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Identification and Functional Characterization of *flgM*, a Gene Encoding the Anti-Sigma 28 Factor in *Pseudomonas aeruginosa*

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We describe here the functional characterization of the putative *flgM* gene of *Pseudomonas aeruginosa*. FlgM of *P. aeruginosa* is most similar to FlgM of *Vibrio parahaemolyticus*. A conserved region is present in the C-terminal half of the FlgM of *P. aeruginosa* and in FlgM homologues of other organisms that includes the σ^{28} binding domain. A role for the *flgM* gene of *P. aeruginosa* in motility was demonstrated by its inactivation. The β-galactosidase activity of a transcriptional fusion of the *fliC* promoter to *lacZ* was upregulated in the *flgM* mutant, suggesting that the activity of FliA, the sigma factor that regulates *fliC*, was increased. Consistent with these results, an increased amount of flagellin was demonstrated in the *flgM* mutant of *P. aeruginosa* strain PAK by Western blot, suggesting that FlgM negatively regulates transcription of *fliC* by inhibiting the activity of FliA. Direct interaction of the *P. aeruginosa* FlgM with the alternative sigma factor σ^{28} was demonstrated by utilizing the yeast two-hybrid system. Three putative consensus σ^{54} recognition sites and one σ^{28} site were found in the *flgM* upstream region. However, analysis of the transcriptional fusion of the *flgM* promoter to *lacZ* in different mutant backgrounds showed that the *flgM* promoter was not entirely dependent on either σ^{28} or σ^{54}. A transcript was detected by primer extension that was 8 bp downstream of the consensus σ^{28}-binding site. Thus, a system for the control of flagellin synthesis by FlgM exists in *P. aeruginosa* that is different from that in the enteric bacteria and seems to be most similar to that of *V. cholerae* where both σ^{28}-dependent and -independent mechanisms of transcription exist.

Chemotactic motility in *Pseudomonas aeruginosa* is facilitated by a single polar flagellum whose biogenesis is dependent on a highly regulated pathway of timed gene expression and protein synthesis. More than 40 genes in *P. aeruginosa* are involved in the flagellum biogenesis, and they are regulated in a complex hierarchy that has been shown to be different from that described in the *Salmonella* system (3, 10, 30). The highest level of transcriptional regulation that has been discovered so far in *P. aeruginosa* is mediated by the alternative sigma factor σ^{54} (RpoN) and by the positive transcriptional regulator FleQ that belongs to the NtrC subfamily of response regulators (3, 35). None of the flagellar genes in *Salmonella enterica* serovar Typhimurium are regulated by σ^{54}, but FleQ homologues and RpoN have been shown to be involved in the regulation of flagellar biogenesis in other organisms such as *Vibrio parahaemolyticus* (20), *Vibrio cholerae* (9), *Helicobacter pylori* (32), *Caulobacter crescentus* (2), and *Campylobacter jejuni* (19). FleQ and σ^{54} regulate the expression of other regulatory genes in *P. aeruginosa*, including the *fleSR* operon that encodes a sensor kinase (FleS) and a transcriptional regulator (FleR), members of a two-component regulatory system (3) that are involved in the regulation of flagellin synthesis. FleN is an additional flagellar regulator that controls flagellar number by exerting an antagonistic effect on FleQ activity (10). Another flagellar gene regulator of *P. aeruginosa* is the alternative sigma factor σ^{28} or FliA that controls flagellin synthesis (33). In contrast to gene regulation described in the *Salmonella* system, where expression of class 3 genes, including flagellin, is positively regulated only by σ^{28}, *fliC* encoding flagellin in *P. aeruginosa* is regulated by both σ^{28} and σ^{54} (35). However, a major regulator of flagellum biogenesis in a variety of organisms (serovar Typhimurium, *Escherichia coli*, and *Bacillus subtilis* [13, 21, 27]), the anti-sigma factor FlgM, has not been demonstrated to date in *P. aeruginosa*. Furthermore, it has been demonstrated in the *Salmonella* flagellar system that negative regulation of σ^{28} by FlgM couples transcription of class 3 promoters such as *fliC* with assembly of the hook basal body complex by secretion of FlgM upon hook basal body completion (14, 17). The existence of similar mechanisms of control in *P. aeruginosa*, while suspected, is unproven at this point.

In this report we functionally characterized the putative *flgM* gene of *P. aeruginosa* that is flanked by the *flgA* gene on the 5′ end and the *flgN* gene at the 3′ end. The *flgM* gene encodes a protein with very limited homology to FlgM of *E. coli* and serovar Typhimurium but demonstrates significant homology to the putative FlgM of *V. parahaemolyticus*. Two lines of evidence strongly suggest that the putative *flgM* gene is indeed the flagellar anti-sigma factor in *P. aeruginosa*. First, the expression of flagellin was upregulated in the *flgM* mutant PAK-M both at the transcriptional level and at the translational level. Second, the putative FlgM of *P. aeruginosa* was shown to interact directly with the alternative sigma factor σ^{28} in the yeast two-hybrid system. Analysis of the promoter region of the *P. aeruginosa flgM* gene demonstrated that the transcription of the *flgM* gene was not entirely dependent on σ^{28}, which is similar to that described for *V. cholerae* (29).

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MATERIALS AND METHODS

Bacteria, yeast strains, plasmids, and media. All bacterial strains, yeast strains, and plasmid vectors used in this study are described in Table 1. The bacterial cultures were grown in liquid Luria broth (L broth) (25) at 37°C with

TABLE 1. Yeast and bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype[a]	Source of reference
S. cerevisiae AH109	*MAT***a** *trp1-901 leu2-3, 112 ura3-52 his3-200 gal4Δ gal80Δ LYS2*::$GAL1_{UAS}$-$GAL1_{TATA}$-*HIS3* $GAL2_{UAS}$-$GAL2_{TATA}$-*ADE2 URA3*::$MEL1_{UAS}$-$MEL1_{TATA}$-*lacZ*	Clontech
E. coli DH5α	*hsdR recA lacZYA* ϕ80 *lacZ*ΔM15	Gibco-BRL
P. aeruginosa		
PAK	Wild-type clinical isolate	D. Bradley
PAK-M	PAK *flgM*::Gm^r	This study
PAK-N1G	PAK *rpoN*::Gm^r	18
PAK-Q	PAK *fleQ*::Gm^r	3
MS540	PAK *fliA*::Gm^r	33
Plasmids		
pCR2.1	Cloning vector, Amp^r Kan^r, LacZα	Invitrogen
pCR2.1-M	pCR2.1 containing a 2.0-kb *Sst*I/*Hind*III fragment including the *flgM* gene from PAK	This study
pCR2.1-MG	pCR2.1-M with a Gm^r gene inserted into a unique *Bam*HI site of the *flgM* gene	This study
pET15bVP	Expression vector, T7 promoter, His tag coding sequence, Amp^r, pBR322 origin, contains a broad-host-range origin of replication *oriV*	3
pET15bVP-M	pET15bVP with a 324-bp *Nde*I/*Bam*HI PCR fragment of the PAK *flgM* gene	This study
pET15bVP-A	pET15bVP with a 744-bp *Nde*I/*Bam*HI PCR fragment of the PAK *fliA* gene	This study
pMMB67HE	Broad-host-range cloning vector, *tac* promoter, $Carb^r$	30
pMMB67HE-M	pMMB67HE with a 1.2-kb *Eco*RI/*Sst*I fragment from pCR2.1-FM containing the *flgM* gene	This study
pDN19lacΩ	Promoterless *lacZ oriV oriT* Tc^r Str^r Ω fragment	34
pDN19lacΩ-M	pDN19lacΩ with a 614-bp *Eco*RI/*Bam*HI fragment of the *flgM* gene promoter region	This study
pMS565	pDN19lacΩ containing the *fliA* promoter region	33
pPT269	pDN19lacΩ containing the *fliC* promoter region	34
pGADT7	Cloning vector, $GAL4_{768–881}$ AD, LEU2, Amp^r, HA epitope tag	Clontech
pGBKT7	Cloning vector, $GAL4_{1–147}$ DNA-BD, LEU2, Kan^r, c-Myc epitope tag	Clontech
pGADT7-M	PAK *flgM* gene of 324-bp inserted as a PCR product into the *Nde*I/*Bam*HI sites of pGADT7	This study
pGADT7-A	PAK *fliA* gene of 744-bp inserted into the *Nde*I/*Bam*HI sites of pGBKT7	This study
pGBKT7-M	PAK *flgM* gene of 324-bp inserted as a PCR product into the *Nde*I/*Bam*HI sites of pGBKT7	This study
pGBKT7-A	PAK *fliA* gene of 744-bp inserted into the *Nde*I/*Bam*HI sites of pGBKT7	This study

[a] Gm^r, gentamicin resistance; Kan^r, kanamycin resistance; $Carb^r$, carbenicillin resistance; Str^r, streptomycin resistance; Tc^r, tetracycline resistance; Amp^r, ampicillin resistance; HA, hemagglutinin.

shaking at 250 rpm or on L agar plates (1.7% agar) with or without antibiotics. The antibiotic concentrations used were as follows: for *E. coli*, ampicillin at 200 μg/ml and gentamicin at 10 μg/ml, and for *P. aeruginosa*, carbenicillin at 300 μg/ml, gentamicin at 100 μg/ml, tetracycline at 100 μg/ml, and streptomycin at 300 μg/ml. The yeast strain AH109 was cultured in yeast extract-peptone-dextrose medium at 30°C with shaking at 250 rpm or on synthetic dropout minimal medium agar plates omitting one or both of the amino acids L-leucine and L-tryptophan (Sigma, St. Louis, Mo.).

Computer analyses. The amino acid sequence of the putative FlgM of *V. parahaemolyticus* was used to search the *Pseudomonas* genome database (www.pseudomonas.com) by using BLASTP (1). A protein annotated as FlgM was found in the *Pseudomonas* genome that was used in a BLASTP search for FlgM homologues in other organisms at the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov). The deduced amino acid sequences of the *P. aeruginosa* FlgM and its homologues in other organisms were subjected to a PILEUP- and PRETTYBOX-generated alignment by using the GCG package (SeqWeb version 1.2, Wisconsin Package version 10.1). An evolutionary analysis that used the Kimura protein distance as correction method, which is based on the number of substitutions per 100 amino acids, was also performed on these proteins by using the same GCG package.

Electroporations and transformations. *E. coli* transformations were performed by a standard procedure (31). The DNA used for electroporation in *P. aeruginosa* was prepared by the alkaline lysis procedure (6), and electroporations were performed as described previously (10).

PCR amplification and site-directed mutagenesis. PCR was performed with *Taq* DNA polymerase (Gibco-BRL, Inc., Gaithersburg, Md.) as described previously (10). Briefly, DNA was initially denatured for 5 min at 95°C, followed by 35 cycles consisting of denaturation for 1 min at 95°C, annealing for 1 min at 55°C, and extension at 72°C for 3 min with primers RER51 and RER52 to generate a 744-bp fragment carrying the *fliA* gene. Annealing and extension at 70°C for 3 min with primers P6Sst and P9Hind were used to generate a 2.0-kb DNA fragment carrying the *flgM* gene, for 2 min 30 s with primers P10Eco and P11Bam to generate the 614-bp *flgM* promoter region, or for 1 min with primers P14Nde and P15Bam to generate the 324-bp *flgM* gene. Site-directed mutagenesis was performed by using the QuickChange Mutagenesis Kit (Stratagene, La Jolla, Calif.) according to the instructions in the user's manual. Specifically, the site-directed mutagenesis PCR was performed with *Pfu* DNA polymerase and primers P12Bam and P13Bam to generate a unique *Bam*HI site at the start of *flgM*, under the following conditions: an initial denaturation for 0.5 min at 95°C, followed by 18 cycles of denaturation for 0.5 min at 95°C, annealing for 1 min at 55°C, and extension for 12 min at 68°C. The nucleotide sequences of the primers will be available upon request.

Plasmid constructions. A 2.0-kb amplification product was obtained by PCR with the primers P6Sst and P9Hind, with PAK genomic DNA as a template, and inserted in cloning vector pCR2.1 (Invitrogen, Carlsbad, Calif.). The resulting plasmid pCR2.1-M was sequenced and demonstrated to contain a complete open reading frame tentatively identified as the *P. aeruginosa flgM* and flanking DNA, including the complete putative *flgN* gene downstream and part of the putative *flgA* gene upstream. PCR2.1-M was digested with *Hin*dIII, followed by religation which removed a *Bam*HI site, and was then used as a template in site-directed mutagenesis PCR. A unique *Bam*HI site was generated in the 5′ end of the *flgM* gene by site-directed mutagenesis that facilitated the insertion of a gentamicin resistance (Gm^r) gene, yielding pCR2.1-MG. Plasmids pET15bVP-M and pET15bVP-A were obtained, respectively, by cloning the *flgM* gene as a 324-bp PCR fragment and the *fliA* gene as a 744-bp PCR fragment into the *Nde*I and *Bam*HI sites of a low-copy-number expression vector pET15bVP (3). PET15bVP-M was used for complementation of the *flgM* mutation in PAK-M. An additional construct containing *flgM* on a broad-host-range plasmid pMMB67HE was made to assess the dose effect of *flgM* on the expression of FliC in the *flgM* mutant PAK-M. A 1.2-kb *Eco*RI/*Sst*I fragment was obtained from the vector pCR2.1-M and cloned into the *Eco*RI and *Sst*I sites of pMMB67HE. The resulting construct pMMB67HE-M included an intact *flgM* gene and part of the *flgA* gene. A 614-bp *Eco*RI/*Bam*HI PCR fragment containing the putative *flgM* promoter region was obtained with the primers P10Eco and P11Bam and with PAK genomic DNA as

a template. This PCR fragment was cloned into the *Eco*RI and *Bam*HI sites of the vector pDN19lacΩ (34) to engineer the promoter fusion construct pDN19lacΩ-M.

Motility assay and EM. Chemotactic motility was assessed by qualitative analysis of the zone formed by the motile bacteria on a 0.3% L agar plate. The plates were inoculated by stabbing the plates with different *P. aeruginosa* strains with a sterile toothpick. The plates were incubated at 37°C for 8 h. For electron microscopy (EM), static cultures were grown overnight at 37°C with the appropriate antibiotics. A drop of the culture was allowed to adhere to a carbon-coated grid for 10 s, and excess culture was drained off; the grid was then rinsed in a drop of saline, and adherent cells were negatively stained with a 2% aqueous solution of phosphotungstic acid for 10 s. Samples were examined with a Hitachi H-7000 transmission electron microscope.

β-Galactosidase assay. Plasmid pPT269 (34) containing the *fliC* promoter region fused to a promoterless *lacZ* was electroporated into *P. aeruginosa* wild-type PAK and the *flgM* mutant PAK-M. The *flgM-lacZ* fusion plasmid pDN19lacΩ-M was electroporated into *P. aeruginosa* strains PAK, PAK-Q, PAK-RG, PAK-N1G, and MS540 (Table 1). β-Galactosidase assays were performed as described previously (25). Cells were grown in L broth with streptomycin to an A_{600} of 0.7 to 1.0, harvested, and assayed for β-galactosidase activity. Two independent experiments were performed in triplicate when the β-galactosidase activity was assessed.

Yeast two-hybrid system. To examine whether there was a direct in vivo interaction between the anti-sigma factor FlgM of *P. aeruginosa* and the alternative sigma factor FliA, a yeast transcriptional assay, the Matchmaker GAL4 Two-Hybrid System 3 (Clontech Laboratories, Inc., Palo Alto, Calif.) was used. Two fusion vectors were used, pGADT7 that expresses proteins fused to the GAL4 activation domain and pGBKT7 that expresses proteins fused to the GAL4 DNA-binding domain. The yeast strain AH109 utilizes three reporter genes that are under the control of GAL4 upstream activating sequences. Construction of gene fusions was made possible by obtaining *Nde*I/*Bam*HI fragments of the entire *flgM* gene (324 bp) and the entire *fliA* gene (744 bp) from the vectors pET15bVP-M and pET15bVP-A, respectively. These fragments were cloned in-frame into the *Nde*I and *Bam*HI sites of both the fusion vectors pGADT7 and pGBKT7, giving the following constructs: pGADT7-M, pGADT7-A, pGBKT7-M, and pGBKT7-A. These constructs, along with proper control vectors, were transformed into the recipient yeast strain AH109 according to the manufacturer's instructions. Transcription of the reporter genes *lacZ*, *ADE2*, and *HIS3* facilitated the assessment of protein interaction by detecting the color reaction of β-galactosidase activity (*lacZ*) and by nutritional selection omitting the amino acids L-adenine (*ADE2*) or L-histidine (*HIS3*) (Sigma). β-Galactosidase activity was detected as described below. Briefly, yeast colonies were patched on filter paper (Whatman no. 1; Whatman International, Ltd., Maidstone, England), allowed to dry, and subsequently immersed in liquid nitrogen for 1 min. The filter paper was placed onto an additional filter paper presoaked with appropriate substrate and buffer and then incubated at 30°C for color reaction.

Western blots. To demonstrate a translated product from the *fliC* gene in the *flgM* mutant PAK-M, Western blotting was performed. After overnight growth of the cultures, identical optical densities of cell suspensions were adjusted, and cells were centrifuged. Proteins from whole-cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 10% gel by using Bio-Rad's Mini-Protean II system (23) and then electrophoretically transferred as described previously (10). Alkaline phosphatase-conjugated immunoglobulin G whole molecule from Sigma was used as secondary antibody, and the alkaline phosphatase activity was detected by color reaction.

Primer extension analysis. RNA was prepared from *P. aeruginosa* strains PAK, PAK-N1G, PAK-Q, and MS540 by using Trizol reagent (Gibco-BRL/Life Technologies, Grand Island, N.Y.) according to the manufacturer's protocol. The primer (7.5 μM) was 5′ end labeled by using [γ-^{32}P]ATP (25 μCi) and T4 polynucleotide kinase at 37°C for 30 min. The enzyme was heat inactivated after the addition of 40 mM EDTA (pH 7.5) and purified by elution through a G-25 spin column (Amersham Pharmacia Biotech, Inc., Piscataway, N.J.). Labeled primer was annealed to 50 μg of RNA in first-strand buffer and RNaseOUT RNase inhibitor at 65°C for 1 h. After a slow cooling to room temperature, deoxynucleoside triphosphate (0.5 mM), dithiothreitol (10 mM), RNaseOUT, and Superscript II (RNase H$^-$ RT) were added for reverse transcription at 42°C for 1 h. After cDNA synthesis, RNase H treatment was given at 37°C for 20 min, followed by phenol-chloroform extractions and ethanol precipitations. Sequencing was done with the same primer (unlabeled) by using [α-^{32}P]dATP and the Sequenase version 2.0 DNA Sequencing kit (U.S. Biochemicals, Cleveland, Ohio). DNA was resolved on an 8 M urea–8% polyacrylamide gel. Two primers were used that were 91 and 64 bp downstream of the consensus σ^{28} binding site, respectively. The start site shown in Fig. 5 was detected by using the primer that was 91 bp downstream of the σ^{28} binding site. In addition to that, six more primers were used in the primer extension analysis to detect any additional start sites. The nucleotide sequences of these primers are available upon request.

Sequencing and nucleotide sequence accession number. DNA sequencing was performed as described previously (10). The nucleotide sequence consisting of 2,048 nucleotides containing the partial sequence of *flgA* and the complete sequences of *flgM* and *flgN* genes have been submitted to GenBank (accession no. AY029221).

RESULTS

Identification and computer analysis of *P. aeruginosa flgM*. Attempts to identify a FlgM homologue in *P. aeruginosa* based on its homology to FlgM in the enteric bacteria *E. coli* and *S. enterica* serovar Typhimurium were unsuccessful. Later, the existence of a putative FlgM protein in *V. parahaemolyticus* was reported (20), and the amino acid sequence of this protein was used to perform a BLASTP search in the *Pseudomonas* genome database. A *P. aeruginosa* homologue was discovered that was initially considered to be a hypothetical protein and then, later during these studies, was deemed to be a putative FlgM protein (confidence level 4) with an unknown function. The putative *P. aeruginosa* FlgM protein consisting of 107 amino acids was further used in a BLASTP search for FlgM homologues in other organisms. The *P. aeruginosa* protein showed 61% similarity and 31% identity to FlgM of *V. parahaemolyticus*, 61% similarity and 28% identity to FlgM of *V. cholerae*, and 57% similarity and 38% identity to FlgM of *Proteus mirabilis*. The *flgM* gene mapped downstream of a chemotaxis gene *cheV* and a flagellar gene *flgA* and upstream of *flgN*. Thus, the location of the *flgM* gene between *flgA* and *flgN* in the *P. aeruginosa* genome is consistent with the gene organization and transcriptional direction in the flagellar system of *V. parahaemolyticus* (20) and in *V. cholerae* (29). The deduced amino acid sequence of *flgM* of *P. aeruginosa* was aligned with FlgM homologues from several other organisms by using the GCG multiple sequence analysis programs PILEUP and PRETTYBOX. As shown in Fig. 1, FlgM of *P. aeruginosa* had weak homology to FlgM proteins of some bacteria, particularly with *E. coli*, *S. enterica* serovar Typhimurium, *Yersinia enterocolitica,* and *Proteus mirabilis* throughout the open reading frame except for a short stretch of amino acids in the C-terminal part of the sequence (amino acids 75 to 101), which was conserved in all bacteria. These results correlated with a computer-generated evolutionary analysis that demonstrated FlgM of *P. aeruginosa* to have the largest genetic distance from that of *E. coli*, serovar Typhimurium, *Y. enterocolitica,* and *Proteus mirabilis* and the closest genetic distance to FlgM of *Vibrio* species (data not shown).

Insertional inactivation and complementation of *flgM*. To examine the function of the putative *flgM* gene, a chromosomal *flgM* mutation was generated in *P. aeruginosa* PAK by allelic replacement. The plasmid pCR2.1-MG that included the inactivated *flgM* gene was electroporated into PAK and replaced the corresponding wild-type gene by double reciprocal recombination, giving rise to the mutant strain, PAK-M. Inactivation of the *flgM* gene was confirmed by PCR with primers P6Sst and P9Hind which gave rise to an ~3.8-kb product in the mutant (data not shown). The *flgM* mutant strain PAK-M and the corresponding wild-type strain were examined on motility

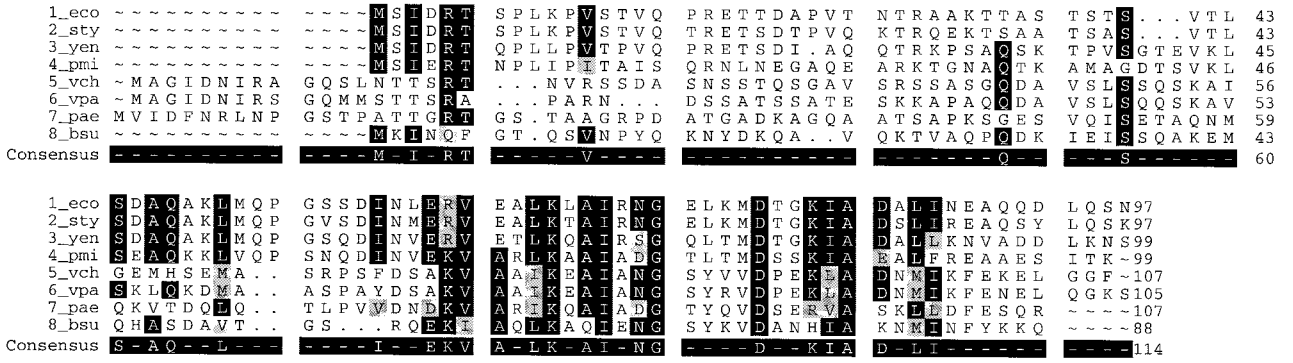

FIG. 1. Computer-generated alignment (PILEUP and PRETTYBOX) based on the deduced amino acid sequence of FlgM from *P. aeruginosa* (7_pae) and those of its homologues from *E. coli* (1_eco), serovar Typhimurium (2_sty), *Y. enterocolitica* (3_yen), *Proteus mirabilis* (4_pmi), *V. cholerae* (5_vch), *V. parahaemolyticus* (6_vpa), and *B. subtilis* (8_bsu) by using GCG multiple alignment sequence programs (SeqWeb version 1.2, Wisconsin Package version 10.1). Black shading indicates identity, and gray shading (light and dark) indicate degrees of similarities among amino acid residues.

plates (0.3% agar). The *flgM* mutant strain PAK-M was weakly motile, as assessed by the significantly lower size of the swarming zone compared with the wild-type PAK after 8 h of incubation (Fig. 2Aa and b). To rule out that this defective phenotype was a result of inactivating downstream genes, an intact copy of the *flgM* gene was cloned as a 324-bp *Nde*I/*Bam*HI fragment into the plasmid pET15bVP, and this construct (pET15bVP-M) was introduced into PAK-M by electroporation. The motility function was restored to that of the wild-type PAK by providing the *flgM* gene in *trans*, while the vector control (pET15bVP) did not restore this phenotype (Fig. 2Ac and d). When the *flgM* gene, carried on the plasmid pMMB67HE-M under the stronger *tac* promoter, was introduced into the *flgM* mutant PAK-M, a nonmotile phenotype was evident, a finding consistent with an explanation that FlgM in excess inhibited transcription of the σ^{28}-dependent *fliC* promoter (Fig. 2Ae and f). Electron microscopy studies revealed that the *flgM* mutant strain PAK-M possessed a single polar flagellum as the wild-type PAK, while the PAK-M carrying the *flgM* gene on plasmid pMMB67HE-M was not flagellated (Fig. 2B). In contrast to this observation, when the *flgM* gene was expressed from the plasmid pET15bVP-M, the bacteria showed a single polar flagellum. These data suggested a dose effect of *flgM* in motility and flagellin accumulation.

Negative regulation of flagellin synthesis by FlgM. Transcription of the class 3 *fliC* promoter has been demonstrated to

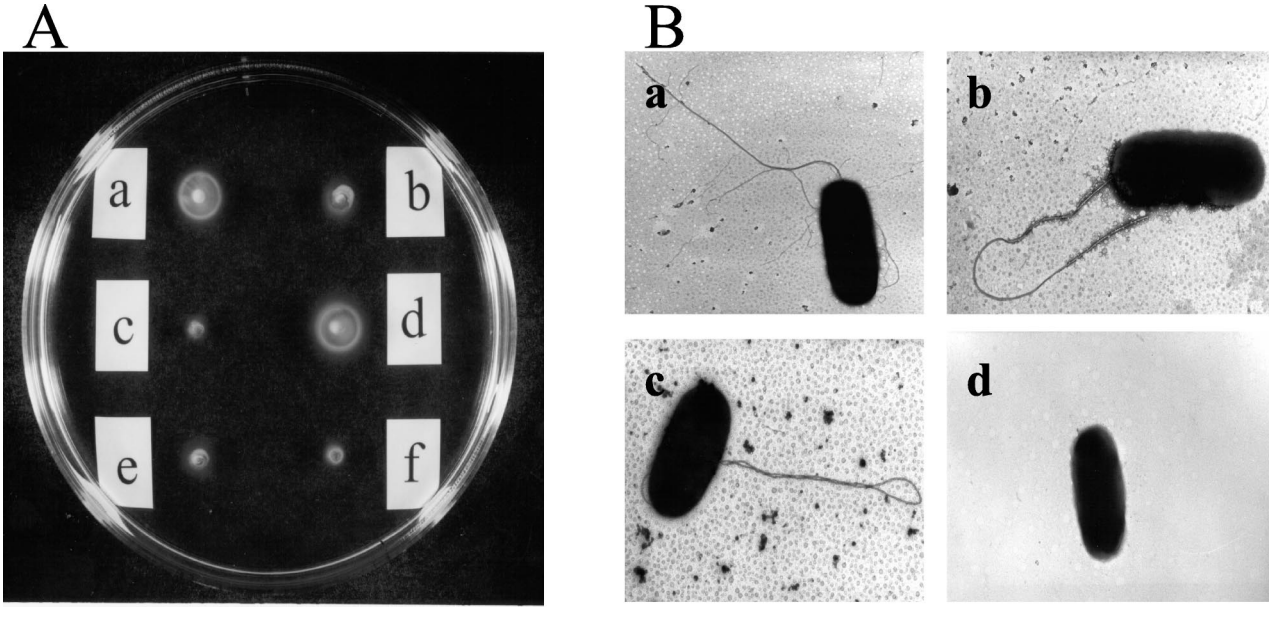

FIG. 2. Dose effect of *flgM* in motility. (A) Chemotactic motility of *P. aeruginosa* strains PAK (a); PAK-M (*flgM* mutant) (b); PAK-M (pET15bVP), which is the *flgM* mutant strain containing empty vector (c); PAK-M (pET15bVP-M) expressing the *flgM* gene at very low levels (d); PAK-M (pMMB67HE), which is another vector control (e); and PAK-M (pMMB67HE-M), which has *flgM* under the *tac* promoter (f). (B) Electron micrographs of wild-type PAK (a), PAK-M (*flgM* mutant) (b), PAK-M (pET15bVP-M) (c), and PAK-M (pMMB67HE-M) (d). Magnifications: a, ×42,500; b, ×50,000; c and d, ×37,500.

TABLE 2. Assessment of transcriptional activities of the *fliC* promoter in strains PAK and PAK-M

Host strain	Plasmid construct	β-Galactosidase activity (Miller units; mean ± SD)
PAK, wild type	placΩ	278 ± 36
PAK, wild type	placΩ*fliC*	2,080 ± 87
PAK-M, *flgM* mutant	placΩ	164 ± 107
PAK-M, *flgM* mutant	placΩ*fliC*	23,948 ± 1065
PAK-M, *flgM* mutant	placΩ*fliC*, pMMB67HE	13,655 ± 677
PAK-M, *flgM* mutant	placΩ*fliC*, pMMB67HE-M	247 ± 64

be repressed by FlgM in serovar Typhimurium (17, 28). By analogy, it is reasonable to expect that the *fliC* promoter would be upregulated in *P. aeruginosa flgM* mutant PAK-M, if FlgM acts as a negative regulator of FliA. Indeed, a ca. 11.5-fold upregulation of the β-galactosidase activity of the flagellin gene (*fliC*) promoter fusion was demonstrated in the *flgM* mutant PAK-M (Table 2). In addition, when an intact copy of the *flgM* gene was introduced in the PAK-M containing the *fliC-lacZ* fusion, the β-galactosidase activity of the *fliC* promoter fusion was completely abrogated (Table 2). These results suggested that an excess of FlgM suppresses transcription of *fliC*. To verify that the demonstrated upregulation of the *fliC* promoter in the *flgM* mutant background also correlated with accumulation of translated *fliC*, whole-cell lysates of wild-type PAK and PAK-M were analyzed by Western blot for their flagellin content. As shown in Fig. 3 (lane 2), an excess of flagellin was demonstrated in the *flgM* mutant PAK-M compared with the wild-type PAK (lane 1). However, flagellin was not expressed when the *flgM* gene was introduced on the plasmid pMMB67HE-M under the control of the *tac* promoter, as shown in Fig. 3, lane 6. This result is consistent with that of the β-galactosidase assays showing no activity of the *fliC* promoter fusion in PAK-M when *flgM* was introduced on pMMB67HE-M, as well as with the results of the motility assay and EM demonstrating a nonmotile, nonflagellated phenotype of PAK-M carrying the same plasmid. The PAK-M strain carrying the empty vector pMMB67HE appeared the same as PAK-M (Fig. 3, lane 5). However, when *flgM* was expressed from pET15bVP-M, flagellin was detected in amounts similar to the wild-type PAK (Fig. 3, lane 4). These results confirmed the findings from the motility and EM analyses that the amount of FlgM affects the amount of intracellular flagellin and thus the

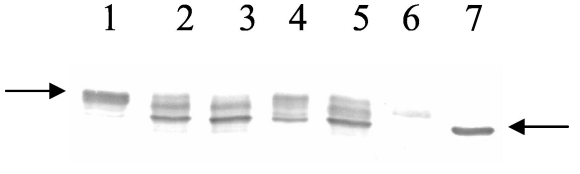

FIG. 3. Flagellin content in whole-cell lysates of wild-type PAK and *flgM* mutant PAK-M, analyzed by Western blot with polyclonal anti-FliC rabbit serum. Lanes: 1, wild-type PAK; 2, PAK-M (*flgM* mutant); 3, pET15bVP vector control in PAK-M; 4, pET15bVP-M plasmid in PAK-M; 5, pMMB67HE vector control in PAK-M; 6, pMMB67HE-M plasmid in PAK-M; and 7, *orfA* mutant that makes nonglycosylated flagellin. The arrow on the left indicates modified flagellin (45 kDa). The arrow on the right indicates nonglycosylated flagellin (~40 kDa).

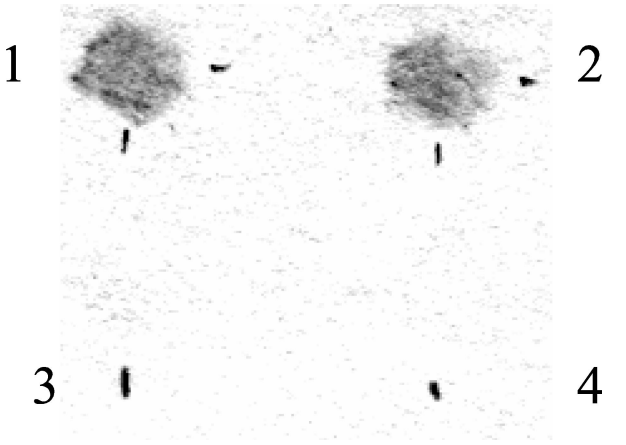

FIG. 4. FlgM-FliA in vivo fusion protein interaction in yeast AH109 transformants. Positive interaction is based on expression of β-galactosidase activity after transcription of the reporter gene *lacZ*. Panels 1 and 2 show positive β-galactosidase activity in yeast strain AH109 carrying plasmids pGADT7-M and pGBKT7-A and plasmids pGADT7-A and pGBKT7-M, indicating positive interaction between the FlgM and FliA proteins. Panels 3 and 4 show pGADT7-M or pGADT7-A carrying the *fliA* or *flgM*, respectively, with the vector control pGBKT7 where no β-galactosidase activity was detected.

presence or absence of a flagellum and chemotactic motility on the plates.

FlgM-FliA in vivo protein interaction. One of the criteria for anti-σ^{28} activity of FlgM is the physical association of FlgM with the alternative sigma factor σ^{28} (FliA) described in serovar Typhimurium (8, 28). In order to confirm this interaction of FlgM with FliA in *P. aeruginosa*, a two-hybrid yeast transcriptional assay was used that allowed in vivo protein interaction to be detected by activating transcription of the reporter genes *lacZ*, *ADE2*, and *HIS3*. A 324-bp *Nde*I/*Bam*HI fragment containing the entire *flgM* gene and a 744-bp *Nde*I/*Bam*HI fragment containing the entire *fliA* gene were cloned in both of the vectors pGADT7 and pGBKT7, giving pGADT7-M, pGADT7-A, pGBKT7-M, and pGBKT7-A. β-Galactosidase activity was detected when both plasmids pGADT7-M and pGBKT7-A, as well as pGADT7-A and pGBKT7-M, were transformed in the yeast strain AH109 (Fig. 4, panels 1 and 2), indicating induced transcription of the *lacZ* gene due to interaction between the fusion proteins FlgM and FliA. No activity was detected in the yeast AH109 transformants with the *flgM* or *fliA* gene fusion constructs with the corresponding vector controls, pGADT7-M with pGBKT7 or pGADT7-A with pGBKT7 (Fig. 4, panels 3 and 4). In addition, growth of yeast strain AH109 on medium that was devoid of L-histidine or L-adenine occurred only when AH109 was transformed with the gene fusion constructs pGADT7-M and pGBKT7-A or the gene constructs pGADT7-A and pGBKT7-M. These results indicate that the putative FlgM protein physically interacts with the alternative sigma factor FliA in *P. aeruginosa*, in agreement with the mechanism of action of FlgM in other organisms.

Transcriptional regulation of the *flgM* gene. The *flgM* upstream region was inspected for the presence of consensus σ^{54} (YTGGCACG-N_4-TTGCW) (5) and σ^{28} (TAAAGTTT-N_{11}-GCCGATAA) (21) recognition sites. Downstream of the *flgA*

A

```
1                          → flgA stop   σ54                        σ54   50
GAACCCGGCACGGTCGAGGTGAATATGTAGAGGGAAAGCCGTGGCCGGTA
E   P   G   T   V   E   V   N   M   *

51        σ54                                                       100
GCGCTGGCGCTACCCCGGCGGGTTTCTCTAAACTGGGAAACGACGCACGA

101                σ28                                        +1     150
AAATAAGTCCTAAAGTTTTTCAAGCCTTCGCCGAAAAGCTAGGCAAGCGT

151                          flgM →                                  200
CCAATACCTTCGAGGTTTACAACCATGGTCATCGACTTCAACCGGCTGAAT
                               M   V   I   D   F   N   R   L   N

                                                σ28 primer
201                                                              250
CCCGGCAGCACGCCAGCCACCACCGGCCGTACCGGCAGCACCGCCGCAGG
P   G   S   T   P   A   T   T   G   R   T   G   S   T   A   A   G

251
TCGCC
  R   P
```

B

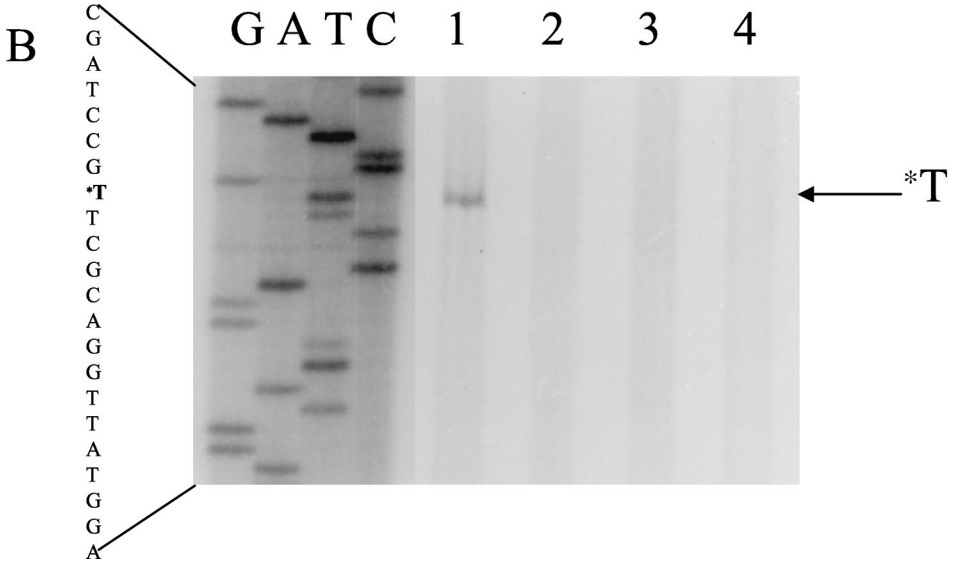

FIG. 5. (A) Partial nucleotide sequence of the 614 bp upstream *flgM* promoter region used for β-galactosidase assays. In this promoter sequence three putative σ^{54} binding sites (GGCACG-N_4-TTGC) and one σ^{28} binding site (TAAAGTTT-N_{11}-GCCGATAA) are indicated in boldface and underlined. The transcriptional start site 8 bp downstream of the σ^{28} consensus sequence is shown as +1, with an arrow showing the direction of transcription. (B) Primer extension analysis of the *flgM* gene. Sequencing ladders GATC are on the left, and primer extension products from *P. aeruginosa* strains PAK (lane 1), PAK-N1G (σ^{54} mutant) (lane 2), MS540 (σ^{28} mutant) (lane 3), and PAK-Q (*fleQ* mutant) (lane 4) RNAs are shown to the right. The arrow denotes the transcriptional start site.

gene, three putative σ^{54} binding sequences were identified which were located 119, 128, and 143 bp upstream of the translational start site of the *flgM* gene (Fig. 5A). The putative σ^{54} binding sequence located 119 bp upstream of the *flgM* gene matched 12 of 17 residues with the consensus σ^{54} binding site, and the additional two upstream putative σ^{54} binding sites matched 11 of 17 and 10 of 17 residues. In addition, a putative σ^{28} consensus site having only a single mismatch with the consensus σ^{28} binding site was located 8 bp upstream of the transcriptional start site (Fig. 5A). To gain further insight into the transcriptional regulation of the *flgM* gene, the putative *flgM* promoter region comprising 614 bp was fused to the promoterless *lacZ* gene. The β-galactosidase activity of the *flgM* promoter was measured in several *P. aeruginosa* strains. As shown in Table 3, the existence of a *flgM* promoter in the *flgM* upstream region was demonstrated in wild-type PAK

TABLE 3. Assessment of transcriptional regulation of the *flgM* promoter in *P. aeruginosa*

Host strain	Genetic background	β-Galactosidase activity (Miller units; mean ± SD)	
		Vector alone	*flgM* promoter
PAK	Wild type	172 ± 4	13,961 ± 379
PAK-N1G	*rpoN* mutant	88 ± 18	9,809 ± 857
PAK-Q	*fleQ* mutant	34 ± 3	6,831 ± 721
MS540	*fliA* mutant	47 ± 26	8,698 ± 1,030

since β-galactosidase activity was ca. 81 times higher than that of the pDN19lacΩ vector control. *P. aeruginosa* strains MS540 (σ^{28} mutant), PAK-NIG (σ^{54} mutant), and PAK-Q (*fleQ* mutant) had high activities of β-galactosidase, although they were reduced compared to that of the wild-type PAK. These data suggested the existence of a promoter element that was not entirely dependent on σ^{28}, σ^{54}, or FleQ. Further analysis of the *flgM* promoter by primer extension revealed a transcriptional start site (Fig. 5) 8 bp downstream of the consensus σ^{28} binding site. However, no σ^{54}-dependent transcripts could be detected when several primers downstream of the three putative σ^{54} consensus binding sites were used. A faint band was, however, found at the same location in all four lanes upstream of the σ^{28} binding site, but there was no recognizable consensus binding site for any known sigma factor just upstream of this location. These results suggest that, in *P. aeruginosa*, the anti-sigma 28 factor FlgM is regulated by a mechanism that is somewhat different from that existing in enteric bacteria, in which *flgM* transcription is solely dependent on σ^{28}. Another unknown sigma factor may also be involved in *P. aeruginosa*. This mode of *flgM* regulation is the closest to that observed in *V. cholerae* (29).

DISCUSSION

One major control mechanism in flagellar biosynthesis that has not yet been accounted for in *P. aeruginosa* is the control of σ^{28}-dependent transcription of *fliC* by the anti-sigma factor FlgM. Here we describe the identification and functional analysis of the anti-sigma factor FlgM in *P. aeruginosa*.

Transcription of *fliC* has been shown to be dependent on the *fliA*-encoded sigma factor σ^{28} in *P. aeruginosa* and in other organisms such as serovar Typhimurium, *E. coli*, and *B. subtilis* (24, 26, 28, 33). Inhibition of σ^{28} by the anti-sigma factor FlgM has also been demonstrated (7, 13, 21). In this context, it was reasonable for us to pursue the identification of and to ascertain whether FlgM was present and acted as the negative regulator of σ^{28} in *P. aeruginosa*. While our previous efforts based on homology to the FlgM of *E. coli* and serovar Typhimurium were unsuccessful in identifying FlgM, we were now able to identify a putative FlgM candidate by searching the *Pseudomonas* genome database by using the annotated FlgM of *V. parahaemolyticus* (20). The PILEUP- and PRETTYBOX-generated alignment of the FlgM of different organisms showed a low degree of homology throughout the open reading frame except for a short stretch of ca. 26 amino acids positioned from amino acids 75 to 101 in the C-terminal half of the protein. Interestingly, it has been demonstrated that this part of FlgM of serovar Typhimurium contains the σ^{28} binding domain that is positioned from amino acids 41 to 97. Mutations in the C-terminal part of FlgM disrupt the activity of the anti-sigma factor (8, 11), indicating that the most conserved region of the FlgM protein is involved in its interaction with σ^{28} in several organisms. Furthermore, in an evolutionary analysis of FlgM of different organisms, *P. aeruginosa* FlgM was most distant from the FlgM of *E. coli* and serovar Typhimurium but was closest to FlgM in *V. parahaemolyticus* and *V. cholerae*, findings correlating with those from the PILEUP- and PRETTYBOX-generated alignment.

Insertional inactivation and complementation of the putative *flgM* gene in *P. aeruginosa* made it possible to analyze the function of *flgM*. Even though EM analysis revealed that the *flgM* mutant PAK-M possessed a single polar flagellum, this mutant was only weakly motile, as observed by the smaller swarming zone compared to wild-type PAK. This may be due to intracellular accumulation of FliC or to the fact that the transcription of genes controlled by σ^{28}-dependent promoters other than *fliC*, e.g., chemotaxis genes, may be affected in this mutant. Thus far, the transcription of chemotaxis genes in *P. aeruginosa* has not been shown to be σ^{28} dependent, although in *B. subtilis* and in the enteric bacteria chemotaxis genes are σ^{28} dependent (12, 16). Motility was restored to normal when the *flgM* gene was provided to the *flgM* mutant on the plasmid pET15bVP-M. However, a nonmotile phenotype was observed when *flgM* was introduced into this strain under the control of the *tac* promoter on the plasmid pMMB67HE-M. Complementation of the motility defect in studies with the low-copy expression vector pET15bVP under noninduced conditions in *P. aeruginosa* has previously been demonstrated (10). The fact that the phenotype was not restored when the *flgM* gene was introduced with the plasmid pMMB67HE-M may be explained by the amount of FlgM exceeding the threshold level leaving no free σ^{28} available for flagellin expression. A Western blot of this complemented mutant supported this conclusion in that the amount of flagellin seen was markedly diminished from the wild-type strain. This repressive effect of FlgM has been suggested in wild-type serovar Typhimurium when FlgM was overexpressed (17). In addition, observations were made that the *flgM* mutant in serovar Typhimurium was weakly motile, possessed longer flagella, and expressed flagella in increased numbers (13, 22). However, the flagellum of the *P. aeruginosa flgM* mutant PAK-M appeared normal under EM.

Besides characterizing the morphology and phenotype of the *flgM* mutant PAK-M, β-galactosidase assays and Western blots were used to assess σ^{28}-dependence of the *fliC* promoter in *P. aeruginosa* at both transcriptional and translational levels. Our results manifested upregulated β-galactosidase activity of the flagellin gene (*fliC*) promoter *lacZ* fusion in the *flgM* mutant PAK-M, which correlated with an increased amount of the translated *fliC* product. In addition, the overexpression of FlgM not only downregulated the *fliC* promoter in the *flgM* mutant PAK-M but also reduced flagellin synthesis. Although it is still unknown if extracellular transportation of FlgM to initiate σ^{28}-dependent transcription of late gene promoters is also a prerequisite in *P. aeruginosa*, identification of *flgM* will facilitate further investigations of this phenomenon to clarify this mechanism. Another unexpected observation was made in the *flgM* mutant that a greater proportion of the flagellin mi-

grated faster than the wild-type strain. This band migrated at the same location as nonglycosylated flagellin (Fig. 3). This is consistent with an interpretation that excess of flagellin accumulates in the cytoplasm and cannot be dealt with by the glycosylation pathway or that there is an actual defect in post-translational glycosylation of the flagellin, a phenomenon recently described in *P. aeruginosa* (4).

Transcription of the *flgM* gene in serovar Typhimurium has been shown to be dependent on the alternative sigma factor σ^{28} and FlhCD, the master regulator (15). The situation in *P. aeruginosa* appears to be somewhat different in that *flgM* appears to be regulated by σ^{28} and an additional factor(s). The transcriptional start site studies definitively implicate σ^{28}, and the absence of a start site in the *fleQ* and *rpoN* mutants suggests that they are also involved. Furthermore, the *fliA*, *fleQ*, and *rpoN* mutants containing a *flgM-lacZ* fusion showed similar reductions in β-galactosidase activity compared to the wild-type strain, suggesting that these regulators may be linked in some way. One explanation may be that *fliA* is regulated by *fleQ* and *rpoN*. However, it has been suggested that *fliA* is not regulated by *rpoN* in *P. aeruginosa*, a suggestion which may have to be reexamined in more extensive studies. Regardless of the role of RpoN and FliA, the residual β-galactosidase activity in these mutants suggests that other regulators are involved. *P. aeruginosa* does not have *flhCD* homologues; therefore, there are as yet no known regulatory candidates. A similar situation was reported for the control of the *flgM* promoter of *V. cholerae* (29), in which the *flgM* promoter exhibited high levels of transcription even in the absence of σ^{28} and σ^{54}. Taken together, identification of *flgM* in *P. aeruginosa* illustrates resemblance to the flagellar system of enterics and other organisms in that FlgM represents a major regulator at a critical point in flagellin synthesis. On the other hand, the regulatory hierarchy of the flagellar system in *P. aeruginosa* has developed differently from that of enteric bacteria and is probably closest to that in *V. cholerae*.

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