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MucR, a Novel Membrane-Associated Regulator of Alginate Biosynthesis in *Pseudomonas aeruginosa*▽

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Alginate biosynthesis by *Pseudomonas aeruginosa* was shown to be regulated by the intracellular second messenger bis-(3′-5′)-cyclic-dimeric-GMP (c-di-GMP), and binding of c-di-GMP to the membrane protein Alg44 was required for alginate production. In this study, PA1727, a c-di-GMP-synthesizing enzyme was functionally analyzed and identified to be involved in regulation of alginate production. Deletion of the PA1727 gene in the mucoid alginate-overproducing *P. aeruginosa* strain PDO300 resulted in a nonmucoid phenotype and an about 38-fold decrease in alginate production; thus, this gene is designated *mucR*. The mucoid alginate-overproducing phenotype was restored by introducing the *mucR* gene into the isogenic Δ*mucR* mutant. Moreover, transfer of the MucR-encoding plasmid into strain PDO300 led to an about sevenfold increase in alginate production, wrinkly colony morphology, increased pellicle formation, auto-aggregation, and the formation of highly structured biofilms as well as the inhibition of swarming motility. Outer membrane protein profile analysis showed that overproduction of MucR mediates a strong reduction in the copy number of FliC (flagellin), required for flagellum-mediated motility. Translational reporter enzyme fusions with LacZ and PhoA suggested that MucR is located in the cytoplasmic membrane with a cytosolic C terminus. Deletion of the proposed C-terminal GGDEF domain abolished MucR function. MucR was purified and identified using tryptic peptide fingerprinting and matrix-assisted laser desorption ionization–time of flight mass spectrometry. Overall, experimental evidence was provided suggesting that MucR specifically regulates alginate biosynthesis by activation of alginate production through generation of a localized c-di-GMP pool in the vicinity of Alg44.

Pseudomonas aeruginosa is a ubiquitous opportunistic pathogen which is responsible for systemic infection of immunocompromised patients and severe chronic infections of the lungs of cystic fibrosis (CF) patients (9, 10). Establishment of a chronic CF lung infection coincides with production of copious amounts of the exopolysaccharide alginate leading to the formation of persistent biofilms which prevent the diffusion of antibiotics and protect cells from the host immune response (9, 30). Early after the onset of infection, *P. aeruginosa* switches to a genetically stable alginate-overproducing mucoid variant capable of forming persistent biofilms (32, 33, 43). Alginates are linear unbranched exopolysaccharides consisting of β-1,4-linked monomers of β-D-mannuronic acid and its C5-epimer α-L-guluronic acid (44, 46). The switch to mucoidity and the complex transcriptional regulation of alginate biosynthesis have extensively been investigated (4).

Recently it has become apparent that an additional post-transcriptional level of regulation is playing a role in alginate biosynthesis in *P. aeruginosa*. The membrane-anchored alginate biosynthesis protein Alg44, which is essential for alginate production, contains a bis-(3′-5′)-cyclic-dimeric-GMP (c-di-GMP) binding/sensing PilZ domain in its C terminus (45). This PilZ domain was demonstrated to bind c-di-GMP and was essential for alginate biosynthesis (1, 34).

The secondary messenger c-di-GMP is a central regulator of bacterial physiology and has been linked to diverse physiological responses, such as exopolysaccharide production, motility, biofilm formation, and the production of adhesive surface organelles (48). *P. aeruginosa* PAO1 contains at least 38 genes encoding proteins shown to be involved in the production (diguanylate cyclases [DGC]) and/or the breakdown (phosphodiesterases [PDE]) of c-di-GMP (26). Sequence analysis of these enzymes indicated the presence of domains involved in signal transduction, which suggested that these proteins function in response to diverse signals. It was also suggested that localized c-di-GMP pools might exist inside the cell, enabling spatially resolved regulation of physiological responses (26). At present it is unclear how or if any of these different proteins influence alginate biosynthesis. Since the c-di-GMP-binding Alg44 protein is localized in the cytoplasmic membrane, presumably as a subunit of the alginate polymerization/secretion multiprotein complex, membrane-anchored DGCs could provide localized pools of c-di-GMP for activation of alginate production. Putative membrane-anchored PA1727 (MucR) comprises a DGC (GGDEF) as well as a PDE (EAL) and a conserved integral membrane-sensing MHYT domain, which was proposed to sense oxygen, CO, or NO (16). DGC activity but not PDE activity has previously been demonstrated (26). Oxygen and NO have previously been shown to influence alginate production (6, 15) In this study, MucR was assessed with respect to its involvement in regulation of alginate production.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains, plasmids, and oligonucleotides used in this study are listed in Table 1. All *Escherichia coli* strains were grown in LB medium at 37°C, and *E. coli* S17-1 (54) was used for

TABLE 1. Bacterial strains, plasmids, and oligonucleotides used in this study

Strain, plasmid, or oligonucleotide	Description or sequence	Source or reference
Strains		
P. aeruginosa		
PAO1	Prototrophic wild-type strain; Alg^-	21
PDO300	*mucA22* isogenic mutant derived from PAO1	33
PAO1ΔPA1727	Isogenic *mucR* (PA1727) deletion mutant derived from PAO1	This study
PDO300ΔPA1727	Isogenic *mucR* (PA1727) deletion mutant derived from PDO300	This study
PDO300Δ*alg8*	Isogenic *alg8* deletion mutant derived from PDO300; cannot produce alginate	47
PAO1Δ*pslA*	Isogenic *pslA* deletion mutant derived from PDO300; does not produce the Psl polysaccharide	40
E. coli		
TOP10	*E. coli* cloning strain	Invitrogen
S17-1	*thi-1 proA hsdR17* (r_K^- m_K^+) *recA1*; *tra* gene of plasmid RP4 integrated in chromosome	54
Plasmids		
pBBR1MCS-5	Gm^r; broad-host-range vector; P_{lac}	23
pBBR1MCS-5:*mucR*	KpnI-ClaI fragment comprising *mucR* inserted into vector pBBR1MCS-5	This study
pBBR1MCS-5:*mucR*his	Translational MucR-hexahistidine tag fusion, inserted into vector pBBR1MCS-5	This study
pBBR1MCS-5:*mucR*lacZ	Translational MucR-LacZ fusion, inserted into vector pBBR1MCS-5	This study
pBBR1MCS-5:*mucR*phoA	Translational MucR-PhoA fusion, inserted into vector pBBR1MCS-5	This study
pBBR1MCS-5:*mucR*(277)lacZ	Truncated translational MucR-LacZ fusion, inserted into vector pBBR1MCS-5	This study
pBBR1MCS-5:*mucR*(277)phoA	Truncated translational MucR-PhoA fusion, inserted into vector pBBR1MCS-5	This study
pBBR1MCS-5:*wspR*	XbaI-SacI fragment comprising *wspR* inserted into vector pBBR1MCS-5	This study
pBBR1MCS-5:*rocR*	XbaI-SacI fragment comprising *rocR* inserted into vector pBBR1MCS-5	This study
pEX100T	Ap^r Cb^r, gene replacement vector containing *sacB* gene for counterselection	20
pEX100TΔ*mucR*Gm	Ap^r Cb^r Gm^r; vector pEX100T with SmaI-inserted *mucR* deletion construct	This study
pPS865	Ap^r Gm^r; source of 1,100-bp BamHI fragment comprising *aacC1* gene flanked by Flp recombinase target site signal sequences	20
pPFLP2	Ap^r Cb^r; broad-host-range vector encoding Flp recombinase	20
pPHO7	Ap^r; *phoA* without signal sequence	17
pJE608	LacZ lacking the first eight amino acids with promoter P*tac* in pMMB67EH	12
Primers		
PA1727C1-Bbr	TATACCACGTGCTGGCGAGCAACTGCTCGGGCATCGGCCTG	
PA1727C2-Ba	TCAATGGATCCGCAGATCGGCGAGAGGGTGCTCGACGAAGC	
PA1727N1-Ba	AAGTGGGATCCGATCACCGCGATCAGGATGGAGAGG	
PA1727N2-Bbr	CTGCTCACGTGCAGGTTCTTGTCGCCTTTTCCCTGATTGTGG	
PA1727C(Cla)	GAGTAATCGATAAATCAGGCGACGATGGCGAGCAACTGCTCG	
PA1727N (KpSDNd)	AGCAAGGTACCAGGAGACGCTCATATGCTTATCAGCAGCTACACCCA GGTTATT	
PA1727C-tga(BamHI-ClaI)	GAGTAATCGATAAATCAAGGATCCGCGGCGACGCTGGCGAGCAACT GCTGCGCCGGCATCGG	
PA1727(277) Fus(BamHI-ClaI)	GAGTAATCGATAAATCAAGGATCCGCGAGGAGCATGCGGTTGGGCA GCTTGGTCAGGTTGTCGTGCAGG	
PA3702N(XbSDNd)	GCGTCGTCTAGAAGGAGAGAGACATATGCACAACCCTCATGAGAGC AAGACCGACC	
PA3702C(SacI)	GGCTGGAGCTCAAATCAGCCCGCCGGGGCCGGCGGCACC	
PA3947N(XbSDNd)	GCGTCGTCTAGAAGGAGGGACCCATATGAATGATTTGAATGTTCTGG TGTTGGAGG	
PA3947C(SacI)	GGCTGGAGCTCAAATCAGGATCCGGAGCAATAGTCGAGAAAGTGC	
LumioHis (BaXbdirect)	GATCCATGTTGTCCTGGCTGTTGCGGTGGCGGCACCGGTCATCATCA CCATCACCATTGAT	
LumioHis (BaXbcomplement)	CTAGATCAATGGTGATGGTGATGATGACCGGTGCCGCCACCGCAACA GCCAGGACAACATG	

conjugative transfer of mob site-containing pBBR1MCS-5 (23) derivatives. When required, antibiotics were added to the media at the following concentrations: ampicillin, 100 μg/ml; and gentamicin, 10 μg/ml. *P. aeruginosa* was cultivated in LB or *Pseudomonas* isolation agar (PIA) medium at 37°C, and, if required, gentamicin was added at a concentration of 100 μg/ml.

Isolation, analysis, and manipulation of DNA. General cloning procedures were performed as described previously (51). Deoxynucleoside triphosphate, *Taq*, and Platinum *Pfx* polymerases were purchased from Invitrogen. DNA sequences of new plasmid constructs were confirmed by DNA sequencing according to the chain termination method using the model ABI310 automatic sequencer.

Construction of *mucR* deletion mutant. Two regions of the *mucR* gene were amplified by using *Taq* polymerase with primers PA17271N2-Bbr, PA17271N1-Ba, PA17272C2-Ba, and PA17272C1-Bbr. Region PA1727N (432 bp) comprised bases 21 to 452, and region PA1727C (555 bp) comprised bases 1493 to 2047, relative to the designated *mucR* coding region (55). Both PCR products were hydrolyzed with BamHI, ligated together, and inserted into vector pGEM-TEasy (Promega). Vector pPS856 (20) was hydrolyzed with BamHI, releasing an about 1,100-bp fragment containing the *aacC1* gene (encoding gentamicin acetyltransferase) flanked by two Flp recombinase target sites. The 1,100-bp BamHI fragment (*aacC1* gene) was inserted into the BamHI site of plasmid pGEM-TEasy:Δ*mucR*NC, resulting in plasmid pGEM-TEasy:Δ*mucR*Gm. The Δ*mucR*Gm-

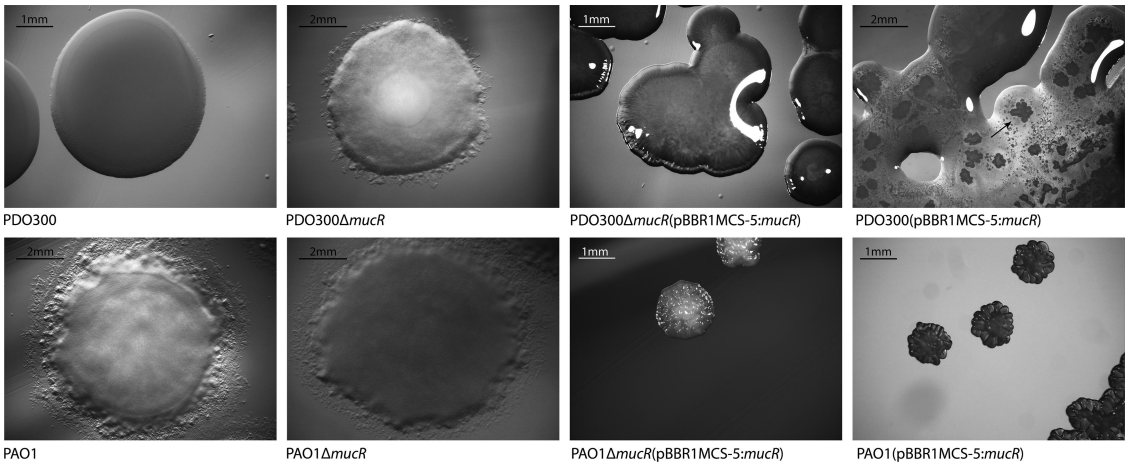

FIG. 1. MucR is essential for the mucoid colony morphology. Colonies of various *P. aeruginosa* strains were grown on PIA medium for 24 h. The top row shows the mucoid strain PDO300 and the strains derived from it; the bottom row shows the nonmucoid strain PAO1 and the plasmids derived from it. Plasmids harbored by certain strains are indicated in parentheses.

comprising DNA fragment was excised using restriction endonuclease BbrP1, and the corresponding 2,087-bp fragment was inserted into the SmaI site of vector pEX100T (20, 53), resulting in plasmid pEX100TΔ*mucR*Gm. *E. coli* S17-1 was used as a donor for the transfer of plasmid pEX100TΔ*mucR*Gm into *P. aeruginosa* strains, and transconjugants were selected on mineral salt medium (52) containing 300 μg of gentamicin/ml and 5% (wt/vol) sucrose. Cells growing on this selective medium should have emerged from double-crossover events. Gene replacement was confirmed after the subculture of cells on PIA medium containing 300 μg of gentamicin/ml and using PCR with primers PA1727up and PA1727down. *E. coli* S17-1 was used to transfer the Flp recombinase-encoding vector pFLP2 (20) into *P. aeruginosa* Δ*mucR*Gm strains, and after 24 h of cultivation on PIA medium containing 5% (wt/vol) sucrose, gentamicin- and carbenicillin-sensitive cells were analyzed by PCR for loss of the gentamicin resistance cassette.

Complementation of the Δ*mucR* mutant. The *mucR* gene of *P. aeruginosa* PAO1 was amplified by PCR using the primers PA1727N(KpSDNd) and PA1727C(Cla). The PCR product was hydrolyzed with KpnI and ClaI and was inserted into the KpnI and ClaI sites of the broad-host-range vector pBBR1MCS-5, resulting in plasmid pBBR1MCS-5:*mucR*. In addition a C-terminally hexahistidine-tagged MucR-encoding plasmid was constructed by using primers PA1727N(KpSDNd) and PA1727C-tga(BamHI-ClaI), and the resulting PCR product was inserted into pBBR1MCS-5 as described above. The resulting plasmid was then hydrolyzed with BamHI and XbaI; a hexahistidine-encoding sequence was ligated into this using the complementary primer dimer pair LumioHis(BaXbdirect) and LumioHis(BaXbcomplement), resulting in plasmid pBBR1MCS-5:*mucR*His. In addition two plasmids carrying two other genes known to influence c-di-GMP levels were constructed. The genes *wspR* (PA3702) (encoding a highly active DGC) and PA3947 (encoding a highly active PDE) were amplified from the *P. aeruginosa* PAO1 genome using the 5′-end primers PA3702N(XbSDNd) and PA3947N(XbSDNd) and the 3′-end primers PA3702C (SacI) and PA3947C(SacI), respectively. These PCR products were then hydrolyzed with XbaI and SacI and inserted into the respective sites of pBBR1MCS-5, resulting in plasmids pBBR1MCS-5:PA3702 and pBBR1MCS-5:PA3947, respectively.

Construction of plasmids encoding reporter enzyme fusion proteins. For the generation of a plasmid encoding full-length MucR fused either to β-galactosidase (LacZ) or alkaline phosphatase (PhoA), the 3′ end of *mucR* (*mucR* minus the stop codon) was amplified by PCR using the 5′-end primer PA1727N(KpSDNd) and the 3′-end primer PA1727C-tga(BamHI-ClaI). The PCR product was hydrolyzed with KpnI and BamHI and was inserted into the respective sites of broad-host-range vector pBBR1MCS-5, resulting in plasmid pBBR1MCS-5:*mucR*(Δstop). XbaI-BamHI fragments of vectors pPHO7 (12) and pJE608 (17) were inserted into XbaI/BamHI-hydrolyzed pBBR1MCS-5:*mucR*(Δstop) to construct translational LacZ (pBBR1MCS-5:*mucR*LacZ) and PhoA (pBBR1MCS-5:*mucR*PhoA) fusions, respectively. In addition, a translational fusion was constructed that encodes the first 277 amino acids of MucR fused to either LacZ or PhoA. The 3′ region was amplified by using the 5′-end primer PA1727N(KpSDNd) and the 3′-end primer PA1727277 (BamHI-ClaI). Fusion protein-encoding plasmids pBBR1MCS-5:*mucR*277LacZ and pBBR1MCS-5:*mucR*277PhoA were constructed as described above.

Subcellular localization of MucR. Strains of *P. aeruginosa* were grown overnight in LB medium containing the appropriate antibiotics. The cells were harvested by centrifugation (1 h at 5,000 × *g*) and washed with 1 volume of 10 mM HEPES (pH 7.4). Cells were placed in 15 ml 10 mM HEPES with Roche Complete Mini EDTA-free protease inhibitor, sonicated on ice for 12 cycles of 15-s sonication, and then cooled for 15 s. Cellular debris and the remaining intact cells were sedimented by centrifugation (1 h at 5,000 × *g*). The supernatant was centrifuged at 100,000 × *g* for 2 h. The supernatant (soluble fraction) was removed, and the sediment was washed in 1 volume of 10 mM HEPES and centrifuged under the same conditions. The resulting sediment represents the envelope fraction, which was dissolved in 10 mM HEPES. The total protein concentration of the respective fractions was determined using the Bradford method (5).

Outer membrane protein analysis. Cells were fractionated as described above. The cytosolic membrane fraction was then selectively solubilized from the total envelope fraction using 0.7% (wt/vol) *n*-lauroylsarcosine. The insoluble (outer membrane) fraction of the envelope fraction was then obtained by centrifugation at 100,000 × *g* for 1 h. Resulting outer membrane proteins were then separated via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and identified by tryptic peptide fingerprinting using matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry.

β-Galactosidase and alkaline phosphatase activity assays. β-Galactosidase and alkaline phosphatase enzymatic assays were performed according to the methods by Miller (37) and Manoil (31), respectively. Between 2 and 20 μl of the various subcellular fractions was added to the reaction mixtures, and the specific activity was determined in U/mg total protein, with 1 U corresponding to the hydrolysis of 1 μmol of substrate (*o*-nitrophenyl-β-galactoside and *p*-nitrophenylphosphate for alkaline phosphatase and β-galactosidase, respectively) per 1 min at 37°C. The results are given as average values of at least four independent experiments.

Uronic acid assays. Alginate concentrations were assayed by a modification of the Blumenkrantz and Asboe-Hansen protocol (3), using purified *P. aeruginosa* PDO300 alginate (100% [wt/wt] uronic acid content) as a standard, as previously described (45). The uronic acid concentrations were determined spectrophotometrically at a wavelength of 520 nm.

Swarming motility. Swarming motility was assessed by the method of Tremblay et al. (57). Briefly, plates consisted of modified M9 medium (20 mM NH_4Cl; 12 mM Na_2HPO_4; 8.6 mM NaCl; 1 mM $MgSO_4$; 1 mM $CaCl_2 \cdot 2H_2O$; and 10 mM dextrose, supplemented with 0.5% [wt/vol] Casamino Acids [Difco]) solidified with 0.5% (wt/vol) Bacto agar (Difco). Autoclaved medium was poured in petri dishes and dried under laminar flow for 60 min. Then swarm plates were immediately inoculated with 5 μl of stationary-phase bacterial culture and incubated at 30°C for 16 h.

Solid surface attachment assay. Attachment to a solid surface was assessed by a modified method described by Merritt et al. (36). Briefly, relevant strains were grown to saturation in LB medium. A total of 100 μl of these cultures was transferred to 4 wells of a sterile 96-well microtiter plate and incubated at 37°C

TABLE 2. Alginate quantification of various *P. aeruginosa* strains

Strain	Alginate production (g/g CDM)[a]	SD[b]
PDO300	0.225	±0.047
PDO300Δ*mucR*	0.006	±0.001
PDO300(pBBR1MCS-5)	0.584	±0.023
PDO300Δ*mucR*(pBBR1MCS-5)	0.386	±0.021
PDO300(pBBR1MCS-5:*mucR*)	1.54	±0.130
PDO300Δ*mucR*(pBBR1MCS-5:*mucR*)	0.958	±0.170
PAO1	0	±0
PAO1Δ*mucR*	0	±0
PAO1(pBBR1MCS-5)	0	±0
PAO1Δ*mucR*(pBBRMCS-5)	0	±0
PAO1(pBBR1MCS-5:*mucR*)	0	±0
PAO1Δ*mucR*(pBBRMCS-5:mucR)	0	±0
PDO300(pBBR1MCS-5:*wspR*)	0.295	±0.006
PDO300(pBBR1MCS-5:*rocR*)	0	±0

[a] CDM, cell dry mass.
[b] SD, standard deviation. Results represent the data of four independent experiments.

for 1 h. Planktonic/nonadherent bacteria were removed by inverting the plate, and the plates were washed with water. A total of 125 μl of 0.1% crystal violet was added to each well, incubated at room temperature for 10 min, subsequently washed twice as described above, and allowed to air dry. Bound crystal violet was solubilized with the addition of 200 μl of 100% dimethyl sulfoxide. Absorbance was measured at 595 nm.

Continuous-culture flow cell biofilms. For biofilm analysis, *P. aeruginosa* strains were grown in continuous-culture flow cells (channel dimensions of 4 mm by 40 mm by 1.5 mm) at 37°C as previously described (7). Channels were inoculated with 0.5 ml of early-stationary-phase cultures containing approximately 2×10^9 cells ml^{-1} and incubated without flow for 4 h at room temperature. Flow was then started with a mean flow of 0.3 ml min^{-1}, corresponding to a laminar flow with a Reynolds number of 5. The flow cells were then incubated at 37°C for 20 h. Biofilms were stained using the LIVE/DEAD BacLight bacterial viability kit (Molecular Probes, Inc., Eugene, OR) and visualized first using phase-contrast microscopy and then confocal laser scanning microscopy (Leica SP5 DM6000B).

Detection and purification of MucR. The envelope fraction of the PDO300 Δ*mucR* strain harboring plasmid pBBR1MCS-5:*mucR*his was solubilized by the addition of 1% (vol/vol) Triton X-100 and incubated at room temperature for 2 h. The solubilized envelope fraction was subjected to affinity purification using Ni-NTA agarose (Qiagen). Briefly, the solubilized envelope fraction from 1 liter of a stationary-phase culture was resuspended in 20 ml of buffer A (50 mM NaH_2PO_4, 300 mM NaCl, 1% Triton X-100, pH 8.0) containing 10 mM imidazole, and 1 ml of a 50% (wt/vol) Ni-NTA agarose (Qiagen) was added and incubated with shaking at 4°C for 1 h. The Ni-NTA agarose was then loaded onto a gravity flow column, and the agarose was washed four times with 5 ml of buffer A containing 20 mM imidazole and four times with 5 ml of buffer A containing 50 mM imidazole. The bound proteins were eluted four times with 500 μl of buffer A containing 250 mM imidazole. Fractions were then subjected to SDS-PAGE analysis and immunoblotting analysis. In order to identify proteins, protein bands of interest were cut off the gel. Proteins were subjected to tryptic peptide fingerprinting. Identification of tryptic peptides was performed by collision-induced dissociation tandem mass spectrometry and enabled identification of proteins. For immunoblotting, proteins were transferred to a nitrocellulose membrane and hexahistidine-tagged proteins were detected using a SuperSignal HisProbe-HRP kit (Pierce).

RESULTS

Primary structure analysis of MucR. The MucR (PA1727) sequence was attained from the complete *P. aeruginosa* PAO1 genome sequence (55). TMHMM (24) predicts that MucR has seven transmembrane regions, all located in the N-terminal 240 residues of the protein, with the N terminus residing in the periplasm and the C terminus residing in the cytosol. SMART (28) and Pfam (13) predicted an MHYT domain (16) in the second to seventh transmembrane regions. This domain consists of six transmembrane segments connected by short arginine-rich cytosolic loops. The second, fourth, and sixth transmembrane segments in the domain have a highly conserved amino acid motif, MHYTXM, located near the outer side of the inner membrane. It has been suggested that the MHYT domain serves as a sensing domain. The C terminus of MucR is predicted to contain a GGDEF domain, common to DGCs, at amino acids 252 to 423 and an EAL domain, conserved among PDE, at amino acids 433 to 679. A sequence database search (BLASTP) revealed the strongest similarity of 73%

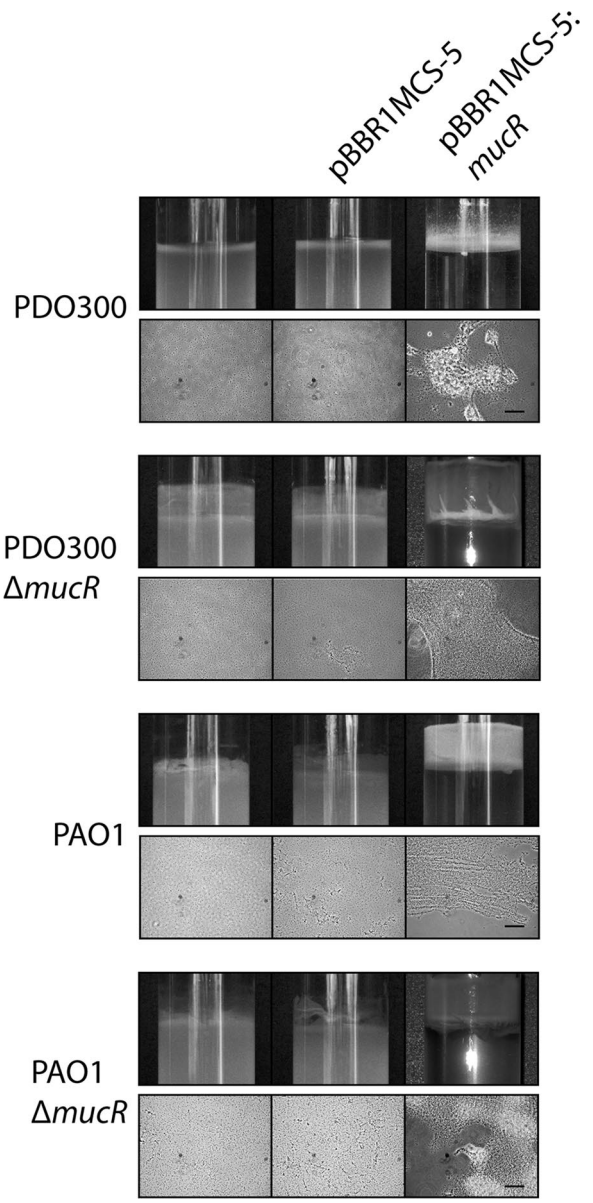

FIG. 2. Multiple copies of *mucR* induce auto-aggregation and the formation of pellicles. Various strains were grown in LB for 16 h. The top row for each strain indicated on the left contains photographs of overnight cultures grown in LB overnight showing pellicle formation. The bottom row for each strain contains phase-contrast microscopic images showing the cell aggregation; the black bars represent 50 μm. Plasmids (where present) are indicated along the top.

TABLE 3. Rapid solid surface attachment assay

Strain	OD_{550}[a]	SD[b]
PDO300(pBBR1MCS-5)	0.088	±0.046
PDO300Δ*mucR*(pBBR1MCS-5)	0.198	±0.179
PDO300Δ*mucR*(pBBR1MCS-5:*mucR*)	0.742	±0.141
PAO1(pBBR1MCS-5)	0.114	±0.078
PAO1Δ*mucR*(pBBR1MCS-5)	0.095	±0.028
PAO1Δ*mucR*(pBBR1MCS-5:*mucR*)	0.983	±0.250

[a] OD_{550}, optical density at 550 nm.
[b] SD, standard deviation. Results represent the data of three independent experiments.

identity with the hypothetical protein ZP_00417207 from *Azotobacter vinelandii* belonging to one of the two bacterial genera (*Pseudomonas* and *Azotobacter*) comprising species capable of alginate production.

The Δ*mucR* mutant is defective in alginate biosynthesis. Deletion of 1,041 bp of the *mucR* gene in the alginate-overproducing *P. aeruginosa* strain PDO300 resulted in a nonmucoid colony phenotype on solid medium (Fig. 1). Interestingly, strains PDO300 and PAO1, each harboring plasmid pBBR1MCS-5:*mucR* and thus additional *mucR* gene copies, showed small wrinkly colonies, while PDO300 was additionally surrounded by copious amounts of transparent extracellular alginate (Fig. 1). Alginate is the primary exopolymeric substance responsible for the mucoid phenotype, which suggested that MucR plays a role in biosynthesis or regulation of alginate biosynthesis in *P. aeruginosa*. To assess whether the Δ*mucR* mutant of strain PDO300 is still able to produce alginate, the extracellular polysaccharide was purified and the uronic acid (alginate) content was determined. The Δ*mucR* mutation caused a reduction in alginate production to nearly undetectable levels (Table 2). To confirm this was not due to polar effects, the *mucR* gene (pBBR1MCS-5:*mucR*) was introduced into the PDO300Δ*mucR* strain, and alginate production was restored and increased by about 4.4-fold compared with that of strain PDO300. The Δ*mucR* mutant harboring only the vector pBBR1MCS-5 showed an about 1.6-fold increase in alginate production compared with strain PDO300 (Table 2).

To assess whether the increase in alginate production was due to a general increase in intracellular c-di-GMP levels based on the presence of multiple *mucR* gene copies or was the result of a more specific function of MucR, additional c-di-GMP-influencing genes were introduced. The DGC gene (*wspR*/PA3702) shown to encode a highly active DGC was expressed in PDO300 (18, 26). This resulted not in an increase but in a twofold reduction in alginate production (Table 2). Overexpression of *rocR* (PA3947), which encodes a highly active PDE, abolished alginate production (26, 27) (Table 2).

MucR impacts colony morphology, auto-aggregation, pellicle formation, attachment to surfaces, and swarming motility. To further assess the function of MucR, the Δ*mucR* mutants were characterized with respect to several phenotypes known to be influenced by c-di-GMP levels.

Strains harboring additional copies of the *mucR* gene showed an altered phenotype. On solid medium these strains typically formed compact wrinkly colonies, which were surrounded by alginate when PDO300 was considered (Fig. 1). In liquid culture, cells harboring plasmids encoding MucR formed large aggregates

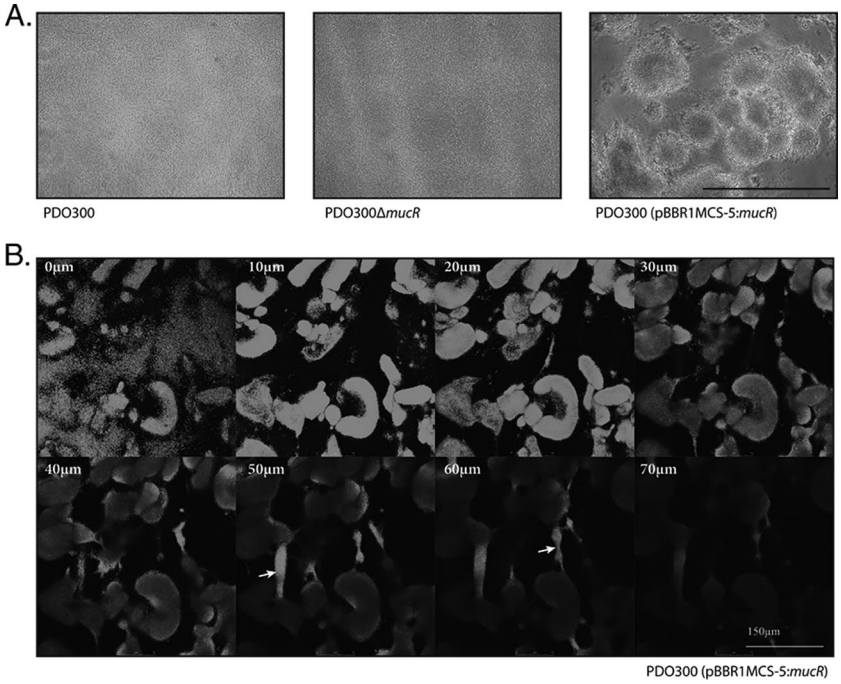

FIG. 3. Multiple copies of *mucR* lead to the formation of highly structured biofilms. (A) Phase-contrast images of 24-h-old biofilms grown in a continuous-culture flow cell. Black bar represents 200 μm. (B) Confocal laser scanning microscopy images of a 24-h-old biofilm of the strain PDO300(pBBR1MCS-5:*mucR*). Each frame represents a picture 10 μm away from the last. The white bar represents 150 μm; the white arrow shows the formation of bridge-like structures connecting adjacent microcolonies.

(pellicles) at the air-liquid interface (Fig. 2). In order to assess if any of these phenotypic changes were due to increased alginate production mediated by MucR, the Δ*alg8* mutant of PDO300, which is defective in alginate biosynthesis, was employed (47). Multiple copies of the *mucR* gene in the alginate-negative Δ*alg8* mutant resulted in small wrinkly colonies and the same autoaggregation phenotype that was observed for the nonmucoid strain PAO1 (data not shown and Fig. 2).

The solid surface assay was applied to assess attachment and biofilm formation. In general, MucR mediated increases in initial attachment of about 8.4-fold and 8.9-fold when produced in PDO300 and PAO1, respectively (Table 3). These results were mimicked by continuous-culture flow cell biofilm analysis. Cellular layers, 1or 2 cells thick, were observed after 20 h on the glass surface for the PDO300 and PDO300Δ*mucR* strains (Fig. 3). Multiple copies of the *mucR* gene in PDO300 resulted in an initial increased attachment which developed into highly structured biofilms after 20 h (Fig. 3).

Recent studies have described a link between c-di-GMP levels and swarming motility in *P. aeruginosa* (25, 35). The alginate-overproducing strain PDO300 did not show a swarming phenotype, whereas PAO1 did. The lack of swarming shown in the PDO300 strains could be due to the presence of alginate. Thus, when the Δ*alg8* mutant of PDO300 was analyzed, this mutant showed swarming motility (Fig. 4). Deletion of the *mucR* gene did not significantly change the level of swarming motility in any of the strains (Fig. 4). In strains harboring only the vector and where gentamicin was added to the media, the swarming phenotype was reduced but detectable (Fig. 4). The presence of plasmids containing the *mucR* gene in the PAO1, PAO1Δ*mucR*, and PDO300Δ*alg8* strains resulted in a strong inhibition of swarming motility (Fig. 4).

MucR affects the outer membrane protein profile. The altered phenotypes described above suggested that MucR influences cell surface properties. Thus, the outer membrane protein profiles were analyzed. The protein profiles differed between the mucoid strain PDO300 and the nonmucoid strain PAO1 with respect to two proteins (Fig. 5). A protein with an apparent molecular mass of 56 kDa was present only in PDO300 and was identified as AlgE by using tryptic peptide fingerprinting employing MALDI-TOF mass spectrometry. AlgE is essential for alginate production, presumably enabling the export of alginate (42, 43). Another protein with an apparent molecular mass of 53 kDa was found in PAO1 and absent in PDO300 (Fig. 5). This protein was identified as FliC, a flagellin type B protein.

In the Δ*mucR* mutant of PDO300, AlgE becomes less abundant while FliC becomes apparent. The PAO1Δ*mucR* showed no significant change in the outer membrane protein profile. Introduction of a plasmid encoding MucR significantly reduced the levels of FliC in the outer membrane of both PAO1 and PDO300 but did not affect the levels of AlgE in PDO300 (Fig. 5).

The GGDEF/EAL domain is required for *mucR* function. To establish whether MucR function in the regulation of alginate production was dependent on the GGDEF/EAL domain containing the C terminus of MucR, a hybrid gene encoding only the first 277 amino acids of MucR was constructed. This region corresponded to the seven-transmembrane putative MHYT-sensing domain but lacked the GGDEF and EAL domains.

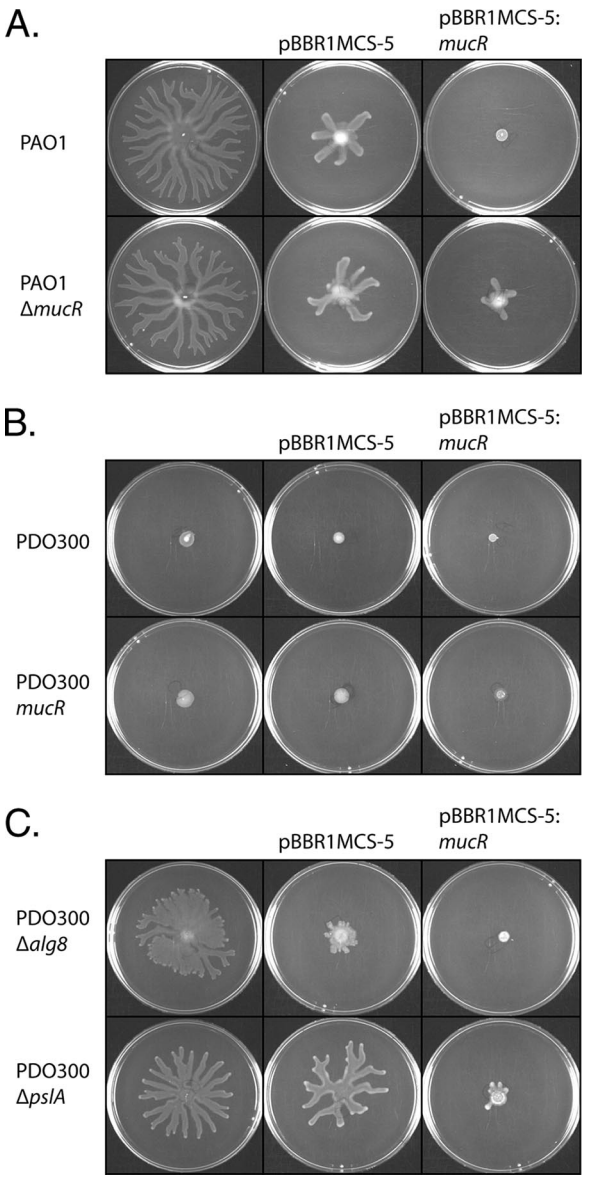

FIG. 4. Loss of *mucR* does not influence swarming motility, but multiple copies of *mucR* inhibit swarming motility. (A) Swarming motility of the nonmucoid *P. aeruginosa* strain PAO1 and its isogenic *mucR* knockout mutant. (B) Swarming motility of the mucoid *P. aeruginosa* strain PDO300 and its isogenic *mucR* knockout mutant. (C) Swarming phenotype of a mutant derived from the mucoid PDO300 strain incapable of producing alginate due to the loss of the *alg8* gene, which is essential for alginate biosynthesis.

This N-terminal region and full-length MucR were each translationally fused to the reporter enzymes LacZ and PhoA. Full-length MucR but not C-terminally truncated MucR restored alginate production to levels exceeding wild-type levels as well as mediated the formation of wrinkly colonies and an autoaggregation phenotype when produced in the Δ*mucR* mutant (Fig. 6; data not shown).

Subcellular localization, membrane topology, and purification of MucR. The fusion proteins described above were employed to test the predicted membrane topology. *P. aeruginosa* cells expressing the various fusion proteins were fractionated

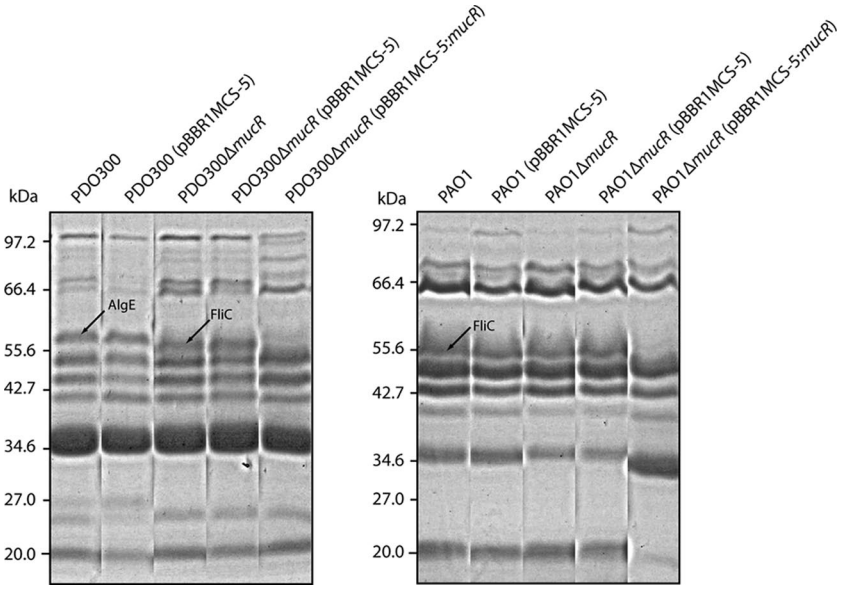

FIG. 5. Outer membrane protein profiles of various *P. aeruginosa* strains. (Left) Outer membrane protein profiles of the Δ*mucR* mutant derived from PDO300; (right) outer membrane protein profiles of the Δ*mucR* mutant derived from PAO1. The arrows indicate the identity of proteins whose levels differed between mutants. Plasmids harbored by certain strains are indicated in parentheses.

into the cell lysate, soluble cytosol, and insoluble envelope fraction. No significant alkaline phosphatase activity could be detected for either the full-length or truncated MucR protein fused to PhoA in any of the fractions (Table 4). β-Galactosidase activity could be detected for both the full-length and truncated MucR-LacZ fusions, with the highest specific activity in the envelope fraction (Table 4).

In addition, plasmid pBBR1MCS-5:*mucR*his was constructed, encoding C-terminally hexahistidine-tagged MucR. The PDO300 Δ*mucR* strain harboring pBBR1MCS-5:*mucR*his showed restored alginate production and was used to detect hexahistidine-tagged MucR via immunoblotting using anti-hexahistidine antibodies (Fig. 7). Immunoblot analysis of the cell lysate, cytosol, and envelope enabled detection of two protein bands in both the cell lysate and the envelope but not in the soluble cytosol fraction. These proteins showed an apparent molecular mass of 65 kDa and 50 kDa, respectively (Fig. 7). The theoretical molecular mass of full-length MucR is 74.4 kDa. The detected proteins could not be separated from the complex mixture of proteins in the gel. To confirm that these proteins corresponded to MucR, the solubilized total membrane fraction was subjected to Ni-NTA affinity purification, resulting in partial purification of the two detected

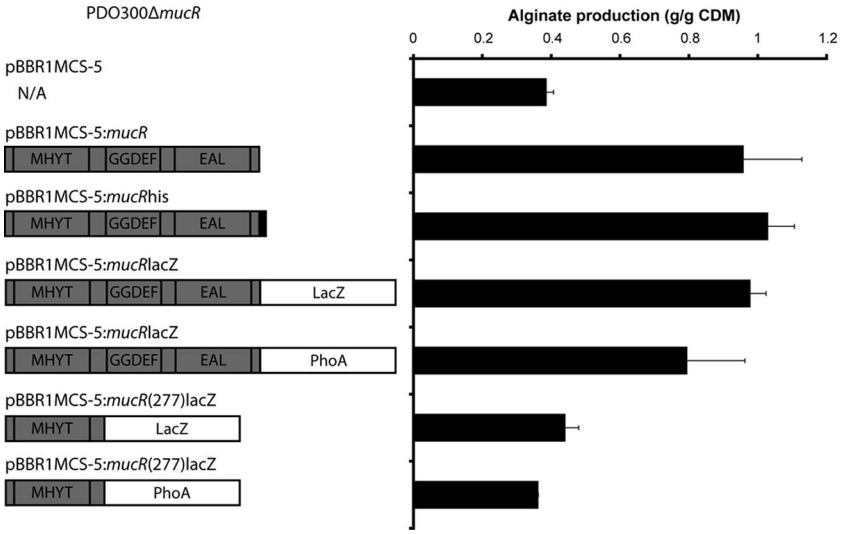

FIG. 6. The alginate stimulating activity of MucR is dependent on the GGDEF and EAL domains containing the C terminus. (Left) Schematic representations of the various MucR fusion proteins. Plasmids harbored by the PDO300Δ*mucR* strain are indicated in parentheses. (Right) Alginate production levels of the PDO300Δ*mucR* strain harboring the genes encoding the various MucR fusion proteins. Right, CDM, cell dry mass. Alginate quantification data represent the results of three independent experiments.

TABLE 4. β-Galactosidase and alkaline phosphatase activities of MucR fusion proteins in various subcellular fractions

Fusion protein	Subcellular fraction[a]	LacZ U (μM/min)/mg ± SD[b]	PhoA U (μM/min)/mg ± SD
MucR	WCL	11.06 ± 0.77	0.01 ± 0.00
	SOL	11.54 ± 1.42	0.01 ± 0.00
	MEM	48.68 ± 0.22	0.03 ± 0.00
MucR(277)	WCL	135.45 ± 2.41	0.11 ± 0.01
	SOL	34.10 ± 3.90	0.12 ± 0.01
	MEM	559.40 ± 20.36	0.07 ± 0.00

[a] WCL, whole-cell lysate; SOL, soluble; MEM, membrane.
[b] SD, standard deviation. Results represent data of three independent experiments.

proteins, enabling identification via tryptic peptide fingerprinting (Fig. 7). Both proteins were identified as MucR; however, only peptides corresponding to the C-terminal half of MucR and none from the transmembrane regions were detected. In addition, a protein with an apparent molecular mass of 330 kDa could be detected by immunoblotting, and peptide fingerprinting analysis confirmed that it belongs to MucR; this is presumably aggregate formation due to the hydrophobicity in the N terminus (Fig. 7).

DISCUSSION

Recently it has been shown that c-di-GMP binding to membrane-anchored Alg44, an essential alginate biosynthesis protein, is required for alginate production in *P. aeruginosa* (1, 34, 45). It has been suggested that some of the DGC and PDE proteins respond to different signals by controlling intracellular c-di-GMP levels in localized pools. Thus, c-di-GMP sensing/binding proteins may be colocalized in the membrane with proteins producing and/or degrading c-di-GMP.

Experimental evidence to support this hypothesis had previously been obtained for *Gluconacetobacter xylinus*, where the DGC DgcA and the PDE PdeA were shown to copurify with the c-di-GMP binding cellulose synthase (50), and for *Caulobacter crescentus*, where the GGDEF-containing protein PleD localized to the base of the flagellum where its DGC activity is required for flagellum ejection (41).

Twenty-two of the 38 DGCs and PDEs contain predicted transmembrane regions, and 14 of these contained domains recognized by Pfam (14). These domains were the PAS and PAC domains, involved in numerous signaling processes sensing diverse signals; the CHASE domains, sensing domains predicted to bind diverse low-molecular-weight ligands, such as the cytokinin-like adenine derivatives or peptides (38); the 7TM-DISM2 domains, thought to act as a receptor for carbohydrates (2); the MASE1 domain, with unknown function; and the MHYT domains, thought to sense oxygen, CO, or NO through the coordinated binding of one or two copper atoms (16). Both oxygen and NO have previously been shown to influence alginate production in *P. aeruginosa* (6, 15, 58, 59). Therefore, the two MHYT domain-containing proteins (MucR and PA3311) were considered. However, in a recent study, PA3311 showed no detectable DGC or PDE activity, whereas MucR showed DGC activity (26). Consequently, in this study MucR was functionally characterized.

The isogenic Δ*mucR* mutant of alginate-overproducing *P. aeruginosa* PDO300 showed a nonmucoid colony morphology and was strongly impaired in alginate production, and restoration of alginate production by the *mucR* gene suggested that MucR is involved in regulation of alginate biosynthesis (Fig. 1 and 2). The observed induction of alginate production by only the vector pBBR1MCS-5 had previously been described to be mediated by gentamicin (45, 47).

Introduction of the *mucR* gene-containing plasmid into PDO300 mediated a wrinkly colony morphology and strongly increased alginate production (Fig. 1 and 2). The small wrinkly colony morphology is very similar to the "small-colony variant" observed with the overexpression of the DGC WspR protein and commonly found among isolates from CF patients (11, 19).

The finding that the introduction of the *wspR* gene, encoding a cytosolic DGC showing 18.5-fold higher activity than MucR, did not cause an increased alginate production (26; Table 2) supports the premise of a specific role of MucR in the regulation of alginate biosynthesis and the hypothesis of localized pools of c-di-GMP.

Previous studies showed that various c-di-GMP concentration-controlling enzymes, e.g., SadC and BifA, inversely regulate attachment/biofilm formation and swarming motility in *P. aeruginosa* (8, 22, 25, 35). In general, DGCs increase intracellular c-di-GMP levels and correlate with enhanced aggregation, attachment, and biofilm formation, but with repressed swarming motility, whereas PDEs lower levels of c-di-GMP and correlate with the contrary. Overproduction of MucR followed this trend, but loss of the *mucR* gene did not result in any change in these phenotypes, as would be expected of a DGC directly involved in regulation of these phenotypic properties.

Although transfer of pBBR1MCS-5:*mucR*, i.e., multiple *mucR* gene copies, into various *P. aeruginosa* strains led to multiple phenotypic changes, many of these phenotypic changes

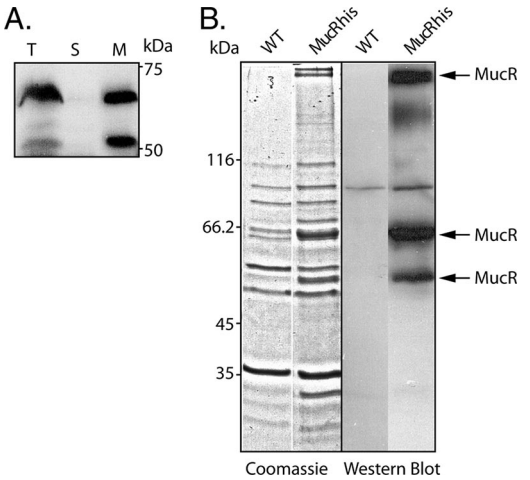

FIG. 7. Subcellular localization and purification of MucR. (A) Immunoblot analysis of whole-cell lysate (T), soluble cytosol (S), and envelope (M) fractions. (B) SDS-PAGE and immunoblot analysis of hexahistidine-tagged MucR (MucRhis) purified from the solubilized membrane fraction of the PDO300Δ*mucR*(pBBR1MCS-5:*mucR*his) strain using Ni-NTA affinity purification. MucR was identified by tryptic peptide fingerprinting and MALDI-TOF mass spectrometry. WT, wild type.

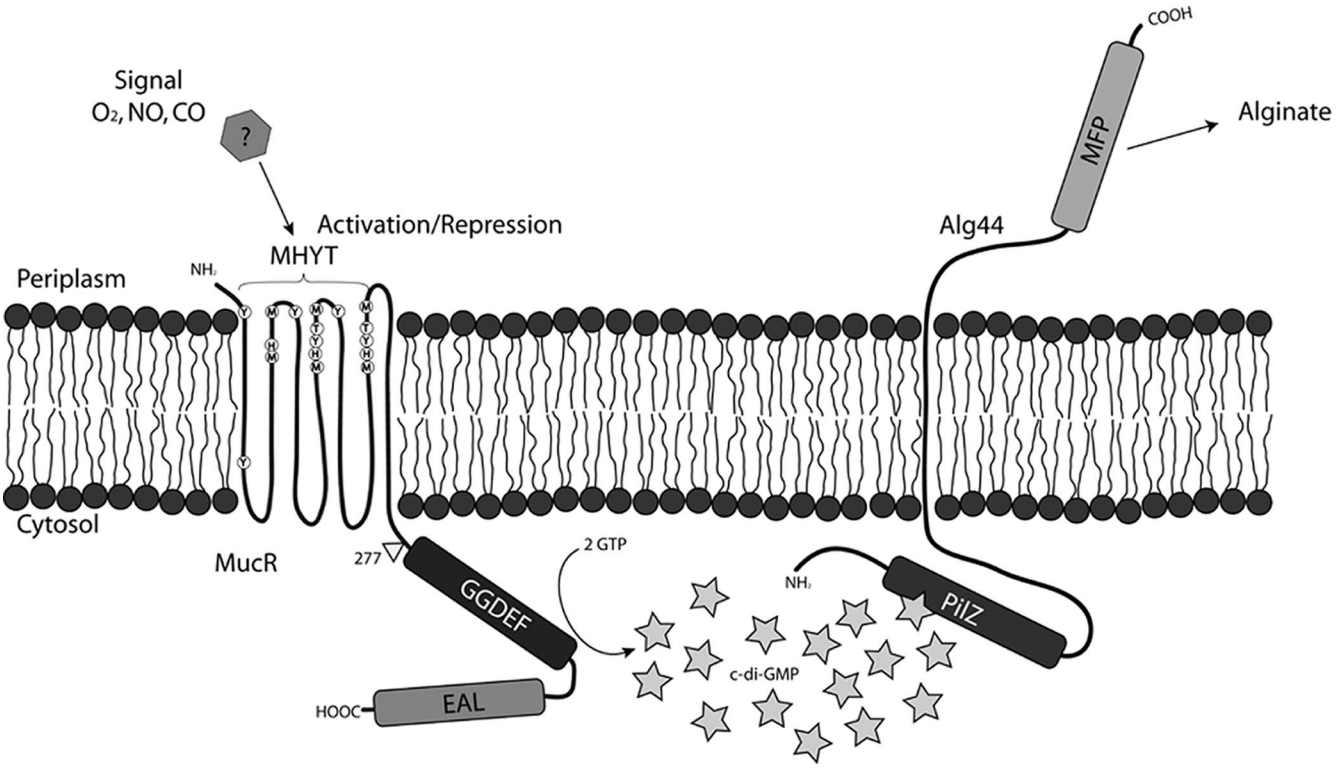

FIG. 8. Proposed model for the MucR-mediated regulation of alginate biosynthesis in *P. aeruginosa*. The membrane topology and domain structure of MucR, as predicted by SMART and Pfam, are shown. The circles represent the conserved MHYT residues suggested to be involved in binding a copper atom and sensing oxygen, NO, or CO. The hexagon represents the putative signal. The stars represent c-di-GMP. Various domains are shown. MFP, membrane fusion protein domain, similar to multidrug efflux systems.

resemble the overexpression of many DGCs and the corresponding increase in c-di-GMP levels (11, 19, 35, 48, 49, 56).

Since MucR impacted phenotypic properties which often depend on proteinaceous cell surface structures, the outer membrane protein profiles of the various strains were analyzed (Fig. 5). The strong decrease of the copy numbers of the flagellar filament protein FliC in strains PAO1 and PDO300 harboring plasmid pBBR1MCS-5:*mucR* correlated with the loss of swarming motility (Fig. 4). FliC has been described to be required for flagellum-based swarming motility (39). However, plasmid pBBR1MCS-5:*mucR* caused not an increase but a slight decrease of the copy numbers of the alginate export protein AlgE, while mediating alginate overproduction (Fig. 5).

The loss of MucR function after removal of the C-terminal region comprising the GGDEF domain suggested that DGC activity is required for MucR function (Fig. 6). As LacZ can fold correctly and become active only when exposed to the cytosol and as PhoA can correctly fold as an active enzyme only in the periplasm (29), the reporter enzyme activities support the prediction that MucR is localized to the cytoplasmic membrane with its GGDEF/EAL C terminus exposed to the cytosol (Table 3).

Overall this study suggested that MucR is a membrane-anchored DGC which is a positive regulator of alginate biosynthesis. In accordance with the recent findings by Merighi et al. (34) that the c-di-GMP-binding PilZ domain of the Alg44 membrane protein is essential for alginate biosynthesis, a model for c-di-GMP-dependent regulation of alginate biosynthesis was developed (Fig. 8). The presence of the proposed localized c-di-GMP pool was supported by the finding that a single gene copy of *mucR* is required for normal levels of alginate biosynthesis in mucoid *P. aeruginosa*, whereas multiple copies of *mucR* led to phenotypes common to overexpression of DGCs (25). Further support that MucR regulated alginate biosynthesis via localized c-di-GMP pools in the vicinity of Alg44 was obtained by the finding that MucR but not the highly active cytosolic DGC WspR protein significantly increased alginate production (Fig. 8). The model proposed that an as yet unidentified signal (possibly CO, NO, or O_2 [16]) is detected via the MHYT domain of MucR. Reception of this signal might influence the activity of MucR by modulating the activity of the DGC, and possibly the PDE, domain of MucR.

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