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The Sodium-Driven Flagellar Motor Controls Exopolysaccharide Expression in *Vibrio cholerae*

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Vibrio cholerae **causes the life-threatening diarrheal disease cholera. This organism persists in aquatic environments in areas of endemicity, and it is believed that the ability of the bacteria to form biofilms in the environment contributes to their persistence. Expression of an exopolysaccharide (EPS), encoded by two *vps* gene clusters, is essential for biofilm formation and causes a rugose colonial phenotype. We previously reported that the lack of a flagellum induces *V. cholerae* EPS expression. To uncover the signaling pathway that links the lack of a flagellum to EPS expression, we introduced into a rugose *flaA* strain second-site mutations that would cause reversion back to the smooth phenotype. Interestingly, mutation of the genes encoding the sodium-driven motor (*mot*) in a nonflagellated strain reduces EPS expression, biofilm formation, and *vps* gene transcription, as does the addition of phenamil, which specifically inhibits the sodium-driven motor. Mutation of *vpsR*, which encodes a response regulator, also reduces EPS expression, biofilm formation, and *vps* gene transcription in nonflagellated cells. Complementation of a *vpsR* strain with a constitutive *vpsR* allele likely to mimic the phosphorylated state (D59E) restores EPS expression and biofilm formation, while complementation with an allele predicted to remain unphosphorylated (D59A) does not. Our results demonstrate the involvement of the sodium-driven motor and suggest the involvement of phospho-VpsR in the signaling cascade that induces EPS expression. A nonflagellated strain expressing EPS is defective for intestinal colonization in the suckling mouse model of cholera and expresses reduced amounts of cholera toxin and toxin-coregulated pili in vitro. Wild-type levels of virulence factor expression and colonization could be restored by a second mutation within the *vps* gene cluster that eliminated EPS biosynthesis. These results demonstrate a complex relationship between the flagellum-dependent EPS signaling cascade and virulence.**

Vibrio cholerae causes the diarrheal disease cholera. This organism is introduced into human populations through the ingestion of contaminated food or water. Within the human, it colonizes the small intestine through the action of a type IV pilus (TCP) and expresses cholera toxin (CT), which causes the electrolyte imbalance and profuse watery diarrhea that is characteristic of this disease. The expression of TCP and CT is coordinated through a complex regulatory cascade that is referred to frequently as the ToxR regulon (for reviews, see references 24 and 31).

V. cholerae is a natural inhabitant of the aquatic environment. Epidemic strains can be found in both fresh- and saltwater locations in areas of endemicity and are the cause for the initiation of new cholera epidemics. *V. cholerae* can form biofilms in the laboratory, and it is believed that this is a likely persistent form of the bacteria within the environment, since biofilms are more resistant to environmental stresses, e.g., chlorine and antibiotics (41, 44). A great deal of interest and research has recently been focused on *V. cholerae* biofilm formation, which can be considered a primitive developmental process.

V. cholerae biofilm development is dependent upon the expression of an exopolysaccharide (EPS). Expression of EPS is believed to occur after the bacteria have attached to an abiotic surface and formed microcolonies, and the EPS allows the bacteria to build three-dimensional structures characteristic of mature biofilms (41, 44). However, natural phase variation can also occur upon passage in the laboratory, which leads to an altered wrinkled colonial variant referred to as a rugose variant, and the rugose phenotype is due to EPS expression (44). The rugose phenotype also occurs in some strains with the inactivation of *hapR* (a *luxR* homologue) (15) and in some strains with the inactivation of flagellar genes (42) (see below). Two large operons encode the *vps* genes necessary for EPS expression (44). A response regulator, VpsR, which has homology with σ^{54}-dependent activators (21), has been identified as a positive regulator of *vps* gene transcription (43), while a CytR homologue has been identified as a repressor of *vps* gene transcription (11). The exact manner in which these factors stimulate EPS expression has not yet been elucidated, but some details of the HapR-dependent signaling cascade have recently been reported.

V. cholerae has multiple signaling cascades that respond to quorum-dependent molecules and ultimately converge on regulating the phosphorylation state of the response regulator LuxO (28, 46), which has homology with σ^{54}-dependent activators (21). LuxO (presumably in the phosphorylated state, as mimicked by constitutive mutant forms [8, 36]) exerts an effect on virulence factor expression by repressing the expression of HapR, which in turn acts as a repressor of the TcpP/ToxR virulence cascade. Thus, CT and TCP expression is reduced in a *luxO* strain and elevated in *hapR* and *luxO hapR* strains (28, 46). The LuxO/HapR quorum-dependent signaling cascade

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also regulates the expression of EPS and biofilm formation (8, 36, 45). HapR represses *vps* gene transcription and biofilm formation, and thus *hapR* strains express elevated amounts of EPS (and exhibit the rugose phenotype, as mentioned above) and form thicker biofilms. The authors of these previous studies have suggested that quorum sensing regulates biofilm development in an unusual manner, in that high cell density (represented by unphosphorylated LuxO and/or high levels of HapR) would appear to promote the dissolution of biofilms; high cell density would also appear to promote the cessation of virulence factor expression by the same rationale. We have found (see below) that the HapR-dependent pathway appears to control EPS expression in a subset of strains, while a flagellum-dependent pathway controls EPS expression in another distinct subset of strains.

Mutations that disrupt flagellar synthesis in the O139 strain MO10 cause elevated EPS expression (and a rugose phenotype) (42). Interestingly, the expression of EPS also causes a decrease in O139 intestinal colonization, and this decrease is specifically due to EPS expression rather than to a lack of motility. Zhu and Mekalanos (45) also found a defect in intestinal colonization of a biofilm-associated *hapR* strain, suggesting that EPS expression interferes in some manner with *V. cholerae* virulence. We have further investigated the flagellum-dependent signaling cascade that controls EPS expression and have found that the sodium-driven motor and active VpsR (presumed to be phospho-VpsR) play important roles in signal transduction that leads to EPS expression. Moreover, we have found that there is a complex relationship between EPS expression, motility, and intestinal colonization.

MATERIALS AND METHODS

Plasmid construction. The Δ*flaA*::Cm, Δ*flrA*::Cm, Δ(*flrBC*)::Cm, pGP704 *'motY'*, pGP704 *'vpsF'*, pGP704 *'rpoN'*, pGP704 *'vpsR'*, and pGP704 *'motB'* plasmids have been described previously (7, 19–21, 42). The Δ*hapR*::Kn, Δ*motA*::Kn, Δ*motX*::Kn, Δ*vpsR*, Δ*fliF*, Δ*fliA*, and Δ*flhF* plasmids were constructed by using the same general strategy. A ~500-bp fragment 5′ of the deletion was PCR amplified with the corresponding primers (primers 1 and 2 [Table 1]) and then cleaved with EcoRI and BamHI or HindIII and ligated into the corresponding sites in pWSK30 (40). A ~500-bp fragment 3′ of the deletion was PCR amplified with the corresponding primers (primers 3 and 4 [Table 1]); digested with BamHI or EcoRI and XbaI, HindIII, or SalI; and ligated into the plasmids that already contained the 5′ fragment of the corresponding gene. The Δ*vpsR*, Δ*fliF*, Δ*fliA*, and Δ*flhF* mutations were designed to be in-frame deletions. The plasmids containing Δ*motX*, Δ*motA*, and Δ*hapR* were then digested with BamHI or EcoRI, and the BamHI or EcoRI Kan^r fragment from pUC4K (Pharmacia) was ligated into this site. Finally, the constructs were ligated into pKEK229 (4), a *pir*-dependent derivative of pCVD442 (5), which resulted in the plasmids listed in Table 2.

The *fliD*::Cm construct was made by PCR amplifying a *'fliD'* fragment with primers FliD1 and FliD2, digesting with BamHI and HindIII, ligating into the corresponding sites in pWSK30 (40), then digesting with EcoRI, which cleaves at a site within the *fliD* sequence, and ligating to an MfeI fragment containing Cm^r (19); the *fliD*::Cm^r fragment was subsequently ligated into pKEK229 as described above. Plasmid pKEK370 was constructed by digesting the 3′ fragment used to generate Δ*hapR* (see above; amplified with HapR3 and HapR4) with EcoRI and SalI and ligating into pGP704 (29) digested similarly; this creates a suicide plasmid with an internal *'hapR'* fragment used to insertionally inactivate the gene. The *vps* promoter transcriptional fusion plasmids were made by PCR amplification with the primer pairs VC0916p1 and -2 and VC0934p1 and -2, digestion with EcoRI and BamHI, and ligation into the corresponding sites in pRS551 (32).

The D59A and D59E alleles of *vpsR* were constructed by a two-step PCR technique in which overlapping PCR fragments containing the mutation of interest were generated and then used as a template in a second PCR. In the first step, two separate fragments were generated by PCR amplification with primers VpsRregionD and VpsRD59A Up or VpsRD59E Up and with primers VpsRregionU and VpsRD59A Down or VpsRD59E Down. In the second step, the two fragments corresponding to the mutation to be generated were used as a template in a second PCR amplification with primers VpsRregionD and VpsRregionU. The corresponding wild-type *vpsR* allele was generated by a single PCR amplification with VpsRregionD, VpsRregionU, and MO10 chromosomal DNA. These amplicons were digested with BamHI and HindIII and then ligated into pWSK30 digested similarly to form plasmids which express the various *vpsR* alleles from the native promoter in a low-copy-number vector.

TABLE 1. Oligonucleotide primer sequences

Primer	Sequence (5′→3′)
HapR1	GCGGATCCGCGACCTCTTGCTCAGAAATC
HapR2	GCGAATTCGCGTTTTTCGATTGATGCGTC
HapR3	GCGAATTCCAAGTCTCCGTTGCAACAGTG
HapR4	GCGCGTCGACGCTGGCCATGTTATCGACATC
MotX1	GCGAATTCGCAAAAACGCTGGCTGAACTG
MotX2	GCGGATCCGGAAGCGGCTACCGTTCGTAG
MotX3	GCGGATCCCGAGCCAAACGCCGAGAAACG
MotX4	GCACTAGTTACAGCTACATTCCTGACAAAG
MotA1	GCGAATTCTCGCCACTTTCTAGCTGTTCG
MotA2	GCGGATCCAACCAGTGTTGCTAAATCCAC
MotA3	GCGGATCCGACGGTGTTTTAGCGATTCAAG
MotA4	GCACTAGTGAATTCCAAGGTTTGTTGGGTG
FliD1	GCGGATCCGATATTATCCGTGGAATCAATGGT
FliD2	GCAAGCTTCGATAAGGTCGCCACCCCATCCAG
FliA1	GCGGATCCAGCAAAGAACATCAAGTTCAAC
FliA2	GCGAATTCGATACGCTTAACCAATACAGAG
FliA3	GCGAATTCGAAATTGGTGAGGTACTTGGAG
FliA4	GCGAAGCTTAGCGGCTTCGATGATTTGCTCAC
FlhF1	GCGGATCCATGGAAATAAAACGATTTTTTGC CAAG
FlhF2	ATTGCTCATGCTGCAGTGCATCGGCATCTTCTT GCAG
FlhF3	TGCCGATGCACTGCAGCATCAGCAATTGTCGA TTTATG
FlhF4	GCGCGTCGACCTAGAATCTCTCTGAATCACTG
FliF1	GCGAAGCTTGAATGGAATTTTTGAGTCAAAC
FliF2	GCGAATTCGCCTGCCATCAAGGCATGCTC
FliF3	GCGAATTCCAGGTTCTGATCGGCACCGTAG
FliF4	GCTCTAGATTAGCCATTTTGCATCCAGTTC
VpsR1	GCGGATCCAGAAATAATCGTGCCAAGTCG
VpsR2	GCGAATTCGGTATCTGAACTGAGCTGCGC
VpsR3	GCGAATTCCTGATCGATGGTGATTTTAAC
VpsR4	GCGAAGCTTCAAAACTTAGAAGTTTTCATC
VC0916p1	GCGAATTCGCAGAGCTCAACCATGAGCTG
VC0916p2	GCGGATCCCTCGAGTACTGATAAACCTTTAAC CTTC
VC0934p1	GCGAATTCATATTGTTCTGTTTTTCCTTTC
VC0934p2	GCGGATCCCTCGAGTATTCTGCTTTTTTCCTT CATC
VpsRregionD	GCGAAGCTTTGAACGATGCTGAAGACCAAG
VpsRregionU	GCGGATCCCCGCAGCCTAATAAGAGGTTAC
D59A Up	CATGGCTTAAAGCCACAATACC
D59A Down	GGTATTGTGGCTTTAAGCCATG
D59E Up	CATGGCTTAACTCCACAATACC
D59E Down	GGTATTGTGGAGTTAAGCCATG

All PCRs were performed with either KOD HiFi DNA polymerase or XL DNA polymerase (Novagen), with MO10 chromosomal DNA as a template. All primer sequences were designed based on the complete *V. cholerae* genome sequence (13).

Bacterial strains and media. The *V. cholerae* strains used in this study are listed in Table 2. Strain construction with pGP704 and pCVD442 derivatives has been described previously (5, 19, 29); the correct construction of all strains was verified by PCR, sequencing, and/or Southern blot analysis. Bacteriophage CP-T1ts-mediated transduction was used to construct some strains, and the protocol of Hava and Camilli (12) was followed. *Escherichia coli* strain DH5α (9) was used for plasmid construction, and SM10λ*pir* (29) was used for propagation of *pir*-dependent plasmids and conjugation into *V. cholerae*.

V. cholerae were grown in Luria-Bertani (LB) broth, supplemented with the following concentrations of antibiotics, when appropriate: 2 μg of chloramphen-

TABLE 2. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype or description	Source or reference
V. cholerae strains		
P27459	Wild-type O1 El Tor	30
KKV1101	P27459 Δ*flaA*::Cm Δ*lacZ*	This study
KKV1507	P27459 Δ*hapR*::Kn Δ*lacZ*	This study
C6706	Wild-type O1 El Tor	35
KKV1599	C6706 Δ*flaA*::Cm	This study
KKV1612	C6706 Δ*hapR*::Kn	This study
C6709	Wild-type O1 El Tor	37
KKV89	C6709 Δ*flaA*::Cm	This study
KKV1562	C6709 Δ*hapR*::Kn	This study
2740-80	Wild-type O1 El Tor	30
KKV1608	2740-80 Δ*flaA*::Cm	This study
KKV1610	2740-80Δ*hapR*::Kn	This study
N16961	Wild-type O1 El Tor	13
KKV832	N16961 Δ*flaA*::Cm	This study
KKV1198	N16961 *hapR*::pGP704 (Amp^r)	This study
E7946	Wild-type O1 El Tor	26
KKV1601	E7946 Δ*flaA*::Cm	This study
KKV1611	E7946 Δ*hapR*::Kn	This study
A1552	Wild-type O1 El Tor	F. Yildiz
KKV1600	A1552 Δ*flaA*::Cm	This study
KKV1613	A1552 Δ*hapR*::Kn	This study
MO10	Wild-type O139	39
KKV927	MO10 *motY*::pGP704 (Amp^r)	42
KKV955	MO10 Δ*flaA*::Cm Δ*lacZ*	42
KKV1004	MO10 Δ*flrA*::Cm Δ*lacZ*	This study
KKV1026	MO10 Δ(*flrBC*)::Cm Δ*lacZ*	This study
KKV1028	MO10 *vpsF*::pGP704 (Amp^r)	42
KKV1029	MO10 Δ*flaA*::Cm *vpsF*::pGP704 (Amp^r)	42
KKV1029	MO10 Δ*flaA*::Cm *vpsR*::pGP704 (Amp^r)	This study
KKV1046	MO10 Δ*fliD*::Cm Δ*lacZ*	This study
KKV1082	MO10 *rpoN*::pGP704 (Amp^r)	This study
KKV1114	MO10 Δ*fliA* Δ*lacZ*	This study
KKV1184	MO10 *motB*::pGP704 (Amp^r)	This study
KKV1273	MO10 Δ*motA*::Kn Δ*lacZ*	This study
KKV1495	MO10 Δ*motX*::Kn Δ*lacZ*	This study
KKV1502	MO10 Δ*flaA*::Cm Δ*motX*::Kn Δ*lacZ*	This study
KKV1504	MO10 Δ*flaA*::Cm *motY*::pGP704 (Amp^r) Δ*lacZ*	This study
KKV1505	MO10 Δ*hapR*::Kn Δ*lacZ*	This study
KKV1520	MO10 Δ*flaA*::Cm Δ*motA*::Kn Δ*lacZ*	This study
KKV1536	MO10 Δ*fliF* Δ*lacZ*	This study
KKV1559	MO10 Δ*flaA*::Cm *motB*::pGP704 (Amp^r) Δ*lacZ*	This study
KKV1561	MO10 Δ*flhF*	This study
KKV1578	MO10 Δ*vpsR* Δ*lacZ*	This study
KKV1579	MO10 Δ*flaA*::Cm Δ*vpsR* Δ*lacZ*	This study
KKV1842	MO10 Δ*motX*::Kn Δ*vpsR* Δ*lacZ*	This study
KKV1843	MO10 Δ*flaA*::Cm Δ*motX*::Kn Δ*vpsR* Δ*lacZ*	This study
KKV1862	MO10 *vpsR*::pGP704 (Amp^r)	This study
Plasmids		
pKEK229	R6K *ori sacB mob* Amp^r	4
pKEK428	Δ*motA*::Kn in pKEK229	This study
pKEK436	Δ*motX*::Kn in pKEK229	This study
pKEK479	Δ*hapR*::Kn in pKEK229	This study
pKEK311	*fliD*::Cm in pKEK229	This study
pKEK374	Δ*fliA* in pKEK229	This study
pKEK518	Δ*vpsR* in pKEK229	This study
pKEK516	Δ*flhF* in pKEK229	This study
pKEK424	Δ*fliF* in pKEK229	This study
pGP704	R6K *ori mob* Amp^r	29
pCG1050	*′motB′* in pGP704	7
PKEK129	*′vpsR′* in pGP704	21
pKEK328	*′motY′* in pGP704	42
pKEK349	*′vpsF′* in pGP704	42
pKEK370	*′hapR′* in pGP704	This study
pRS551	Transcriptional *lacZ* fusion vector, Amp^r Kan^r	32
pKEK343	*VC0916* promoter in pRS551	This study
pKEK396	*VC0934* promoter in pRS551	This study
pWSK30	pSC101 *ori* Amp^r	40
pKEK725	*vpsR* WT allele in pWSK30	This study
pKEK662	*vpsR* D59A allele in pWSK30	This study
pKEK663	*vpsR* D59E allele in pWSK30	This study

icol per ml for smooth strains, 20 μg of chloramphenicol per ml for rugose strains, 25 μg of kanamycin per ml, 100 μg of streptomycin per ml, and 50 μg of ampicillin per ml. For counterselection with *sacB*-containing plasmids, LB broth without NaCl and with 10% sucrose was used. For virulence-factor-inducing conditions, strains were grown in modified AKI medium (1.5% tryptone, 0.4% yeast extract, 0.5% NaCl) overnight at 37°C and then normalized to identical densities based on the optical density at 600 nm (OD_{600}), and 100 μl was inoculated into a 10-ml tube filled completely to the top with modified AKI medium. These tubes were incubated statically at 37°C for 4 h, and then 5 ml was removed and added to a 25-ml culture tube, which was then incubated on a roller drum at 37°C for 18 to 20 h.

Biofilm assay. The basic biofilm protocol used previously (42) was followed, with some modifications. Strains were grown overnight in LB broth and then normalized to identical densities based on OD_{600}, and 5 μl was inoculated into 500 μl of LB broth in 10-ml borosilicate glass tubes. The tubes were then incubated statically at 30°C for 22 h. The tubes were rinsed with distilled water, incubated with 600 μl of 0.1% crystal violet for 30 min, and rinsed again with distilled water. One milliliter of dimethyl sulfoxide was then added, the tube was vortexed and allowed to stand for 10 min, and the OD_{570} measured.

β-Galactosidase assays. Δ*lacZ V. cholerae* strains were transformed with plasmids pKEK343 and pKEK396 (pBR322 derivatives) (33) and then grown in LB broth, harvested at OD_{600} of ~0.2 to 0.4, permeabilized with chloroform and sodium dodecyl sulfate, and assayed for β-galactosidase activity according to the method of Miller (27).

In vitro and in vivo virulence assays. CT was measured by GM_1-ganglioside enzyme-linked immunosorbent assay, as described previously (34). For TCP detection, whole-cell lysates were matched by OD_{600}, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and probed with TcpA polyclonal antiserum by utilizing ECL detection reagent (Amersham Pharmacia). Mouse intestinal competition assays to measure colonization have been described previously (7). The inocula consisted of ~10^5 wild-type and ~10^5 mutant organisms.

RESULTS

EPS expression is regulated differently in *V. cholerae* O1 El Tor strains. We previously reported (42) that flagellar mutations which produce nonflagellated cells caused EPS expression and a rugose colony phenotype in the *V. cholerae* O139 strain MO10. The O139 serogroup is believed to have arisen from an O1 El Tor strain following acquisition of the O139 biosynthetic gene cluster (2, 38). We therefore reasoned that a lack of flagellar synthesis would likely lead to EPS expression and a rugose phenotype in O1 El Tor strains. A mutation in *flaA*, which encodes the "core" flagellin of the flagellum, results in a nonflagellated cell (19) and causes a rugose phenotype in MO10 (Fig. 1, top panel), as previously reported (42). We introduced a Δ*flaA*::Cm mutation into a panel of O1 El Tor strains via CP-T1ts-mediated transduction (12) and found that a *flaA* mutation in the O1 El Tor strains P27459, C6706, and E7946 also caused a rugose colonial phenotype (Fig. 1, middle panel, and data not shown). However, *flaA* mutants of the O1 El Tor strains N16961, A1552, 2740-80, and C6709 maintained a smooth colony phenotype, even though these strains were nonflagellated (not shown). The MO10, P27459, and C6706 *flaA* strains were clearly rugose after 24 to 48 h of growth on LB agar, while the E7946 *flaA* strain was not obviously rugose until approximately 72 h of growth.

Jobling and Holmes (15) reported that a mutation in *hapR*, which encodes the transcriptional regulator of the HA protease gene, results in a rugose colonial phenotype in the O1 El Tor strain 3083. We introduced a Δ*hapR*::Kn mutation via CP-T1ts-mediated transduction into strains MO10, P27459, C6709, C6706, E7946, A1552, 2740-80, and N16961. The C6709, A1552, C6706, and 2740-80 *hapR* strains displayed a rugose phenotype (Fig. 1, bottom panel, and data not shown), while

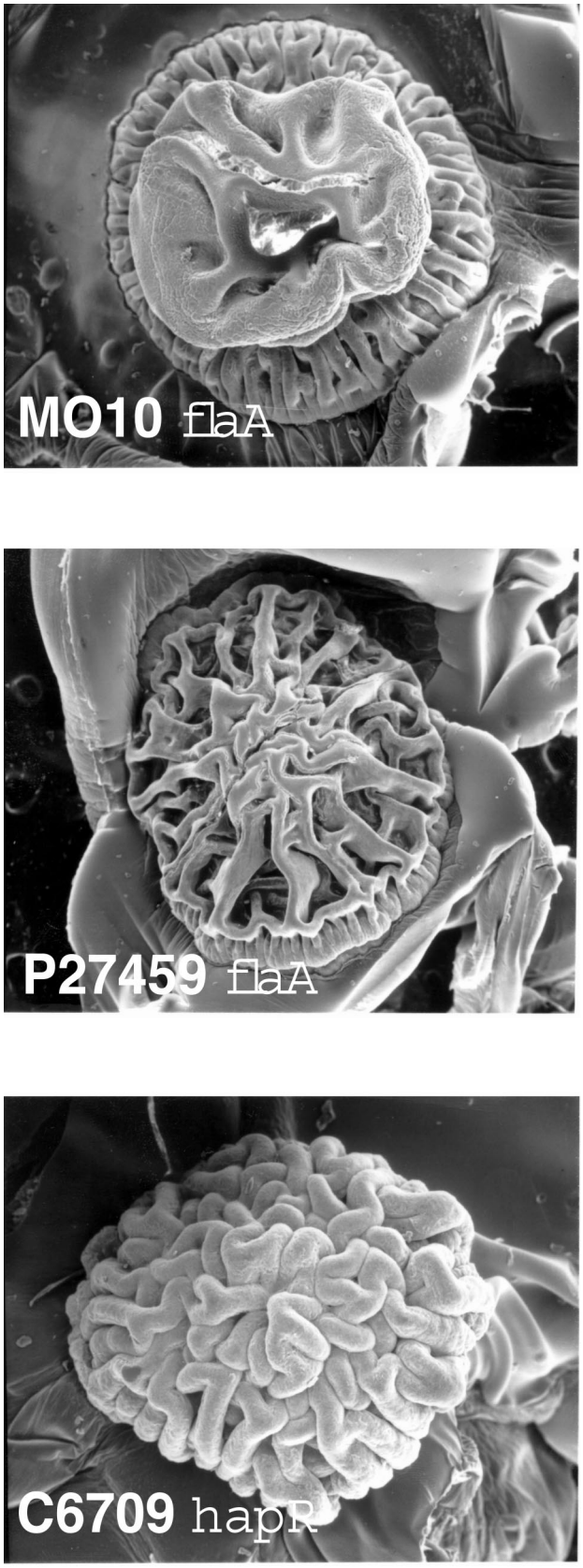

FIG. 1. Rugose colonial phenotypes. Colonies of the MO10 *flaA* (KKV955) (top), P27459 *flaA* (KKV1101) (middle), and C6709 *hapR* (KKV1562) (bottom) strains were visualized by scanning electron microscopy. The rugose colonial phenotype is well-preserved by this technique, but the underlying agar medium becomes somewhat corrugated upon dehydration.

the other *hapR* mutant strains remained smooth (not shown). The C6709, A1552, and 2740-80 *hapR* strains were clearly rugose after 24 h of growth on LB agar, while the C6706 *hapR* strain was not obviously rugose until approximately 48 h of growth. The rugose phenotype of the C6706 *hapR* strain is consistent with the enhanced biofilm development reported for this strain (8, 45).

The rugose phenotype in the P27459 *flaA* and C6709 *hapR* strains is caused by expression of the *vps* genes encoding the EPS, because the introduction of a polar mutation into one of the EPS biosynthetic gene clusters (in *vpsF*) in these strains restored a smooth phenotype (not shown), as we had shown previously for the MO10 *flaA* strain (42). While neither the *flaA* nor the *hapR* N16961 strain was rugose, it has been demonstrated previously that rugose variants of this strain can be isolated upon nutrient starvation (43). Our results demonstrate that there are apparently at least three distinct genetic pathways for rugose EPS expression in *V. cholerae* O1 El Tor strains: one initiated by a lack of flagellar synthesis (seen in the O139 strain MO10 and O1 El Tor strains C6706, P27459, and E7946), one initiated by a lack of HapR (seen in the O1 El Tor strains C6706, C6709, A1552, and 2740-80), and one independent of both flagellum- and HapR-dependent pathways (seen in spontaneous rugose variants of O1 El Tor strain N16961; this strain carries a frameshift mutation in *hapR*, so no HapR-dependent pathway was anticipated). Interestingly, both HapR- and flagellum-dependent pathways seem to be operational in strain C6706.

The HapR-dependent pathway to EPS expression in strain C6706 has been the subject of recent investigations in several laboratories (8, 45). We have undertaken a more in-depth analysis of the flagellum-dependent pathway to EPS expression, utilizing MO10 as our model strain.

Mutations in the sodium-driven motor or VpsR reduce EPS expression and biofilm formation in nonflagellated *V. cholerae*. We had previously demonstrated that a mutation in *motY*, which encodes one of the components of the sodium-driven flagellar motor, results in flagellated but nonmotile MO10 cells which have a smooth colonial phenotype and do not express EPS (42). We have subsequently constructed MO10 strains with mutations in the other three motor genes, *motA*, *motB*, and *motX*, and all three of these strains are flagellated but nonmotile and maintain a smooth phenotype (not shown). These results are consistent with our previous hypothesis that the lack of a complete flagellum, rather than a lack of motility, stimulates EPS expression. However, we had not considered an alternative possibility, namely, that perhaps the sodium-driven motor has a dual function: as an integral motility component and also as a component of the signal transduction cascade that leads to EPS expression.

Interestingly, the introduction of any of the *mot* mutations (*motA*, *motB*, *motX*, or *motY*) into a *flaA* MO10 strain results in reversion to a smooth colonial phenotype, suggestive of reduced EPS expression (Fig. 2), similar to the phenotype of a

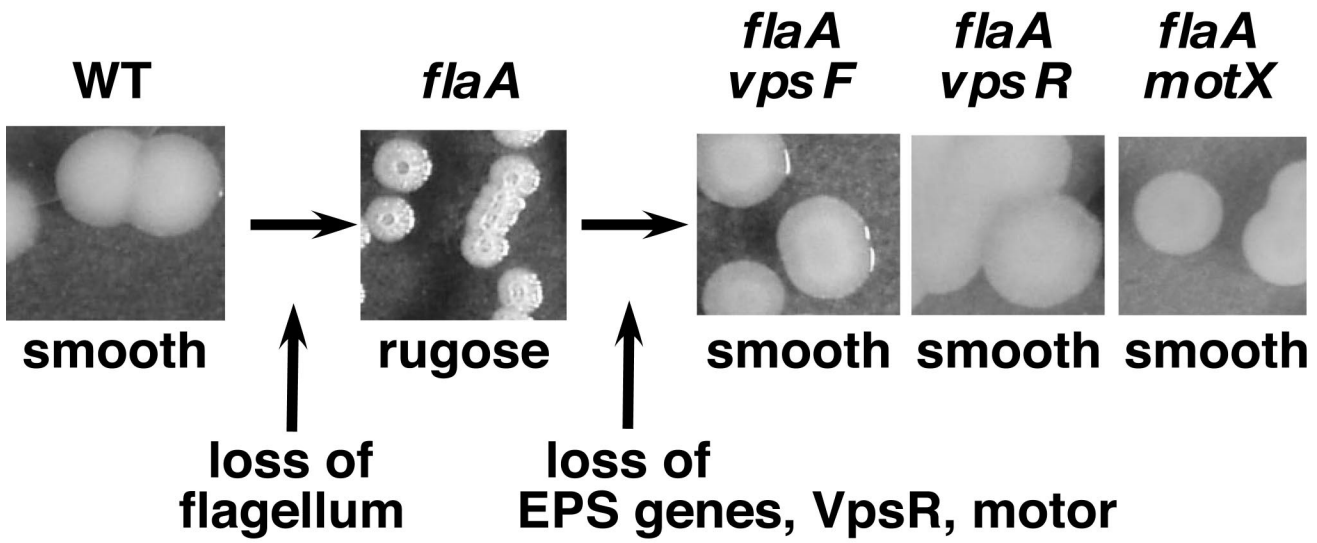

FIG. 2. Mutations in EPS, *vpsR*, and *mot* genes suppress the rugose phenotype of a *flaA* strain. Shown are photographs of the colonial phenotypes of MO10 (wild type [WT]), KKV955 (*flaA*), KKV1029 (*flaA vpsF*), KKV1579 (*flaA vpsR*), and KKV1502 (*flaA motX*).

flaA strain that has been disrupted in one of the *vps* gene clusters (*flaA vpsF*). Considering that these strains already lack a complete flagellum, this indicates that the sodium-driven motor may participate in transduction of the EPS inducing signal, in addition to its role in flagellar rotation.

The MO10 *flaA* strain can form a biofilm (Fig. 3A), as demonstrated previously (42). Presumably, high-level EPS expression in this strain overcomes any need for motility and microcolony formation, because the strain forms aggregates in solution that likely settle onto the surface and serve as microcolonies to initiate mature biofilm development. However, the *flaA motA* and *flaA motX* strains are defective for biofilm development. The *motX* and *motA* mutant strains are also defective for biofilm development, as we showed previously for a *motY* mutant strain (42). These results are consistent with an important role for the sodium-driven motor in EPS expression and biofilm formation by both flagellated and nonflagellated MO10 cells.

Yildiz et al. have identified a response-regulatory protein, VpsR, that is important for biofilm formation in the O1 El Tor strain A1552 (43). To determine whether VpsR is involved in biofilm formation in nonflagellated MO10 cells, we constructed a *flaA vpsR* MO10 strain. This strain demonstrated a smooth colonial phenotype (Fig. 2) and had greatly reduced biofilm formation (Fig. 3A). A *vpsR* mutation in a wild-type (i.e., flagellated) background also led to a reduction in biofilm formation. These results suggest an important role for VpsR in EPS expression and biofilm formation of both flagellated and nonflagellated MO10 cells.

VpsR and the sodium-driven motor are essential for high-level *vps* transcription. Yildiz and Schoolnik (44) identified a number of polysaccharide biosynthetic genes (*vps*) that are necessary for EPS expression and biofilm formation. The *vps* genes are organized in two large operons corresponding to VC0916-VC0928 and VC0934-VC0939. It was shown (43) that VpsR was necessary for high-level transcription of the two *vps*

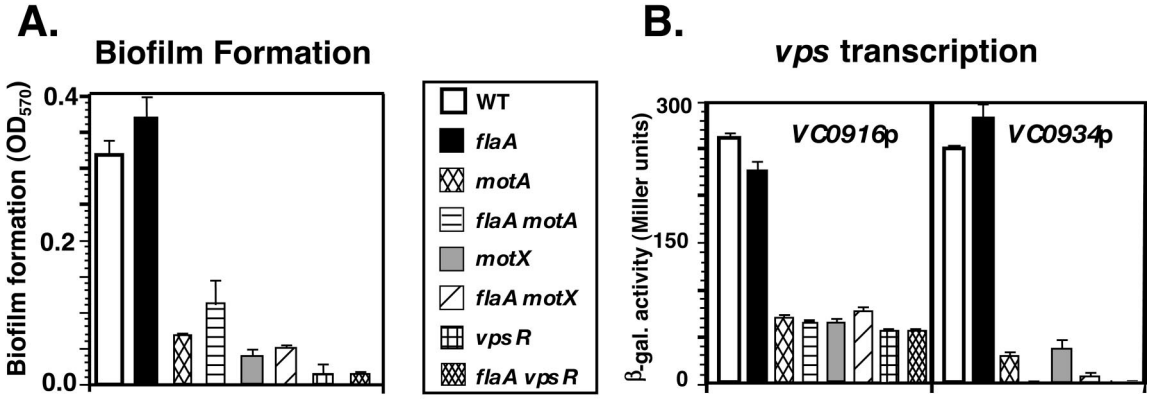

FIG. 3. The sodium-driven motor and VpsR are necessary for enhanced biofilm formation and high-level *vps* transcription. Biofilm formation (A) and VC0916p and VC0934p transcription (B) in the MO10 (wild type [WT]), KKV955 (*flaA*), KKV1273 (*motA*), KKV1520 (*flaA motA*), KKV1495 (*motX*), KKV1502 (*flaA motX*), KKV1578 (*vpsR*), and KKV1579 (*flaA vpsR*) *V. cholerae* strains were measured as described in Materials and Methods. β-gal., β-galactosidase. Error bars indicate standard deviations.

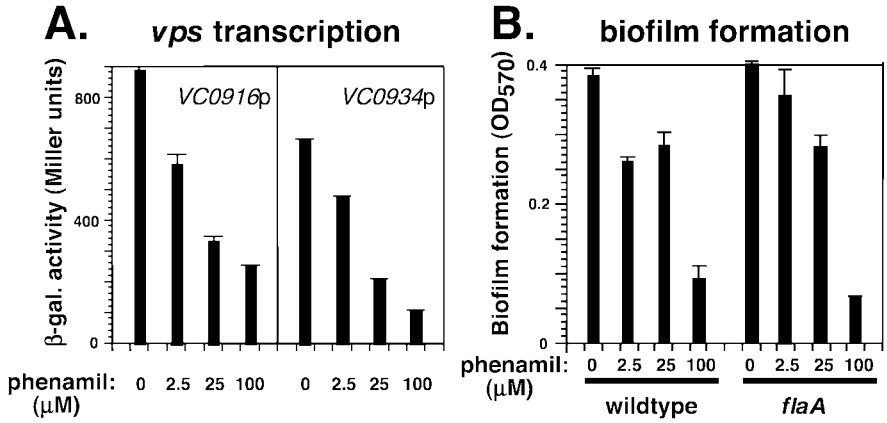

FIG. 4. Phenamil inhibits *vps* transcription and biofilm formation. (A) Transcription of VC0916p and VC0934p in the wild-type MO10 strain with increasing concentrations of phenamil was measured as described in Materials and Methods. β-gal., β-galactosidase. (B) Biofilm formation by the MO10 (wild-type) and KKV955 (*flaA*) strains was measured as described Materials and Methods. Error bars indicate standard deviations.

operons in A1552 O1 El Tor cells. To determine whether transcription of the two *vps* operons correlated with the colonial phenotype and biofilm-forming abilities of *mot* and *vpsR* MO10 strains, we measured *vps* transcription from the two *vps* operon promoters (corresponding to VC0916p and VC0934p) (Fig. 3B).

Our results showed a strong correlation between the ability of the strains to form biofilms (Fig. 3A) and high levels of *vps* gene transcription (Fig. 3B). Interestingly, the wild-type (smooth) MO10 strain had high levels of transcription of both *vps* gene clusters, which were not altered by the introduction of a *flaA* mutation (causing the rugose phenotype). This suggests that the smooth-to-rugose transition is not caused by an increase in *vps* transcription, unlike in a spontaneous rugose O1 El Tor A1552 strain (43) or the O1 El Tor C6706 *hapR* strain (45). However, transcription of both *vps* gene clusters in *motX*, *motA*, and *vpsR* mutant strains was reduced, either in a flagellated (wild-type) or nonflagellated (*flaA*) background. Our results demonstrate that high-level *vps* transcription may be necessary, but not sufficient, for EPS expression and that VpsR and the sodium-driven motor are necessary for high-level *vps* gene transcription.

Phenamil inhibits *vps* transcription and biofilm formation. Phenamil specifically poisons the sodium-driven motor and inhibits motility in *Vibrio* spp., including *V. cholerae* (16, 23). We have confirmed that phenamil reduces the motility of the wild-type (flagellated) MO10 strain in a dose-dependent manner, as determined in a motility assay (not shown). Transcription of the two *vps* operons in wild-type and *flaA* MO10 strains is inhibited by phenamil in a dose-dependent manner (Fig. 4A). Likewise, biofilm formation by wild-type and *flaA* MO10 strains is inhibited by phenamil in a dose-dependent manner (Fig. 4B). These observations are consistent with the function of the sodium-driven motor being critical for *vps* transcription and biofilm formation in both flagellated and nonflagellated MO10 cells.

Mutant forms of VpsR suggest that phosphorylation is necessary for EPS expression and biofilm formation. As shown above, the response-regulatory protein VpsR is necessary for high-level *vps* transcription, EPS expression, and biofilm formation in the MO10 O139 strain, as was shown previously for spontaneous rugose colonies of the O1 El Tor strain A1552 (43). VpsR has a response-regulatory domain in its amino terminus with the conserved aspartate residue (D59) that is predicted to be the site of phosphorylation. Because VpsR is an "orphan" response regulator (i.e., no gene encoding a histidine kinase is located nearby) and its cognate histidine kinase has not yet been identified, we have no biochemical proof that D59 is the site of phosphorylation in VpsR. However, it has been shown for numerous response regulators that an alteration of this conserved aspartate residue to an alanine (D59A) prevents phosphorylation and results in a protein that represents the unphosphorylated state. Interestingly, the substitution of a glutamate residue (D59E) can mimic aspartyl-phosphate even though it prevents phosphorylation (i.e., it can act as a "constitutive" mutation), at least in some response regulators that share homology with σ^{54}-dependent activators (e.g., VpsR, NtrC, and LuxO) (4, 6, 22); this is the "locked-on" constitutively active mutation that has been used extensively to analyze LuxO function (6, 8). Thus, a D59A allele would be predicted to behave like unphosphorylated VpsR, while a D59E allele would be predicted to behave like phospho-VpsR.

To determine the effect of substitutions at the putative phosphorylation site of VpsR on the flagellum-dependent EPS signaling pathway, we constructed *vpsR* alleles containing alanine and glutamate substitutions (D59A and D59E, respectively) and complemented Δ*vpsR*, *flaA* Δ*vpsR*, Δ*motX* Δ*vpsR*, and Δ*flaA* Δ*motX* Δ*vpsR* strains with these alleles expressed from the native *vpsR* promoter in a low-copy-number vector. We complemented these same strains with the wild-type *vpsR* allele in the same manner.

The ability of these strains to form biofilms was measured (Fig. 5). Provision of the wild-type VpsR protein expressed from the plasmid stimulated biofilm formation in both a wild-type and a *flaA* mutant background, and to approximately the same level as when the wild-type VpsR protein is expressed from the chromosome, as expected. Strains complemented with the D59A mutant VpsR behaved similarly to a Δ*vpsR* strain in both wild-type and *flaA* backgrounds; i.e., this allele fails to stimulate biofilm development. Interestingly, complementation with the D59E mutant VpsR allowed biofilm formation at a level two- to threefold greater than that for complementation with the wild-type VpsR in both wild-type

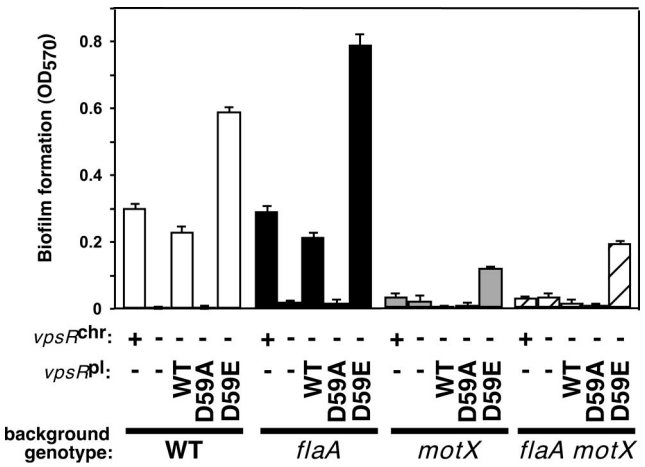

FIG. 5. Mutant forms of VpsR predicted to affect phosphorylation alter biofilm formation. Biofilm formation in the following sets of strains was measured as described Materials and Methods. (i) MO10 (wild type [WT]), KKV955 (*flaA*), KKV1495 (*motX*), and KKV1502 (*flaA motX*) contain the native *vpsR* allele on the chromosome and are designated $vpsR^{chr}$ +. (ii) KKV1578 (WT), KKV1579 (*flaA*), KKV1842 (*motX*), and KKV1843 (*flaA motX*) additionally carry an in-frame deletion within the chromosomal *vpsR* allele and are designated $vpsR^{chr}$ −. (iii) Strains KKV1578, KKV1579, KKV1842, and KKV1843 were transformed with low-copy-number plasmids carrying either the wild-type *vpsR* allele (pKEK725, WT $vpsR^{pl}$), the D59A *vpsR* allele (pKEK662, D59A $vpsR^{pl}$), or the D59E *vpsR* allele (pKEK663, D59E $vpsR^{pl}$). Error bars indicate standard deviations.

and *flaA* mutant backgrounds. Our results suggest that phosphorylation of VpsR at aspartate 59 is necessary for the flagellum-dependent EPS signaling cascade that leads to biofilm formation (due to inhibition of biofilm formation by the D59A allele) and that the D59E mutant protein is active, i.e., mimics phospho-VpsR.

Provision of the wild-type or D59A VpsR protein failed to stimulate significant biofilm formation in the *motX* or *flaA motX* background, as expected, since we have shown above that the motor is an important component of the flagellum-dependent EPS signaling cascade. Interestingly, the D59E VpsR protein stimulated biofilm development in both *motX* and *flaA motX* backgrounds, at a level 12- to 20-fold greater than that for the wild-type VpsR protein. This suggests that VpsR is unphosphorylated in the absence of the sodium-driven motor. Thus, the requirement for the sodium-driven motor for biofilm development is in part likely due to its stimulation of the phosphorylation of VpsR, which can be bypassed by a constitutive (D59E) *vpsR* mutation.

The colonial phenotypes of the nonmotile strains, indicative of the level of EPS expression, correlated with their abilities to form biofilms. The Δ*flaA* Δ*vpsR* strain complemented with either the wild-type or D59E *vpsR* allele was rugose, while complementation of this strain with the D59A *vpsR* allele resulted in a smooth phenotype (not shown). Complementation of the Δ*motX*, and Δ*flaA* Δ*motX* strains with the D59E *vpsR* allele also led to a modest rugose phenotype (rough center and smooth edges). All strains complemented with the D59A *vpsR* allele remained smooth. These results suggest that phosphorylation of VpsR is necessary for the rugose phenotype and hence for EPS expression.

Effects of VpsR and the sodium-driven motor on in vivo colonization and in vitro virulence factor expression. We have previously shown that a *flaA* strain (rugose, nonmotile) is defective for intestinal colonization in the infant mouse competition assay (42) and that this defect is specifically due to the expression of EPS, since a *flaA vpsF* mutant strain (smooth, nonmotile) colonizes similarly to the wild-type strain (Fig. 6A). Mutations in the sodium-driven motor and *vpsR* also diminish EPS expression in a nonflagellated (*flaA*) strain, as shown above. To determine whether *mot* and *vpsR* mutations can restore intestinal colonization by the *flaA* strain, similar to a *vpsF* mutation, we measured the ability of *motX*, *flaA motX*, *vpsR*, and *flaA vpsR* strains to colonize the infant mouse intestine in a competition assay (Fig. 6A).

The *motX* and *flaA motX* mutants were defective for intestinal colonization; the *motX* strain colonized similarly to a *flaA* strain, while the *flaA motX* mutant colonized worse than either single mutant strain. These strains are smooth and nonmotile and thus would be predicted to colonize the intestine similarly to the smooth, nonmotile *flaA vpsF* strain. However, the *motX* mutation likely alters sodium flux across the membrane, which Hase and Mekalanos have shown can alter virulence factor expression (10); this may explain the colonization deficiencies of these strains. The *vpsR* mutant was competent for colonization, even slightly outcompeting the wild-type strain, indicating that VpsR is not essential for intestinal colonization. However, the *flaA vpsR* mutant showed a defect for colonization that was similar to that of the *flaA* strain, demonstrating that the *vpsR* mutation, while able to disrupt EPS expression in the *flaA* strain, was unable to restore wild-type levels of colonization to the *flaA* strain.

These strains were grown under in vitro conditions that promote virulence factor expression (AKI growth conditions) (14) (Fig. 6B); the inability of mutant strains to colonize the infant mouse intestine is frequently correlated with a lack of in vitro expression of CT and TcpA, the major component of TCP. Growth of the wild-type MO10 strain under identical AKI-inducing conditions results in detectable CT and TcpA expression, and there is no detectable expression of either CT or TcpA when MO10 is grown under noninducing conditions. The in vitro CT and TcpA expression of the *flaA* and *flaA vpsF* strains could be correlated with their colonization patterns; i.e., the *flaA* strain failed to express CT or TcpA in vitro, but the introduction of the *vpsF* mutation into this strain, which abolishes EPS expression, restored wild-type levels of both CT and TcpA expression. There was also a good correlation between the very low levels of CT and TcpA expression in vitro by the *flaA motX* strain and poor colonization in vivo and between detectable levels of CT and TcpA expression in vitro by the *vpsR* strain and wild-type levels of colonization in vivo.

Interestingly, the *motX* and *flaA vpsR* strains expressed wild-type levels of CT and detectable TcpA in vitro yet colonized poorly in vivo. This discrepancy between virulence factor expression in vitro and virulence in vivo emphasizes the difficulty in replicating the intestinal environment in a test tube, as suggested by Lee et al. (25). Perhaps although the *flaA vpsR* strain is nonrugose and thus should be competent for colonization, VpsR is required to regulate some other factor that facilitates intestinal colonization by nonflagellated (but not flagellated) cells. Our results suggest a complex relationship

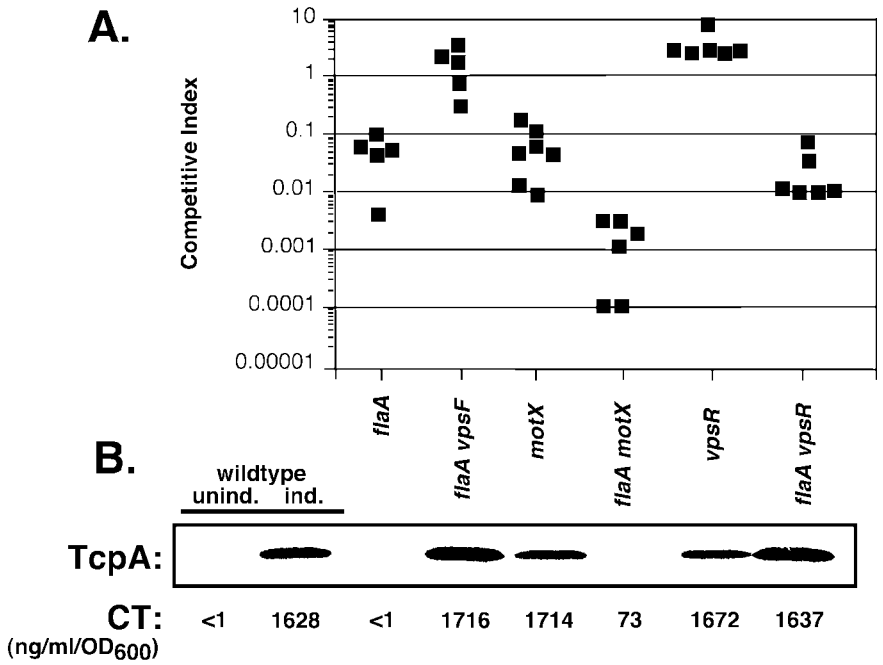

FIG. 6. Effects of the flagellum, sodium-driven motor, VpsR, and EPS on intestinal colonization and virulence factor expression. (A) Infant mouse intestinal colonization competition assay. Strains KKV955 (*flaA*), KKV1029 (*flaA vpsF*), KKV1495 (*motX*), KKV1502 (*flaA motX*), KKV1862 (*vpsR*), and KKV1029 (*flaA vpsR*) were coinoculated with MO10 perorally into infant mice at ratio of ~1:1; intestinal homogenates were recovered at 24 h postinoculation, and the CFU of wild-type and mutant strains were determined. The competitive index is given as the output ratio of mutant to wild type divided by the input ratio of mutant to wild-type; each value shown is from an individual mouse. Strains KKV955, KKV1495, KKV1502, and KKV1029 colonized the intestine significantly less than the wild-type strain ($P < 0.01$ as determined by Student's two-tailed *t* test). (B) In vitro expression of CT and TcpA. The same strains as in panel A were grown under AKI-inducing conditions, and values correspond to the strain designations in panel A. TcpA was detected by Western immunoblotting with anti-TcpA antiserum, and CT in the supernatant was measured by GM_1-ganglioside ELISA. Also shown is CT and TcpA detection for the wild-type MO10 strain grown under noninducing (unind.) and AKI-inducing (ind.) conditions.

between flagellar synthesis, motor function, EPS expression, and intestinal colonization.

DISCUSSION

The ability of *V. cholerae* to form biofilms has been postulated to contribute to cholera epidemics by enhancing environmental persistence of the organisms in aquatic reservoirs. Expression of the EPS encoded by the *vps* genes is necessary to form the mature biofilms seen when *V. cholerae* is grown under the laboratory conditions utilized in this study (42, 44). A recent report (17) has shown that this particular EPS may be utilized only by the O139 strain MO10 found in freshwater biofilms, while a *vps*-independent MO10 biofilm, dependent on the O139 antigen, appears to form in saltwater environments (18). Considering that cholera infections are frequently derived from freshwater sources, especially in areas of endemicity, understanding the regulation of *vps*-dependent EPS expression is likely to be directly relevant to understanding the environmental persistence of epidemic strains.

Some of the details of the induction of EPS in *V. cholerae* are beginning to be understood. Two recent reports (8, 45) have demonstrated that a quorum-sensing signaling cascade controls EPS expression in the *V. cholerae* O1 El Tor C6706 strain. This cascade converges on controlling the expression of HapR, a LuxR homologue that represses both virulence factor expression and *vps* gene transcription (28, 46). Thus, a *hapR* mutant of this strain is derepressed for both virulence factor and EPS expression, explaining the rugose phenotype associated with a *hapR* mutant (15). However, natural frameshift mutations in *hapR* have been found in several (smooth) clinical isolates of *V. cholerae* which can still induce EPS and form biofilms in the laboratory (13, 43), suggesting that there are HapR-independent pathways for this process.

We previously identified a second pathway that leads to EPS expression and biofilm formation in the O139 MO10 strain (42). We have shown here that a flagellum-dependent pathway also regulates EPS expression in several O1 El Tor strains, and thus this signaling pathway is not unique to O139 *V. cholerae*. The absence of the flagellum is the inducing signal for EPS expression, which leads to the question of how an intracellular signaling cascade can recognize the lack of an extracellular organelle. In the studies presented here, we have identified the sodium-driven motor as an essential component of this signal cascade, since mutations in the sodium-driven motor abolish *vps* gene transcription, EPS expression, and biofilm formation. Phenamil, a specific poison of the sodium-driven motor, has the same effect as a mutation in one of the motor components, suggesting that there is a functional, rather than structural, role for the motor in this EPS signal cascade. While our studies identify the sodium-driven motor as a component in the EPS signaling cascade, it still remains unclear how the loss of the flagellum stimulates EPS expression, since the two *vps* operons are transcribed at high levels even in the smooth wild-type

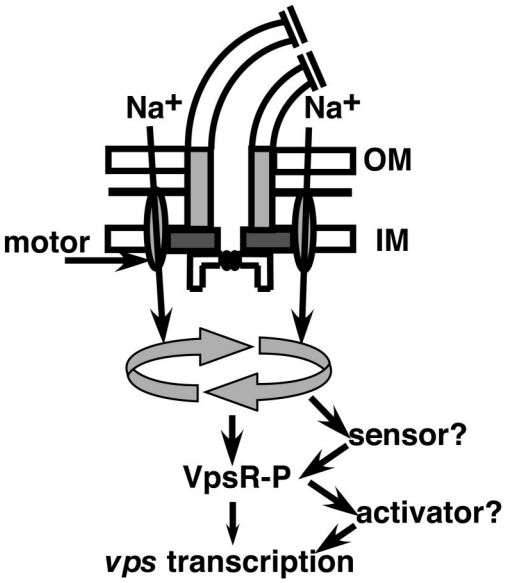

FIG. 7. Proposed flagellum-dependent EPS signaling cascade. The sodium-driven motor couples the flux of Na^+ across the membrane to flagellar rotation (depicted as circular arrows). Our results suggest that the motor may act as a mechanosensor, possibly by altering Na^+ flux, which stimulates the phosphorylation of VpsR, which in turn stimulates *vps* transcription and EPS production. The sensor responsible for phosphorylation of VpsR, as well as the direct activator of *vps* transcription, has not yet been identified (see text for details). OM, outer membrane; IM, inner membrane.

strain. We hypothesize that *vps* transcription is necessary but not sufficient for EPS expression and that transcription of an additional necessary gene(s) is stimulated by the lack of a flagellum.

A sodium gradient exists across the *Vibrio* membrane, and the sodium-driven motor allows an influx of sodium ions, which is coupled to flagellar rotation (1). Flux of sodium ions through the sodium-driven motor of *Vibrio parahaemolyticus*, which is predicted to change upon a decrease in the flagellar rotation rate, has been shown to be coupled to the induction of lateral flagellum transcription (16); the authors of that study characterized the motor as a mechanosensor. Our results suggest that the *V. cholerae* motor also acts as a mechanosensor to induce EPS expression. We hypothesize that the function of the sodium motor as a mechanosensor of flagellar rotation has been conserved among *Vibrio* spp. and has been adapted to induce appropriate behavior on solid surfaces, e.g., swarming behavior in *V. parahaemolyticus* and biofilm formation in *V. cholerae*.

One of the downstream events involved in this EPS signaling cascade appears to be the phosphorylation of the regulatory protein VpsR. Because VpsR with an alteration predicted to prevent phosphorylation (D59A) failed to stimulate biofilm formation, our results suggest that VpsR must be phosphorylated to induce biofilm formation. Also, VpsR with an alteration predicted to mimic phosphorylation (D59E) stimulated biofilm formation even in the absence of the motor. These results suggest that the motor is involved in stimulating the formation of phospho-VpsR, which in turn stimulates *vps* gene transcription and biofilm formation (Fig. 7). Because no cognate histidine kinase for VpsR has been identified, it is unclear what phosphorylates VpsR and whether this responds directly to sodium influx through the motor. We have also been unable to demonstrate a direct effect of VpsR at the two *vps* gene cluster (VC0916 and VC0934) promoters; chromosomal transcriptional reporter fusions of these promoters in a heterologous system failed to be transcribed in the presence of the constitutive D59E VpsR allele (not shown). Therefore, we hypothesize that phospho-VpsR may directly activate the transcription of some other factor that, in turn, activates *vps* gene transcription; this unknown factor may be the recently identified VpsT (3).

While the flagellum-dependent EPS signaling cascade is operational in some O1 El Tor and O139 strains, other O1 El Tor strains regulate EPS expression via the HapR-dependent pathway. The presence of two distinct EPS signaling pathways in these closely related isolates seems odd, and we suspect that the pathways are linked in some manner. One manner in which the two pathways may converge would be for both signaling pathways to regulate the phosphorylation of VpsR; this hypothesis is currently being tested. Our evidence already suggests that the sodium-driven motor does not control the HapR-dependent signaling pathway, because we have inactivated a *mot* gene in a rugose *hapR* strain (C6709), which had no effect on the rugose phenotype.

Evidence also suggests that the MO10 strain has a functional HapR. Sequence analysis revealed that the MO10 HapR contains a single R12L substitution (compared to the functional HapR from strain 3083) (15). Expression of MO10 *hapR* from its native promoter in a low-copy-number plasmid in the (rugose) C6709 *hapR* strain or in strain N16961 (which has a natural frameshift mutation in *hapR*) complements these strains for increased HA protease expression and causes a reversion of C6709 *hapR* to the smooth phenotype (not shown). Moreover, the MO10 *hapR* strain shows decreased protease expression that can be complemented back to wild-type levels by providing *hapR* from MO10 or C6709 on a plasmid, indicating that the MO10 *hapRR12L* allele is functional. Interestingly, Hammer and Bassler (8) identified an R12Q *hapR* mutation as a suppressor of a *luxO* mutant C6706 strain that demonstrated reduced protease activity and increased EPS expression, suggesting that mutation of R12 to Q results in decreased activity; perhaps changing this residue to L is less deleterious to HapR function.

The flagellum-dependent EPS signaling cascade affects the virulence of *V. cholerae* in some unexpected ways. We had previously shown that the nonflagellated rugose *flaA* MO10 strain is defective for intestinal colonization and that this defect was specifically due to EPS expression, since a nonrugose but still nonflagellated *flaA vpsF* strain could colonize to wild-type levels (42) (Fig. 6A). One reason for this may be that the rugose strain forms an aggregate that is unable to effectively contact the intestinal epithelia to result in productive colonization. However, our in vitro results (Fig. 6B) suggest that induction of virulence factor expression is defective in the rugose strain and that this defect can be alleviated by disruption of the EPS (via mutation of *vpsF*). Likewise, disruption of EPS in the *flaA* strain via mutation of *vpsR* also allows for CT and TCP expression in vitro. Thus, the EPS itself likely disrupts the ToxR/TcpP signaling cascade that induces TCP and CT, perhaps by altering the microenvironment surrounding the cell.

Mutations in the sodium-driven motor also disrupt EPS expression in a nonflagellated strain, so one might expect that a *flaA motX* strain, like a *flaA vpsF* strain, would colonize similarly to a wild-type strain. However, the *motX* mutation in both flagellated and nonflagellated cells leads to decreases in intestinal colonization. This defect is clearly not due to a lack of motility, since the nonmotile *flaA vpsF* strain can colonize at wild-type levels, but rather might be linked to the altered sodium signaling induced by the lack of the motor. Hase and Mekalanos (10) found that disruptions in the sodium motive force across the membrane alter transcription of the virulence-regulatory gene *toxT*, suggesting that virulence factor expression would also be altered. However, this effect of the motor on virulence factor expression may occur only in vivo, since the *motX* strain expressed detectable CT and TCP under in vitro inducing conditions.

A strain with a mutation in *vpsR* exhibited normal intestinal colonization and in vitro virulence factor expression, yet the introduction of the *vpsR* mutation into the *flaA* strain did not restore normal intestinal colonization, even though this mutation disrupts EPS expression in this strain (similar to the *vpsF* mutation) and allows for normal induction of CT and TCP in vitro. Since VpsR is a regulatory factor, its absence may have pleiotropic effects that may not be evident except in certain genetic backgrounds (e.g., nonflagellate). Thus, perhaps the lack of EPS allows the *flaA vpsR* strain to induce CT and TCP under inducing in vitro conditions, like the *flaA vpsF* strain, but the lack of some other VpsR-dependent factor causes a reduction in colonization of the nonflagellated strain; this VpsR-dependent factor is not necessary for colonization by flagellated cells. While this scenario is speculative, it suggests a role for VpsR in *V. cholerae* virulence, at least under certain circumstances. Given the homology of VpsR and LuxO with σ^{54}-dependent activators (21), our results and the recent results of others (28, 46) suggest the involvement of σ^{54} in multiple aspects of *V. cholerae* virulence that are distinct from flagellar regulation, as we had previously hypothesized (20).

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REFERENCES

1. **Atsumi, T., L. McCarter, and Y. Imae.** 1992. Polar and lateral flagellar motors of marine *Vibrio* are driven by different ion-motive forces. Nature **355:**182–184.
2. **Bik, E. M., A. E. Bunschoten, R. D. Guow, and F. R. Mooi.** 1995. Genesis of the novel epidemic *Vibrio cholerae* O139 strain: evidence for horizontal transfer of genes involved in polysaccharide synthesis. EMBO J. **14:**209–216.
3. **Casper-Lindley, C., and F. H. Yildiz.** 2004. VpsT is a transcriptional regulator required for expression of *vps* biosynthesis genes and the development of rugose colonial morphology in *Vibrio cholerae* O1 El Tor. J. Bacteriol. **186:**1574–1578.
4. **Correa, N. E., C. M. Lauriano, R. McGee, and K. E. Klose.** 2000. Phosphorylation of the flagellar regulatory protein FlrC is necessary for *Vibrio cholerae* motility and enhanced colonization. Mol. Microbiol. **35:**743–755.
5. **Donnenberg, M. S., and J. B. Kaper.** 1991. Construction of an *eae* deletion mutant of enteropathogenic *Escherichia coli* by using a positive-selection suicide vector. Infect. Immun. **59:**4310–4317.
6. **Freeman, J. A., and B. L. Bassler.** 1999. A genetic analysis of the function of LuxO, a two-component response regulator involved in quorum sensing in *Vibrio harveyi*. Mol. Microbiol. **31:**665–677.
7. **Gardel, C. L., and J. J. Mekalanos.** 1996. Alterations in *Vibrio cholerae* motility phenotypes correlate with changes in virulence factor expression. Infect. Immun. **64:**2246–2255.
8. **Hammer, B. K., and B. L. Bassler.** 2003. Quorum sensing controls biofilm formation in *Vibrio cholerae*. Mol. Microbiol. **50:**101–104.
9. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**577–580.
10. **Hase, C. C., and J. J. Mekalanos.** 1999. Effects of changes in membrane sodium flux on virulence gene expression in *Vibrio cholerae*. Proc. Natl. Acad. Sci. USA **96:**3183–3187.
11. **Haugo, A. J., and P. I. Watnick.** 2002. Vibrio cholerae CytR is a repressor of biofilm development. Mol. Microbiol. **45:**471–483.
12. **Hava, D. L., and A. Camilli.** 2001. Isolation and characterization of a temperature-sensitive generalized transducing bacteriophage for *Vibrio cholerae*. J. Microbiol. Methods **46:**217–225.
13. **Heidelberg, J. F., J. A. Eisen, W. C. Nelson, R. A. Clayton, M. L. Gwinn, R. J. Dodson, D. H. Haft, E. K. Hickey, J. D. Peterson, L. Umayam, S. R. Gill, K. E. Nelson, T. D. Read, H. Tettelin, D. Richardson, M. D. Ermolaeva, J. Vamathevan, S. Bass, H. Qin, I. Dragoi, P. Sellers, L. McDonald, T. Utterback, R. D. Fleishmann, W. C. Nierman, O. White, S. L. Salzberg, H. O. Smith, R. R. Colwell, J. J. Mekalanos, J. C. Venter, and C. M. Fraser.** 2000. DNA Sequence of both chromosomes of the cholera pathogen *Vibrio cholerae*. Nature **406:**477–483.
14. **Iwanaga, M., K. Yamamoto, N. Higa, Y. Ichinose, N. Nakasone, and M. Tanabe.** 1986. Culture conditions for stimulating cholera toxin production by *Vibrio cholerae* O1 El Tor. Microbiol. Immunol. **30:**1075–1083.
15. **Jobling, M. G., and R. K. Holmes.** 1997. Characterization of *hapR*, a positive regulator of the *Vibrio cholerae* HA/protease gene *hap*, and its identification as a functional homologue of the *Vibrio harveyi luxR* gene. Mol. Microbiol. **26:**1023–1034.
16. **Kawagishi, I., M. Imagawa, Y. Imae, L. McCarter, and M. Homma.** 1996. The sodium-driven polar flagellar motor of marine Vibrio as the mechanosensor that regulates lateral flagellar expression. Mol. Microbiol. **20:**693–699.
17. **Kierek, K., and P. I. Watnick.** 2003. Environmental determinants of *Vibrio cholerae* biofilm development. Appl. Environ. Microbiol. **69:**5079–5088.
18. **Kierek, K., and P. I. Watnick.** 2003. The *Vibrio cholerae* O139 O-antigen polysaccharide is essential for Ca2+-dependent biofilm development in sea water. Proc. Natl. Acad. Sci. USA **100:**14357–14362.
19. **Klose, K. E., and J. J. Mekalanos.** 1998. Differential regulation of multiple flagellins in *V. cholerae*. J. Bacteriol. **180:**303–316.
20. **Klose, K. E., and J. J. Mekalanos.** 1998. Distinct roles of an alternative sigma factor during both free-swimming and colonizing phases of the *Vibrio cholerae* pathogenic cycle. Mol. Microbiol. **28:**501–520.
21. **Klose, K. E., V. Novick, and J. J. Mekalanos.** 1998. Identification of multiple σ^{54}-dependent transcriptional activators in *Vibrio cholerae*. J. Bacteriol. **180:**5256–5259.
22. **Klose, K. E., D. S. Weiss, and S. Kustu.** 1993. Glutamate at the site of phosphorylation of nitrogen-regulatory protein NTRC mimics aspartyl-phosphate and activates the protein. J. Mol. Biol. **232:**67–78.
23. **Kojima, S., K. Yamamoto, I. Kawagishi, and M. Homma.** 1999. The polar flagellar motor of *Vibrio cholerae* is driven by an Na^+ motive force. J. Bacteriol. **181:**1927–1930.
24. **Krukonis, E. S., and V. J. DiRita.** 2003. From motility to virulence: sensing and responding to environmental signals in Vibrio cholerae. Curr. Opin. Microbiol. **6:**186–190.
25. **Lee, S. H., D. L. Hava, M. K. Waldor, and A. Camilli.** 1999. Regulation and temporal expression patterns of *Vibrio cholerae* virulence genes during infection. Cell **99:**625–634.
26. **Mekalanos, J. J., D. J. Swartz, G. D. Pearson, N. Harford, F. Groyne, and M. de Wilde.** 1983. Cholera toxin genes: nucleotide sequence, deletion analysis and vaccine development. Nature **306:**551–557.
27. **Miller, J. H.** 1992. A short course in bacterial genetics, 2nd ed. Cold Spring Harbor Laboratory Press, Plainview, N.Y.
28. **Miller, M. B., K. Skorupski, D. H. Lenz, R. K. Taylor, and B. L. Bassler.** 2002. Parallel quorum sensing systems converge to regulate virulence in *Vibrio cholerae*. Cell **110:**303–314.
29. **Miller, V. L., and J. J. Mekalanos.** 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. J. Bacteriol. **170:**2575–2583.
30. **Pearson, G. D., A. Woods, S. L. Chiang, and J. J. Mekalanos.** 1993. CTX genetic element encodes a site-specific recombination system and an intestinal colonization factor. Proc. Natl. Acad. Sci. USA **90:**3750–3754.
31. **Reidl, J., and K. E. Klose.** 2002. Vibrio cholerae and cholera: out of the water and into the host. FEMS Microbiol. Rev. **26:**125–139.
32. **Simons, R. W., F. Houman, and N. Kleckner.** 1987. Improved single and multicopy *lac*-based cloning vectors for protein and operon fusions. Gene **53:**85–96.
33. **Sutcliffe, J. G.** 1979. Complete nucleotide sequence of the *Escherichia coli* plasmid pBR322. Cold Spring Harbor Symp. Quant. Biol. **43:**77–90.
34. **Svennerholm, A. M., and J. Holmgren.** 1978. Identification of the *Escherichia coli* heat-labile enterotoxin by means of a ganglioside immunosorbent assay (GM_1-ELISA) procedure. Curr. Microbiol. **1:**19–23.
35. **Thelin, K. H., and R. K. Taylor.** 1996. Toxin-coregulated pilus, but not mannose-sensitive hemagglutinin, is required for colonization by *Vibrio cholerae* O1 El Tor biotype and O139 strains. Infect. Immun. **64:**2853–2856.

36. **Vance, R. E., J. Zhu, and J. J. Mekalanos.** 2003. A constitutively active variant of the quorum-sensing regulator LuxO affects protease production and biofilm formation in *Vibrio cholerae*. Infect. Immun. **71:**2571–2576.
37. **Wachsmuth, I. K., G. M. Evins, P. I. Fields, O. Olsvik, T. Popvic, C. A. Bopp, J. G. Wells, C. Carrillo, and P. A. Blake.** 1993. The molecular epidemiology of cholera in Latin America. J. Infect. Dis. **167:**621–626.
38. **Waldor, M. K., R. Colwell, and J. J. Mekalanos.** 1994. The *Vibrio cholerae* O139 serogroup antigen includes an O-antigen capsule and lipopolysaccharide virulence determinants. Proc. Natl. Acad. Sci. USA **91:**11388–11392.
39. **Waldor, M. K., and J. J. Mekalanos.** 1994. ToxR regulates virulence gene expression in non-O1 strains of *Vibrio cholerae* that cause epidemic cholera. Infect. Immun. **62:**72–78.
40. **Wang, R. F., and S. Kushner.** 1991. Construction of versatile low-copy-number vectors for cloning, sequencing and gene expression in *Escherichia coli*. Gene **100:**195–199.
41. **Watnick, P. I., and R. Kolter.** 1999. Steps in the development of a *Vibrio cholerae* biofilm. Mol. Microbiol. **34:**586–595.
42. **Watnick, P. I., C. M. Lauriano, K. E. Klose, L. Croal, and R. Kolter.** 2001. The absence of a flagellum leads to altered colony morphology, biofilm development, and virulence in *Vibrio cholerae* O139. Mol. Microbiol. **39:**223–235.
43. **Yildiz, F. H., N. A. Dolganov, and G. K. Schoolnik.** 2001. VpsR, a member of the response regulators of the two-component regulatory systems, is required for expression of *vps* biosynthesis genes and EPS(ETr)-associated phenotypes in *Vibrio cholerae* O1 El Tor. J. Bacteriol. **183:**1716–1726.
44. **Yildiz, F. H., and G. K. Schoolnik.** 1999. Vibrio cholerae O1 El Tor: identification of a gene cluster required for the rugose colony type, exopolysaccharide production, chlorine resistance, and biofilm formation. Proc. Natl. Acad. Sci. USA **96:**4028–4033.
45. **Zhu, J., and J. J. Mekalanos.** 2003. Quorum sensing-dependent biofilms enhance colonization in *Vibrio cholerae*. Dev. Cell **5:**647–656.
46. **Zhu, J., M. B. Miller, R. E. Vance, M. Dziejman, B. L. Bassler, and J. J. Mekalanos.** 2002. Quorum-sensing regulators control virulence gene expression in *Vibrio cholerae*. Proc. Natl. Acad. Sci. USA **99:**3129–3134.