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Identification and Characterization of a *Vibrio cholerae* Gene, *mbaA*, Involved in Maintenance of Biofilm Architecture

Natalia Bomchil, Paula Watnick,† and Roberto Kolter*

Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115

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The formation of biofilms is thought to play a key role in the environmental survival of the marine bacterium *Vibrio cholerae*. Although the factors involved in *V. cholerae* attachment to abiotic surfaces have been extensively studied, relatively little is known about the mechanisms involved in the subsequent maturation of the biofilms. Here we report the identification of a novel gene, which we have named *mbaA* (for maintenance of biofilm architecture), that plays a role in the formation and maintenance of the highly organized three-dimensional architecture of *V. cholerae* El Tor biofilms. We demonstrate that although the absence of *mbaA* does not significantly affect the initial attachment of cells onto the surface, it leads to the formation of biofilms that lack the typical structure, including the pillars of cells separated by fluid-filled channels that are evident in mature wild-type biofilms. Microscopic analysis indicates that the absence of *mbaA* leads to an increase in the amount of extracellular matrix material in the biofilms. The predicted *mbaA* product is a member of a family of regulatory proteins, containing GGDEF and EAL domains, suggesting that MbaA regulates the synthesis of some component of the biofilm matrix.

Bacteria living in association with surfaces are referred to as biofilms (6). In these surface-attached communities, bacteria are often protected from external challenges, including environmental stresses and predation (13). Biofilm formation therefore emerges as an important process for microbial survival in the environment and within host organisms (6). Biofilms generally show a distinct architecture characterized by pillar- or mushroom-like cell assemblages separated by fluid-filled channels. These channels allow nutrients to reach all levels of the biofilm and allow toxic waste products to diffuse out (5). Several reports have shown that alterations in the architecture of the biofilms can greatly affect the physiology of the biofilm-associated cells (7, 11, 29).

Vibrio cholerae, the causative agent of cholera, a human diarrheal disease, survives in both marine and freshwater environments, where it can form biofilms on diverse surfaces (4). Under laboratory conditions, the developmental pathway leading to *V. cholerae* El Tor biofilm formation has been dissected into its constituent steps through genetic and microscopic analyses (26, 27). Bacteria first swim towards the surface using their polar flagella and establish cell-to-surface interactions. These interactions are greatly enhanced by the presence of the mannose-sensitive hemagglutinin (MSHA) type IV pilus (25). Once contact with the surface has been made, bacteria move along the surface by means of their flagella, recruit additional planktonic cells, and divide, resulting in the formation of microcolonies. Attached cells synthesize an exopolysaccharide (EPS), produced by the *vps* genes, which is thought to be a major component of the extracellular matrix that stabilizes the mature biofilm (26, 30, 31). Overproduction of EPS leads to alterations in the biofilm architecture that correlate with an increased resistance of the cells to osmotic and oxidative stresses as well as killing by biocides such as chlorine (24, 30, 31), suggesting that the maintenance of biofilm architecture may play an important role in the environmental survival of *V. cholerae*.

Many of the environmental signals and genes involved in the early events of biofilm formation in many species have been elucidated through genetic analyses (17, 27). However, relatively little is known about the genes involved in the maintenance of the three-dimensional architecture of mature biofilms. In this report we describe the identification of a novel gene that plays a role in the maturation of *V. cholerae* El Tor biofilms. We have named this gene *mbaA* (for maintenance of biofilm architecture).

MATERIALS AND METHODS

Bacterial strains and media. Bacterial strains used in this study are described in Table 1. Unless otherwise indicated, all strains were grown statically at room temperature in Luria-Bertani (LB) broth. Where appropriate, streptomycin (25 μg/ml) and ampicillin (150 μg/ml) were added to the medium. All *V. cholerae* strains described in this work are isogenic with N16961Sm, a streptomycin-resistant derivative of an El Tor strain isolated during the seventh pandemic.

Plasmid constructs. Plasmids used in this study are described in Table1. pNB4 was generated as follows. Primers mbaA1-F (5′-GCATTCTAGAGTTCGCTACGATATGCTG-3′) and mbaA1-R (5′-GCATGAATTCGCGTTGATAGGTTGAATG-3′) were used to amplify ′mbaA1′, an internal 516-bp region of the chromosomal *mbaA* gene corresponding to amino acids 167 to 334. Similarly, primers mba2-F (5′-GCATTCTAGAGAATTCCGCCATTTTTTGGATACACTC-3′) and mbaA2-R (5′-GCATCCCGGGGGATCCTTCCACAATCATCCACGCAAAGG-3′) were used to amplify ′mbaA2′, a 535-bp fragment which included the 3′ end of the *mbaA* coding sequence (amino acids 696 to 791) and the 220-bp region downstream of *mbaA*. PCR-amplified fragments ′mbaA1′ and ′mbaA2′ were digested with the restrictions enzyme combinations *Xba*I and *Eco*RI or *Bam*HI and *Eco*RI and cloned into the vector pBluescriptII KS+, forming plasmids pNB1 and pNB3, respectively. The *Sac*I/*Eco*RI 534-bp insert of pNB1 and the *Bam*HI/*Eco*RI 517-bp insert of pNB3 were then ligated simultaneously into vector pWM91, which had been previously digested with *Sac*I and *Bam*HI, to form plasmid pNB4.

* Corresponding author. Mailing address: Department of Microbiology and Molecular Genetics, Harvard Medical School, 200 Longwood Ave., Boston, MA 02115. Phone: (617) 432-1776. Fax: (617) 738-7664. E-mail: rkolter@hms.harvard.edu.

† Present address: Division of Geographic Medicine and Infectious Disease, New England Medical Center, Boston, MA 02111.

TABLE 1. Bacterial strains and plasmids

Strains or plasmid	Genotype	Reference or source
V. cholerae		
N16961Sm	El Tor strain isolated from the 7th pandemic	Laboratory collection
KFV11	Δ*mshA1*	25
ZK2736	*flgF*::Tn*10*Km	26
ZK2987	Δ*mbaA*	This study
ZK2988	Δ*mbaA* Δ*mshA1*	This study
ZK2989	Δ*mbaA flgF*::Tn*10*Kan	This study
ZK2985	*Plac*::*gfp*::*lacZ*	J. Zhu and J.J. Mekalanos
ZK2979	Δ*mshA1 Plac*::*gfp*::*lacZ*	This study
ZK2982	*flgF*::Tn*10*Kan *Plac*::*gfp*::*lacZ*	This study
ZK2984	Δ*mbaA Plac*::*gfp*::*lacZ*	This study
ZK2990	Δ*mbaA* Δ*mshA1 Plac*::*gfp*::*lacZ*	This study
ZK2991	Δ*mbaA flgF*::Tn*10*Kan *Plac*::*gfp*::*lacZ*	This study
ZK3046	*vps59*::*pGp704*	This study
ZK3047	*vpsR*::*pGp704*	This study
ZK3048	Δ*mbaA vps59*::*pGp704*	This study
ZK3049	Δ*mbaA vpsR*::*pGp704*	This study
E. coli		
DH5α λpir	*endA1 hdR17 supE44 thi-1 recA1 gyrA relA1* Δ*(lacZYA-argF) U169* (ϕ*80lacZ*ΔM15) λ*pirR6K*	W. Lin and J.J. Mekalanos
SM10 λpir	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu λ*pir*R6K Km^r	15
Plasmids		
pBluescriptII KS+	cloning vector; Ap^r	Laboratory collection
pCVD442	*ori*R6K *mob*RP4 *sacB* Ap^r	8
pJZ111	*Plac*::*gfp*::*lacZ* in pCVD442	J. Zhu and J.J. Mekalanos
pKEK349	*′vps59′* in pGp704	28
pNB1	*′mbaA1′* in pBluescriptII KS+	This study
pNB3	*′mbaA2′* in pBluescriptII KS+	This study
pNB4	Δ*mbaA* in pWM91	This study
pvpsR	*′vpsR′* in pGp704	31
pWM91	*ori*R6K *ori*T *sacB lacZ*α Ap^r	14

Generation of mutants. The Δ*mbaA* chromosomal mutation was constructed in *V. cholerae* strains N16961, KFV11, and ZK2736 by using the suicide plasmid pNB4, generating strains ZK2987, ZK2988, and ZK2989, respectively (Table1). Plasmid pNB4 was introduced into the recipient *V. cholerae* strains by mating, by using the donor *Escherichia coli* SM10λ*pir*. The recombinants *V. cholerae* colonies were selected on LB plates supplemented with streptomycin and ampicillin. The streptomycin-ampicillin-resistant colonies were then grown on nonselective medium (i.e., LB plates supplemented with streptomycin) to allow growth of cells in which excision of plasmid pNB4 had occurred through homologous recombination between its flanking regions. Cells that had been cured of the plasmid were selected for their ability to form colonies on sucrose, based on the toxicity of the plasmid-encoded *sacB* gene during growth on sucrose (8). PCR amplification with primers mbaA1-F and mbaA2-R was used to identify the clones harboring an in-frame deletion in the chromosomal *mbaA* gene.

Mutants with an insertion in the *vps59* gene were constructed in the wild-type (WT) (N16961) and Δ*mbaA* (ZK2987) backgrounds by using suicide plasmid pKEK349, generating strains ZK3046 and ZK3048, respectively. Strains ZK3047 and ZK3049 (Table1) were generated in a similar manner, by insertional inactivation of the *vpsR* gene of strains N16961 and ZK2987 by using suicide plasmid pvpsR. The suicide plasmids were introduced by mating by using the donor *E. coli* SM10λ*pir*. *V. cholerae* recombinants were selected on LB plates supplemented with streptomycin and ampicillin.

Generation of *gfp*-labeled strains. The *gfp* gene was introduced into the *lacZ* locus of the chromosome of *V. cholerae* strains KFV11, ZK2736, ZK2987, ZK2988, and ZK2989 by using the suicide plasmid pJZ111, generating strains ZK2979, ZK2982, ZK2984, ZK2990, and ZK2991, respectively (Table1). Plasmid pJZ111 was introduced into the recipient *V. cholerae* strains by mating, by using the donor *E. coli* SM10λ*pir*. The recombinants *V. cholerae* colonies were selected on LB plates supplemented with streptomycin and ampicillin. The streptomycin-ampicillin-resistant colonies were then grown on nonselective medium (i.e., LB plates supplemented with streptomycin) to allow growth of cells in which excision of plasmid pJZ111 had occurred through homologous recombination between its flanking regions. Cells that had been cured of the plasmid were selected for their ability to form colonies on sucrose, based on the toxicity of the plasmid-encoded *sacB* gene during growth on sucrose (8). X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (40 μg/ml) was added to the sucrose plates in order to identify white clones in which the *lacZ* gene was replaced by the *gfp* gene. The expression of the *gfp* gene from the chromosome was confirmed by epifluorescence by using an Optiphot-2 (Nikon) microscope equipped with a Nikon mercury lamp.

DNA manipulations and analysis. Plasmid and chromosomal DNA preparation, DNA ligation, bacterial electroporation, agarose gel electrophoresis, and PCR amplifications were carried out by using standard techniques (20). Restriction enzymes were from New England Biolabs, Inc.

Biofilm assays. Cells from colonies grown overnight on LB agar plates at room temperature were resuspended in LB broth at an optical density at 600 nm (OD_{600}) of ~0.3. Three microliters of the cell suspension was added to 300 μl of LB in 10- by 75-mm borosilicate glass tubes (VWR). Cultures were incubated at room temperature without shaking for various times. At the desired end-point, nonadherent cells were removed by rinsing with distilled water. Biofilms were stained by the addition of 350 μl of 1% crystal violet (Sigma) for 25 min followed by rinsing with distilled water, as described elsewhere (16). The cell-associated dye was solubilized in 400 μl of dimethyl sulfoxide (DMSO) and quantified by measuring the OD_{570} of the resulting solution. All assays were performed in triplicate.

Phase contrast microscopy. Cells from overnight colonies grown on LB agar plates at room temperature were resuspended in LB broth at an OD_{600} of ~0.3. Sixty microliters of the cell suspension was added to 6 ml of LB in 50-ml tubes (Falcon). Biofilms were formed at room temperature on borosilicate coverslips placed in the Falcon tubes. At the desired end-point, the coverslip was rinsed with LB broth to remove nonadherent bacteria and placed over a concave microscope slide filled with LB broth. Attached bacteria were then examined at 600× magnification by using an Optiphot-2 microscope (Nikon). Images were captured by using a CCD video camera system (Photometrics) and processed by

using the Metavue software (Universal Imaging Corp.). The images in the figures are representative of what was observed in at least 10 fields and in at least three independent experiments.

Confocal scanning laser microscopy. *gfp*-tagged *V. cholerae* strains were incubated with borosilicate coverslips in LB medium at room temperature and placed on a concave microscope slide as described above. For all confocal microscopy observations, 65-h biofilms were used. A MRC-1024 confocal microscope (Bio-Rad) set at a wavelength of 488 nm was used to collect z-section images of the biofilms (magnification, 600×). The images in the figures are representative of what was observed in 10 random fields in each of three independent experiments.

Scanning electron microscopy (SEM). *V. cholerae* strains were incubated on borosilicate coverslips in LB medium at room temperature. The resulting biofilms were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 1 h and then postfixed with 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.2), dehydrated with ethanol, critical point dried, and coated with gold palladium alloy. Samples were examined with a scanning image observing device equipped with an electron microscope (Autoscan Etec). The images in the figures are representative of what was observed in 10 random fields in each of three independent experiments.

RESULTS AND DISCUSSION

Isolation and characterization of a mutant that forms more robust biofilms. A screen for biofilm-deficient mutants of *V. cholerae* El Tor N16961 was previously performed in our laboratory (26). Those studies showed that flagellar motility, the presence of MSHA, and the synthesis of EPS were essential for the development of a *V. cholerae* El Tor biofilm (25, 26, 27). In the course of the same genetic screen, we obtained a mini-Tn*10* mutant strain that produced more robust biofilms, as quantified by using the crystal violet biofilm assay (see Materials and Methods). The intensity of the crystal violet ring formed by the Tn*10* mutant showed a threefold increase relative to that of the WT parent (Fig. 1A), suggesting that the mutant was forming unusually large biofilms.

Using arbitrary PCR (16) and DNA sequencing, we determined that the mini-Tn*10* transposon in this *V. cholerae* mutant was inserted at position 752679 of the *V. cholerae* genome (10). This position is in the 315th codon of a 2,376-bp open reading frame (The Institute for Genomic Research locus no. VC0703) coding for a protein with no known function. The results of our work showed that the VC0703 locus was involved in the maintenance of the biofilm architecture, and therefore, we have designated this gene *mbaA*, for maintenance of biofilm architecture. Sequence analysis of the *mbaA* region indicated that *mbaA* is likely the second gene of a three-gene operon, with putative open reading frames upstream and downstream (VC0704 and VC0702, respectively) (Fig. 1B). To determine whether the biofilm phenotype observed in the transposon mutant was a consequence of a transcriptional polarity effect upon transposon insertion, we constructed a chromosomal in-frame deletion in the *mbaA* gene (Fig. 1B). The phenotype of the Δ*mbaA* mutant was indistinguishable from the phenotype of the *mbaA*::Tn*10* insertion mutant. Both mutant strains displayed greatly enhanced biofilm formation on borosilicate glass after 30 h compared to the WT strain (Fig. 1A). The same phenotype of enhanced biofilm formation was observed for the *mbaA* mutants on plastic surfaces, such as polyvinylchloride and polystyrene (data not shown). The growth rates of planktonic cells of the *mbaA*::Tn*10* and Δ*mbaA* mutants were similar to that of the WT (data not shown). These data confirm that the increased capacity of the mutant to form biofilms is a direct result of the inactivation of the *mbaA* gene and that this effect is not due to an increase in the growth rate of the mutants.

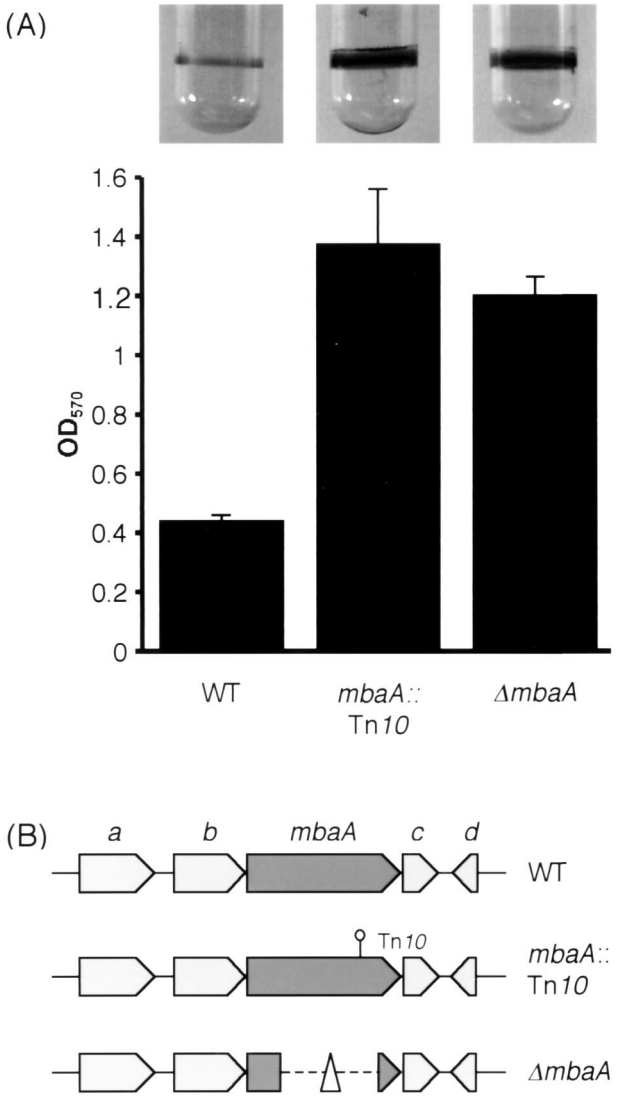

FIG. 1. Disruption of a novel gene named *mbaA* in *V. cholerae* N16961 leads to increased biofilm formation. (A) Crystal violet-stained borosilicate tubes and quantification of the DMSO-solubilized dye are shown for the indicated strains after 30 h of growth in LB broth at room temperature. All assays were performed in triplicates. (B) Genetic organization of the *mbaA* region on the chromosome of *V. cholerae* N16961. The *a*, *b*, *mbaA*, *c*, and *d* open reading frames correspond to loci VC0705, VC0704, VC0703, VC0702, and VC0701, respectively, based on the DNA sequences obtained from The Institute for Genomic Research microbial genome database. Open reading frames *a*, *mbaA*, and *c* might constitute an operon. The Tn*10* insertion in *mbaA* and the extent of the in-frame deletion created in *mbaA* are indicated.

Flagella and MSHA pili requirements for early biofilm development are not bypassed in the absence of MbaA. It has been previously shown that flagella and MSHA type IV pili are important for the early steps of biofilm formation in *V. cholerae* (25, 26). The *flgF*::Tn*10* mutants were unable to develop a biofilm, even after prolonged incubation. The Δ*mshA* mutants

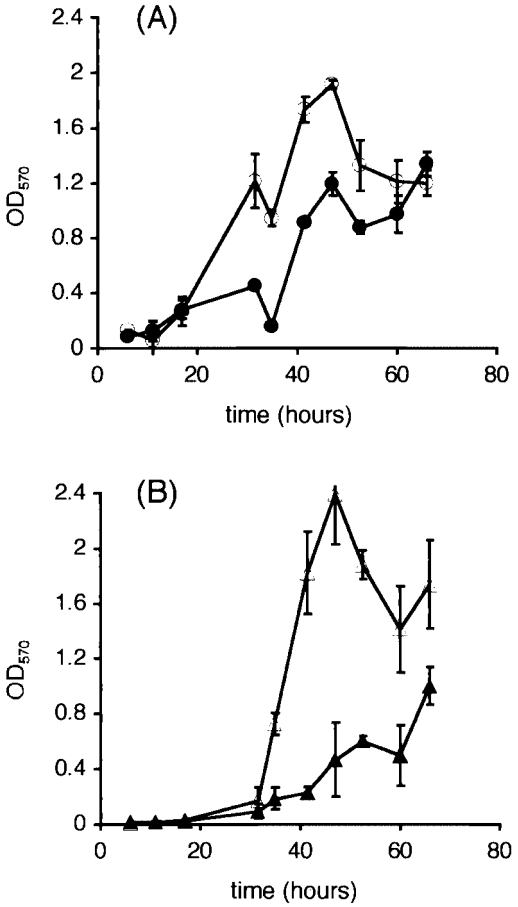

FIG. 2. Flagella and MSHA pili are required for biofilm formation in the absence of *mbaA*. Biofilm development of the indicated *V. cholerae* strains was followed in LB medium at room temperature over the course of 65 h. Cells that attached to borosilicate tubes after various periods of incubation were stained with crystal violet. The OD_{570} was measured to quantify the amount of DMSO-solubilized dye. All assays were performed in triplicates. Shown are the biofilm developments of the WT (closed circles) and Δ*mbaA* (open circles) strains (A) and those of the Δ*mshA* (closed triangles) and Δ*mbaA*-Δ*mshA* (open triangles) mutant strains (B).

were severely delayed in their initial attachment to the surface but developed normal biofilms when given enough time (27). To characterize the effects of an *mbaA* mutation on biofilm formation, we compared the biofilm formation kinetics of the WT and Δ*mbaA* strains by crystal violet staining over the course of 65 h (Fig. 2A). In addition, to determine if biofilm formation in the Δ*mbaA* mutant occurred through the same genetic pathway as in the WT, we constructed Δ*mbaA*-Δ*mshA* and Δ*mbaA*-*flgF*::Tn*10* double mutants and compared their biofilm formation kinetics to those of the single mutants over the course of 65 h (Fig. 2B and data not shown).

During the early attachment steps, WT and Δ*mbaA* strains behaved similarly, with detectable biofilms forming between 10 and 20 h of incubation (Fig. 2A). Between 20 and 30 h, the WT and Δ*mbaA* strains exhibited significantly different rates of biofilm formation. By 30 h, the Δ*mbaA* mutant showed levels of biofilm formation that were nearly threefold greater than that of the WT. By 48 h, both strains had reached their maximum levels of crystal violet staining and the difference between the two strains remained significant. Later time points indicated a decrease in the amount of cell-associated dye for both strains. These results suggest that the absence of *mbaA* does not affect the early steps of surface attachment but rather impacts later stages in biofilm development.

In both the Δ*mshA* and Δ*mbaA*-Δ*mshA* mutants, the early attachment phase was delayed: biofilms could be detected only until after 35 h of incubation, compared to 10 h for the WT (compare Fig. 2A and 2B). After the initial attachment phase, Δ*mbaA*-Δ*mshA* strain formed significantly more robust biofilms than the Δ*mshA* strain, with levels of crystal violet staining that were eightfold greater in the Δ*mbaA*-Δ*mshA* strain at 40 h (Fig. 2B). These results indicate that MSHA pili are required for the early events of biofilm formation in the Δ*mbaA* mutant but that they are not involved in the later stages of the development of the mutant biofilm.

Both the *flgF*::Tn*10* and Δ*mbaA*-*flgF*::T*n10* strains were unable to develop a biofilm over the course of 65 h of observation (data not shown). In addition, we found that Δ*mbaA* strains displayed flagellar motility comparable to that of the WT, as assayed on 0.3% agar plates (data not shown). These results indicate that the loss of *mbaA* did not bypass the requirement for a flagellum.

Taken together, these data show that both the MSHA pili and the flagellum are required for the early events of biofilm formation in the Δ*mbaA* mutant, indicating that biofilm formation in the Δ*mbaA* strain occurs through the same pathway as in the WT. Furthermore, our results suggest that the absence of *mbaA* affects later stages of biofilm development.

The increased-biofilm phenotype of Δ*mbaA* depends on the production of EPS. While flagella and pili are required for the initial attachment of the *V. cholerae* cells onto the surface, EPS production is essential at later stages of biofilm formation (26, 27). To determine whether the enhanced biofilm formation observed in the Δ*mbaA* mutant was dependent on the production of EPS, we constructed mutants of *V. cholerae* N16961 carrying an insertion in the *vps59* gene in the WT or Δ*mbaA* background (Table1). *vps59* belongs to a cluster of genes that are involved in producing the EPS that is essential for biofilm development and for the rugose colony morphology of *V. cholerae* El Tor (1, 30) and O139 (28). We followed the biofilm development of each of these strains by crystal violet staining and by microscopy (Fig. 3). Neither mutant formed biofilms, even after 48 h of incubation, as determined by crystal violet staining (Fig. 3, right panel). In agreement with previous observations (26, 28), the *vps59*::*pGp704* mutant attached as a monolayer on the glass surface but never developed into mature microcolonies, as determined by phase contrast microscopy (Fig. 3, left panels). Moreover, the absence of *mbaA* did not suppress this defect in biofilm formation, as both the *vps59*::*pGp704* and Δ*mbaA*-*vps59*::*pGp704* mutants formed only a monolayer on the glass surface after 48 h. Similar results were obtained by following the biofilm development of the *vpsR*::*pGp704* single mutant in parallel with that of the Δ*mbaA*-*vspR*::*pGp704* double mutant. The *vpsR* gene encodes a response regulator that is required for the expression of the *vps* cluster (31). No biofilm was formed in the absence of the *vpsR* gene, regardless of whether the strain harbored a mutant

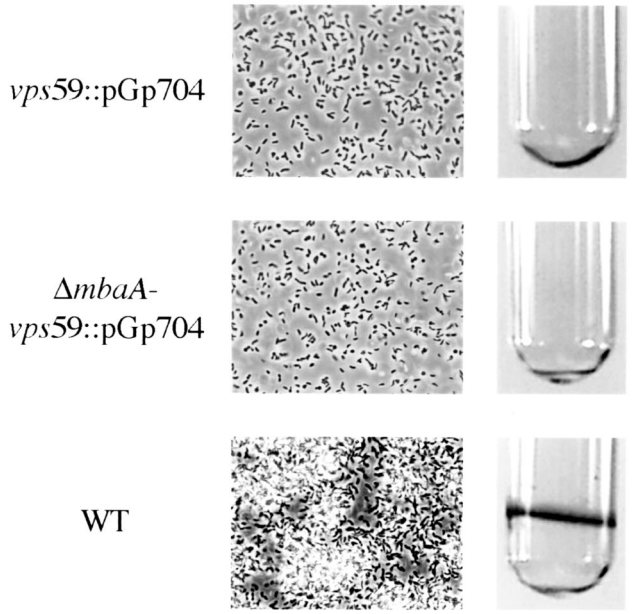

FIG. 3. The production of EPS is required for biofilm formation in the absence of *mbaA*. WT, *vsp59*::*pGp704*, and Δ*mbaA-vps59*::*pGp704* mutant cells of *V. cholerae* N16961 were incubated in LB broth at room temperature for 40 h. Phase contrast micrographs of biofilms formed on borosilicate coverslips are shown in the left panels. Crystal violet-stained borosilicate tubes are shown in the right panels.

or WT allele of *mbaA* (data not shown). These results show that the production of EPS is required for the development of a biofilm in the absence of MbaA.

The results presented this far show that the disruption of *mbaA* does not trigger an alternative pathway for biofilm formation and suggest that the *mbaA* gene plays a role during the maturation of the biofilms, when the three-dimensional architecture is formed and maintained.

The *mbaA* gene is important for the maintenance of biofilm architecture. In order to learn more about the role of *mbaA* in biofilm maturation, we sought to compare the spatial distribution of cells within the biofilms by microscopy analyses (Fig. 4). WT and Δ*mbaA* mutant strains of *V. cholerae* N16961 expressing the *gfp* gene from the chromosomal *lac* promoter were incubated with borosilicate coverslips in LB medium at room temperature, and samples were observed periodically by using a phase contrast microscope. No differences were seen between WT and Δ*mbaA* strains during the first 8 h, when the initial steps of cell attachment occurred (Fig. 4A). However, at later time points, the Δ*mbaA* cells were much more abundant than the WT bacteria on the glass surface. After 40 h, the Δ*mbaA* cells covered the entire surface, while the WT microcolonies remained scattered (Fig. 4B). We also characterized the three-dimensional architecture of mature biofilms by using confocal scanning laser microscopy (Fig. 4C). In these experiments, *gfp*-tagged WT and Δ*mbaA* cells were incubated on borosilicate coverslips for 65 h. We found that biofilms reached their maximum thickness at 65 h under these experimental conditions instead of reaching the 48-h peak observed when biofilms were grown in borosilicate glass tubes (Fig. 2A). As shown in Fig. 4C, the WT biofilms formed in this static system showed the characteristic architecture of pillars of cells interspersed by water channels, which are typical of mature biofilms formed in continuous flow systems (5). In contrast, the mutant biofilms lacked these architectural details. Instead, they covered the entire surface and had no visible fluid-filled channels. These data demonstrate that the absence of *mbaA* does not affect the initial attachment events but that it promotes the accumulation of a larger amount of biomass on the surface at later stages of development, resulting in dramatic differences in the architecture of the mature biofilms. The *mbaA* gene therefore appears to be essential for the maintenance of the three-dimensional structure of the biofilms.

The *mbaA* gene appears to control the amount of extracellular matrix within the biofilms. A key element of the architecture of the biofilms is the presence of an extracellular matrix surrounding the cells (22). We therefore sought to compare the amount of matrix material in the WT and mutant biofilms by SEM (Fig. 5). WT and Δ*mbaA* mutant strains of *V. cholerae* N16961 were incubated with borosilicate coverslips during 65 h in LB medium at room temperature, and samples were observed after fixation by using a scanning electron microscope. The extracellular matrix of the biofilms can be visualized on the micrographs on Fig. 5 as a white fibrous network among the cells. In the WT biofilms, a small amount of that material was detected among the discrete microcolonies. In contrast, the surface of the Δ*mbaA* biofilms was covered with a layer of contiguous bacterial cells embedded within a much more abundant polymeric matrix. These data suggest that the absence of the *mbaA* gene results in the overproduction of some extracellular polymeric substance that accumulates within the matrix of the mutant biofilms.

In order to gain some insight into the possible function of the *mbaA* product, we analyzed the sequence of the gene. The 2,376-bp *mbaA* gene is predicted to encode a protein (MbaA) of 791 amino acids and 90.8 kDa. Two putative transmembrane domains were identified by using the Kyte Doolittle hydropathy scale (12). Sequence comparisons of MbaA using the PSI-BLAST (2) revealed that the carboxy terminal region contained the so-called GGDEF and EAL domains (9). The roles of the GGDEF and EAL domains remain to be elucidated. Nonetheless, it has been suggested that they might constitute a novel widespread bacterial signal transduction system, based on the presence of these domains in over 50 putative sensor-regulator proteins (9).

Interestingly, the GGDEF domain has been found in several proteins involved in the regulation of extracellular cellulose production in various organisms (19); among them are PdeA and Dgc from *Gluconacetobacter xylinus* (also known as *Acetobacter xylinus*), which also contain an EAL domain (23). The other cellulose biosynthesis regulators of the GGDEF family are AdrA from *Salmonella enterica* serovar Typhimurium and *E. coli* (18, 32), CelR2 from *Rhizobium leguminosarum* bv. trifolii (3), and WspR from *Pseudomonas fluorescens* (21).

The chemical nature of the EPS component of the *V. cholerae* biofilm matrix remains unknown. Given that cellulose is a common component of bacterial biofilms (19) and that MbaA is similar to several cellulose biosynthesis regulators, it could be possible that MbaA controls the biosynthesis of extracellular cellulose production in *V. cholerae* and thereby plays a role

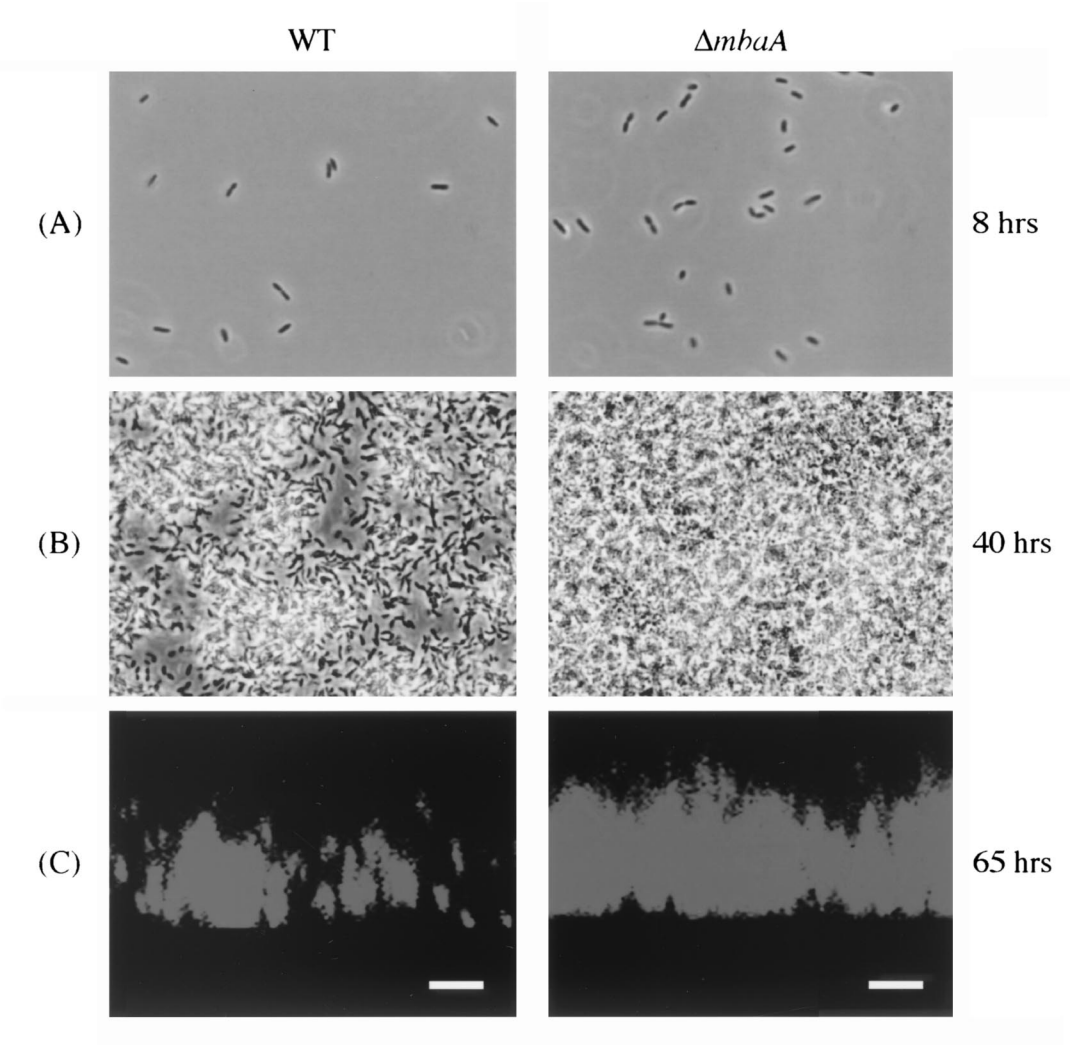

FIG. 4. The absence of *mbaA* leads to alterations in the biofilm architecture. WT and *gfp*-tagged Δ*mbaA* mutant cells of *V. cholerae* N16961 were incubated with borosilicate coverslips in LB broth at room temperature. Biofilm formation on the coverslips was observed at the indicated times. Shown are phase contrast micrographs of biofilms formed after 8 (A) and 40 h (B) and confocal scanning laser micrographs of biofilms formed after 65 h of incubation (C). Micrographs represent optical sections in the *x-z* plane, with the substratum located at the bottom. Bar, 5 μm.

in biofilm maturation. However, several lines of evidence suggest that extracellular cellulose is not produced by *V. cholerae*: neither the WT nor the *mbaA* mutant strains had the ability to bind Congo red and calcofluor, two dyes known for their characteristic staining of cellulose (results not shown) (19). In addition, homologues of the conserved *bcs* cellulose biosynthetic genes (19) are not present in the *V. cholerae* genome (10). Lastly, all of our efforts to quantify differences in the amounts of EPS produced by the WT and Δ*mbaA* strains were unsuccessful. Consistent with a prior report (30), the levels of EPS produced by our WT *V. cholerae* El Tor strain were below the level of detection of standard EPS assays, such as ruthenium red staining and/or anthrone reaction. Further studies will therefore be necessary in order to identify the specific matrix component(s) whose production is controlled by MbaA protein in *V. cholerae*.

In summary, we have identified a novel *V. cholerae* gene which plays a role in the development and maintenance of the

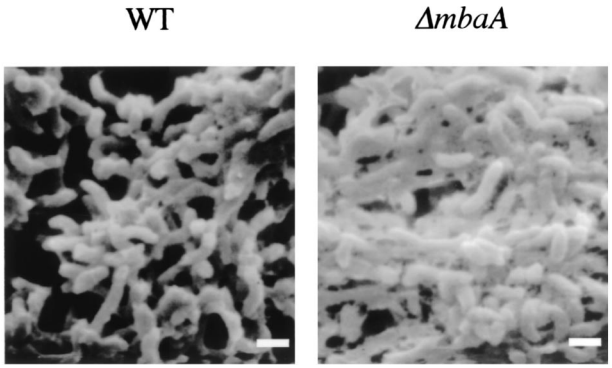

FIG. 5. The absence of *mbaA* leads to accumulation of a larger amount of biofilm matrix material. WT and Δ*mbaA* mutant strains of *V. cholerae* N16961 were incubated with borosilicate coverslips during 65 h in LB medium at room temperature. Biofilms were observed after fixation by SEM. The extracellular matrix of the biofilms appears on the micrographs as a white fibrous network among cells. Bar, 1 μm.

highly organized three-dimensional architecture of *V. cholerae* El Tor biofilms. We have demonstrated that although the absence of *mbaA* did not significantly affect the early steps of biofilms formation, it led to the accumulation of a greater amount of matrix material, resulting in biofilms that lacked the typical structure consisting of pillars of cells separated by fluid filled channels. The exact nature of the overproduced matrix component(s) remains to be determined by further biochemical characterization.

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