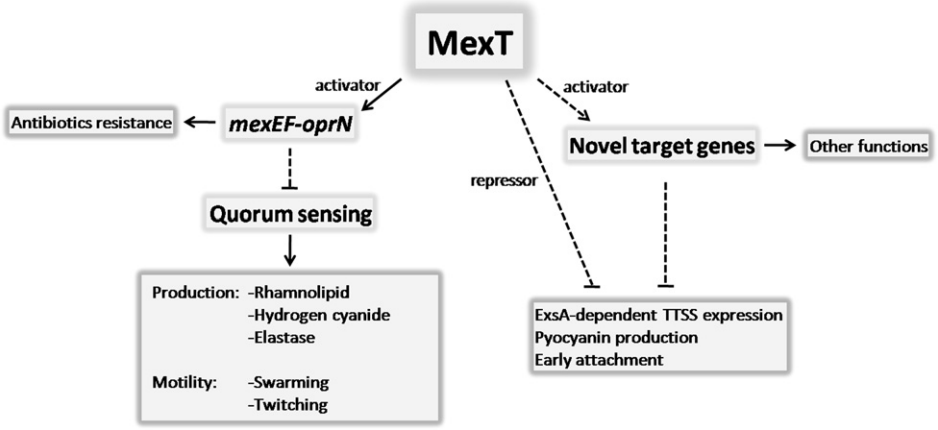

Fig. 4. A proposed model illustrating the global regulatory role of MexT on virulence phenotypes. Arrows indicate a positive effect, and bars a negative effect. Solid lines indicate already known links, and dashed lines indicate links to be determined.

been amplified by PCR with primers (5′-GGAATTCCATCTCCACCGCCATG-3′) and (5′-GGGGTACCTGTTCCATGCTTGACTCC-3′) was linked to a 0.9-kb KpnI–BamHI fragment containing from 119th to 408th codons of *mexF*, which had been amplified by PCR with primers (5′-GGGGTACCGAAGCTCCCGGAAGAAGTG-3′) and (5′-CGGGATCCACCACGATGCCGATGGC-3′). The resulting 1.8-kb fragment was cloned into the suicide plasmid pEX18Tc digested with EcoRI and BamHI. A KpnI fragment containing the *FRT* gentamicin-resistance (Gm) cassette from plasmid pPS856 [14] was then inserted in-between the flanking regions on the plasmid. The *mexEF* of PAO1 was then replaced with the plasmid as described by Hoang et al. [14]. The Gm-resistance sequence in the chromosome was removed by introducing plasmid pFLP2, which carries the Flp recombinase gene [14]. Correct insertion in the constructed mutant was verified by PCR.

To obtain plasmid pME6032-*mexT*, the *mexT* gene was amplified by PCR with the primers 5′-GTAGTAGACGCTGGCCTCCAC-3′ and 5′-GTGAATTCGTCCCACTCGTTC-3′ and cloned into pCR-2.1TOPO vector (Invitrogen). 1.9-Kb EcoRI fragment was subcloned into pME6032 [15] in an orientation driven by *tac* promoter, yielding pME6032-*mexT*. It has been noticed that this construct can overexpress *mexT* even without IPTG induction as shown in Fig. 1.

3.3. Antibiotic resistance profiles

The minimum inhibitory concentration (MIC) of diverse antibiotics was determined for PAO1 and PAO1Δ*mexEF*, containing either pME6032-*mexT* or pME6032 by microtitre broth dilution. Overnight cultures of each strain were diluted 1:100 in fresh Mueller–Hinton broth containing 50 μg ml^{-1} tetracycline and grown to mid-log phase (OD_{600} of 0.4–0.6), harvested and washed in PBS. An inoculum of 10^4–10^5 cfu/ml was added to wells containing a dilution series of each antibiotic in Mueller–Hinton broth. The MIC was defined as the concentration at which bacterial growth was completely inhibited after static incubation for 20 h at 37 °C.

3.4. Semi-quantitative reverse transcription PCR for rhlA, lasB and hcnB

Total RNA was isolated from PAO1 and PAO1Δ*mexEF* containing either pME6032-*mexT* or pME6032 when grown in LB broth to an OD_{600} nm of 0.5. Residual DNA was removed by treatment with the Rnase-free Dnase I (Ambion). RNA samples were reverse transcribed using random primers (Promega). The amplifications were performed with primers specific for the selected genes: for *rhlA* (5′-TCAACGAGACCGTCGGCAAATAC-3′ and 5′-TCCGCTCCAGGCAAGCCAAGT-3′); for *lasB* (5′-TGCCCGACCAACACCTACAAGC-3′ and 5′-AGCGACACCAGCGGATAGAACAT-3′); for *hcnB* (5′-GCTGCTGAACAAGCCGCAACTGT-3′ and 5′-TGAAGCCATAGCCGACCGCCAGGGT-3′) and for *rpsL* (5′-GCAAGCGCATGGTCGACAAGA-3′ and 5′-CGCTGTGCTCTTGCAGGTTGTGA-3′). Expression of the *rpsL* gene was measured as an internal control that ensured that equal amounts of RNA were used in all samples.

3.5. Generation of exoS promoter-lacZ fusion and β-galactosidase assay

Chromosomal *exoS* transcriptional fusion was generated by cloning the promoter region of *exoS* into the vector pUC18miniTn7T-Gm-*lacZ*, which integrates into the PAO1 chromosome at a neutral integration site downstream of the *glmS* gene [16]. The *exoS* promoter region was PCR amplified using primers (5′-GAGAGCTCGTCGGGAGATCGAGAGCGAG-3′ and 5′- GACCCGGGGGCTGGTGCAATTCGACGGC-3′) with *Sac*I and *Sma*I restriction sites. Following TA cloning into pCR-2.1TOPO vector, the *exoS* promoter region was excised and ligated into pUC18miniTn7T-Gm-*lacZ* cut with *Sac*I and *Sma*I to produce pUC18miniTn7T-Gm-*exoS-lacZ*. Plasmids were electroporated into strains of interest through the procedures described previously [16].

P. aeruginosa cultures were grown in LB or TTSS-inducing media (LB10 mM NTA, nitrilotriacetic acid, supplemented with 5 mM $MgCl_2$) at 37 °C, and β-galactosidase assays were performed as described by Miller [17]. Data are the mean of three independent experiments with triplicate samples.

3.6. Quantitative real-time PCR for exsA, exsC and exsD

Total RNA was isolated, using an RNeasy kit (QIAGEN), from PAO1 and PAO1Δ*mexEF* containing either pME6032-*mexT* or pME6032 when grown in TTSS-inducing (LB10 mM NTA) and non-inducing (LB) conditions to an OD_{600} nm of 1.5. RNA samples were reverse transcribed using random primers (Promega). Real-time RT-PCR was performed using SYBR Green Quantifast (QIAGEN) and primers specific for *exsA* (Forward; 5′-ATGTCGGTCCTGCGGCAACTGAGC-3′, Reverse; 5′-GCGCGGCGAAACCCCATAGACACT-3′), *exsC* (Forward; 5′-AGCGCGAGCGTCTGTTGCTGGAG-3′, Reverse; 5′GGGTCAGTTGCGCTGCGAGAATCT-3′) and *exsD* (Forward; 5′-GCCGGGTTACGCATCGAGCACTTT-3′, Reverse; 5′-CGCCGCGCAGAGGAGAATC-3′) and normalized to the housekeeping gene *proC*.

3.7. Pyocyanin quantification

Overnight cultures were grown in LB, washed in PBS and diluted to an OD of 0.01 in 20 ml glycerol–alanine media (112 mM DL-alanine, 2% glycerol, 0.8 mM K_2HPO_4, 0.4 mM ferric citrate 0.02 M $MgCl_2$ and 0.1 M Na_2SO_4) in 100 ml conical flasks. After 24 h incubation at 37 °C, pyocyanin was quantified in stationary phase cultures by HCL extraction as previously described [18].

3.8. Early attachment assay

PAO1 and PAO1Δ*mexEF*, containing either pME6032-*mexT* or pME6032, were grown overnight in M9 minimal medium (0.2% glucose, 10 mM ammonium sulfate as carbon and nitrogen source respectively). Cells were diluted 1:100 into fresh M9 medium. These bacteria were allowed to grow to mid-log phase (OD_{600} of 0.4–0.7) before dilution to an OD600 of 0.1 in 20 ml of M9 medium. 1 ml cultures were incubated at 37 °C in 24-well polystyrene plates for 2 h. Unattached cells were removed and attached cells were stained with 0.25% crystal violet for 30 min, washed twice with PBS and air dried. 2 ml ethanol was added to the wells to extract crystal violet and levels of attachment were quantified at OD_{595} nm.

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