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Mutations in the Extracellular Protein Secretion Pathway Genes (*eps*) Interfere with Rugose Polysaccharide Production in and Motility of *Vibrio cholerae*

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***Vibrio cholerae* is the causal organism of the diarrheal disease cholera. The rugose variant of *V. cholerae* is associated with the secretion of an exopolysaccharide. The rugose polysaccharide has been shown to confer increased resistance to a variety of agents, such as chlorine, bioacids, and oxidative and osmotic stresses. It also promotes biofilm formation, thereby increasing the survival of the bacteria in the aquatic environments. Here we show that the extracellular protein secretion system (gene designated *eps*) is involved directly or indirectly in the production of rugose polysaccharide. A Tn*phoA* insertion in *epsD* gene of the *eps* operon abolished the production of rugose polysaccharide, reduced the secretion of cholera toxin and hemolysin, and resulted in a nonmotile phenotype. We have constructed defined mutations of the *epsD* and *epsE* genes that affected these phenotypes and complemented these defects by plasmid clones of the respective wild-type genes. These results suggest a major role for the *eps* system in pathogenesis and environmental survival of *V. cholerae*.**

Vibrio cholerae is a gram-negative bacterium that causes the diarrheal disease cholera, which continues to be a global threat. Seven pandemics have been recorded in the history of cholera, and a novel epidemic strain, O139 Bengal, has emerged recently (15). In countries where cholera is endemic, such as Bangladesh and India, cholera occurs in seasonal peaks intermittent with an endemicity baseline (8). The survival of *V. cholerae* during interepidemic periods has long been a key question. It has been proposed that *V. cholerae* can enter into a nonculturable state but remain viable and capable of producing disease (35, 42). It has also been shown that *V. cholerae* cells remain associated with plankton, which may be reservoirs of this bacterium (13).

V. cholerae can switch to another survival form, known as the rugose phenotype, while retaining virulence (28, 34, 49). The rugose phenotype, as originally described by Bruce White in 1938, is characterized by wrinkled colony morphology associated with the secretion of copious amounts of extracellular polysaccharide (49, 50). Under normal growth conditions, the shift from smooth to rugose or vice versa occurs at a low frequency that can be increased by growth in alkaline peptone water for 2 to 3 days at 37°C (28) or by starvation in M9 salts at 16°C (45, 46). *V. cholerae* serogroup O1 El Tor, serogroup O139, and non-O1 strains have been shown to switch to the rugose phenotype, although O1 classical strains have not (28, 52).

The rugose form of *V. cholerae* exhibits increased resistance to chlorine, acid, serum killing, and oxidative and osmotic shocks (28, 34, 45, E. W. Rice, C. J. Johnson, R. M. Clark, K. R. Fox, O. J. Reasoner, M. E. Dunnigan, P. Panigraphi, J. A. Johnson, and J. G. Morris, Jr., Letter, Lancet **340:**740, 1992). The increased resistance is probably due to aggregation of cells in the polysaccharide matrix 28, 34; Rice et al., Lancet **340:**740, 1992). Consistent with this idea, rugose polysaccharide has been shown to promote biofilm formation that might result in an increased survival of *V. cholerae* in aquatic environments, although the presence of rugose *V. cholerae* in aquatic environments has not yet been confirmed (52). The genetic basis of rugosity and its role in biofilm formation are just beginning to be unraveled. Recently, a genetic region (*vps*, for vibrio polysaccharide synthesis) involved in the synthesis of rugose polysaccharide, termed EPS^{ETr}, has been identified. Mutations in this region abolished the formation of rugose material, and addition of purified rugose polysaccharide to the mutant bacteria conferred resistance to chlorine (52). A search for the genes responsible for biofilm formation has identified three groups of genes involved in (i) biosynthesis and secretion of mannose-sensitive hemagglutinin type IV pilis (MSHA), (ii) vibrio polysaccharide synthesis (*vps* genes), and (iii) flagellar motility (47, 48).

Epidemic cholera is caused by *V. cholerae* strains of serogroup O1 or O139 that secrete cholera toxin (CT) (15). CT secretion requires a general secretory pathway (GSP) encoded by the *eps* (extracellular protein secretion) operon, comprising 12 genes, *epsC* to *-N*. This pathway is also essential for the secretion of chitinase and protease (30, 38–40).

A diverse group of gram-negative bacteria utilize GSP homologs to secrete either soluble proteins such as cellulase, pectinase, protease, pullulanase, and chitinase (9, 19, 21, 32, 33, 40) or cell surface-associated appendages such as type IV pili (25) and S-layers (44). GSP systems are divided into two branches. Transport of specific signal-associated proteins across the inner membrane involves Sec proteins, while assembly and transport of proteins across the outer membrane re-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference(s)
V. cholerae strains		
C6706	Wild type, smooth, serogroup O1 El Tor biotype	34
C6706 R	C6706 rugose variant	This study
569B	Wild type, smooth, serogroup O1 Classical biotype	12
N16961	Wild type, smooth, serogroup O1 El Tor biotype	28
N16961 R	N16961 rugose variant	This study
NS1	Derivative of N16961 R, smooth, *epsD*::Tn*phoA*, Kan^R	This study
AA10	N16961 R, *epsD*::Kan^r cassette	This study
AA1	N16961 R, *epsE*::Kan^r cassette	This study
E. coli strains		
DH5α	*recA* Δ*lacU169* ϕ80d *lacZ*ΔM15	Gibco BRL
S17-1 λ pir	*pro hsdR hsdM*$^+$ Tmp^r Str^r	7, 27
SM10 λ pir	*thi thr leu tonA lacY supE recA*::RP4-2Tc::Mu Kan^r	7
Plasmids		
pBluescript	High-copy-number vector, Amp^r, *ori* ColE1	Stratagene
pCVD442	Suicide vector, *ori* R6K, Amp^r, *sacB*	7
pHC79	Low-copy-number cosmid vector, Amp^r, *ori* R6K	11
pRT733	Tn*phoA* delivery vector, *ori*R6K, *mob* RP4, Kan^r Amp^r	43
pUC18 K	High-copy-number vector, Amp^r, *ori* ColE1, Kan^r cassette mutagenesis vector	26
pUC18 K2	High-copy-number vector, Amp^r, *ori* ColE1, Kan^r cassette mutagenesis vector	J. B. Kaper
pAA5	pHC79 containing a 38-kb *Sau*3 AI fragment of NS1, Amp^r Kan^r	This study
pAA13	pBluescript SK containing a 4.3-kb Tn*phoA* insertion junction sequence from pAA5, Amp^r	This study
pDSK-2	pBluescript KS containing a 2.4-kb *Eco*RI-*Bam*HI PCR fragment (*epsD*) from *V. cholerae* 569B	This study
pAA26	pBluescript SK containing a 2.4-kb *Bam*HI-*Eco*RI PCR fragment (*′epsD-5′-epsE′*) from the N16961 rugose strain	This study
pAA29	pAA26::Kan^r, a 0.8-kb Kan^r cassette from pUC18K inserted into the unique *Nhe*I site at the 3′ end of *epsD*	This study
pAA35	pCVD442 containing a 3.2-kb *Bam*HI-*Eco*RI fragment from pAA29	This study
pAA40	pBluescript SK containing 2.3-kb *Bam*HI-*Kpn*I PCR fragment (*′epsD-epsE-epsF′*)	This study
pAA42	pAA40 containing Tet^r cassette (*Eco*RI-*Ava*I fragment of pBR322) cloned in the *Xmn*I site	This study
pAA46	pAA42Δ*eps*E::Kan^r, deletion of a *Hin*cII (bp 4117–4263) fragment and insertion of a 0.8-kb Kan^r cassette from pUC18K2 into that site	This study
pAA48	pCVD442 containing 3.1-kb *Bam*HI-*Eco*RI fragment of pAA46 cloned into the *Sal*I site	This study

quire a terminal branch (32). GSP terminal branches, like *eps*, consist of 12 to 14 genes and are present in both animal and plant pathogens (37). *Escherichia coli* filamentous bacteriophages such as M13, fd, and f1 carry some of these GSP gene homologs, which are involved in the secretion of matured phage particles (36). GSP proteins are highly specific in secretory functions and cannot be substituted for each other despite a high degree of similarity among the proteins of different bacterial origins (32, 37). While the majority of these proteins are localized in the cytoplasmic membrane, GspD is an integral outer membrane protein (9). It has been suggested that GspD is a multimeric complex of 12 monomers that forms a large pore allowing transport of macromolecules such as S-layers, pili, mature filamentous phages, and extracellular proteins such as pectinase, hydrolase, CT, chitinase, and protease (9, 37). We report here the involvement of the *V. cholerae eps* operon in the production of rugose polysaccharide and motility as well as secretion of hemolysin and CT. To the best of our knowledge, this is the first report of the involvement of a type II secretion system in polysaccharide production.

MATERIALS AND METHODS

Bacterial strains, plasmids, and primers. The bacterial strains and plasmids used in this study are listed in Table 1, and the oligonucleotide primers are listed in Table 2. Media and growth conditions were as previously described (28).

Isolation of Tn*phoA* mutants. Tn*phoA*, on suicide vector pRT733 (43), was introduced by conjugation into rugose isolates of strains C6706 and N16961. Transconjugants were plated on Luria-Bertani (LB) agar containing kanamycin and polymyxin B to select for cells that acquired the Tn*phoA* and were screened for smooth colonies. These colonies were grown in alkaline peptone water (31) to check whether the shift from the rugose to smooth phenotype is not due to phase variation but is due to the inactivation of a gene essential for the rugose phenotype.

Mapping of the Tn*phoA* insertions. To determine the number and locations of the insertions in each mutant, pulsed-field gel electrophoresis (PFGE) (4) of *Sfi*I-digested chromosomal DNAs of wild-type and mutant strains was carried out on a CHEF-DRII mapper (Bio-Rad Laboratories). The DNA was transferred onto nylon membrane (MSI, Westboro, Mass.) by capillary transfer and hybridized with a ^{32}P-labeled 2.6-kb *Bgl*II Tn*pho*A fragment under stringent conditions (22). Since *Sfi*I does not cut within Tn*pho*A, each hybridized band corresponded to a single insertion.

Cosmid cloning. Cloning and recombinant DNA techniques were carried out as previously described (22). Chromosomal DNA of NS1 was partially digested with *Sau*3A1, and fragments in the range of 30 to 35 kb were purified. These fragments were ligated into the *Bam*HI site of cosmid pHC79 (11), packaged using Gigapack (Stratagene, Inc., La Jolla, Calif.), and transduced into *E. coli* DH5α, and the transductants were selected on LB plates containing 50 μg of kanamycin per ml.

Cloning of the *epsD* gene. Two convergent PCR primers (TD001 and TD002 [Table 2]) based on the published nucleotide sequence of the *eps* operon (from bp 850 to 3217) were used to amplify the *epsD* gene from *V. cholerae* 569B (40). The resulting 2.4-kb amplicon was gel purified, digested with *Bam*HI, and partially digested with *Eco*RI, as *epsD* has an internal *Eco*RI site, before ligation into *Eco*RI-*Bam*HI-digested pBluescript vector. The ligated DNA was transformed into *E. coli* DH5αF′*tet*, and transformants were selected on LB plates containing 100 μg of ampicillin per ml. One of the transformants, designated pDSK-2, was shown to contain the *epsD* gene by restriction enzyme analysis.

Construction of in-frame insertion mutants. The *epsD* gene was PCR amplified using primers M-157 and M-158 and cloned into pBluescript, resulting in pAA26. A Kan^r cassette from pUC18K was inserted into the *Nhe*I site of *epsD* (pAA29). The entire insert was transferred into a suicide vector, pCVD442 (pAA35), that contains a sucrose selection system. Transfer of the *epsD* knockout into the *V. cholerae* chromosome was done by conjugation and selection for ampicillin resistance. Derivatives with chromosomal integration of the Kan^r cassette were selected on 5% sucrose plates (7). Further analysis of the sucrose-

TABLE 2. Sequences of the oligonucleotide primers

Primer	Location	Nucleotide sequence	GenBank accession no.
J-238	IS*50*R	5′-GAAAGGTTCCGTTCAGGA-3′	U25548
M-157	*epsE*	5′-CGGAATTCCATATCGCGCAGCCGAGTCAC-3′	L33796
M-158	*epsD*	5′-CGGGATCCGATTTCTGGCGATCCTAAAGTG-3′	L33796
M-202	*epsD*	5′-CGGGATCCGTAAATACAACTACATCCGC-3′	L33796
M-203	*epsF*	5′-GGGGTACCCAGCGCACCAGATAAATCAG-3′	L33796
M-229	*epsD*	5′-GCAAGAAGTCTCGAACGTG-3′	L33796
M-230	*epsE*	5′-CGAGCGCCTCCATCGACAAC-3′	L33796
M-231	IS*50*L	5′-AGCCCGGTTTTCCAGAAC-3′	U25548
M-355	*epsE*	5′-CTCTTTGTCCGCCTCGTA-3′	L33796
M-357	*epsE*	5′-GATCTGCACAGTTTGGGTATG-3′	L33796
TD001	*epsC*	5′-GAAATCCGCAGGAAATTT-3′	L33796
TD002	*epsE*	5′-AGAGATCACCATTTCGG-3′	L33796

resistant colonies (Kan^r Amp^s) by PCR and DNA sequencing was carried out in order to verify the chromosomal knockout of the *epsD* gene. The *epsE* gene was cloned similarly in pBluescript using primers M-202 and M-203 (pAA40). A Kan^r cassette from pUC18 K2 was inserted into the gene at the *Hin*cII sites (pAA46), and in the next step the *epsE* gene with the Kan^r cassette was transferred into pCVD442 (pAA48). The knockout was introduced into the chromosome as described above.

Other techniques. CT assay was done by GM1 ganglioside enzyme-linked immunosorbent assay (5). Hemolysin activity was measured by streaking out the test strains on 5% rabbit blood agar plates and incubating the plates at 37°C overnight. Motility assays were carried out by inoculation of the test strains on motility agar plates containing 0.3% agar followed by incubation at 37°C for 4 to 5 h, at the end of which time the zone of motility was measured (17). DNA sequencing was done with an ABI automated sequencer at the University of Maryland Biopolymer Laboratory. DNA sequence analyses were performed using the DNASIS program (Hitachi Software Corp.), and sequence homology searching was performed using the National Center for Biotechnology Information BLAST program (1).

RESULTS

Isolation of Tn*phoA* mutants with an altered rugose phenotype. To identify the genetic regions involved in rugosity, we isolated mutants following Tn*phoA* mutagenesis. Tn*phoA* (23), on suicide vector pRT733 (43), was introduced by conjugation into rugose variants of *V. cholerae* O1 El Tor strains C6706 and N16961. Kan^r and polymyxin B-resistant exconjugants were screened for smooth colonies. Smooth colonies were tested in alkaline peptone water and were found to remain smooth, indicating the linkage of Tn*phoA* to the smooth phenotype.

The locations of Tn*phoA* insertions in each mutant were mapped by PFGE analysis. Seven of the 16 stable smooth mutants analyzed had multiple Tn*phoA* insertions (Table 3). The remaining nine mutants had single insertions in a total of

TABLE 3. Secretion phenotypes of wild-type and mutant *V. cholerae* O1 El Tor rugose strains

Strain[a]	Antibiotic resistance[b]	No. of Tn*phoA* insertions	Map location of Tn*phoA* insertion(s) (kb of *Sfi*I fragment)[c]	Hemolysis[d]	Motility[e]	CT activity (% of total) in:	
						Medium	Cells
S1	Kan^r Amp^r	1	677	+++	+++	99	1
S2	Kan^r Amp^r	1	145.5	+++	++	99	1
S3	Kan^r Amp^r	2	291, 339	+	+	80	20
S4	Kan^r Amp^r	2	291, 339, 388	−	−	66	34
S7	Kan^r Amp^r	1	291	+++	+++	99	1
S8	Kan^r Amp^r	1	677	+++	+++	99	1
S10	Kan^r Amp^r	1	165	+++	+	99	1
S11	Kan^r Amp^r	2	291, 490	+++	+++	70	30
S13	Kan^r Amp^r	2	291, 490	+++	++	99	1
S14	Kan^r Amp^r	3	291, 490, 525	++	+++	99	1
S16	Kan^r Amp^r	2	291, 280	+++	+++	53	47
S17	Kan^r Amp^r	1	291	+++	+++	86	14
S18	Kan^r Amp^r	2	80, 425	+++	+++	75	25
S20	Kan^r Amp^r	1	291	+++	+++	99	1
NS1	Kan^r Amp^r	1	291	−	−	28	72
NS25	Kan^r Amp^r	1	435	+++	+++	ND[f]	ND
N16961 R	Kan^r Amp^r	None		+++	+++	99	1
AA16	Kan^r Amp^r	None		+++	+++	98	2
AA10	Kan^r	None		−	+	36	64
AA10/pDSK-2	Kan^r Amp^r	None		+	+++	99	1
AA1	Kan^r	None		−	−	87	13
AA1/pAA44	Kan^r Amp^r	None		+	++	99	1

[a] Strains S1 to S20 were derived from C6706 rugose; NS1 and NS25 were derived from N16961 rugose
[b] Amp^r is probably due to cointegration of the transposon delivery vector
[c] In PFGE analysis
[d] Hemolytic activity on 5% rabbit blood agar plates after 24 h of incubation at 37°C. +++, ++, +, and −, complete, moderate, weak, and no lysis, respectively.
[e] Motility determined after 4 to 6 h of incubation at 37°C on motility agar. +++, ++, +, and −, high, moderate, weak, and nonmotile phenotypes, respectively.
[f] ND, not determined.

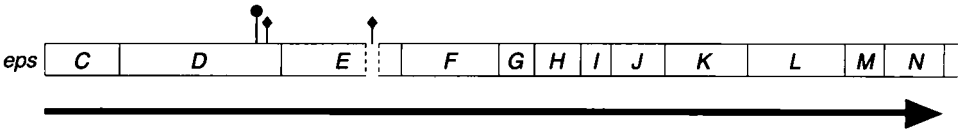

FIG. 1. The *eps* operon of *V. cholerae*. The *eps* cluster contains the genes *epsC* to *epsN*, and the entire region is 12,077 bp in length. The transcriptional orientation of the *eps* cluster is indicated by the arrow below the genes. The site of the Tn*phoA* insertion in *epsD* is indicated by a filled circle, and those of the in-frame Kan^r cassettes are indicated by the filled diamonds. A 146-bp deletion in the *epsE* gene at the site of Kan^r insertion is indicated by the broken box.

five unique fragments (145.5, 165, 291, 435, and 677 kb), indicating the possible association of these fragments with the rugose phenotype. The 291-kb fragment had an insertion in the majority (ten) of the mutants. Of these, four mutants (S7, S17, S20, and NS1) had a single insertion. Therefore, strain NS1 with a Tn*phoA* insertion in the 291-kb fragment was selected for further analysis.

Subcloning, mapping, and sequencing of NS1 Tn*phoA* insertion junctions. The Tn*phoA*-containing fragment was cloned in a cosmid vector as described in Materials and Methods. One Kan^r transductant, designated pAA5, was characterized further. To map the Tn*phoA* insertion junctions in pAA5, cosmid DNA was purified, digested with *Eco*RI or *Sal*I, and hybridized with a 2.6-kb *Bgl*II Tn*phoA* probe. *Eco*RI cuts upstream of the *kan* gene in Tn*phoA*, and the fragment that hybridizes to the probe is the 3′ Tn*phoA-Vibrio* junction fragment. *Sal*I cuts downstream of *kan* gene in Tn*phoA*, and the two fragments that hybridize are the 5′ and 3′ Tn*phoA-Vibrio* junction fragments. As expected, this probe hybridized with a single *Eco*RI fragment, identifying the 3′ Tn*phoA* insertion junction, and with two *Sal*I fragments, identifying both the 5′ and 3′ Tn*phoA* insertion junctions (data not shown).

The 4.3-kb *Sal*I fragment from pAA5 containing the 3′ Tn*phoA-Vibrio* insertion junction was cloned into the *Sal*I site of pBluescript SK(+) to yield pAA13. The cloned fragment was sequenced using primer J-238 (Table 2), derived from the 3′ end of Tn*phoA*. A DNA sequence homology search revealed that the Tn*phoA* was inserted 148 bp upstream of the stop codon of *epsD*, the second gene in the *eps* operon (Fig. 1). We verified the integrity of the *epsD* sequence at the 5′ Tn*phoA-Vibrio* junction, since a deletion or other rearrangements at this end could also result in the mutant phenotype. To rule out this possibility, we PCR amplified the 5′ Tn*phoA-Vibrio* junction sequence from NS1 chromosomal DNA using two convergent primers, M-158 and M-231 (Table 2). The resulting 1.2-kb PCR product was sequenced using a Tn*phoA* reverse primer, M-231, and an *epsD* primer, M-229 (Table 2). The DNA sequence data did not reveal any evidence of a deletion or other rearrangement at the 5′ Tn*phoA* junction except for a 9-bp direct repeat at the 5′ and 3′ ends of the Tn*phoA* insertion site. Tn*5* is known to create a 9-bp target duplication (3). These results indicated that the Tn*phoA* insertion at the 3′ end of *epsD* gene (corresponding to 49 amino acid residues from the carboxyl-terminal end of the EpsD protein) was responsible for the reversion of the rugose to the smooth phenotype in the NS1 mutant.

Complementation of the *epsD*::Tn*phoA* mutant by a cloned *epsD*$^+$ gene. Although the Tn*phoA* insertion in the NS1 mutant resulted in the loss of the rugose phenotype, we could not conclude whether the phenotypic change was due exclusively to inactivation of *epsD* or was caused by a polar effect on the genes downstream of *epsD* within the *eps* operon. We therefore attempted to complement the *epsD* mutation in NS1. A plasmid carrying *epsD*$^+$, pDSK-2, was electroporated into NS1, and ampicillin-resistant transformants were observed for colony morphology. No rugose colonies were seen on initial isolation, but on passage in LB medium without the antibiotic, rugose colonies were recovered at a high frequency (20 to 50%). All the rugose revertants had lost the Kan^r marker. One such rugose strain, AA16, selected for further study, exhibited sensitivity to both kanamycin and ampicillin, and furthermore, plasmid DNA could not be detected (data not shown). These results suggested the loss of both the Tn*phoA* insertion and pDSK-2 from AA16 and reversion to the wild-type rugose phenotype. This probably happened by allelic exchange between the chromosomal *epsD*::Tn*phoA* and the pDSK-2 (*eps*$^+$). The loss of Tn*phoA* and restoration of the wild-type *epsD* sequence in AA16 were confirmed by DNA sequence analysis. DNA sequences flanking the Tn*phoA* insertion site were amplified using primers M-157 and M-158 (Table 2), purified, and sequenced using primers M-229 and M-230. Sequence analysis showed that AA16 contained the *epsD*$^+$ allele, indicating the loss of Tn*phoA* along with the 9-bp direct repeat. In our hands, under identical conditions the reversion of rugose to smooth or vice versa or with NS1 containing a control plasmid (pBluescript) lacking *epsD* occurs at a very low frequency (ca. 10^{-4}). These results indicated that the reversion in AA16 is not due to Tn*phoA* excision but is attributable to homologous recombination.

In-frame insertion mutations in *epsD* and *epsE* genes affect rugose polysaccharide production. In order to eliminate the possibility of a polar effect and to examine whether genes other than *epsD* in the *eps* operon are involved in the rugose phenotype, we constructed an in-frame insertion of a Kan^r cassette in *epsD*. The resulting mutant, AA10, exhibited a time-dependent defect in the expression of rugose polysaccharide; i.e., the mutant remained rugose for 24 h but turned smooth on prolonged incubation (Fig. 2A). Unlike NS1, this mutant could be complemented by pDSK-2; i.e., AA10 containing pDSK-2 was rugose (Fig. 2A).

NS1 exhibited a severe defect in rugose polysaccharide production (i.e., smooth even after several days of growth) compared to AA10. We interpret this to mean that in NS1 the Tn*phoA* insertion had polar effects on the expression of genes downstream of *epsD* in the *eps* operon in addition to a partially defective *epsD* gene. In order to test this hypothesis, we introduced an in-frame insertion in the *epsE* gene. A 146-bp deletion of *epsE* was created and a Kan^r cassette was inserted in-frame at this position, resulting in the strain AA1. This mutation abolished the production of rugose polysaccharide, indicating that *epsE* also is involved in rugose polysaccharide synthesis or secretion. A plasmid carrying an *epsE*$^+$ allele complemented this mutant, restoring rugosity (Fig. 2B).

Other phenotypes affected in *eps* mutants. Sandkvist et al. have found that mutations in six of the 12 genes of the *eps* operon (*epsC*, *-E*, *-F*, *-G*, *-L*, and *-M*) resulted in defects in secretion of CT, protease, and chitinase (38–40). They also noted that the rest of the genes of this operon might be required for extracellular secretion. We were interested in determining whether *epsD* and *epsE* mutants are defective in

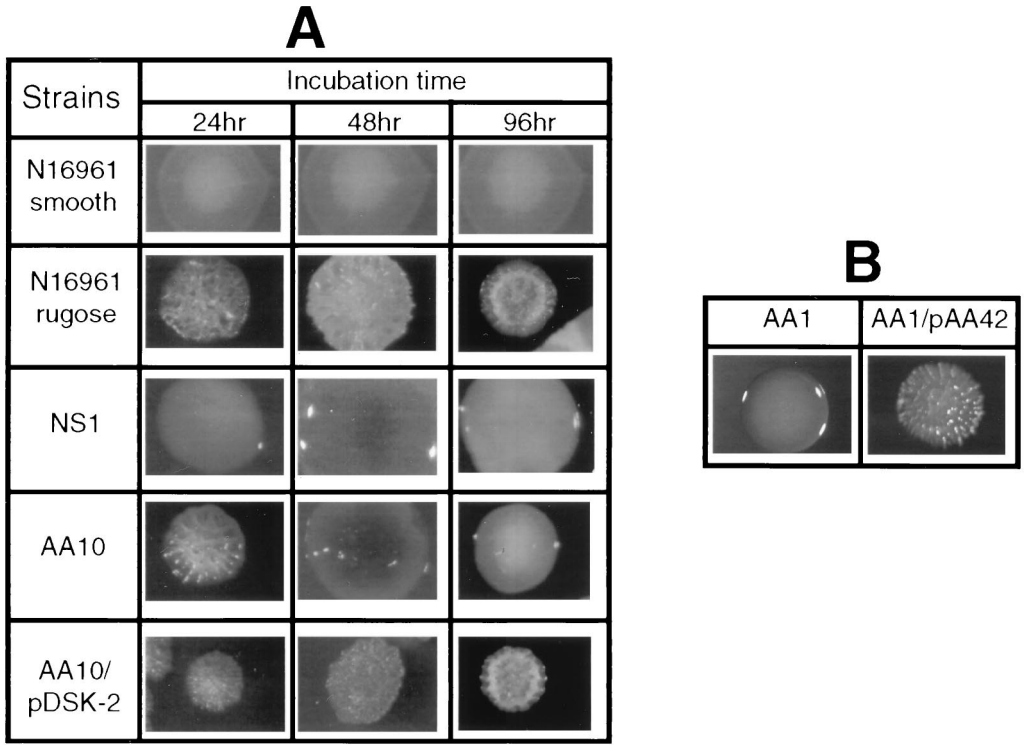

FIG. 2. (A) Smooth and rugose colony morphologies of the wild-type and mutant strains. The various strains were grown to different times, as indicated, in LB agar plates with or without the respective antibiotics. (B) Strain AA1 (*epsE*::Kan^r) and the complemented strain (*epsE*::Kan^r/pAA42) after overnight growth.

secretion of CT, hemolysin, and extracellular structures such as flagella. In addition to its defect in rugose expression (Fig. 2), NS1 exhibited a severe defect in secretion of CT and hemolysin as well as in motility (Table 3 and Fig. 3). Reversion of *epsD*::Tn*phoA* in NS1 to the wild type (AA16) restored secretion of CT and hemolysin, motility, and rugosity to wild-type levels (Table 3). Similarly, AA10 (*epsD*::Kan^r) and AA1 (*epsE*::Kan^r) exhibited defects in these phenotypes, although AA1 showed a less severe defect in CT secretion. These defects could be complemented by plasmid clones of the respective wild-type genes (Table 3). The nonmotile phenotype of these mutants is not an indirect effect due to growth defects. The mutants NS1 and AA10 do not show any significant growth defect compared to the wild-type strain, N16961 R (generation time, 35 to 40 min). Although AA1 (*epsE*::Kan^r) grows more slowly than N16961 R, there was no evidence of motility even after prolonged incubation (24 h) on motility agar plates. These results indicated that the *epsD* and *epsE* genes are involved in rugose polysaccharide production and formation of the polar flagella in addition to secretion of CT and hemolysin.

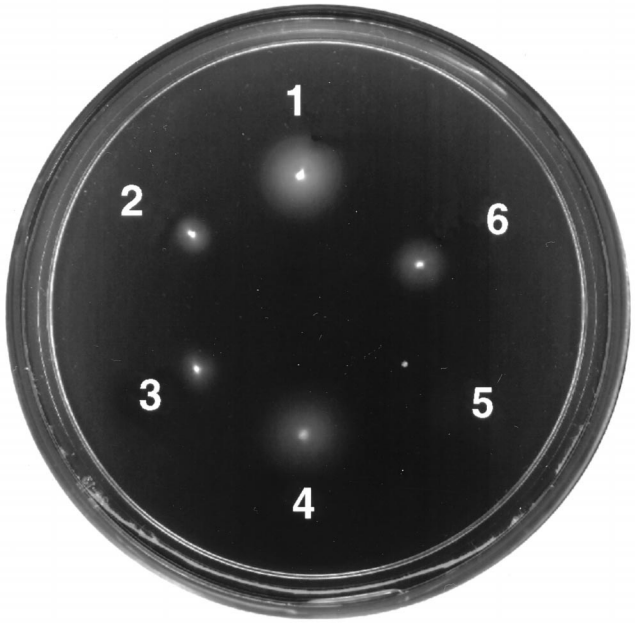

FIG. 3. Swarming behavior of wild-type and mutant *V. cholerae* strains on motility agar plates. 1, N16961 R (wild type); 2, NS1 (*epsD*::Tn*phoA*); 3, AA10 (*epsD*::Kan^r); 4, AA10/pDSK-2 (*epsD*$^+$); 5, AA1 (*epsE*::Kan^r); 6, AA1/pAA42 Tet^r (*epsE*$^+$)

DISCUSSION

We were interested to decipher the genetic basis of rugosity. We hypothesized that there are separate genetic regions responsible for the synthesis, control, phase variation, and secretion to the extracellular milieu of the rugose polysaccharide. Indeed, the mutations in the Tn*phoA* mutants isolated in this study mapped to five distinct *Sfi*I fragments, suggesting that there may be five different regions involved in rugosity. Recently, Yildiz and Schoolnik (52) identified one of the regions (*vps*) involved in the synthesis of rugose polysaccharide. Here we report a second region (*eps*) involved in the synthesis or secretion of the polysaccharide. Identification of the other regions is in progress. Most of the Tn*phoA* mutants isolated in this study had multiple insertions, and many had the suicide delivery vector cointegrated at the insertion site. This phenom-

enon has been previously observed, and it is one of the drawbacks of this transposon delivery vector (43).

The data presented here demonstrate that a 291-kb *Sfi*I fragment involved, directly or indirectly, in the production of rugose polysaccharide contains the *eps* operon. Interestingly, the recently described rugose polysaccharide biosynthesis region, *vps*, contains an (exopolysaccharide (EPS) transport-associated gene (*exoP*) (52). The role of this gene needs to be determined in light of our finding that the *eps* operon may be involved in the secretion of rugose polysaccharide. Perhaps the two systems are involved in the transport and secretion of rugose polysaccharide to different cellular compartments. For example, *exoP* may be involved in transport of the polysaccharide from the cytoplasm across the inner membrane, analogous to the Sec-dependent transport of proteins. The *eps* pathway may be involved in the subsequent transport across the outer membrane and secretion into the external medium.

The Tn*phoA* insertion at the 3′ end of *epsD* in the N16961 rugose strain resulted in stable smooth colonies. Although EpsD has been predicted to be the channel-forming outer membrane protein of the GSP system, this report presents the first experimental evidence for the involvement of this protein in CT secretion as well as other secretion phenotypes in *V. cholerae*. Introduction of a wild-type *epsD*$^+$ gene on a multicopy plasmid (pDSK-2) resulted in a high-frequency allelic exchange with the chromosomal *epsD*::Tn*phoA* copy. The failure of complementation in trans may be due to overexpression of *epsD* from pDSK-2 compared to the genes downstream of *epsD*, which are probably underexpressed because of polarity of the chromosomal *epsD*::Tn*phoA* insertion. The resulting alteration in the stoichiometry of the EPS proteins is probably detrimental to the cell, leading to reciprocal recombination that eliminated the *epsD*$^+$ plasmid. This is consistent with the findings of Sandkvist et al. that EpsL and EpsM interact with each other (39, 41), and EpsD probably interacts with one or more of the EPS proteins in a stoichiometric fashion in the multiprotein secretion apparatus. Additional support for this argument is based on the construction of an in-frame insertion mutant of *epsD* (AA10) that could be complemented by pDSK-2. AA10 exhibits a delayed smooth phenotype. The colonies, after overnight growth, show rugose morphology, but they turn smooth on continued incubation. We speculate that this is due to a partial defect in secretion of rugose polysaccharide and that the synthesis of rugose polysaccharide is probably shut down when secretion is partially blocked. AA10 could be complemented by pDSK-2, and this indicated that the failure of complementation in NS1 is not due to lack of expression of *epsD* on pDSK-2.

Previously the *eps* system was shown to mediate the secretion of CT, chitinase, and protease (38–40). The data presented here indicate its involvement in the secretion of additional substrates, including hemolysin and complex structures such as rugose polysaccharide and flagella. However, not all the Tn*phoA* mutants (rugose to smooth) are defective in secretion of CT and hemolysin. The mutants with multiple Tn*phoA* insertions need to be separated to understand which of the insertions causes the defects. Even in isolates with a single Tn*phoA* insertion there is not a strict correlation of the phenotypes. For example, S7, S17, S20, and NS1 all have a Tn*phoA* in a 291-kb *Sfi*I fragment. However, only NS1 exhibits all the secretory defects. The majority of the mutants that show defects in CT secretion (S3, S4, S11, S16, S17, and NS1) have at least one insertion in the 291-kb fragment. CT secretion is severely attenuated in *epsD* mutants NS1 and AA10. For reasons not well understood, in AA1 (*epsE*::Kan^r), CT secretion is less severely affected.

The mutants S3, S4, and NS1 showed complete loss of hemolytic activity. *E. coli* hemolysin is secreted via the type I secretory pathway (32). In *V. cholerae* serogroup O1, biotype El Tor, hemolysin is secreted extracellularly as prohemolysin and requires cleavage by a secreted protease to become biologically active (29, 51). Hence, the *epsD*::Tn*phoA* mutation in NS1, and presumably that in S4 as well, may be directly inhibiting the secretion of hemolysin or indirectly inhibiting hemolysis by affecting protease secretion.

The loss of motility in some mutants (S2, S3, S4, S10, and NS1) suggests a role for *eps* system in flagellar biosynthesis. The defects in motility of the *epsD* and *epsE* mutants are not due to growth defects. Preliminary electron microscopic analyses indicate that NS1 and AA1 lack a polar flagellum (A. Ali and S. Sozhamannan, unpublished data). It is interesting that unlike other systems where flagellar biogenesis involves dedicated pathways, in *V. cholerae* the GSP may play a direct or indirect role.

We are currently examining whether the *eps* mutants affect the formation of other extracellular structures such as toxin-coregulated pili (TCP) or mannose-sensitive hemagglutinin type IV pili as well. This is particularly relevant because some GSP homolog proteins are involved in assembly and maturation of filamentous phages (fd, f1 and M13) (36); the TCP pilus has been suggested to be the major coat protein of a single-stranded phage, VPI ϕ (16). It would be of interest to see if the *epsD* and *epsE* mutants are defective for the secretion of single-stranded phages of *V. cholerae* such as CTX ϕ and VPI ϕ.

It is surprising that the *eps* system, involved in transporting proteins across the outer membrane, can also affect polysaccharide secretion. It may be that the rugose polysaccharide is coated with proteins during translocation. Alternatively, the *eps* system may affect polysaccharide synthesis or processing rather than secretion. Our future studies will be aimed at distinguishing these possibilities.

It is conceivable that *eps* mutants affect the production of rugose polysaccharide indirectly by affecting the secretion of an outer membrane protein that positively regulates polysaccharide synthesis. The positive regulation may be through a two-component sensor-transducer regulatory cascade that relays an environmental signal. It has been shown that the *eps* mutants have a modified cell envelope due to defects in the production of certain outer membrane proteins (24, 40). This membrane perturbation might affect the signaling pathway that regulates the transcription of polysaccharide biosynthesis genes. In other species polysaccharide production and biofilm formation are regulated at the transcriptional level by LuxR and LuxI homologs (2, 6, 10, 20). Indeed, a LuxR homolog, HapR, has been identified recently in *V. cholerae* as a positive regulator of hemagglutinin and protease gene expression. Interestingly, HapR seems to negatively regulate rugose polysaccharide formation (14). Although the mechanism of this regulation is not known, it might involve quorum sensing. It may be that HapR itself is an outer membrane protein that requires a functional *eps* system for proper membrane localization. In this case, *eps* mutants would be derepressed for rugose expression. Although in a serogroup O139 smooth strain background, an *epsD* mutant is not phenotypically HapR$^-$ with respect to rugose expression (A. Ali and S. Sozhamannan, unpublished observation), there may be other strain-specific differences in this regulatory network.

Similar indirect effects of the mutations in *eps* genes on flagellar biogenesis may result in nonmotile mutants. Regulatory genes, such as *rpoN* (encoding σ^{54}) and the cognate transcriptional activators (*flrA* to *-C*) of flagellar biosynthesis have been identified (18), and it is possible that their ability to

regulate flagellar gene transcription might also depend on a functional *eps* system. Our future studies will focus on elucidating the mechanism(s) by which the *eps* pathway affects phenotypes such as flagellar biogenesis and polysaccharide production. The significant finding of this work, however, is that the *V. cholerae* GSP system, *eps*, seems to affect directly or indirectly multiple aspects of *V. cholerae* pathogenesis and environmental survival.

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