Molecular Genetics and Physiology of the Rugose Phenotype of *Vibrio cholerae*

Mohammed Harunur Rashid

Department of Epidemiology and Preventive Medicine
University of Maryland School of Medicine
Department of Microbiology
University of Dhaka

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Declaration Certificate

I here by, humbly declare that, the topic of my thesis is "**Molecular Genetics and Physiology of the Rugose Phenotype of *Vibrio cholerae***" and this thesis is based on work carried out by me and me only. No part of this work has previously been presented any where for any degree. This research work has been carried out in the Department of Epidemiology & Preventive Medicine, University of Maryland School of Medicine, USA under the active supervision and guidance of Professor Dr. Chowdhury Rafiqul Ahsan, Dept of Microbiology, University of Dhaka.

18-11-06

Dr. Chowdhury Rafiqul Ahsan

Professor & Supervisor
Department of Microbiology
University of Dhaka

(Mohammed Harunur Rashid)

Ph.D. Candidate
Reg. No. 60/2000-2001
Dept. of Microbiology
University of Dhaka

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switch, transposon mutagenesis of a smooth *V. cholerae* strain was used to identify mutants that were unable to shift to the rugose phenotype under inducing conditions. I identified (i) *vpsR*, *galE* and *vps*, which were previously associated with the rugose phenotype, and (ii) genes not previously associated with the rugose phenotype; e.g., *rfbD* and *rfbE*, which have roles in LPS (lipopolysaccharide) synthesis, and *aroB* and *aroK* , which have roles in aromatic amino acid synthesis. In addition, a mutation in *amiB*, which encodes an N-acetylmuramoyl-L-alanine amidase caused defects in the smooth to rugose switch, motility and cell morphology. Also, I found that a gene encoding a novel regulatory protein (designated "RocS" [regulation of cell signaling]) containing GGDEF and EAL domain, and apparently associated with c-di-GMP levels in the cell, is important for expression of the rugose phenotype, biofilm formation and motility. As such, manipulation of c-di-GMP and its level could be used to regulate various phenotypes and potentially control biofilm and virulence. VpsR has previously been reported to up-regulate *Vibrio* polysaccharide (VPS) genes and the synthesis of extracellular EPS (VPS). In that regard, my *in vivo* studies with ligated rabbit ileal loops indicated that VpsR mutants had attenuated reactogenicity, and intestinal colonization studies with infant mice suggested that VPS production, the rugose phenotype and VpsR have a role in the pathogenesis of cholera, and that VpsR may regulate genes with roles in virulence. Rugose strains appear to be a subpopulation of cells that may act as a 'helper' phenotype that promotes the virulence of some strains. In conclusion, the results of my studies provide insight into the potential role of VPS, the rugose phenotype, VpsR and new regulatory mechanisms in the pathogenesis of epidemic strains of *V. cholerae*.

A. General introduction

1. Brief history of cholera:

Cholera is an old infectious diarrheal disease that has killed millions of people over the years and continues to be a major public health concern worldwide. Cholera affects more than 75 countries and every continent (Communicable Disease Surveillance and Response, World Health Organization). A total of 293,121 cholera cases and 10,586 deaths were reported to WHO in 1998 (119). The disease has a high morbidity and mortality in developing countries because of poor sanitation, lack of education, crowded living conditions, and underlying malnutrition. It is characterized by massive explosive secretory diarrhea, vomiting, muscle cramps, abdominal pain, and severe dehydration that may cause death if prompt and appropriate treatment is not initiated (79). The term cholera appears to have been derived from the Greek words *chole* (bile) and *rein* (flow). Other investigators have suggested (79, 93) that the term cholera may have been derived from the Hebrew words for "bad disease." Historically, there have been seven recorded cholera pandemics since 1807 (93); however, there have been reports of cholera-like illness in the form of epidemic/endemic disease reported both in the Sanskrit and Greek literature since 400-500 BC. Thus, it is most likely that cholera was present and common long before the first cholera pandemic was officially recorded in 1807 (15). The current and seventh cholera pandemic started in Indonesia in 1961, and it is still continuing to this day with outbreaks in South America (94, 174) and in other endemic region such as Bangladesh and India (79). During the past 40 years, much progress has been made in understanding the pathogenesis, prevention and treatment of cholera, including the use of Oral Rehydration Saline (ORS) to treat cholera patients. In fact, the treatment of cholera

patients with ORS has drastically reduced the disease's mortality rate from >50% to < 1%. In addition, the adoption and maintenance of good sanitary practices have virtually eliminated cholera epidemics in developed countries (94, 94), and vaccine candidates to prevent cholera are under active development in various parts of the world (79).

2. *Vibrio cholerae*: the causative organism of cholera:

The etiologic microorganism responsible for human cholera is *Vibrio cholerae*, a gram-negative, facultatively anaerobic, oxidase-positive and usually O129 (a vibriostatic compound)-sensitive bacterium. *V. cholerae* is ubiquitous and autochthonous to aquatic environments, including fresh, estuarine and marine waters (20, 21, 79). There are more than 200 known O-antigen serogroups (140) of *V. cholerae*, however, only the O1 serogroup and the recently described O139 serogroup have been associated with typical clinical cholera cases and epidemics. While they do not cause diarrheal disease cholera, other non-O1 serogroups of *V. cholerae* can cause extraintestinal infections (including septicemia and wound infections) and sporadic gastroenteritis (113). *V. cholerae* serogroup O1 is traditionally divided into three serotypes (Ogawa, Inaba and Hikojima) based on their patterns of three lipopolysaccharide antigens A, B and C (150). *V. cholerae* O1 are further divided into two biotypes, classical and El Tor that are distinguished by differences in various metabolic properties. The 6th pandemic (classical biotype) strain began in 1899 and caused significant outbreaks through 1923 (126). The 6th pandemic (the classical biotype) of *V.* cholerae is presumed to be the cause of the first six pandemics, however the biotype and serotype designations for strains causing the earlier pandemics are not known (79, 93). The current 7th pandemic (El Tor biotype) strain emerged in 1961 on the island of Sulawesi in Indonesia and then rapidly spread

throughout Asia (12, 78) and worldwide resulting in significant epidemics particularly in Africa (40, 50) and Latin America (151, 153). In 1992, a novel strain with a previously unrecognized serogroup (O139) emerged in India (130). The term *V. cholerae* "O139 Bengal" was given to these novel strains to signify their geographic isolation from coastal cities along the Bay of Bengal (146). O139 Bengal strains have the ability to produce a capsule and appear to have evolved from a 7th pandemic El Tor strain following exchange mainly of the O antigen gene cluster (1, 16, 23, 39, 53, 55, 76, 127, 132, 143, 149, 160).

3. Regulation of virulence in *V. cholerae*:

A principal virulence factor of *V. cholerae* is CT, a heterodimeric protein exotoxin consisting of a single, enzymatically active A subunit non-covalently associated with five identically-sized B subunits (46). During assembly of cholera holotoxin, A and B subunits enter the periplasmic space after cleavage of signal peptides, and assemble there into a holotoxin which is secreted across the outer membrane by a type II secretion system (65, 141). The B subunit pentamer binds holotoxin to its eukaryotic cell receptor, GM_1 ganglioside while the A subunit is proteolytically cleaved to its functional A1 and A2 fragments by a protease. The genes encoding CT (*ctxAB*) (101, 102), are carried by a lysogenic filamentous bacteriophage designated CTXΦ (161). A second distinguishing characteristic of toxigenic *V. cholerae* strains is their ability to colonize the human intestine. The TCP gene cluster in both classical and El Tor strains functions as an essential colonization factor in both animal and human volunteer studies (63, 155) and as the CTXΦ receptor (161). The TCP gene cluster and *toxT* that regulates *ctxAB* and *tcpA*, the major protein subunit of TCP, are encoded on the *Vibrio* Pathogenicity island (VPI) that is present in all epidemic strains and typically absent from nonpathogenic strains (80, 83,

89). The synthesis, secretion and binding of CT to the epithelial cell surface subsequently triggers a sequence of events that eventually results in massive secretion of fluids and electrolytes into the intestinal lumen, which causes the life-threatening diarrhea characteristic of cholera.

In El Tor strains, production of CT and TcpA occurs under very limited *in vitro* growth conditions (i.e., at 37°C). The *in vitro* conditions for optimal (albeit limited) virulence gene expression in El Tor strains have been termed AKI conditions (73, 74). In classical biotype strains, expression of CT *in vitro* is not constitutive but is specifically regulated by environmental growth conditions, such as pH, temperature, osmolarity and amino acids (107, 109). The optimal temperature and pH for virulence gene expression in classical biotype strains is 30°C and pH 6.5 and most of the regulation studies of *V. cholerae* virulence have been performed using classical biotype strains. The virulence of *V. cholerae* is under the control of the "ToxR regulon" in which the *toxR* gene encodes a transmembrane protein which regulates metabolic genes present in all strains and virulence genes present only in pathogenic strains (105, 107). A second regulatory gene, *toxS*, is transcribed in an operon with *toxR* and encodes a periplasmic protein that possibly stabilizes ToxR (35, 37, 108). ToxR binds to the repeated DNA sequence TTTTGAT which is present in multiple copies upstream of the *ctxA* promoter (107). Although ToxR can activate *ctxAB*, it is not able to directly activate transcription of *tcpA* (17, 107). In this case, ToxR activates transcription of *toxT* whose protein directly activates *ctxAB* and *tcpA* (34, 35, 64, 117). It has recently been shown that *tcpP* and *tcpH* also act as important regulators of

virulence expression by affecting *toxT* transcription (18, 56) and that *tcpPH* expression is regulated by *aphA* and *aphB* (90, 91, 148).

4. Environmental survival and persistence:

The primary reservoir for *V. cholerae* is the aquatic and estuarine environment. Thus, contaminated water and food serve as the primary vehicles for transmitting the bacterium to humans (20, 47). Although *V. cholerae* is known to adapt and persist in a variety of aquatic niches, the basis for its persistence in those ecological niches has not yet been elucidated. In endemic areas, such as Bangladesh and Peru, an epidemic occur seasonally and *V. cholerae* appears during the epidemics and disappears during interepidemic periods (43, 70). The underlying basis for the appearance and disappearance of *V. cholerae* during and after cholera epidemics is not clear. Until the early 1980s, *V. cholerae* was believed to be human host-adapted and incapable of surviving for more than a few hours in natural waters. Consequently, cholera epidemics were believed to be impossible if contamination of the water by human feces did not occur (21). However, it is now well established that *V. cholerae* can persist indefinitely in aquatic environments, although the basis for this survival, particularly during interepidemic periods in cholera endemic areas, is not well understood. Franco *et al.* (43) have reported that CT- positive strains of *V. cholerae* (i) appeared in the aquatic environment 2-3 months before the onset of cholera epidemics in Peru, which suggests amplification of *V. cholerae* in water in response to an as-yet-unknown environmental stimulus, and (ii) were not detected during the interepidemic period (winter season), which indicates that epidemic strains of *V. cholerae* survive during the interepidemic period by some as-yet-unknown mechanism.

Colwell *et al.* (21) have demonstrated that *V. cholerae* can enter a viable but non-culturable (VBNC) state in response to adverse environmental conditions (e.g., nutrient deprivation, elevated salinity, and/or reduced temperature), while retaining full virulence (21, 22, 70, 176). However, attempts to resuscitate VBNC *V. cholerae* have been unsuccessful so far. Furthermore, the importance of the VBNC state in the epidemiology of cholera remains unclear. In studies investigating the environmental survival mechanisms of *V. cholerae*, Colwell and other investigators (20, 70, 72) also reported that *V. cholerae* binds to phytoplankton and zooplankton in the environment, and that cholera epidemics follow plankton blooms, suggesting some association with plankton is involved in the endemicity of *V. cholerae*.

Although epidemic *V. cholerae* strains are known to persist for many years in the marine environment while regularly causing significant cholera epidemics (12, 40, 50, 78, 130, 151, 153), the specific mechanism underlying their adaptation to long-term persistence is still unknown at the present time. Several studies have proposed that the persistence of *V. cholerae* in the environment is due to a viable but nonculturable (VBNC) state elicited by starvation and cold (22, 139, 145, 176), however, it is not known whether the VBNC phenotype promotes persistence in endemic regions and under conditions other than starvation and cold. Alternatively, another hypothesis is that production of EPS and the rugose phenotype might promote the survival and persistence of *V. cholerae* in aquatic environments.

5. *V. cholerae* Exopolysaccharide:

In 1938 Bruce White first described another potential survival form of *V. cholerae* (170). As originally described, *V. cholerae* exhibits a "rugose-mediated" survival phenotype characterized by wrinkled (rugose) colony morphology that is distinct from that of rough strains (170, 171, 172). Studies of the rugose phenotype of *V. cholerae* lapsed due to Bruce White's death and to an inability to distinguish smooth and rugose colonies on selective growth media such as thiosulphate-citrate-bile salts- sucrose (TCBS) agar that was typically used for isolating *V. cholerae* from environmental and clinical samples. However, recent studies in our laboratory and by the Environmental Protection Agency (Cincinnati, Ohio) have demonstrated that O1, O139 and some other non-O1 *V. cholerae* can produce exopolysaccharide (EPS) and assume the rugose phenotype during passage in alkaline peptone water (APW) while retaining their virulence for various in *vitro* and *in vivo* models (112, 114, 133). The differences in colony morphology between the smooth and rugose phenotypes of *V. cholerae* is illustrated in Figure 1 and 2. The switch from smooth to rugose phenotype is reversible in the laboratory; however, its frequency is highest during incubation in APW for 2-4 days at 37°C. Cells in the rugose state are smaller than smooth cells, and they form aggregates of cells embedded in EPS matrix. Furthermore, the rugose phenotype of El Tor strains has been studied and found that it exhibits (probably due to cell aggregation and exopolysaccharide production) increased resistance to chlorine, UV light, acid pH, oxidative and osmotic stress, and serum/complement-mediated killing (112, 133, 134). The chlorine resistance of the rugose variants of virulent *V. cholerae* strains may have

important implications for the bacterium's ecology and epidemiology since chlorine-treatment is used to eliminate pathogenic bacteria from contaminated water supplies.

6. Significance and Importance of *V. cholerae* EPS and the rugose phenotype:

Based on the data from studies to date and the characteristics of *V. cholerae* producing EPS and exhibiting the rugose phenotype, the following generalizations can be made:

1. The EPSs that *V. cholerae* cells secrete promotes and essential for biofilm formation. In turn, the biofilm may (i) help to promote endemicity and maintain *V. cholerae* cells indefinitely in natural environments, and (ii) promote the release of sessile or planktonic cells, which also help the bacterium to persist in the aquatic environment and serve as a potential continued source of infection for humans. Furthermore, while the importance of EPS and the rugose phenotype in the environment is preserved, the role of EPS and the rugose phenotype in virulence is not well understood.

2. The smooth phenotype of *V. cholerae* can switch reversibly to the rugose phenotype. This switch is in part associated with production of EPS. Thus, understanding the "switch" at the molecular genetic level may serve as a paradigm for other related bacteria that express EPS and switch to a rugose phenotype. Although the mechanisms are not well understood, EPS production may be induced by various environmental cues and its synthesis may require a cascade of regulatory proteins similar to those used in two-component signal transduction pathways indicating a complex mechanism of regulation.

3. The resistance of the *V. cholerae* rugose phenotype to various environmental stresses such as chlorine, acid pH and UV light may be associated with epidemiology of cholera because these factors are commonly used to eliminate pathogenic bacteria from contaminated water. Epidemic strains that express EPS and the rugose phenotype and which promote resistance to these factors are therefore of public health concern and need to be studied.

7. Specific objectives of this project:

In order to improve our understanding of EPS production, the switch to the rugose phenotype and the role of EPS and the rugose phenotype in the epidemiology and pathogenic cycle of cholera, this project was designed to

(i) Identify conditions promoting EPS production and the switch to the rugose phenotype in shortest period of time.

(ii) Determine whether epidemic strains of *V. cholerae* have better potential to undergo rugose production than environmental strains.

(iii) Characterize the EPSoff / EPSon molecular regulatory switch in *V. cholerae.*

(iv) Study the role of EPS and the rugose phenotype in the virulence and pathogenesis of *V. cholerae.*

B. Rugose exopolysaccharide (rEPS) of *V. cholerae*

1. Discovery of a medium that promotes high frequency rEPS production:

The rugose colony phenotype was first reported and described in both sixth (classical biotype) and seventh (El Tor biotype) pandemic strains of *V. cholerae* in the late 1930s by Bruce White (170, 171) and is characterized by wrinkled colonies expressing copious amounts of EPS. Since then, various studies (110, 112. 158, 170, 171, 178) have demonstrated that "smooth" *V. cholerae* cells can, under nutrient-limiting conditions, spontaneously shift - at a low frequency - to express EPS and exhibit wrinkled ("rugose") colonies.

Recent studies have demonstrated three important characteristics associated with the rugose phenotype of *V. cholerae*: (i) rugose cells are virulent in the rabbit ileal loop model and in human volunteers (112, 133) (ii) rugose cells are resistant to a variety of environmental stresses; e.g., chlorine, UV light, acid pH, osmotic and oxidative stress, and serum/complement-mediated killing, (iii) the shift from smooth to rugose phenotype increases dramatically during growth in APW, and (iv) the expression of the rugose phenotype is inhibited during growth on TCBS agar (79, 112, 158). As *in vitro* studies have demonstrated that rugose cells are significantly more resistant to various noxious environmental conditions than are smooth cells, the former have been hypothesized to survive better than the latter in their natural aquatic reservoirs. However the prevalence of this phenotype in *V. cholerae* and the genetic basis, underlying this phenotype have not yet been rigorously explored.

TCBS is widely used as a primary isolation medium for *V. cholerae*. Unfortunately, the rugose variant of *V. cholerae* is difficult to detect on TCBS agar (87) because this medium inhibits the expression of the rugose phenotype. Therefore, in order to determine the true incidence of the rugose phenotype of *V. cholerae* in both environmental and clinical samples, and to understand the possible clinical and ecological significance of this phenotype, it is first essential to maximize the isolation rate of rugose *V. cholerae* from both clinical and environmental samples.

The biochemical and genetic basis for EPS production and the rugose phenotype are not well understood since early studies were hampered by the low frequency of switching to the rugose phenotype under the experimental conditions used. For example, Morris *et al.* (112) reported the isolation of only low numbers spontaneous rugose variants from a variety of *V. cholerae* strains after 3-4 days incubation in standard alkaline peptone water (APW) and plating on L agar at 37°C. Also, Wai *et al.* (158) subsequently reported that a *V. cholerae* O1 El Tor strain spontaneously shifted at a low frequency to the rugose phenotype under starvation conditions. They isolated a few spontaneous rugose variants after smooth colonies were starved in M9 salts for 2 months at 16°C, and then were plated on L agar at 37°C. Also, Yildiz and Schoolnik (178) found that the nutrient (carbon source) limiting conditions they used (i.e., 30°C incubation in M9 minimal media supplemented with 0.02% glucose) enabled smooth El Tor strains to shift to the rugose phenotype, after 20 days of culture, at a frequency of only ~1%. Therefore the importance of rugose variant and its population biology in the context of its environmental persistence and human infection has not been well studied due to the lack of a suitable medium promoting EPS production and a higher frequency of switching

from the smooth variant to the rugose variant. In order to address this problem, studies were initiated to identify a medium that can promote high-frequency rugose production.

2. Analysis of EPS produced by clinical strains of *V. cholerae*:

Recent studies (4, 165, 178) have examined the importance and role of EPS production in the survival of *V. cholerae* in the environment. A large variety of EPS types are synthesized by bacteria and many are believed to be important in virulence such that (i) EPS enables bacterial attachment to surfaces and promotes biofilm, (ii) EPS production and subsequent biofilm formation improves nutrient acquisition; and (iii) protects cells against environmental stresses and host defenses (156). EPS may form a capsule composed of a high-molecular-weight polysaccharide that is tightly attached to the cell surface or it may exist as a slime layer that is either loosely attached to the cell surface or secreted and released by the cell into the surrounding medium. In either case, EPS is known to be essential for biofilm formation. The biofilm concept has drawn attention to the structures that are important for ecology and persistence of *V. cholerae* in aquatic environments and possibly for its ability to colonize the gut of humans (25, 26, 27, 28). Furthermore, it is now well-accepted that bacteria respond to changes in their environment by exhibiting profound phenotypic changes in enzymatic activity, cell wall composition (144), and surface structure (9). As such, EPS production might offer a selective advantage under specific conditions.

Several previous studies have examined the compositional and linkage analyses of the EPS from two independent El Tor strains and found that it is unrelated to previously described *V. cholerae* O1 serogroup carbohydrates. However, until now, no studies have analyzed the structure and composition of EPS in a classical biotype strain.

3. Importance of EPS in biofilm formation:

V. cholerae cells can exist in a planktonic (free-swimming) form and in surface-attached communities called biofilms (71). Bacteria in aquatic environments are predominantly found in biofilms; therefore, the ability to form biofilms rapidly and efficiently may provide a competitive advantage in those environments, promote resistance to various stresses and increase cell survival and persistence (26). Biofilms are a specialized and highly adapted form of surface growth characterized by assemblages of bacteria that form pillars or mushroom-like structures separated by fluid-filled channels. The pillars, in turn, consist of an EPS matrix and the bacteria that secrete it. Since EPS accounts for about 85% of the biofilms depth, the production of EPS is critical for the development of a mature biofilm (29, 88).

V. cholerae O1 El Tor, in common with many aquatic bacterial species, forms a typical three-dimensional biofilm on a variety of abiotic surfaces (165, 178). Biofilm formations may be important in the life cycle of virulent *V. cholerae* strains because it helps the bacteria to reside within their natural aquatic habitats during interepidemic periods. In that regard, the rugose variant of *V. cholerae* forms a thicker and more differentiated biofilm than does the smooth variant (178) and this capacity is associated with the production of an EPS by rugose strains. This EPS has been shown to promote chlorine resistance (178) and to protect the organism from the bactericidal action of hydrogen peroxide (159). Open reading frames (ORFs) required for the biosynthesis of *V. cholerae* EPS are clustered in a 30.7-kb segment on the *V. cholerae* O1 chromosome. Their putative protein products are homologous to capsular or EPS biosynthetic enzymes

of other species; therefore, the corresponding *Vibrio* genes were designated as *vps*, for *Vibrio* polysaccharide (178).

In *Escherichia coli*, *Pseudomonas aeruginosa and V. cholerae* O1 El Tor, initial bacterial attachment to a surface is accelerated by functional flagella, which suggests that motility may overcome some energetic barrier encountered by the bacterium en route to the surface (120, 128, 165). Type IV pili are also responsible for a surface-associated motility and are important for biofilm formation (142, 169, 175, 162, 173). In this type of motility, the pilus is thought to anchor to a surface and then propel the bacterium by retracting the pilus against the surface. In *V. cholerae* O1 El Tor, the mannose-sensitive haemagglutinin (MSHA) type IV pilus also accelerates attachment to surface (166).

There is evidence that flagellin and EPS synthesis are inversely regulated in several bacterial species. For example, biofilm-associated *E. coli* represses the transcription of their flagellin-encoding genes and increases the transcription of their colanic acid biosynthetic genes (129). Furthermore, recent observations with *P. aeruginosa* suggest that alginate and flagellin syntheses are inversely regulated (45). Taken together, those results suggest that some bacteria present in a biofilm repress flagellin synthesis and increase EPS synthesis, and that those two functions may be coordinately regulated during biofilm development to promote attachment and biofilm formation. Interestingly, for rugose *V. cholerae* O1 El Tor strains, wild type expression levels of both motility and EPS depend on genes involved in the type II secretion of proteins (3) suggesting that in *V. cholerae* O1 El Tor, motility and EPS production may share common steps in their regulatory and biosynthetic pathways. Thus, the level of biofilm formation and motility in smooth and rugose *V. cholerae* appears to be important

for biofilm formation but has not been well studied, particularly in classical biotype strains.

C. Identification of the genes involved in the switch from the smooth to the rugose phenotype of *V. cholerae*

We recently found that *V. cholerae* can switch at high frequency from an EPSoff (i.e., smooth colony morphology) to an EPSon (rugose phenotype) in which the cells are embedded in EPS and demonstrate a wrinkled colony morphology (170, 171). As mentioned above, the rugose phenotype exhibits significantly better biofilm forming ability than smooth strains suggesting that EPS production and the rugose phenotype are advantageous under certain conditions (112, 133, 166). High frequency switching occurs more commonly in epidemic strains than in nonpathogenic strains (4) and further suggests that the EPS production and the rugose phenotype is important in the epidemiology of *V. cholerae*. Also, the rugose variant is highly chlorine resistant and has increased resistance to acid pH, UV light, and serum/complement-mediated bactericidal activity (112, 133, 166). Therefore, switching to the EPSon phenotype might be important in niche specialization and in promoting survival in stressful environments.

Previous studies have shown that production of rEPS is linked to the type II general extracellular protein secretion pathway which is involved in the secretion of important virulence factors (3, 32) and that the *vps* (*Vibrio* polysaccharide) gene cluster in *V. cholerae* is thought to be comprised of two closely located but separate operons in which *vpsA* and *vpsL* represent the first gene of each operon (178, 179). Transcription of *vpsA* and *vpsL* is regulated by VpsR (a homolog of σ54 transcriptional activators), by a mechanism that is not well understood (179). VpsR has a high homology to NtrC, AlgB, and HydG bacterial enhancer-binding protein that activates transcription after

phosphorylation of its receiver domain by an associated sensor kinase protein (84). Previous studies have also found that HapR in some *V. cholerae* strains is linked to the rugose phenotype by some unknown mechanism (75) and that CytR represses transcription of *vps* genes and the associated biofilm formation (57). We have also found that switching to the rugose phenotype is independent of ToxT, LuxS and RpoS (4).

We recently identified conditions that promote rapid switching (up to ~80%) to the rugose phenotype in a process we called "high frequency rugose production" (HFRP) (4). Also, we found that, HFRP is more common in epidemic *V. cholerae* strains than in nonpathogenic strains (4). Thus, previous observations support the working hypothesis that the ability to switch to the rugose phenotype and form biofilms, under certain stressful conditions, is an important adaptive advantage to epidemic strains of *V. cholerae*. Although the physiological significance of rugosity can be surmised from many adaptive features conferred by the EPS, the environmental signals that triggers its production and the genes involved are still poorly understood. Unfortunately, the genes involved in the switch to the rugose phenotype are still not well understood. As such, the rugose inducing conditions promoting the switch to the rugose phenotype were exploited in order to identify genes involved in the switch between the smooth (EPSoff) and rugose (EPSon) phenotypes of *V. cholerae*.

D. Role of the rugose phenotype and *vpsR* in the pathogenesis of cholera

Some of the important, sequential steps in the pathogenesis of cholera include: (i) ingestion of a virulent toxigenic strain of *V. cholerae* in contaminated food or water, (ii) passage of the etiologic agent through the gastric acid barrier of the stomach, (iii) adherence of the bacteria to, and penetration through, the small intestines' mucus lining, (iv) adherence of the etiologic agent to GM1 receptors on the small intestines' epithelial cells, and (v) bacterial multiplication and CT production (67). During their transition from the external environment to the human body, the bacteria are exposed to a series of changes in temperature, pH, osmolarity, etc., and they must survive in the small intestines, which is an environment that contains bacterial growth-inhibitory substances; e.g., bile salts and organic acids. In addition, they are exposed to antimicrobial components of the innate immune system; e.g., complement factors secreted by intestinal epithelial cells (7), and defensins produced by Paneth cells (98). Several *V. cholerae* gene products have been shown to be important for colonization of the small intestines including the toxin-coregulated pili (155), accessory colonization factors (124), regulatory proteins (e.g., ToxR/ToxS, TcpP/TcpH, and ToxT), outer membrane proteins (56, 147), metabolic factors, biotin, purine biosynthetic genes, and the O antigen of the lipopolysaccharide (19).

V. cholerae can switch from the EPSoff (smooth) phenotype to the EPSon (rugose) phenotype characterized by cell aggregation wrinkled, rugose colonies and increased biofilm formation (4, 112, 170, 171). The EPS is believed to provide a

gelatinous matrix that enhances the ability of the rugose variant to survive under *in vivo* conditions (110. 133, 158, 159). The virulence of the rugose variant is unclear since, the results of one study performed here at the University of Maryland (112) with human volunteers demonstrated that a rugose El Tor strain is virulent in humans; i.e., its oral administration elicited severe diarrheal disease, however another study found that rugose O139 strains are defective in colonization in infant mice (168).

Therefore, the virulence of rugose variants of epidemic *V. cholerae* strains is not well understood. In order to improve our understanding of the role of EPS and the rugose phenotype of *V. cholerae* in cholera pathogenesis, *in vitro* and *in vivo* studies were performed.

A. Bacterial strains and plasmids

The bacterial strains and plasmids used in this study, and their relevant characteristics, are listed in Table 1. All of the bacterial strains were stored (-70°C) in LB broth supplemented with 30% glycerol.

TABLE 1. Bacterial strains and plasmids used in this study

Strains and Plasmid	Genotype and phenotype	Origin/source[a] (year) and/or reference
***V. cholerae* strains**		
N16961S (DK 81)	Smooth, serogroup O1, El Tor biotype	Bangladesh, C (1971), (95)
N16961R (DK 81R)	Rugose, serogroup O1, El Tor Biotype	This study
NCTC6585S (DK 64)	Smooth, serogroup O1, classical biotype	India, C (1943)
NCTC6585R (DK 64R)	Rugose, serogroup O1, classical biotype	This study
AMS20A73	Serogroup O1, classical biotype	China, C (1945),
1803	Non-O1 serogroup	Brazil, C (1992)
P44	Non-O1 serogroup	Peru, E (2000)
1085-93	Serogroup O37	Germany, E (1993)
141-94	Serogroup O70	Germany, E (1994)
928-93	Serogroup O6	Argentina, E (1993)
C6706S	Wild-type, smooth, serogroup O1, El Tor biotype	(133)
C6706R	Wild-type, rugose, serogroup O1, El Tor biotype	This study

C6709	Wild-type, smooth, serogroup O1, El Tor biotype	A. Camilli
Aldova	Serogroup O37	Czechoslovakia, C (1965)
569B	Smooth, serogroup O1, classical biotype	(66)
AI 1837	Serogroup O139, opaque	Laboratory strain
DK568	VC0243, RfbD, mini-Tn5 mutant of N16961 (DK 81)	This study
DK623	VC0244, RfbE, mini-Tn5 mutant of N16961 (DK 81)	This study
DK578	VCA0744, GalE, mini-Tn5 mutant of N16961 (DK 81)	This study
DK589	VC0920, Vps (EpsF), mini-Tn5 mutant of N16961 (DK 81)	This study
DK576	VC0921, Vps (Wzx), mini-Tn5 mutant of N16961 (DK 81)	This study
DK588	VC0922, Vps, mini-Tn5 mutant of N16961 (DK 81)	This study
DK562	VC0665, VpsR, mini-Tn5 mutant of N16961 (DK 81)	This study
DK614	VC2628, AroB, mini-Tn5 mutant of N16961 (DK 81)	This study
DK625	VC2629, AroK, mini-Tn5 mutant of N16961 (DK 81)	This study
DK630	VC0344, AmiB, mini-Tn5 mutant of N16961 (DK 81)	This study
DK567	VC0653, RocS, mini-Tn5 mutant of N16961 (DK 81)	This study
***E. coli* strains**		
DH5α	*recA*1 Δ*lacU*169 φ80d*lacZ*ΔM15	Invitrogen
E. coli S17 λpir	pUT/mini-Tn5 Km	(96)

Plasmids

pWSK29	Low-copy-number vector, Ap^r, pSC101 *ori*	(163)
pBR322	Medium-copy-number vector, Ap^r, Tc^r, ColE1 *ori*	New Englad Biolab

[a]C: Clinical isolate, E: Environmental isolate

TABLE 2. Sequences of oligonucleotides used as primers

Primer	Nucleotide sequence
KAR486	5′-CGG GAT CCC GCT AAG TCA GAG TTT TTA TCG C-3′
KAR487	5′-TCC CCG CGG GTC GGT GGT TTT GAT CGT GT-3′
KAR514 (Arb1)	5′-GGC CAC GCG TCG ACT AGT CAN NNN NNN NNN GAT AT-3′
KAR518 (Arb2)	5′-GGC CAC GCG TCG ACT AGT AC-3′
KAR515 (Arb6)	5′-GGC CAC GCG TCG ACT AGT CAN NNN NNN NNN ACG CC-3′
KAR516	5′-CGT TGC GCT GCC CGG ATT AC-3′
KAR517	5′-CCT CTA GAG TCG ACC TGC AG-3′
KAR519	5′-GCA CTT GTG TAT AAG AGT CAG-3′
KAR520	5′-CCT AGG CGG CCA GAT CTG AT-3′

B. Reagents

Taq DNA polymerase, Restriction endonucleases, T4 polymerase, and T4 ligase were purchased from Invitrogen (Carlsbad, California). Plasmid extraction kits (for mini, midi and maxi preparations) were obtained from QIAGEN (Valencia, California). Chromosomal DNA isolation and purification was performed with Wizard genomic DNA purification kit obtained from Promega (Madison, Wisconsin). The DNA in solutions and agarose gels was rapidly purified with a GENECLEAN Spin Kit (Q-BIOgene, Vista, California). IPTG (isopropyl-β-D-thiogalactopyranoside) and X-Gal (5-bromo-4-chloro-3-indolyl-B-D-galactopyranoside), purchased from the Sigma Chemical Co. (St. Louis, Missouri), were used at the following concentrations: IPTG, 0.1 mmol; X-Gal, 40 μg/ml.

C. Media

Traditional culture media were purchased from Difco Laboratories (Detroit, Michigan) and prepared according to the manufacturer's recommendations. *V. cholerae* strains were grown, as required, in LB broth or on LB agar plates with appropriate antibiotic selection where appropriate. Antibiotics (Sigma) were used at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; streptomycin, 100 μg/ml; polymyxin B, 50 IU/ml; and tetracycline, 12.5 μg/ml (*E. coli*) and 5 μg/ml (*V. cholerae*).

Composition of commonly used media

1. **TCBS** agar (Difco): Thiosulfate-citrate-bile-sucrose agar (for isolating *V. cholerae* and other enteropathogenic vibrios). Eighty-nine grams of the powder are suspended in 1L of purified water, the suspension is boiled for 1 min (with stirring) to dissolve the powder, and the solution is cooled to 40°-50°C and dispensed immediately. TCBS was not autoclaved.

Approximate formula per liter:

Yeast extract	5.0 g
Proteose peptone No. 3	10.0 g
Sodium citrate	10.0 g
Sodium thiosulfate	10.0 g
Oxgall	8.0 g
Saccharose	20.0 g
Sodium chloride	10.0 g
Ferric ammonium citrate	1.0 g
Bromthymol blue	0.04 g
Thymol blue	0.04 g
Agar	15.0 g

Final pH = 8.6 ± 0.2

2. **LB agar** (Miller): Luria Bertani agar. Fourty grams of the powder are suspended in 1 L of purified water, the suspension is boiled for 1 min (with stirring) to dissolve the powder, and the solution is autoclaved (121°C, 15 min).

Approximate formula per liter:

Tryptone	10.0 g
Yeast extract	5.0 g
Sodium chloride	10.0 g
Agar	15.0 g

Final pH = 7.0 ± 0.2

3. **LB broth** (Miller): Twenty-five grams of the powder are suspended in 1 L of purified water, the suspension is heated (with stirring) to dissolve the powder, and the solution is autoclaved (121°C, 15 min).

Approximate formula per liter:

Tryptone	10.0 g
Yeast extract	5.0 g
Sodium chloride	10.0 g

Final pH = 7.0 ± 0.2

4. **TSB** (Difco): Tryptic Soy Broth. Thirty grams of the powder are suspended in 1 L of purified water, the suspension is heated (with stirring) until the powder is dissolved, and the solution is autoclaved (121°C, 15 min).

Approximate formula per liter:

Pancreatic digest of casein	17.0 g
Enzymatic digest of soybean meal	3.0 g
Dextrose	2.5 g
Sodium chloride	5.0 g

Dipotassium phosphate 2.5 g

Final pH = 7.3 ± 0.2

5. **APW#3** (Alkaline peptone water # 3) Mix ingredients thoroughly and warm gently until solution is complete. Adjust the solution's pH to 8.5, and autoclave (121°C, 25 min).

Approximate formula per liter:

Proteose peptone #3 (Difco) 1.0 g

NaCl (Difco) 1.0 g

Deionized water 1 L

pH = 8.5

6. **AKI** broth

Bacto peptone (Difco) 15.0 g

Yeast extract (Difco) 4.0 g

NaCl 5.0 g

Deionized water 1 L

Autoclave the broth (30 min, 121°C, at 15 psi), use a sterile-filtered solution of $NaHCO_3$ to adjust the pH of an aliquot (10 ml) of the broth to 7.3-7.4, and add the amount of sterile $NaHCO_3$ solution required adjusting the rest of the prepared medium to a pH of 7.3-7.4.

D. Methods

1. DNA techniques

Chromosomal DNA from *V. cholerae* was purified as recommended by Promega, and plasmids from *E. coli* and *V. cholerae* were extracted and purified using reagents and instructions from QIAGEN (QIAGEN, Inc., Chatsworth, California). Plasmid and chromosomal DNA was digested with restriction enzymes, and the fragments were visualized after agarose gel electrophoresis and purified using a GENECLEAN Spin Kit (Q-Biogene Inc., Vista, California). Recombinant plasmids were transformed into *E. coli* after the bacteria were treated with $CaCl_2$ (osmotic shock) or into *V. cholerae* by electroporation with buffer B described by Marcus et al. (99). DNA sequencing was performed with an Applied Biosystems 373A automated DNA sequencer, sequence analyses were performed with DNASIS software, and an NCBI BLAST server was used to perform a sequence homology search.

PCR was performed with a PCR Express apparatus from Hybaid (Franklin, Massachusetts). In amplifications using the PCR Express, the final reaction mixtures contained 0.4 nmol of each oligonucleotide per ml and 0.1 μg of chromosomal DNA per ml. Typical conditions for the PCR reaction were: pre-denaturation at 94°C for 5 min and denaturation at 94°C for 45 seconds, annealing at 51°C for 45 seconds, and extension at 72°C for 1 min (for 35 cycles), and post-elongation at 72°C for 5 min. The amplified products were resolved by agarose gel (0.7%) electrophoresis, and they were purified with a GENECLEAN Spin Kit. The standard PCR reaction mixture (50 μl in a 0.5 ml PCR reaction tube) consisted of: 2 μl of the template DNA preparation, 5 μl of 10X

concentrated PCR buffer, 1.5 µl of 50 mM $MgCl_2$, 1 µl of the 10 mM dNTP mixture, 1 µl of Primer 1 (forward), 1 µl of Primer 2 (reverse), 0.25 µl of the Taq DNA polymerase preparation, and 38.25 µl MilliQ H_2O. A preparation containing molecular weight markers consisted of 7 µl of a 1 kb plus DNA ladder mixture (Invitrogen), 6 µl of 10X concentrated Reaction 2 buffer (Invitrogen), 10 µl of 6X concentrated loading dye (Sigma), and 37 µl of deionized H_2O. Five to-8 µl of the preparation were used in order to observe clear, sharp bands of the molecular weight markers during agarose gel electrophoresis.

2. Colony PCR

Homogeneous suspensions of fresh, large bacterial colonies from LB agar were prepared by vortexing the selected, individual bacterial colonies with 50 µl of 1X TAE buffer (BIOFLUIDS) in 1.5-ml capacity microcentrifuge tubes. The bacterial suspensions were heated in a boiling water bath for 7 min, and the cells were sedimented by centrifugation (13,000 rpm, 2 min). Aliquots (30 µl) of the supernatant fluids were placed in fresh tubes, and 1-2 µl was used as a template for colony PCR (50 µl reaction mixture, as described above).

3. Isolation and purification of genomic DNA

One ml of a 5-ml overnight shaking bacterial culture was centrifuged (13,000 rpm, 3 min) in a 1.5-ml capacity microcentrifuge tube, the pellet was suspended in Nuclei Lysis Solution (600 µl) and the mixture was incubated (80°C water bath, 5 min) in order to lyse the cells, and the suspension was cooled to room temperature. An RNase solution

(3 µl, RNase/ml) was added to the cell lysate, the preparation was mixed well by inverting the tube 2 to 5 times, and the mixture was incubated (37°C, 60 min) and then cooled to room temperature. A protein precipitation solution (200 µl) was added to the digested preparation, and the mixture was vortexed vigorously for 20 seconds, incubated on ice for 5 min, and centrifuged (14,000 rpm, 3 min). The DNA-containing supernatant fluids were transferred to a clean 1.5-ml capacity microcentrifuge tube containing isopropanol (600 µl, at room temperature), and the contents of the tube were mixed gently (by inverting the tube several times) until thread-like strands of DNA formed a visible mass. The suspension was centrifuged (14,000 rpm, 2 min), the supernatant fluids were carefully decanted, and the tube was drained on clean absorbent paper. The DNA pellet was gently washed (by inverting the tube several times) with 70% ethanol (600 µl), the suspension was centrifuged (14,000 rpm, 2 min), and the ethanol was blotted away by draining the tube on clean absorbent paper for 15 min. DNA rehydration solution (100 µl) was added to the tube, the mixture was incubated at 65°C for 1 hour (periodically, the solution was mixed by gently tapping the tube), and the purified DNA solution was stored at -20°C.

4. Rapid isolation of 0.2-300 kb DNA from solution by using GENECLEAN SPIN protocols (from Q-Biogene)

A GENECLEAN spin glassmilk suspension (400 µl, prepared according to the manufacturer's instructions) was added to a GENECLEAN spin filter, and the solution (300 µl) containing the DNA to be purified was added to the suspension. The mixture was incubated (room temperature, 5 min) and was repeatedly inverted (4-5 times/min) to

prevent the settling of the matrix. The liquid was removed from the filter by centrifugation (14,000 rpm, 1 min) and was collected in a catch tube (the tube was emptied, as required). The filter was washed twice with aliquots (500 µl) of GENECLEAN SPIN NEW wash and it was centrifuged for 2 minutes to dry the pellet. The spin filter was transferred to a fresh catch tube, spin elution solution (10-25 µl) was added to the filter, the solution was centrifuged (14,000 rpm, 1 min) to transfer the eluted DNA to the catch tube, and the tube was capped.

5. Rapid isolation of 0.2-300 kb DNA from agarose gels

A GENECLEAN spin glassmilk suspension (400 µl) was added to a GENECLEAN spin filter with a slice of agarose gel (300 mg maximum per filter) containing the DNA to be purified. The mixture was heated (55°C, 5 min) to melt the gel, and the tube was flicked to mix its contents. The solution was inverted at least once every minute (to prevent settling of the matrix), and the liquid was removed by centrifuging (14,000 rpm, 1 min) the filter (the catch tube was emptied as needed). The filter was washed twice with aliquots (500 µl) of GENECLEAN SPIN NEW wash. The catch tube was made empty and centrifuged for 2 minutes to dry the pellet. The spin filter was transferred to a fresh catch tube, spin elution solution (10-25 µl) was added to the filter, the solution was centrifuged (14,000 rpm, 1 min) to transfer the eluted DNA to the catch tube, and the tube was capped.

6. QIAprep spin miniprep kit protocol (from QIAGEN)

This protocol was designed to purify up to 20 µg of high-copy plasmid DNA from the bacteria in 1-5 ml of overnight cultures of *E. coli* and *V. cholerae* in LB broth. The bacterial cells collected by centrifugation and suspended in 250 µl of Buffer P1 (250 µl) were transferred to a microcentrifuge tube, Buffer P2 (250 µl) was added to the tube, and the tube was gently inverted 4-6 times (or more) until the solution became viscous and slightly clear (the lytic reaction was not allowed to proceed for more than 5 min). Buffer N3 (350 µl) was added to the tube, the preparation was immediately and gently mixed (to avoid localized precipitation) by inverting the tube until its contents appeared cloudy (4-6 times), and the tube was centrifuged (14,000 rpm, 10 min). The supernatants were pipetted onto a QIAprep spin column, and the column was washed with Buffer PB (0.5 ml) and centrifuged for 1 min at 14,000 rpm (the flow-through was discarded). The column was washed again by adding 0.75 ml of Buffer PE and centrifuging (14,000 rpm, 1 min), the flow-through was discarded, and the column was centrifuged for an additional 2 minutes to remove residual wash buffer. The column was then placed in a clean 1.5-ml capacity microcentrifuge tube, and the bound DNA was eluted and collected by adding Buffer EB or H_2O to the center of the column and centrifuging (14,000 rpm, 1 min) the column 1 minute later.

7. QIAfilter plasmid midi protocol (from QIAGEN)

LB broth (3 ml) containing an appropriate selective antibiotic was inoculated with a single colony from a freshly streaked selective plate, and the 3-ml starter culture was incubated (37°C, overnight) with vigorous shaking (~250 rpm). The starter culture was

diluted (1/500 to 1/1000) with the selective LB medium and, for low copy plasmids, 100 ml of the medium were inoculated with the diluted culture and incubated (37°C, overnight) with vigorous shaking (~250 rpm). The bacteria were harvested by centrifugation (6,000 rpm, 15 min) at 4°C, the supernatant fluids were removed, and the bacterial pellet was suspended in Buffer P1 (4 ml). Buffer P2 (4 ml) was added and the preparation was mixed gently but thoroughly (by inverting the tube 4-5 times) and incubated at room temperature for 5 min. Four ml chilled P3 buffer was added, the tube's contents were immediately and gently mixed by inverting the tube several times, and, after storage on ice for 15 minutes, the preparation was mixed again and centrifuged (13,000 rpm, 30 min, 4°C). The supernatants were applied (by gravity flow) to a QIAGEN-tip 100 column equilibrated with 4 ml of QBT buffer, the column was washed twice with QC buffer, and the DNA was eluted with QF buffer and precipitated by adding 3.5 ml of room-temperature isopropanol (Sigma). The precipitated DNA preparation was immediately sedimented by centrifugation (11,000 rpm, 30 min, 4°C), the supernatants were decanted, and the DNA pellet was washed with 70% ethanol (2 ml, at room temperature) and collected by centrifugation (11,000 rpm, 10 min, 4°C). The supernatants were decanted without disturbing the pellet, the pellet was air-dried for 20 minutes, and the DNA was dissolved in 100µl of sterile water or TE buffer (pH 8.0).

8. Preparation of calcium chloride competent cells

LB broth (4 ml) inoculated with a colony of *E. coli* or any other desired cell was incubated (37°C, overnight), 2 ml of the culture were added to 200 ml of LB broth, and the inoculated medium was incubated at 37°C, with shaking (250 rpm), until it had an

OD_{600} of ~ 0.3. The culture was transferred into a 250-ml capacity Nalgene centrifuge bottle, and the bottle was kept on ice for 10 min and centrifuged (3,000 rpm, 10 min, 4°C). The supernatants were discarded and the pellet was suspended in an autoclaved, ice-cold solution containing 100 mM $CaCl_2$ and 15% glycerol (20 ml per 200 ml of culture). The suspension was centrifuged (3,000 rpm, 10 min, 4°C), the supernatants were discarded, and the pellet was suspended again in ice-cold $CaCl_2$-glycerol solution (20 ml per 200 ml of culture) and kept on ice for 10 min. After centrifugation (3,000 rpm, 10 min, 4°C), the supernatants were discarded and the pellet was suspended in ice-cold $CaCl_2$-glycerol solution (4 ml per 200 ml of culture. Aliquots (100 µl) of the cell suspension were dispensed into pre-chilled microcentrifuge tubes and stored at -80°C.

$CaCl_2$ solution:

1 M $CaCl_2$	12 ml
Glycerol	30 ml
Deionized water	158 ml
Total	200 ml

9. Competent cells for electroporation

Unless otherwise mentioned, all steps were performed at 4°C. A selected strain was subcultured, from a glycerol containing frozen (-80°C) specimen, by incubation (37°C, overnight) on an appropriate agar-based medium. LB broth (5 ml) was inoculated with a colony and incubated overnight at 37°C with shaking (250 rpm). One ml of the seed broth culture was added to 100 ml of LB broth in a flask, and the flask was incubated at 37°C, with agitation (250 rpm) until the culture had an OD_{600} of 0.4-0.5

(usually about 1.5-2.5 h). The bacteria were sedimented by centrifugation (6,000 rpm, 10 min), suspended in 100 ml of 137 mM sucrose, collected by centrifugation (6,000 rpm, 5 min), and washed twice with 50 ml of the sucrose solution. After the final washing, the bacteria were suspended in 2 ml of 10% glycerol, collected by centrifugation (8,000 rpm, 5 min) in a microcentrifuge tube, and suspended in 0.5 ml of 10% glycerol. Aliquots (100 µl) of the suspension of competent cells were stored in sterile microcentrifuge tubes at -80°C.

10. Heat shock transformation

For transformation, 50µl competent cells and 3-5 µl respective plasmids were kept on ice for at least 30 minutes and then heat shock was given at 42°C for 90 seconds to 2 minutes. The transformants were than kept on ice for 10 minutes and then 500 µl of LB or SOC medium was added and incubated at 37°C, shaking for 1 hour and plated on selective plates to obtain stable transformants.

11. Transfer of plasmids into competent cells by electroporation

A tube of the competent cell suspension was thawed in an ice-water bath, and the electroporation cuvette was chilled prior to use. An aliquot (6 µl) of the plasmid DNA preparation was gently mixed (to avoid air bubbles) with the competent cells on ice, and the mixture was subjected to electroporation at 2.5 kV for *V. cholerae* or 40 kV (for *E. coli*) and a capacitance of 25. After electroporation, the mixture was incubated (37°C, 1.5 h, 250 rpm) in a microcentrifuge tube containing 1 ml of SOC medium, and the bacteria

were collected by centrifugation, plated on an LB agar medium containing appropriate antibiotics, and incubated overnight at 37°C.

12. Isolation of exopolysaccharide from smooth (sEPS) and rugose (rEPS) strains

Smooth and rugose variants of *V. cholerae* were added to separate 250-ml flasks containing 45 ml 1XAPW#3, the flasks were statically incubated for 3 days at 37°C, and the cultures were filtered with a large pore size filter (10 μm). The biofilm was allowed to remain on the filter for a few minutes (to remove as much medium as possible), and the planktonic cells were removed by gently rinsing the biofilm once with normal saline. The biofilm was gently transferred to a 500 ml beaker containing 3-mm diameter glass beads, and the mixture was shaken well for about 5 minutes and centrifuged (20,000 rpm, overnight, 4°C). The supernatant fluids were passed through a Detoxi Gel Affinity column (Polymixin B column) (PIERCE, Rockford, IL), and the effluent was incubated (37°C, 4 h) with DNase and RNase (both at 0.1 mg/ml). The DNA- and RNA-degraded mixture was incubated (overnight at 37°C, followed by 15 min at 60°C) with Proteinase K (0.1 mg/ml), and the preparation was mixed with 3 volume of cold 95% ethanol. After storage at 4°C overnight, the precipitate was (i) sedimented by centrifugation (12,000 rpm, 20 min), (ii) washed sequentially with 80% ethanol and 96% ethanol, (iii) freeze-dried, and (iv) subjected to glycosyl composition analysis from Complex Carbohydrate Research Center, University of Georgia, Atlanta.

agar, and postfixed with 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.2) overnight at 4°C. After postfixing, the samples were (i) sequentially dehydrated in 30, 50, 70 and 90% ethanol (10 min at each concentration), and twice in 100% ethanol (15 min each time), (ii) treated twice with propylene oxide (15 min each time), (iii) infiltrated with a 1:1 solution of propylene oxide and Epon for 2 h at room temperature and then overnight with 3:1 Epon-propylene oxide, (iv) embedded in Epon, and (v) thin-sectioned (50- to 80-nm-thick sections). The sections were sequentially stained with uranyl acetate and lead citrate (both for 20 min), and they were examined with a JEOL 1200 EX II transmission electron microscope at 80 kV. TEM was performed with the help of facilities from Dental School, UMB.

15. Growth curve

The growth curves of *V. cholerae* N16961 and its mutants (*rocS* and *amiB*) were determined by two different methods. In one method, aliquots (3 ml) of LB broth were inoculated with single colonies and incubated, with agitation (250 rpm), overnight at 37°C. The cultures were diluted to 10^{-4} and aliquots (100 µl containing ca. 10^4 cells) were added to 3-ml portions of fresh LB broth (thus, the starting CFU/ml was ~10^3), which were subsequently incubated statically at 37°C for 10 hours. At hourly intervals the OD_{600} and CFU/ml were determined, (from 0-10 h postinoculation), and the data were plotted. In the second method, LB broth (3 ml) also was inoculated with a single colony and incubated, with shaking (250 rpm), overnight at 37°C. The next day the cultuere was diluted to 10^{-4}, ~10^4 CFU/ml were added to 3 ml of fresh LB broth, and the broth was

incubated for 6 hours at 37°C (shaking at 250 rpm). The CFU/ml and OD_{600} were determined at 30-min intervals, and the values were plotted.

16. Generation of spontaneous rugose variants of *V. cholerae*

Smooth *V. cholerae* strains were grown statically in 1XAPW#3 for 1-3 days at 37°C, and the broth cultures were plated (for isolated colonies) on LB agar and incubated at 37°C. The isolated rugose variants were stored at –80°C in LB broth supplemented with 30% glycerol.

17. High frequency rugose exopolysaccharide production (HFRP)

V. cholerae was streaked to obtain isolated colonies from glycerol stock cultures stored at -80°C. A smooth colony was added to 3 ml of 1XAPW#3 in a glass test tube or to 45 ml of 1XAPW#3 in a 250-ml flask, and the culture was incubated statically for 24 to 72 h at 30°C or 37°C. After incubation, sterile glass beads (4-mm diameter) were added to the culture, and the mixture was vortexed to disrupt any aggregates of rugose cells. Appropriate dilutions of the culture were plated on LB agar, and the number of rugose CFU/ml was determined by standard plate counts.

18. Reversion studies

A rugose colony was added to 3 ml of LB broth, and the broth was incubated at 30°C and 37°C, for 5 to 7 days, with continuous shaking at 250 rpm. Serial dilutions of the culture were plated on LB agar and the number of smooth and rugose colonies was

determined. Dividing the total number of colonies by the number of rugose colonies, and multiplying by 100 determined the percentage of rugose to smooth reversion.

19. Stress resistance assay (Chlorine resistance)

Chlorine resistance was assayed (four independent experiments) by using a 1:50 dilution of an overnight culture of strain NCTC 6585 smooth and rugose cells in 3 ml of fresh LB (Miller) broth. Cultures were then incubated statically at 37°C for 3 h until reaching a density of ~2 x 10^8 CFU/ml; the cells were then harvested by centrifugation and suspended in phosphate-buffered saline (pH 7.2) containing 3 mg of free chlorine per liter (sodium hypochlorite; Sigma). After 5 min of exposure in 3 mg of chlorine per liter, cultures were serially diluted and plated on LB agar to determine the number of surviving bacterial cells.

20. Stress resistance assay (Hydrogen peroxide resistance assay for *V. cholerae* strains)

Stress resistance was assessed by using a 1:1,000 dilution of an overnight culture of NCTC 6585 smooth and NCTC 6585 rugose in 3.0 ml of PBS (Phosphate buffer saline). The diluted cultures were grown to approximately 2×10^9 cells per ml, which corresponded to an A_{600} of 0.4, and cells were harvested by centrifugation and resuspended PBS (pH 7.0) which contained 20 mM H_2O_2 (oxidative stress). Survival was measured at 0 and 5 minutes. Percent survival was calculated by dividing the number of CFUs at a given time by the number of CFUs at time zero and multiplying the result by 100. One hundred percent viability was taken to be approximately 2×10^9 cells per ml

and represents the viable counts obtained immediately before challenge. Percent survival of NCTC 6585 smooth and that of NCTC 6585 rugose were compared.

21. Biofilm assay

A single fresh colony was grown in LB broth (5 ml) overnight at 37°C in an orbital shaker (250 rpm). An aliquot (5 µl) of the overnight culture was added to 500 µl of LB broth in a 13 × 100 mm borosilicate glass tube, and the new culture was incubated at room temperature for 24 hours. The culture was discarded, the tube was gently rinsed with MilliQ H_2O or PBS, and 600 µl of a crystal violet solution (0.1%, Sigma) was added to the tube. The crystal violet-containing tube was incubated at room temperature for 30 min, rinsed twice with MilliQ H_2O or PBS, and inverted on a paper towel to blot away the liquid. Finally, the stained biofilm was dissolved in 600 µl of dimethyl sulfoxide (DMSO, Sigma), and the crystal violet's absorbance (a marker for the amount of biofilm) was determined at 570 nm.

22. Motility test

Bacterial motility was determined by a traditional swarm plate assay that measures the swarm diameter (zone) visible after stabbing an equal amount of *V. cholerae* cells (grown in LB broth) into semisoft LB agar (containing 0.3% agar) and incubating at 37°C for 4 h (86).

23. Transposon mutagenesis and screening for genes involved with switching to the rugose phenotype

The previous identification of culture conditions that promote high frequency switching of *V. cholerae* to its rugose phenotype; e.g., growth in 1XAPW#3 (for its formula, see section C of Materials and Methods), was exploited to develop an assay that identifies genes involved in the molecular switch from the smooth to the rugose phenotype (4). To identify the genes involved in the molecular switch from the smooth (EPSoff) to the rugose (EPSon) phenotype, mini-Tn5km2 mutagenesis (62, 96) was used. Tn5 is contained on the R6K-based plasmid pUT/mini-Tn5 Kan (or pUTKm) that is derived from suicide vector pGP704 (109), and it can only be maintained in donor strains (e.g., a λpir lysogen of *E. coli*) that produce the R6K-specified λpir protein, which is an essential replication protein for R6K and plasmids derived from R6K. It also carries the origin of transfer (oriT) of plasmid RP4, which enables efficient conjugal transfer. Delivery of the donor plasmid pUTKm into recipient cells is mediated by the cognate transposes encoded on the plasmid at a site external to the transposon. An advantage of this mutagenesis system is the stability of the Tn5 insertion, since the cognate transposes is not carried with the transposon during transposition. Thus, each mutant has only a single Tn5 insertion to screen.

During my studies, I mated *E. coli* S17 λpir (pUT/mini-Tn5 Km) with *V. cholerae* smooth N16961 cells, and obtained and stored 14,500 mini-Tn5 mutants from 30 independent conjugations. I then performed a high throughput screen for HFRP mutants. Each transposon mutant was added to 200 µl of 1XAPW#3 in wells of microtiter plates,

incubated for 48 h, and replica plated onto LB agar and incubated for 24-48 h, after which time its colony morphology was visually determined. This approach identified 43 mutants, operationally defined as HFRP-negative that did not produce rugose colonies. Studies during which colonies of the mutants were statically grown in 1XAPW#3 for 48 h at 37°C confirmed that they were stable and defective in switching to the rugose phenotype under HFRP-inducing conditions. In another study, sterile glass beads (4-mm diameter) were vortexed with the cultures in order to disrupt any aggregates of rugose cells. Appropriate dilutions of each culture were plated on LB agar, and the total number of CFU/ml and the number of rugose CFU/ml were determined by standard plate counts. The 43 mutants identified and tested by these screening methods did not produce detectable rugose colonies under rugose-inducing (HFRP) conditions, and they were further studied.

24. Sequencing of transposon insertion sites and identification of disrupted genes

To identify the transposon insertion site in the mutants described above, I used a non-laborious, arbitrary primed PCR method followed by DNA sequencing similar to that described previously (11). Arbitrary PCR was performed in two steps. In the first reaction, the mutant's chromosomal DNA was used as a template for PCR using primers reading out from both sides of the transposon and two arbitrary primers. These primary reactions yielded numerous amplicons, including some that were derived from the junction of the transposon insertions. The products of the first-round PCR were purified with a GENECLEAN spin kit, and the purified products were amplified using a second

pair of outward transposon primers external to the first pair and an arbitrary primer corresponding to the constant region of the original arbitrary primers. This second PCR serves specifically to amplify products of the first PCR that include transposon junctions. The amplified fragments' sizes ranged from 100- to 800-bp. The products that showed the strongest bands were from agarose gels, and they were sequenced using the same transposon and arbitrary primers used in the second-round PCR. Sequencing was performed, according to the manufacturer's instructions, with an automated DNA sequencer (model 373A, Applied Biosystems) and a Prism ready reaction dye deoxy termination kit (Applied Biosystems).

25. Cloning of vpsR

The *vpsR* gene of *V. cholerae* N16961 was obtained on a 2.61-kb PCR fragment, using PCR primers KAR486 (5'-CGGGATCCCGCTAAGTCAGAGTTTTTATCGC-3') and KAR487 (5'-TCCCCGCGGGTCGGTGGTTTTGATCGTGT-3'). The fragment was digested with *Bam*HI and *Sac*II, and it was suitably cloned into the low copy vector pWSK29 (164), creating plasmid pDK104.

26. Microscopic analysis of the *amiB* mutant strain

Individual 18-h-old colonies (on LB agar plates) of wild-type N16961 and the *amiB* mutant DK630, were suspended in small volumes (1 ml/tube) of PBS, and aliquots (50 µl) of each suspension were spread on glass slides, heat-fixed and stained with crystal violet (0.1%) for 30 sec. The slides were rinsed with deionized water and dried, and the cell morphology was observed with a Zeiss Axioskop epifluorescence microscope (Carl

Zeiss, Inc., New York) and was recorded with an AxioCam Mrm camera (Carl Zeiss, Inc., New York).

27. Cholera toxin assay

The level of cholera toxin (CT) production was studied in smooth and rugose variants of *V. cholerae* strain N16961 and in two independent *vpsR* mutants (DK562 and DK581) (131) grown under AKI conditions (73, 74). Briefly, a fresh colony of each strain was added to 15-ml capacity tubes containing AKI broth (4 ml) composed of 1.5% Bacto peptone, 0.4% yeast extract and 0.086 M NaCl, and adjusted (with filter-sterilized 1M $NaHCO_3$), just prior to use, to pH 8.0. In addition, for the vpsR mutants, neomycin (50 μg/ml) was included in the culture medium. The 4-ml broth cultures were incubated overnight, with shaking (200 rpm), at 30°C. The following day, aliquots (100 μl) of them were added to 15-ml tubes containing 10 ml of AKI broth (antibiotics were added, where appropriate), and the cultures were incubated statically, at 30°C, until they reached an OD_{600} of 0.3-.4 (ca. 3 h). The 10-ml broth cultures were then transferred to 250-ml flasks and incubated (30°C, 18 h, 250 rpm), and aliquots (3.6 ml) were removed and centrifuged (8,000 rpm, 5 min). The culture supernatant fluids obtained from each culture were assayed for CT by a GM-1 ELISA (enzyme-linked immunosorbent assay). The ELISA consisted of the following steps: (i) 100 ng of GM1 ganglioside in PBS (i.e., 100 μl of a solution containing 1 μg/ml) were added to the wells of Immulon 2HB ("U" bottom) microtiter plates (Fisher Scientific), and the plates were stored overnight at 4°C; (ii) The wells were washed three times with PBS, 200 μl of PBS containing 5% heat-inactivated fetal calf serum (FCS) were added to each well, the plates were incubated (37°C, 1 h),

and the wells were washed with PBS/0.05% Tween-20; (iii) Aliquots of the culture supernatant fluids to be assayed for CT were added to the wells (serial dilutions of CT (Sigma) were included to establish a standard curve, and PBS served as a negative control), the plates were incubated (37°C, 2 h), and the wells were washed with PBS/0.05% Tween-20; (iv) Aliquots (100 µl) of a rabbit anti-CT antiserum (from the Center for Vaccine Development, diluted 1:3,200 in PBS/Tween-20 with 1% FCS) were added to the wells, and the plates were incubated for 1 h at 37°C; (v) The wells were washed with PBS/Tween-20, 100 µl of a solution (diluted 1:5,000, in PBS containing 1% FCS) of goat anti-rabbit IgG alkaline phosphatase conjugate (Kirkegaard and Perry Laboratories) were added to the wells, and the plates were incubated for 1 h at 37°C; (vi) The wells were washed with PBS/Tween-20, 100 µl of a solution prepared by dissolving a Sigma Fast p-nitrophenyl phosphate substrate tablet in sterile Milli-Q water were added to the wells, and the plates were incubated for 45 min at 37°C; and (vii) The color reaction was stopped by adding 3N NaOH (50 µl) to the wells, and the OD_{405nm} was determined with a plate reader (Spectra MAX250,). The final results were based on data obtained with three independent samples for each strain's culture supernatant fluids (prepared on three different days), which were then tested six times. The data were analyzed by a one-way analysis of variance (ANOVA) or t-test.

28. Collagenase and elastase assays

The *V. cholerae* strains to be tested were grown (37°C, overnight, 250 rpm) in brain heart infusion (BHI) broth, and their culture supernatant fluids (to be assayed for collagenase and eslastase acyivity) were obtained by centrifugation and filter-sterilized

(0.22 μm pore-size filters). Collagenase activity was determined with the EnzChek Collagenase kit (Molecular Probes), using collagen type I from bovine skin, and collagen type IV from human placenta, as the substrates. Elastase activity was determined with the EnzChek Elastase kit (Molecular Probes), using DQ elastin (soluble bovine neck ligament elastin) as the substrate.

29. Infant mouse colonization studies

To determine whether the EPS, the rugose variant of *V. cholerae* N16961 and *vpsR* have a role in intestinal colonization, a multiple co-infection assay based on the method of Baselski and Parker (13) was performed using the suckling, infant mouse model. A single colony of either the smooth strain, the strain's rugose variant, or the *vpsR* mutant of strain N16961 (DK581) was added to a test tube containing LB broth (3 ml), and the culture was incubated (37°C, overnight, 250 rpm). Aliquots (5 μl) of each strain's overnight culture were diluted (1:1,000, with LB broth), and the dilutions were used alone (for individual assays), or aliquots (1 ml) were mixed with an equal volume of the culture (diluted 1:1,000) of the strain to be tested via the co-infection assay. Aliquots (50 μl, see below) of each specimen (individual and mixed) were supplemented with Evans blue dye before being orally administered to suckling mice.

Five-day-old, suckling CD-1 mice was inoculated orally, using a 25-g syringe, with ca. 50 μl of diluted cultures of individual strains or mixtures containing ca. 10^5 CFU (confirmed by plate counts). After 5 or 24 h, the mice were sacrificed (by using CO_2 gas) and their small and large intestines were removed, placed in 2 ml of PBS, mechanically homogenized using a Virsonic 60 (Virtis) blender, serially diluted and plated on LB agar

supplemented with 50 U polymixin B/ml (and, when appropriate, 50 μg neomycin/ml), to enumerate the number of *V. cholerae*/ml. Bacterial colonies were confirmed as *V. cholerae* by subsequent culturing on TCBS agar. The competition index was calculated by dividing the rugose variant's or *vpsR* mutant's output number by the wild-type strain's output number. Strains with an equivalent ability to colonize are expected to have a competition index of approximately 1.0.

30. Rabbit ileal loop studies

The rabbit ileal loop model (33) was used to perform other preliminary, *in vivo* studies examining whether the EPS, the rugose phenotype, and *vpsR* might have roles in the pathogenesis of cholera. Wild-type smooth and rugose variants of strain N16961, a *VpsR* mutant (DK562), and a *vps* mutant (DK589) was streaked (from frozen glycerol stocks) on LB agar plates. A single colony was added to 4 ml AKI broth (containing antibiotics, where appropriate), incubated at 37°C, overnight at 250 rpm, and the culture's OD_{600} was adjusted to 1.2 with fresh AKI broth. An aliquot (100 μl) of the culture was transferred to a 250-ml flask containing 10 ml of AKI broth (supplemented with antibiotics, where appropriate), and the inoculated culture was incubated (37°C, 250 rpm) until it had an OD_{600} of ca. 1.8 (ca. 3 h). One ml of the culture was centrifuged (8,000 rpm, 4 min), the pellet was washed twice with PBS (1.5 ml) and suspended in 10 ml of PBS (final OD_{600}~0.13-0.16), and 3 ml of the suspension was diluted with 7 ml of PBS. The number of CFU/ml of the diluted suspension was estimated (by plate counts) to be in the range of $4\text{-}8\times10^7$/ ml.

Prior to surgery, three New Zealand white, male rabbits (ca. 2 kg each; obtained from Covance Research Products) were fasted for 24 h and fed only water ad libitum. They

were anesthetized, a laparotomy was performed, and each rabbit's small intestines were tied off into four loops (each was ca. 5-cm-long) with interloops (1-cm-long) between each main loop. Each loop was injected, using a 25-gauge needle, with 1ml of bacterial suspension (prepared as described above) containing ca. 10^7 cells of either the smooth, rugose, *vpsR*, or *vps* strains (or PBS as a control). The intestine was returned to the peritoneal cavity, the incision was closed, and the rabbits were returned to their cages and given water ad libitum. Approximately 18 h post injection, the rabbits were sacrificed, the peritoneal cavity was opened, the small intestines was removed, and the injected loops were examined macroscopically. The fluid in each loop was removed and its volume was measured, and a diluted aliquot of the fluid was spread on plates of an appropriate, antibiotic-containing medium, to determine the number of viable *V. cholerae*/ml. The colonies obtained were subcultured on TCBS agar, to confirm their identity as *V. cholerae*. The FAR (fluid accumulation ratio) was estimated by dividing the volume (ml) of fluid in the loop by the length (cm) of the loop.

1. Discovery of a culture medium that promotes high frequency rugose exopolysaccharide (rEPS) production

1.1. Frequency of rEPS production in *V. cholerae*

My interest in characterizing the factors and processes involved in the emergence, virulence, and persistence of epidemic *V. cholerae* strains prompted studies to determine the prevalence of rEPS production by various *V. cholerae* strains. These strains represented O1 and non-O1 strains and included 19 clinical isolates and 16 environmental isolates that were collected over several decades and from several continents.

While comparing several conventional media that are known to promote the growth of *V. cholerae*, a nutrient-poor medium and a modified form of alkaline peptone water (APW), designated APW # 3 (composed of 1% proteose peptone #3 [Difco], 1% NaCl, pH 8.5), was discovered to result in a high frequency of switching (up to 80%) in smooth strain N16961 cells to the rugose phenotype. At least eleven independent experiments revealed that after 24 to 48 h of growth at 37°C (in glass tubes), approximately 60-80% of the smooth cells had switched to the rugose phenotype. Accordingly, this phenotype was designated the "high-frequency rugose production" (HFRP) phenotype. I subsequently examined the ability of additional clinical and environmental isolates to undergo rugose production under the experimental conditions described above. Only 6 of the 19 clinical strains (32%) and one of the 16 (6%) environmental strains exhibited HFRP, which suggested that the HFRP phenomenon is more common in clinical strains than in environmental isolates and that the phenomenon

is strain-specific. (Table 3 and Fig. 1, 2). In order to examine whether the stress response regulator (RpoS), quorum sensing regulator (LuxS) and cholera toxin regulator (ToxT) have any role in HFRP, wild type strains and their mutants were tested under the growth conditions previously observed to elicit HFRP. I found that HFRP was independent of LuxS, RpoS, and ToxT (data not shown).

TABLE 3. Frequency of rugose EPS production by *V. cholerae* strains[a]

Strain[b]	Serogroup/ Biotype	Origin	Source (year)[c]	% Rugose colonies [d, e, f]			
				Flask		Tube	
				30°C	37°C	30°C	37°C
N16961	O1/El Tor	Bangladesh	C (1971)	31 (1.5)	47 (0.9)	70 (0.7)	74 (1.9)
C6709	O1/ElTor	Peru	C (1991)	1	23	15	70
NCTC 6585	O1/classical	India	C (1943)	43 (1.4)	44 (0.2)	0	0
AMS20A73	O1/classical	China	C (1945)	3	4	0	0
Aldova	O37	Czechoslovakia	C (1965)	0	1	72 (0.2)	41 (2.8)
1803	non-O1	Brazil	C (1992)	0	0	16	87
1837	O139	Bangladesh	C (1992)	0	0.2	0	0-2
P44	non-O1	Peru	E (2000)	12	0	0	2
1085-93	O37	Germany	E (1993)	0	0	0.1	0
141-94	O70	Germany	E (1994)	0	0	0.3	0
928-93	O6	Argentina	E (1993)	0	0.2	0.4	0

[a] A smooth colony was inoculated into glass tubes containing 3 ml of APW#3 or into flasks containing 45 ml of APW#3, and the cultures were incubated statically for 48 hours and 72 hours at 30°C and 37°C, respectively.

[b] Only strains showing HFRP or spontaneous rugose colonies under each condition are listed.

[c] C, clinical; E, environmental.

[d] Values represent the mean percentages of cells shifting to a rugose colony morphology.

[e] Readings were obtained at 72 h postincubation for all strains except N16961, for which the reading was taken at 48 h.

[f] Values in parenthesis represents standard errors and they are only shown for strains demonstrating high levels of HFRP.

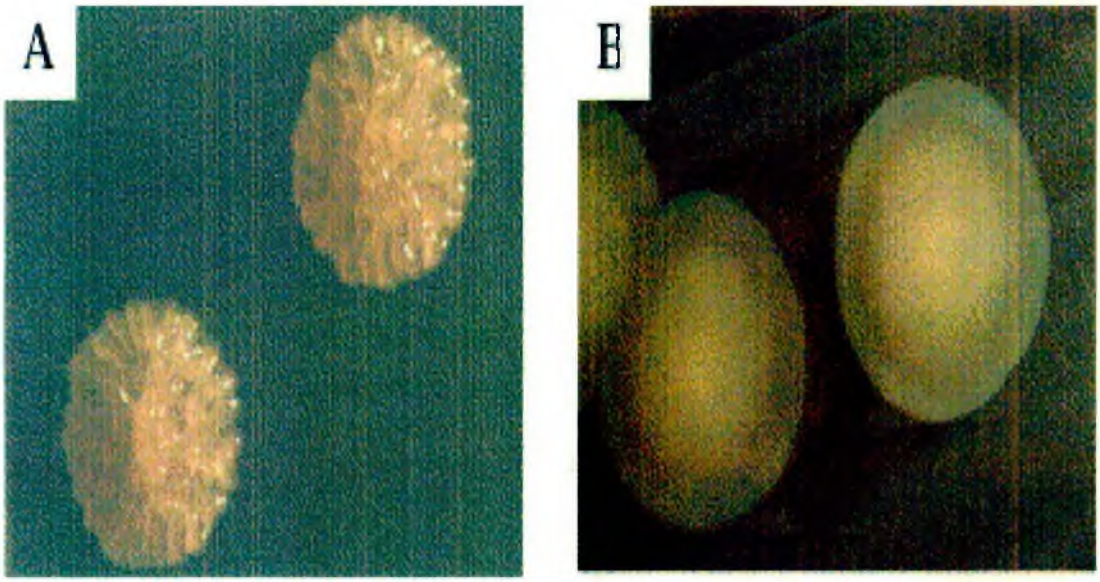
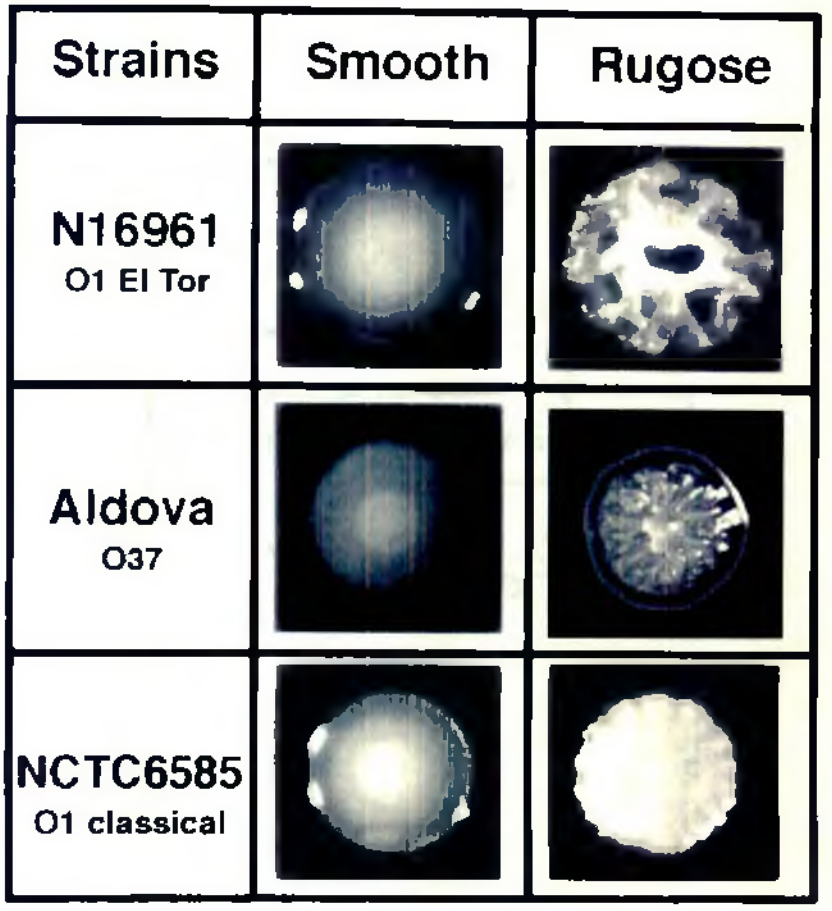


FIG.1. Colony morphology of rugose and smooth variants of *V. cholerae*. (A) Rugose colonies at 72 h postincubation. (B) Smooth colonies at 24 h postincubation.

A



B

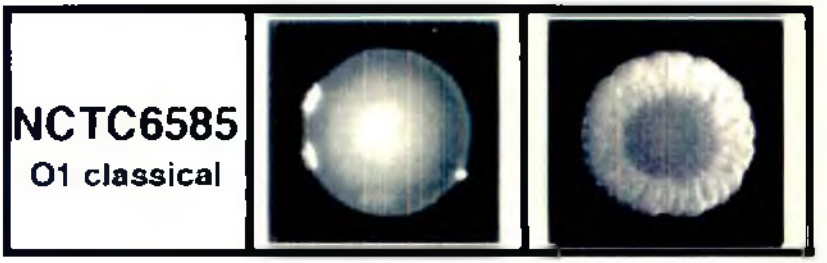


FIG.2. Smooth and rugose colony morphologies of *V. cholerae* strains

1.2. Time course for HFRP in APW # 3

Time course studies analyzing aliquots of the culture every 2 hour revealed that strain N16961 did not demonstrate HFRP during growth in APW # 3 until at least 14 h of incubation, at which time I observed a sudden increase in the percentage of rugose variants (0 to 50%). After this initial increase in HFRP, the percentage of rugose cells increased in the culture, resulting in 60 to 70% rugose cells at 24 h.

1.3. Rugose stability and frequency of reversion to the smooth cell phenotype

A single colony, obtained by plating a fresh glycerol stock of a rugose variant of *V. cholerae* strains N16961, NCTC 6585 and Aldova, was inoculated into glass tubes containing 3 ml LB broth and incubated for 5-7 days at both 30°C and 37°C (Table 4). The cultures were examined and plated after 5 and 7 days of incubation in order to detect the reversion to the smooth phenotype. LB broth was chosen as it is optimal medium for stable maintenance and growth of the smooth phenotype as well as rugose variants. Under these growth conditions, strains N16961 and NCTC 6585 showed a very low (<1%) level of rugose-to-smooth (R-S) reversion. Interestingly, statistical analysis using the two-sided Wilcoxon rank sum test showed that the R-S reversion frequency was significantly higher for the Aldova strain than for N16961 and NCTC 6585 under all conditions tested including 30°C static ($P = 0.01$ and $P = 0.02$, respectively), 30°C shaking ($P = 0.03$ and $P = 0.04$, respectively), 37°C static conditions ($P = 0.002$ and 0.006, respectively), and 37°C shaking conditions (t test; $P < 0.0001$). These findings

imply that different culture conditions regulate phenotypic switching. These findings also indicate that the El Tor and classical strains can form a stable rugose phenotype that might be relevant for their ability to persist under certain conditions and might be linked to epidemicity. In particular, for Aldova rugose cells incubated at 37°C shaking, we observed a 77.7% reversion to the smooth phenotype. In addition, we found that 37°C shaking significantly inhibits the viability of the NCTC 6585 rugose phenotype. Using the two-sided Wilcoxon rank sum test, we found a significant difference (1,000-fold reduction) in the number of CFU/ml for NCTC 6585 compared with N16961 ($P = 0.03$) and the Aldova ($P = 0.02$) while the molecular basis underlying this finding is not yet known. This data implies that this particular condition inhibits the viability of the rugose variant of strain NCTC 6585.

TABLE 4. Rugose stability and frequency of reversion to the smooth cell phenotype[a]

Strain	% of cells undergoing R-S reversion[b]			
	30°C		37°C	
	Static	Shaking	Static	Shaking
N16961	0.8 ± 0.2 ($5.3x10^8$)	0.4 ± 0.1 ($1.7x10^8$)	0.9 ± 0.3 ($1.7x10^8$)	0 ($5.8x10^7$)
NCTC6585	0.9 ± 0.2 ($4.1x10^8$)	0.2 ± 0.1 ($4.0x10^7$)	0.5 ± 0.2 ($3.9x10^8$)	0 ($3.4x10^4$)
Aldova	4.4 ± 0.9 ($4.6x10^8$)	27.5 ± 2.3 ($1.5x10^8$)	27.2 ± 2.2 ($1.9x10^8$)	77.7± 2.2 ($4.1x10^7$)

[a] A rugose colony was inoculated into LB broth and the cultures were incubated (with shaking or statically) for three days at 30°C or 37°C. LB was chosen because it provides stable maintenance of both rugose and smooth cells (112). Cultures were plated and colonies were counted to determine the relative numbers of rugose and smooth colonies. The results presented are the mean values of at least four independent experiments.

Values in parentheses represent the total CFU/ml 3-day-old cultures.

[b] The ± values represent the standard errors, and the values in parenthesis represent the total CFU/ml in 3-day-old cultures.

1.4. TCBS masks rugose colony morphology

Thiosilfate-citrate-bile salts-sucrose (TCBS) agar is universally used to isolate *V. cholerae* from both clinical and environmental samples. To determine whether this primary selective medium supports the appearance and growth of the rugose phenotype, a *V. cholerae* N16961 rugose variant was subcultured first onto LB agar and a rugose colony then subcultured on TCBS agar and incubated overnight at 37°C. The subculturing of the rugose colonies on TCBS resulted in the appearance "smooth" colonies. However, subculturing of those "smooth" colonies from TCBS onto LB agar produced typical rugose colonies. Thus, TCBS agar appears to inhibit (or temporarily mask) the rugose colony morphology. This results suggests that studies using TCBS aimed to isolate *V. cholerae* from the environment or from clinical sources will not detect the rugose variant. Therefore, the presence of the rugose phenotype probably has been previously underestimated in those environments and might be more common than previously thought.

1.5. The rugose variant of *V. cholerae* classical biotype (sixth pandemic) strain NCTC 6585 produces an extracellular glycocalyx

NCTC 6585, a 6th pandemic (classical biotype) strain, consistently shows HFRP in flasks cultures (33 to 48%, 30°C; 44 to 45%, 37°C). In addition, another 6th pandemic strain (AMS20A73) exhibits HFRP, albeit at a lower frequency (3 to 4%), with HFRP being defined as a >3% shift to the rugose phenotype. A previous study (178) reported

that 6th pandemic strains possess homologous sequences; however these investigators were unable to demonstrate rEPS expression by their strains. My study utilizing transmission electron microscopy of ruthenium red-stained the thin sections demonstrates that the rugose classical biotype strain NCTC 6585 over expresses the rEPS (Fig. 3, 7).

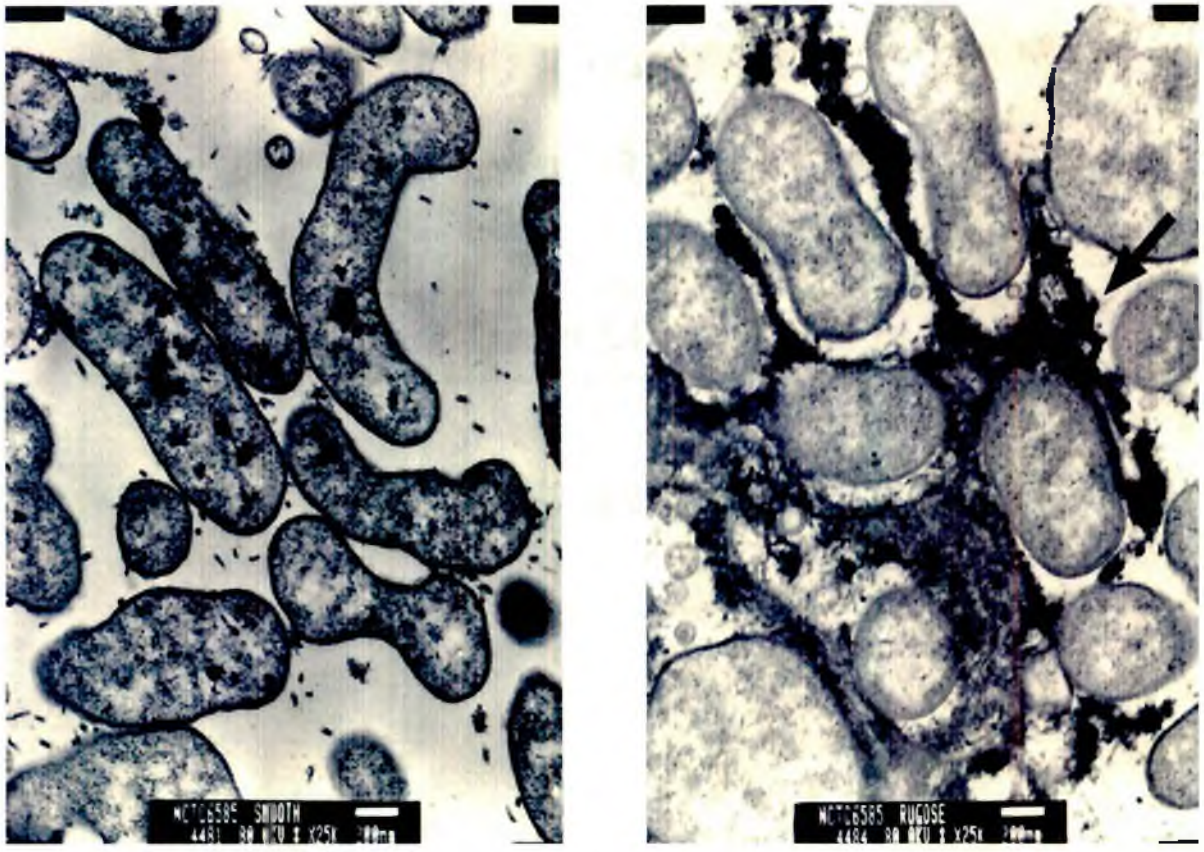


FIG.3. The rugose variant of *V. cholerae* classical biotype (sixth pandemic) strain NCTC 6585 produces an extracellular glycocalyx. The panels show transmission electron micrographs of ruthenium red-stained thin sections of smooth (left panel) and rugose (right panel) cells of strain NCTC 6585. Note the absence of a stained matrix between the smooth cells and the presence of a densely stained matrix between the rugose cells (arrow, right panel).

1.6. Stress resistance

1.6.a. Chlorine resistance

In order to determine whether rEPS production by classical strain NCTC 6585 promoted resistance to chlorine (an environmental stress), we compared the levels of resistance to chlorine exposure between a smooth-cell strain and a rugose variant. We found that the rugose variant of classical strain NCTC 6585 was 10,000-fold more resistant to chlorine (5 min of exposure to 3 mg/liter) than its smooth-cell counterpart (Fig. 4).

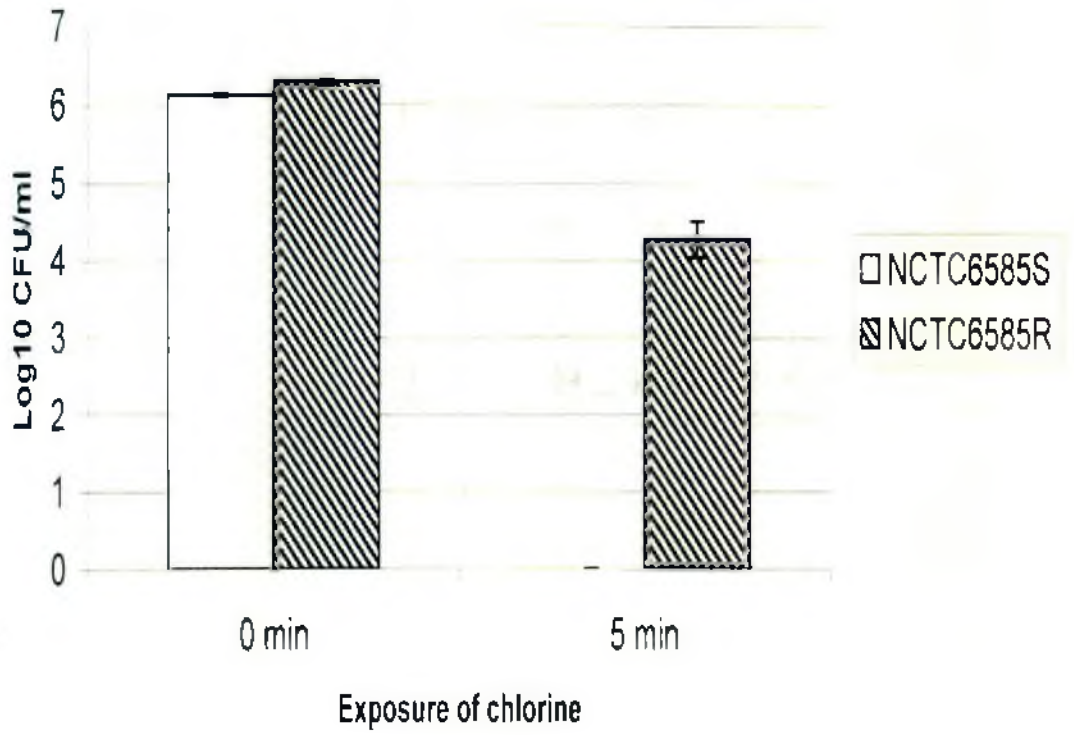


FIG.4. Effect of chlorine (3 ppm) on survival of smooth and rugose variants of *V. cholerae* O1 classical biotype strain NCTC 6585

1.6.b. Hydrogen peroxide resistance

In order to determine whether rEPS production by classical strain NCTC 6585 promoted resistance to hydrogen peroxide (another environmental stress), we compared the levels of resistance to hydrogen peroxide between a smooth-cell strain and a rugose variant. We found that the rugose variant of classical strain NCTC 6585 was 10-fold more resistant to hydrogen peroxide (5 min of exposure to 20 mM H_2O_2) than its smooth-cell counterpart (Fig. 5).

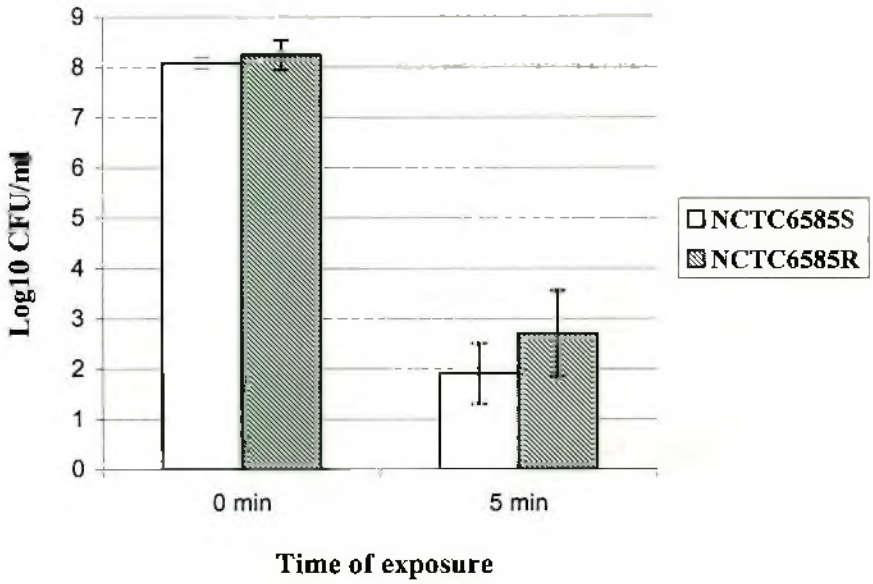


FIG.5. Effect of Hydrogen peroxide on survival of smooth and rugose variants of *V. cholerae* classical biotype NCTC 6585.

1.7. Biofilm formation by smooth and rugose colony variants of *V. cholerae*

EPS is known to be important for biofilm formation; therefore, I compared the biofilm-forming abilities of the rugose and smooth variants of several *V. cholerae* strains. Smooth strains of NCTC 6585 and N16961 incubated statically in APW # 3 at 37°C for 24 h did not produce visible biofilms; however, the rugose variants of both strains produced biofilm at the air-broth interface (Fig. 7). The biofilm-forming ability of rugose variants of El Tor strain N16961, classical strain NCTC 6585, and the O37 Aldova strain also was demonstrated by the quantitative method of Watnick et al. (168) (Fig. 6).

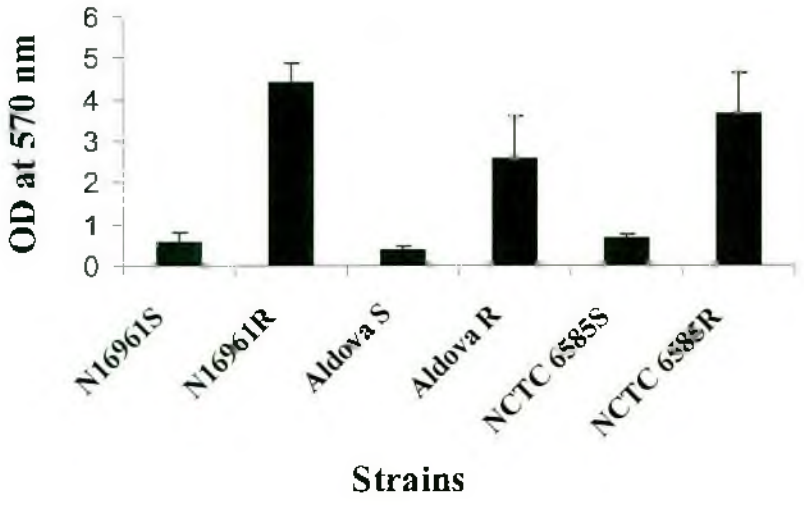


FIG.6. Biofilm formation by smooth and rugose colony variants of *V. cholerae*. LB broth (500 µl) in glass test tubes was inoculated with a 1:100 dilution of an overnight cultures of N16961, NCTC 6585, and Aldova O37 strains and incubated statically at room temperature for 24 h. Quantitative biofilm assays were performed, with minor modifications, as described previously (168).

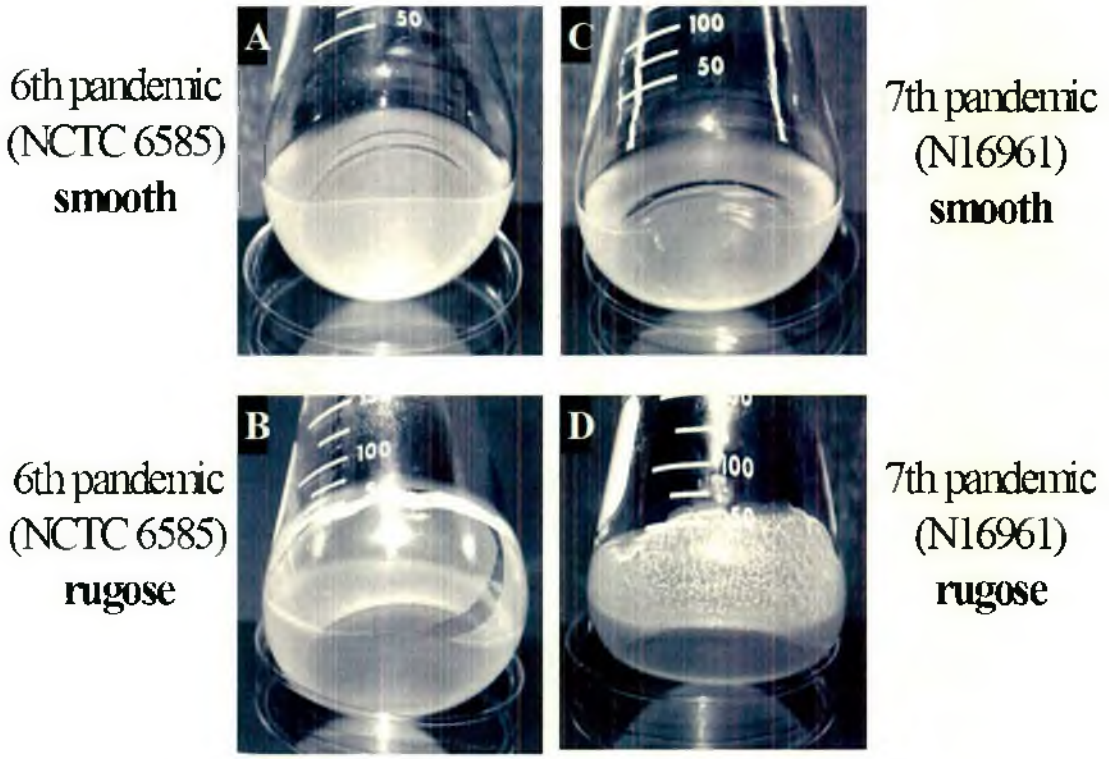


FIG.7. Biofilm formation by *V. cholerae* strains. The biofilm was induced by *V. cholerae* strains by growing them in APW # 3, incubated statically at 37°C for 24 hours. A: Smooth variants of *V. cholerae* classical strain NCTC 6585 B: Rugose variants of *V. cholerae* classical strain NCTC 6585 C: Smooth variants of *V. cholerae* El Tor strain N16961 D: Rugose variants of *V. cholerae* El Tor strain N16961.

1.8. Association between rEPS production and reduced motility

Motility testing of the smooth and rugose variants of strain N16961, on a semisolid medium containing 0.3% agar suggests an association between rEPS production and reduced motility. The average diameter of the smooth cells' motility zone (22 mm) was ca., 2.5 fold less than that of the rugose variant (8.5 mm). Interestingly, both smooth and rugose cells obtained from fresh colonies were flagellated, as were rugose planktonic cells and rugose cells in a biofilm from 48-h LB broth cultures incubated statically at 37°C (data not shown).

2. Carbohydrate and glycosyl linkage analysis of rEPS of clinical strains of *V. cholerae*

My initial studies confirmed that the classical biotypes of *V. cholerae* undergo HFRP despite the fact that classical rugose variants produce a distinct rugose phenotype than an El Tor biotype (Fig. 2). To determine the relationship of the rEPS to other polysaccharides produced by El Tor strains of *V. cholerae*, compositional and linkage analyses of the extracellular carbohydrate produced by classical biotype strain *V. cholerae* NCTC 6585 were performed (Complex Carbohydrate Research Center, University of Georgia, Atlanta). The analyses indicated that the rEPS and the smooth EPS (sEPS) are very similar; i.e. there are only subtle differences between them (Table 6). However, the carbohydrate composition in this classical strain is different to that reported for an El Tor strain (178). This indicates there are differences in structure between *V. cholerae* strains.

TABLE 5. Glycosyl composition and linkage analysis of EPS

Glycosyl composition		Glycosyl linkage	
Glycosyl residue	**Percent***	**Glycosyl residue**	**Relative ratio†**
Glucose	52.6	Terminal glucose	0.19
Galactose	37.0	Terminal galactose	0.06
GlcNAc	5.1	3-linked galactose	0.21
Mannose	3.8	4-linked galactose	0.89
Xylose	1.5	4-linked glucose	1.0
		3,4-linked galactose	0.19
		3,4-linked glucose	0.17
		2,4-linked galactose	0.17
		4,6-linked glucose	0.11
		4,6-linked galactose	0.09

*Values are expressed as the weight percent of total carbohydrate.

†Values represent the ratios of the GC peak areas with terminal 4-linked glucose set to 1.0.

The table was reproduced from the published paper (178)

TABLE 6. Glycosyl composition and linkage analysis of EPS produced by classical strain NCTC 6585

Sample	Glycosyl composition		Glycosyl linkage	
	Glycosyl residue	%	Glycosyl residue[a]	%
rEPS	Rhamnose	8.92	terminal linked –fucosyl residue	9.8
	Fucose	10.46	terminal linked –glucosyl residue	7.9
	Mannose	4.68	3 linked –glucosyl residue	8.8
	Galactose	18.71	2 linked –glucosyl residue	15.0
	Glucose	9.29	4 linked –manosyl residue	14.2
	GalNAc	16.86	4 linked –galactosyl residue	24.8
	GlcNAc	27.65	2,3,4 linked –fucosyl residue	7.7
			2,3 linked –manosyl residue	11.8
sEPS	Rhamnose	4.05	terminal linked –fucosyl residue	12.3
	Fucose	5.75	terminal linked –glucosyl residue	12.4
	Mannose	5.52	3 linked –glucosyl residue	10.9
	Galactose	18.23	2 linked –glucosyl residue	13.8
	Glucose	13.90	4 linked –manosyl residue	13.4
	GalNAc	22.02	4 linked –galactosyl residue	23.1
	GlcNAc	23.54	2,3,4 linked –fucosyl residue	0.0
			2,3 linked –manosyl residue	14.1

[a] All residues are in the pyranose (p) form.

rEPS denotes rugose EPS (all rugose cells) and sEPS denotes EPS from smooth cells.

3. Identification of the genes involved in the switch from the smooth to the rugose phenotype of *V. cholerae*

3.1. Transposon mutagenesis

In order to identify the genes involved in the switch from the smooth to rugose phenotype of *V. cholerae*, smooth cells of N16961 were subjected to transposon mutagenesis (see Materials and Methods). Approximately 15,000 transposon mutants were generated and screened for potential mutants defective for rEPS production. This screening resulted in 43 putative mutants defective for rugose production. Using multiple sets of degenerate primers and transposon specific primers, I successfully sequenced and identified the transposon insertion sites in 43 *V. cholerae* mutants that are unable to switch from the smooth to the rugose phenotype. A BLAST search against the published *V. cholerae* N16961 genome (58) was performed to identify the disrupted genes (Table 7).

TABLE 7. Representative HFRP mutants of *V. cholerae* N16961

Mutant[a]	Locus[b]	Predicted protein	Predicted function
DK568 (2)	VC0243	RfbD	LPS biosynthesis, GDPmannose 4,6-dehydratase
DK623 (1)	VC0244	RfbE	LPS biosynthesis, perosamine Synthase
DK578 (2)	VCA0744	GalE	LPS biosynthesis, UDPglucose 4-epimerase
DK589 (1)	VC0920	Vps (EpsF)	EPS biosynthesis, glycosyl transferase
DK576 (2)	VC0921	Vps (Wzx)	EPS, polysaccharide export, flippase
DK588 (7)	VC0922	Vps	EPS, hypothetical protein
DK562 (13)	VC0665	VpsR	EPS biosynthesis, σ54 transcriptional activator
DK614 (10)	VC2628	AroB	aromatic amino acid synthesis, 3-dehydroquinate synthase
DK625 (1)	VC2629	AroK	aromatic amino acid synthesis, shikimate kinase
DK630 (1)	VC0344	AmiB	N-acetylmuramoyl-L-alanine amidase
DK567 (3)	VC0653	RocS	regulatory, contains GGDEF and EAL domains

[a]Numbers in brackets indicate the number of mini-Tn5 mutants with insertions in same locus.

[b]Loci and predicted proteins derived from the *V. cholerae* N16961 TIGR sequencing project.

3.2. VpsR has an essential role in switching to the rugose phenotype

VpsR is known to positively regulate the *vps* biosynthetic genes in *V. cholerae*, thus resulting in increased production of VPS proteins in rugose background than does in smooth background. I identified several vpsR mutant during this study; therefore, to confirm the role of *vpsR* in rugose production, I studied several *vpsR* mutants, (designated DK562 and DK581) defective for rugose production. Supplying the *vpsR* (on plasmid pDK104) restored the mutants' ability to switch to the rugose phenotype. Motility tests performed with the *vpsR* mutants (DK562 and DK581) revealed that their motility was consistently ~50% less than that of parental strain N16961 (Table 8).

3.3. The AmiB encoded amidase has a role in switching to the rugose phenotype

N-acetylmuramoyl-L-alanine amidase is encoded by the *amiB* gene (locus VC0344). Since rugose strains of *V. cholerae* are known to have reduced motility (4, and confirmed in this thesis), I examined whether a mutation in *amiB* results in reduced motility. The motility of an *amiB* mutant (DK630) was consistently reduced by >50% (10 mm diameter zone of motility) compared to its parent N16961 (22 mm zone) (Fig. 8).

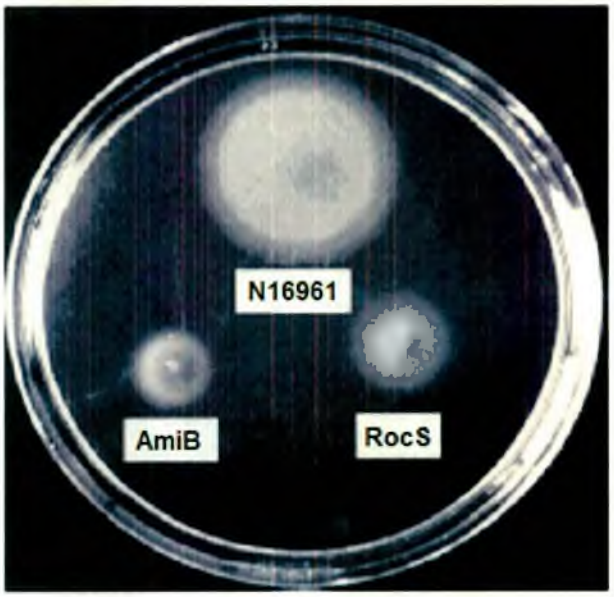


FIG.8. Swarm plate assay comparing the motility of *V. cholerae* N16961 (wild-type), an *amiB* mutant (DK630) and a *rocS* mutant (DK567). The plate contains LB broth supplemented with 0.3% agar and it was incubated at 37°C for 4 h.

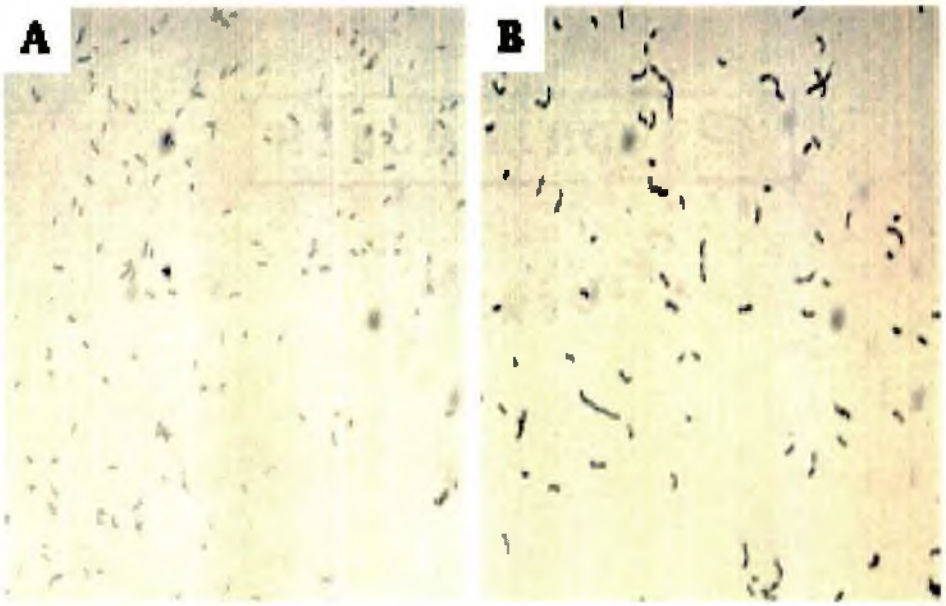


FIG.9. Effect of an *amiB* mutation on the cellular morphology of *V. cholerae* N16961. A: Wild-type cells; B: *amiB* mutant (DK630). The *amiB* cells have a different morphology, show an increase in overall cell size and form chains during growth. Magnification 1000×.

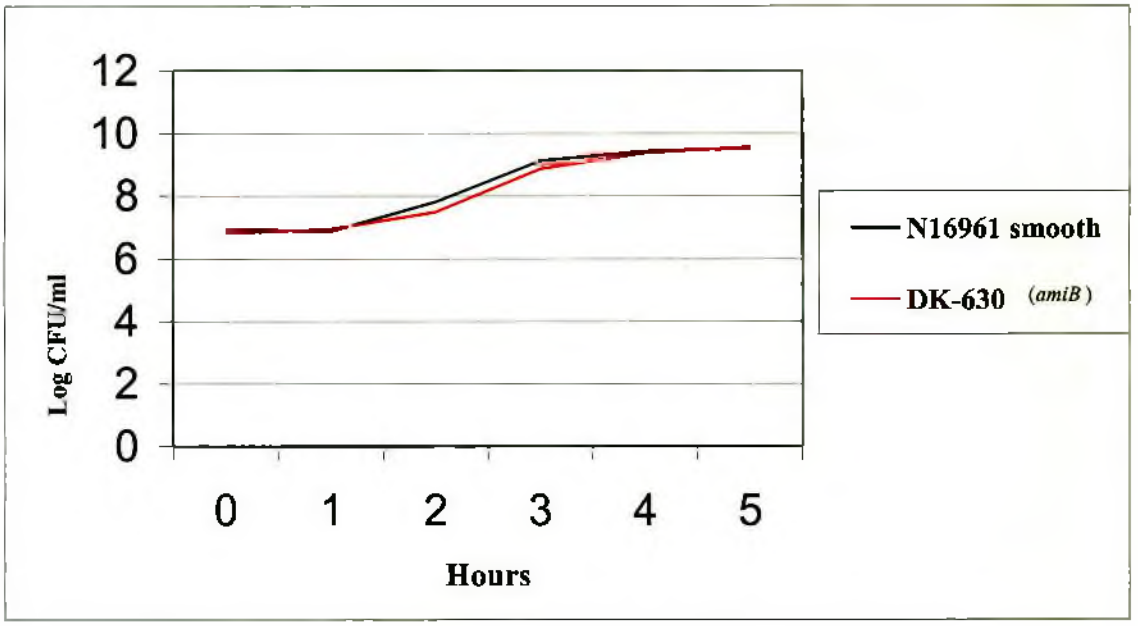


FIG.10. Growth rate of N16961 and its Tn5 mutant DK 630 (*amiB*)

TABLE 8. Motility of representative HFRP mutants of *V. cholerae* N16961

Mutant	Locus	Predicted Protein	Motility[a] (zone diameter in mm)
N16961 (wild-type)			22
DK568	VC0243	RfbD	14
DK623	VC0244	RfbE	13
DK578	VCA0744	GalE	5
DK589	VC0920	Vps (EpsF)	20
DK576	VC0921	Vps (Wzx)	6
DK588	VC0922	Vps	19
DK562	VC0665	VpsR	5
DK614	VC2628	AroB	22
DK625	VC2629	AroK	19
DK630	VC0344	AmiB	10
DK567	VC0653	RocS	7

[a] Motility results are the mean of those obtained by three independent experiments for each strain on motility agar (0.3% agar).

3.4. *V. cholerae* RocS: a conserved regulatory protein with a GGDEF and EAL domain regulates the rugose phenotype and motility

Three independent mutants containing mutations in *rocS* were isolated from three independent conjugations. The defect in the mutants' ability to express the rugose phenotype was not explained by differences in growth rate between the wild-type N16961 and *rocS* (DK567) cells (Fig. 11,12). The finding that *V. cholerae rocS* mutants appear to be defective in switching to the rugose phenotype and therefore biofilm formation, prompted me to test the motility of one of the mutants (DK567). Its motility was consistently reduced by >50%; i.e., the diameter of its motility zone was ca. 12 mm compared to 22 mm for the parental wild-type strain (Table 8). This suggests *rocS* has a role in regulating various phenotypes including biofilm formation, motility and potentially virulence.

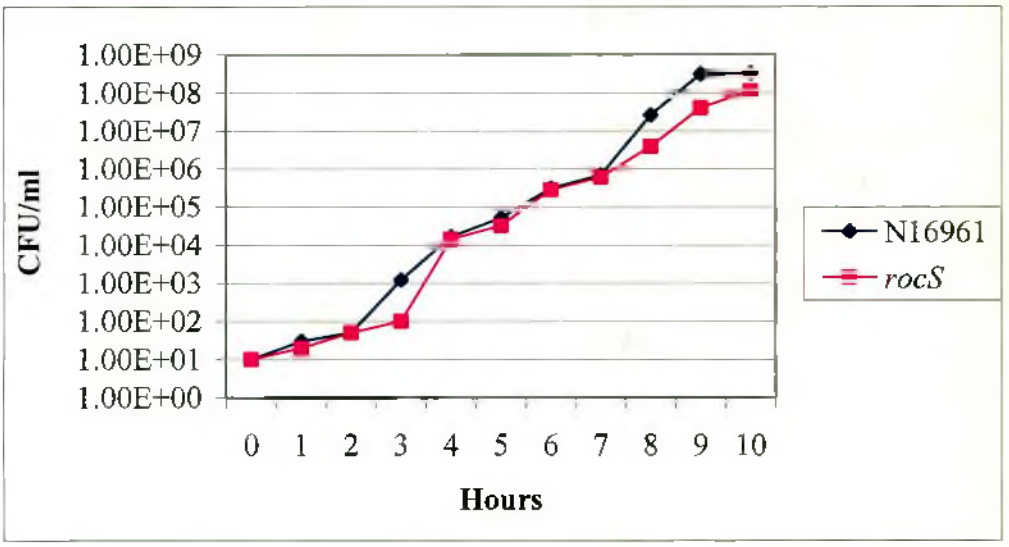


FIG.11. Growth curves of *V. cholerae* N16961 (DK81) and *rocS* (DK567) in separate LB broth cultures.

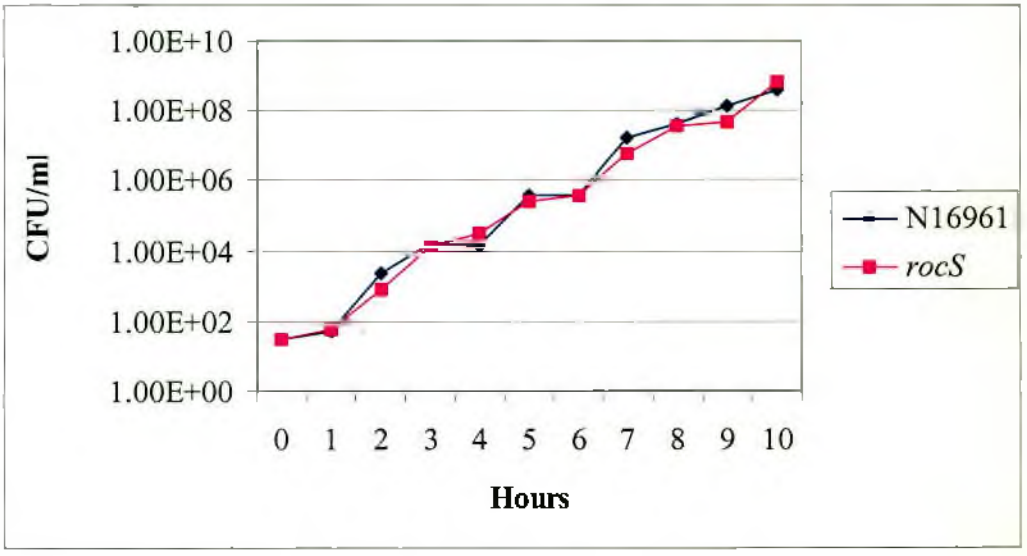


FIG.12. Growth curves of *V. cholerae*N16961 (DK 81) and *rocS* (DK 567) when grown in the same LB broth culture.

4. Role of the rugose phenotype and *vpsR* in the pathogenesis of cholera

4.1. The role of rugose and *vpsR* in the pathogenesis of *V. cholerae*

In addition to *V. cholerae*, several bacterial species - including *Salmonella enterica* Enteritidis (125), *S. enterica* typhimurium (8), *Pseudomonas aeruginosa* (31) and *Enterobacter sakazakii* (38) - are known to express a rugose like phenotype. In addition, it is now becoming increasingly recognized that rugose variants may have an important role in the pathogenesis of disease caused by *V. cholerae* and by some of the above-mentioned species. The recent finding of conditions that promote high frequency switching to the rugose phenotype in *V. cholerae* and that switching appears to be more common in epidemic strains than in non-pathogenic strains suggests that VPS production and the rugose phenotype is important in the epidemiology of cholera (4). To better understand the molecular basis of switching, I reported the identification of genes involved in switching from the smooth to the rugose phenotype (131). Although rugose variants of *V. cholerae* are known to be highly resistant to various stressful conditions *in vitro* and to form large biofilms *in vitro*, the role of VPS, the rugose phenotype and *VpsR* in the variants' ability to cause cholera is not well understood. The *in vitro* and *in vivo* studies presented in this section of my thesis were performed in order to advance knowledge in that area.

4.2. CT production by smooth and rugose strains

To the best of my knowledge, previous studies have not compared the amount of CT produced by smooth and rugose hyperbiofilm variants of *V. cholerae*. Therefore, I quantitated cholera toxin production by smooth and rugose variants of N16961, as well as its mutants. Although extracellular VPS is increased in rugose strains, significant differences in CT production was not found between smooth and rugose strains of N16961 or between wild-type N16961 and two independent *VpsR* mutants (DK562 and DK581) under the conditions tested.

4.3. Studies of collagenase and elastase production

In order to determine whether the rugose phenotype inhibits the production and/or secretion of major extracellular degradative enzymes; i.e. collagenase and elastase, I compared the production and/or secretion of those enzymes by smooth and rugose variants of *V. cholerae* N16961. I found that the rugose strains of *V. cholerae* N16961 (El Tor biotype) and NCTC 6585 (classical biotype) are defective (compared to their smooth variants) in the production of both collagenase I and collagenase IV (Table 9). In addition, the smooth variant of strain N16961 produced approximately three-fold more collagenase I and collagenase IV than did the smooth variant of strain NCTC 6585. Interestingly, although the smooth and rugose variants of El Tor N16961 produced similar amounts of elastase, the classical biotype strain NCTC 6585 did not produce detectable amounts of extracellular elastase.

TABLE 9. Extracellular collagenase and elastase activity of smooth and rugose strains of *V. cholerae*[a]

Strain	Collagenase I	Collagenase IV	Elastase
N16961 smooth	152	86	17
N16961 rugose	4	0	13
NCTC 6585 smooth	57	28	0
NCTC 6585 rugose	0	20	0

[a] Enzyme assay were performed with culture supernatant fluids obtained by membrane filtration (0.22 μm pore-size) of overnight BHI cultures. All results are expressed as a percent of positive controls and blank. Positive controls with specific inhibitors have been subtracted from the data. We subtracted positive controls with enzyme inhibitor in order to eliminate bias due to non-specific proteases.

4.4. Studies with the rabbit ileal loop model of infection

In order to determine whether VPS, the rugose phenotype, and *vpsR* have any role in cholera pathogenesis, I compared the production of cholera toxin in the rabbit ileal loop model, between a smooth and a rugose variant of *V. cholerae*. Obvious differences were not observed between the fluid accumulation ratios (FAR) of loops 18-h postinoculation with the smooth strain, rugose strain, *vps* and *vpsR* mutants (Table 10). This result is consistent with our earlier finding that there is no difference in CT between the strains. Also, plating the fluid contents from ileal loops did not reveal obvious phenotypic switching between the smooth and rugose phenotypes. This finding suggests that if phenotypic switching occurs *in vivo*, it is at a frequency below the detection limit of the methods used in this study. Although there was no significant difference in the total number of *V. cholerae* CFU cultured from the intestinal fluid of each loop, gross examination of the 18-h loops revealed differences in tissue damage (i.e. serosal hemorrhage and necrotic areas) elicited by the variants and mutants (Fig. 13). Ileal loops inoculated with the wild-type smooth or rugose variants showed obvious scattered regions of serosal hemorrhage and necrosis. However, the *vps* mutant DK589 (defective in VPS production) appeared to be slightly less reactogenic than its wild-type smooth parent. In addition the *vpsR* mutant (DK562) was consistently much less reactogenic than its smooth wild-type parent; i.e. the loops inoculated with it showed considerably less tissue damage. Although significant differences were not noted in the number of CFU in the ileal loop fluids, I did not enumerate the number of variant and mutant of *V. cholerae* adhering to the loops' mucosa. Therefore, I cannot say, at this time, whether the observed

reduction in the mutants' reactogenecity was a function of their reduced ability to colonize the loops.

TABLE 10. Fluid accumulation and tissue damage in rabbit ileal loops inoculated with smooth and rugose variants, and with mutants of *vpsR* and *vps*, of *V. cholerae* N16961

Sample	Effect on rabbit ileal loops			
	Volume (ml)	Length (cm)	FAR (volume/length)[a]	Tissue damage
N16961 smooth	19.6	9.6	2.0	++
N16961 rugose	17.5	9.0	1.9	+++
DK562 (*vpsR*)	17.0	8.4	2.0	-
DK589 (*vps*)	24	10.9	2.2	+/-

[a]See Fig 13. For the visual appreance of the loops.

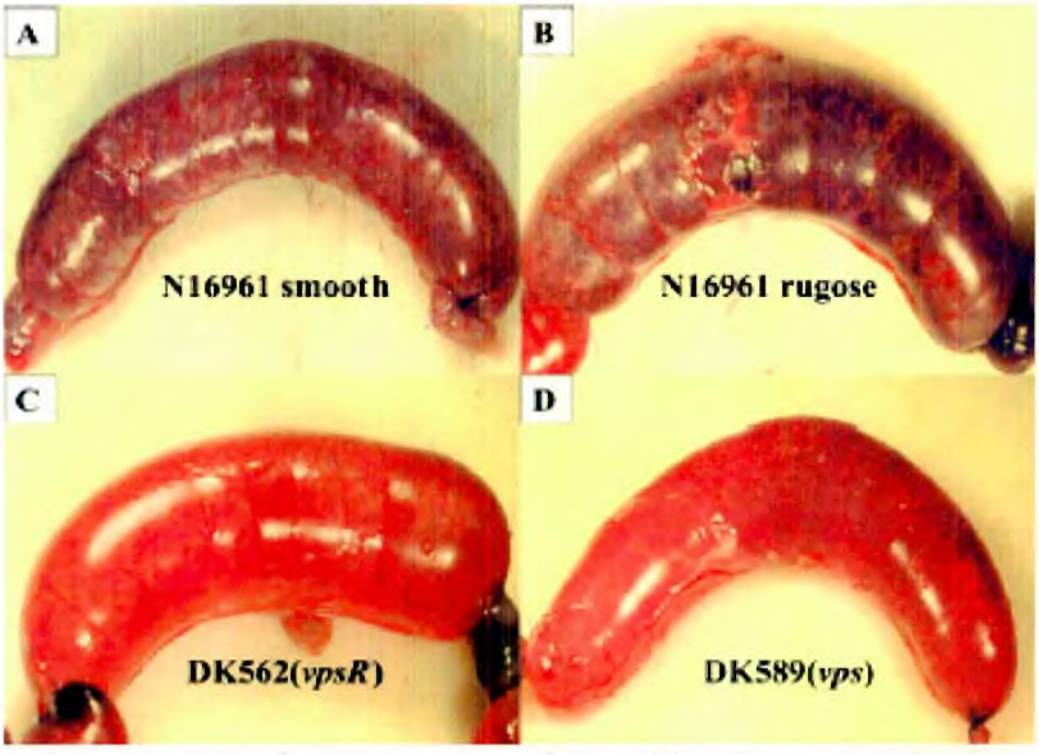


FIG.13. Rabbit ileal loops 18 h postinoculation with *V. cholerae*. Note the reduction in fluid accumulation in the loop containing the *vspR* mutant DK562, compared to the loop containing the wild-type smooth strain N16961. These results were observed in three rabbits inoculated with the strains.

4.5. Studies with the infant mouse colonization model

The *in vivo* studies presented in this section of my thesis explored whether the rugose phenotype, *vps* or *vpsR* is required for intestinal colonization. Significant differences were not detected in the level of TcpA production by smooth and rugose variants of *V. cholerae* strain N16961 (data not shown). However, since the rugose phenotype markedly enhances biofilm formation in vitro (4, 178, 165 and this thesis) I hypothesized that it might also enhance colonization *in vivo*. Therefore, I compared the ability of smooth and rugose variants, and a vpsR mutant (DK581), to colonize the intestinal tract of 5-day-old infant mice after oral administration, either alone or in standard co-infection assays. Although plate counts of the bacterial suspensions used to inoculate the mice showed that there was not a significant difference between the strains' average viable counts (smooth=3.5×10^5 ml-1; rugose=4.8×10^5 ml-1; *vpsR*=4.0×10^5 ml-1), and multiple *in vitro* co-infection assays did not reveal significant differences between the strains after 5 h and 24 h of co-infection (data not shown), significant differences were observed between the strains' intestinal colonization ability (Table 11 and Fig. 14,15).

TABLE 11. Colony counts and co-infection studies of *V. cholerae* in suckling infant mice

Strains	5 h		24 h	
	(CFU/ ml)	CI	(CFU/ ml)	CI
Smooth	ND	ND	2.28×10^{7}	ND (n= 10)
Rugose	ND	ND	9.54×10^{6}	ND (n= 14)
vpsR	ND	ND	3.70×10^{6}	ND (n= 17)
Rugose/smooth	$4.2\times10^{4}/2.2\times10^{5}$	0.19 (n= 7)	$5.1\times10^{6}/2.3\times10^{7}$	0.22 (n=27)
vpsR/smooth	$9.7\times10^{3}/1.8\times10^{4}$	0.53 (n= 8)	$2.1\times10^{6}/2.2\times10^{6}$	0.95 (n=19)
vpsR/rugose	$5.4\times10^{5}/4.3\times10^{4}$	12.55 (n= 8)	$1.1\times10^{7}/1.1\times10^{6}$	10 (n=22)

CI=competitive index; n=number of mice in-group; ND=not done.

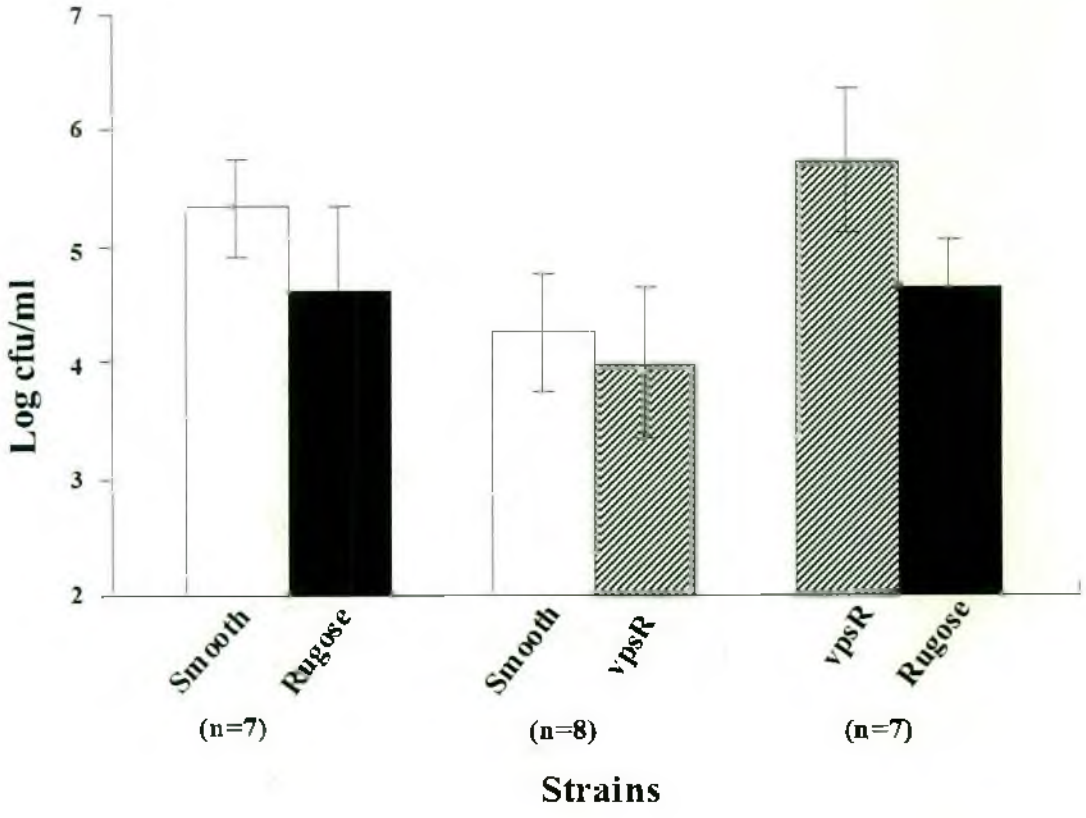


FIG.14. Colonization of infant mice by smooth variant, rugose variant, and vpsR mutant of *V. cholerae* N16961. Colonization rates 5 h postincubation. Colony counts (CFU ml-1) represent the mean values obtained where n represents the number of mice used in the study.

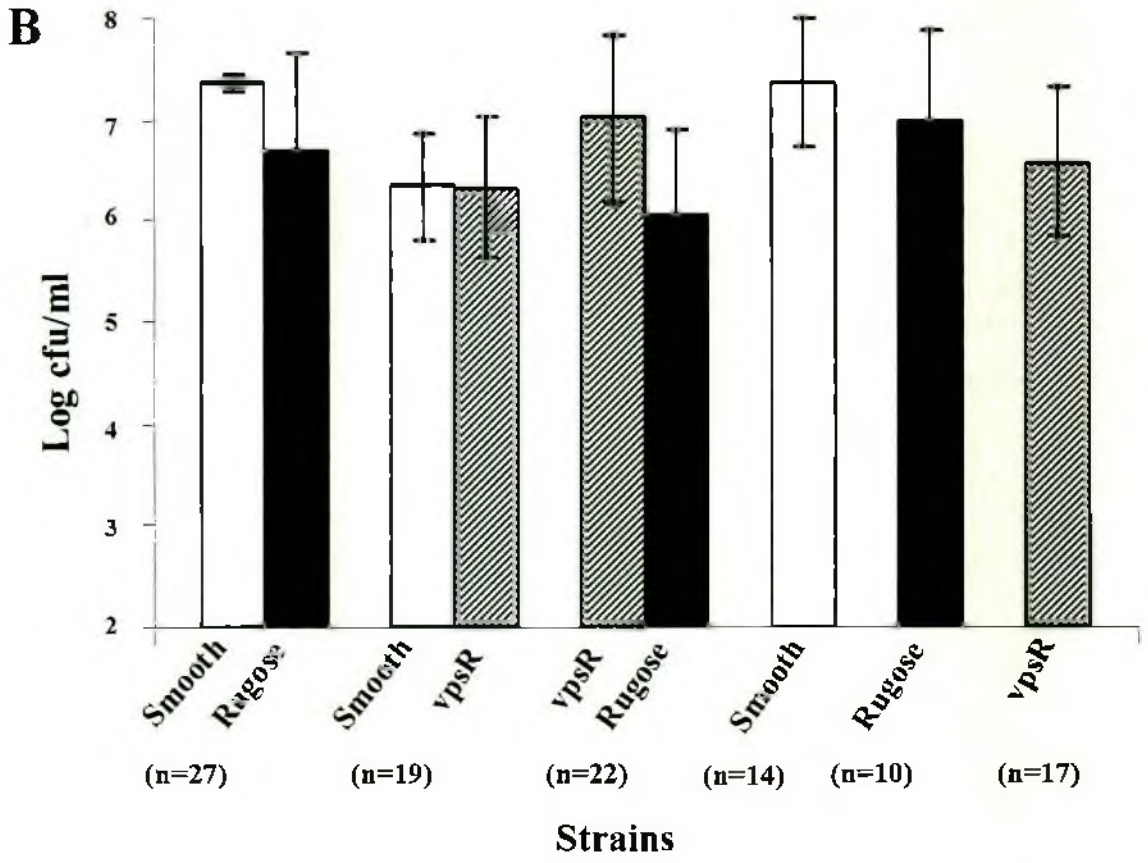


FIG.15. Colonization of infant mice by smooth variant, rugose variant, and vpsR mutant of *V. cholerae* N16961. Colonization rates after 24 h postincubation. Colony counts (CFU ml-1) represent the mean values obtained where n represents the number of mice used in the study.

4.5.a. Intestinal colonization studies (5 h)

Intestinal colonization assays were performed 5 h postinoculation, to determine whether the vpsR mutant colonized the infant mice better than did the wild-type, smooth variant of *V. cholerae* N16961.Co-infection assays performed 5 h postinoculation revealed that the colonization abilities of the smooth variant and vpsR mutant were not significantly different (Table 11 and Fig. 14). However, the smooth variant and the vpsR mutant colonized 5-fold and 12-fold better, respectively, than did the rugose strain. Interestingly, when the smooth variant or vpsR mutant was used together with the rugose variant to co-infect mice, the colonization rate for the smooth variant and the *vpsR* mutant was 12- and 55-fold higher, respectively, than it was in the smooth/*vpsR* co-infection assay.

4.5.b. Intestinal colonization studies (24 h)

In order to compare the variants' and mutants' abilities to colonize infant mice during the later stages of infection, I performed multiple 24 h individual and co-infection assays. Initially, I observed statistically significant differences between the variants' and mutant's colonizing ability when they were used to singly infect mice (P=0.0004; Kruskal–Wallis test) (Table 11 and Fig. 15). The results revealed that the smooth variant colonized ~10-fold better than did the rugose variant. In addition, the *vpsR* mutant's (VPS-negative and unable to switch to the rugose phenotype) ability to colonize the infant mice was significantly lower than that of the wild-type smooth and rugose variants.

After using single infection assays to compare the variants' and vpsR mutant's ability to colonize infant mice at 24 h postinfection, I performed co-infection assays 24 h postinfection with the smooth and rugose variants' and *vpsR* mutant (Table 10 and Fig. 15). Significant differences were not observed between the colonization rates of the smooth variant and the *vpsR* mutant (P=0.36; Wilcoxon signed rank test). However consistent with the results of the co-infection assays performed at 5 h postinfection, the smooth variant's colonization rate was significantly (five-fold) greater than that of the rugose variant (P=0.0034; Wilcoxon signed rank test). In addition, the colonization rates of the smooth and rugose variants significantly decreased (P=0.006 and P=0.012, respectively; Kruskal –Wallis test) when either of them was used to co-infect mice with the *vpsR* mutant, compared to when mice were singly infected with the smooth and rugose variants. Importantly, and again consistent with the results of the assays performed 5 h postinfection, during co-infection with the smooth and rugose variants, the smooth variant's colonizing ability was ca. 10-fold better than during co-infection with the vpsR mutant. Likewise, the the *vpsR* mutant's colonizing ability was ca. 5-fold better during co-infection with the rugose variant than when it was used to singly infect mice (P=0.027; Wilcoxon test).

DISCUSSION

V. cholerae persists for centuries in diverse aquatic and ecological niches, including freshwater, estuarine, sewage and marine environments. Despite its remarkable ubiquity, the underlying genetic and physiologic basis for that bacterium's long-time survival in its aquatic reservoirs is not clearly understood. The isolation rate of *V. cholerae* from aquatic environments - using currently available techniques - is extremely low, even during epidemics in cholera-endemic countries such as Bangladesh and India. This observation suggests that *V. cholerae* persist in its aquatic reservoirs by an as-yet-unknown mechanism(s). Currently, there are two models available regarding *V. cholerae*'s persistence. The first is the bacterium's ability to exist in a viable but nonculturable state (VBNC), which has been proposed by Colwell and her coworkers. According to this model, *V. cholerae* can persist in a VBNC state during nutrient starvation and exposure to cold temperatures (22, 139, 145, 176). However, the state's role in the survival and persistence of *V. cholerae* is controversial due to (i) a lack of molecular genetic evidence supporting the VBNC phenomenon, and (ii) the existence of another model for persistence; i.e., the rugose phenotype/variant *V. cholerae* - originally proposed by Bruce White in 1938. According to this model, the smooth variant of *V. cholerae* can spontaneously shift to a rugose variant under nutrient-starved growth conditions (170, 171). Further studies of the rugose phenotype by several investigators (4, 112, 158, 178) have supported the hypothesis that it is important in the aquatic survival of *V. cholerae*. However, additional physiologic and genetic studies are needed to clarify the rugose phenotype's role in the persistence of *V. cholerae*.

The primary objectives of the studies described in my thesis included (i) developing a medium that promotes the smooth (S) to rugose (R) conversion in the shortest period of time, (ii) determining whether epidemic strains of *V. cholerae* are more able to shift to the rugose phenotype than are environmental strains, (iii) identifying the genes involved in the S to R shift, and (iii) determining whether rugose variants are more virulent than smooth variants of *V. cholerae*.

During the course of my studies, a medium (alkaline peptone water [APW] #3) was developed that promoted both a low frequency S to R shift as well as a high-frequency rugose production (HFRP) state in some strains of *V. cholerae* belonging to the El Tor and classical biotypes. Consistent with previous observations (112), several strains exhibited a low frequency (<0.5%) shift to the rugose phenotype. However, only 6 of 19 (32%) clinical isolates and 1 of 16 (6%) nonpathogenic (environmental) strains shifted to the HFRP state under the conditions tested (t test; $P < 0.05$) (Table 1). Among the strains tested, strain N16961 consistently exhibited the highest HFRP in flask cultures (24 to 38% at 30°C; 42 to 51% at 37°C) and tube cultures (68 to 74% at 30°C; 60 to 80% at 37°C) (Table 1). The mechanism for HFRP, which may be regulated by phase variation, is presently being studied. Phase variation, which occurs *via* DNA inversion, DNA recombination and slipped-strand mispairing, is known (61, 92) to control the expression of several surface structures of Gram-negative bacteria; e.g., fimbriae, flagella, outer membrane proteins, lipopolysaccharide, and capsular polysaccharide.

Although all of the epidemic strains I examined were not capable of expressing HFRP (which indicates that HFRP is not essential for their persistence), my results suggest that the rEPS has a role in *V. cholerae*'s virulence, and that HFRP may be more

common in clinical strains than previously recognized. Thus, the possible importance of the HFRP phenotype with some epidemic strains of *V. cholerae* is being studied further. Chronic *Pseudomonas aeruginosa* lung infections during cystic fibrosis are only caused by the subpopulation of *P. aeruginosa* strains that have the ability to express copious amounts of EPS and form biofilms in the lung (51). Thus, rEPS and HFRP production by a subpopulation of *V. cholerae* strains may provide them with an evolutionary or adaptive advantage in a particular environment(s).

HFRP was not restricted to clinical *V. cholerae* O1 strains (Table 1); e.g., HFRP was consistently observed with the Aldova strain (serogroup O37). Interestingly, that strain was responsible for epidemics in Czechoslovakia in 1965 (2) and in the Sudan in 1968 (180), but has not caused any major outbreaks since that time. In addition to the above-mentioned O37 strain, a previous study (110) reported rEPS production by an O139 serogroup strain (MO10). Also, although I was unable to detect HFRP by an O139 strain (strain 1837), I observed that up to 2% of the strain's cells could shift to a rugose colony phenotype similar to that of the rugose colonies produced by strain N16961. I also detected HFRP by strain 1803, another non-O1 (unknown serogroup) clinical isolate. Interestingly, only 1 of the 16 (12%) environmental strains tested (P44; unknown serogroup), which included many different serogroups, showed HFRP (12%).

There were differences between the examined strains' colony morphologies and in the conditions that induced HFRP (Table 1 and Fig. 1). For example, the rugose colonies of classical strains NCTC 6585 and AMS20A73 (24 to 48 h after plating) were not as pronounced in structure as the rugose colonies of strain N16961 or an O139 strain (Fig. 1). Although it has been known, for more than 60 years, that rugose colony

morphology is the result of copious EPS production (170, 171), the observation of different colony morphologies and different conditions triggering HFRP suggests that there are differences in the regulation and genetics of rEPS production by various strains. Consistent with previous studies (158, 178) reporting that rugose El Tor cells are surrounded by EPS, TEM confirmed the presence of EPS between rugose cells of strain NCTC 6585 and the absence of that material between the strain's smooth cells (Fig. 2).

The results of the carbohydrate composition and linkage analyses of a 6th pandemic strain (NCTC 6585) differed markedly from those obtained with the rEPS isolated from a 7th pandemic strain (92A1552) which has glucose as the predominant sugar (178), and from the strain TSI-4 rEPS, which has mannose as the predominant sugar (158). The compositionl analysis results also indicate that the extracellular polysaccharides described here is different from the O1 LPS, which typically contains large amounts of perosamine and quinovosamine (131-b). In contrast to the results previously reported (178) for El Tor strain 92A1552, in which 4-linked galactose and 4-linked glucose are the dominant linkages, the glycosyl linkage analysis (using gas chromatography-mass spectrometry) performed during my studies revealed that the predominant linkage is a 4-linked galactosyl residue which may represent the backbone of the NCTC 6585 saccharide. Also, a possible quantitative difference was noted in the amount of 2,3,4 linked-fucosyl residues in the two samples, which suggests possible differences in branching. However, the observed difference in the 2,3, 4 linked-fucosyl residues might have resulted from undermethylation and, therefore, may not represent an actual difference in the linkages. Interestingly, some *Klebsiella pneumoniae* strains express different EPS structures containing the same sugar residues, only in different

molar ratios or in different linkages (18-b). Although EPS was clearly induced in APC, I never detected rugose cells after plating any APC cultures. This observation may be explained by the production of EPS variants or by the production of identical EPS structures whose induction is triggered by different signals. These alternate possibilities, and whether the analyzed rEPS contained other constituents or impurities, may be examined by further structural analysis; e.g., by NMR. The results of my reversion studies indicate that there are significant differences between the S to R shift/conversion and the R to S shift/reversion (Table 2). Thus, reversion may be strain- and growth condition-specific.

Previous studies have reported an association among production, reduced motility, and biofilm formation. I observed that smooth and rugose cells obtained from fresh colonies were flagellated, as were planktonic rugose cells and rugose cells in a biofilm from 48-h LB broth cultures incubated statically at 37°C. At the present time, I speculate that the flagellar movement of rugose cells is inhibited by copious amounts of rEPS, or that motility is defective due to some other mechanism. Although motility is required for biofilm formation by smooth cells of *V. cholerae* (168), particularly during the early stages of biofilm formation, perhaps the initial attachment required for biofilm by rugose *V. cholerae* is mediated by rEPS instead of by flagella.

As previously observed (158, 178) with the El Tor biotype of *V. cholerae*, I found that when a strain (regardless of its serotype and biotype) shifts to the rugose phenotype (i.e., elevated rEPS production), it produces several folds more biofilm than its corresponding smooth variant. The quantitative biofilm data (Fig. 3) show that the biofilm-forming abilities of rugose variants of the N16961, NCTC 6585, and Aldova

strains are ca. 7.7-, 6.6-, and 5.5-fold greater, respectively, than those of their corresponding smooth variants. These results clearly indicate that rEPS production in El Tor, classical, and non-O1 strains is associated with increased biofilm-forming ability.

Production of the rEPS has been shown (112, 133, 165, 178) to promote *V. cholerae*'s resistance to a variety of environmental stresses, such as chlorine, UV light, hydrogen peroxide and complement. Consistent with the findings obtained with rugose variants of El Tor strains (178), I found that the rugose variant of classical strain NCTC 6585 was 10,000-fold more resistant to chlorine (5 min of exposure to 3 mg/liter) than was its smooth-cell counterpart.

Previous transposon mutagenesis studies (5, 165, 178) identified gene mutations that result in stable R to S mutants (5, 165, 178). In contrast, taking advantage of conditions that promote switching to the rugose phenotype, I performed transposon mutagenesis on a smooth strain of N16961 and screened for stable mutants that were unable to switch to the rugose phenotype under rugose-inducing conditions. In addition to detecting mutants with defects in genes previously identified as having roles in the rugose phenotype; i.e., biosynthesis and regulatory genes (the *vps* operon and *vpsR*, respectively), and a lipopolysaccharide synthesis-encoding (LPS) gene (*galE*), my screening protocol identified mutants sustaining insertions in previously unidentified genes. The newly identified mutants contained mutations in genes encoding (i) enzymes involved in LPS synthesis (*rfbD* and *rfbE*), in which the enzymes might have roles in catalyzing the addition of certain sugar linkages; thus, impairment of the LPS structure might be linked to the shutdown of the rugose (EPSon) phenotype, (ii) enzymes involved in aromatic amino acid synthesis (*aroB* and *aroK*); thus, aromatic amino acid synthesis

might be directly or indirectly associated with the rugose phenotype, (iii) an enzyme involved in cell wall hydrolysis and septation (*amiB*), and (iv) a putative protein containing GGDEF and EAL domains. The protein, encoded by a novel locus (VC0653), is designated "*pde*-like" in the N16961 genomic database, and I now call it "RocS" (for regulation of cell signaling). The exact function of GGDEF and EAL domains is not well understood; however, proteins containing those domains are widespread in prokaryotic species, and they might play a key role in regulating several phenotypes, including those with roles in the virulence and the persistence of the species.

VpsR, encoded by the locus VC0665, is a predicted 444-amino acid-containing protein that is very similar to the members of a family of σ54 response regulators; e.g., NtrC, AlgB, and HydG (5, 179). Supplying *vpsR* on a plasmid restored the ability of the *vpsR* mutant to switch to the rugose phenotype, which confirmed that the mutant's defect was a function of a mutation in *vpsR*. Since *V. cholerae* cells are typically motile, and motility is important for virulence (135, 177), it is tempting to speculate that VpsR might also have a role in the virulence of *V. cholerae*. Although VpsR is important in regulating EPS (*vps*) biosynthesis genes and, potentially, other phenotypes, the conditions promoting VpsR expression and its mechanism of regulating *vps* genes are not well understood.

An N-acetylmuramyl-L-alanine amidase, designated AmiB, is encoded by the *amiB* gene (locus VC0344). Rugose strains of *V. cholerae* have previously been reported (4) to have reduced motility. During my studies, motility assays revealed that the motility of an *amiB* mutant (DK630) of strain N16961 was consistently <50% that of the

parental strain's motility (Fig. 6). These results suggest that AmiB affect *V. cholerae*'s motility and the expression of its rugose phenotype.

The bacterial cell wall is typically composed of a heteropolymer known as murein or peptidoglycan. Many Gram-negative bacteria degrade up to 50% of their murein per generation and recycle it to form new murein (48, 49, 121). In that regard, N-acetylmuramyl-L-alanine amidases are often associated with autolysis or microbial cell wall hydrolysis. Surprisingly, the importance of enzymes that cleave the Gram-negative bacterial septum, such as AmiB, has only recently been studied in a few species. For example, AmiB mutants of *E. coli* have been reported (60, 68) to grow as long chains of unseparated cells (60, 68). Also, an N-acetylmuramyl-L-alanine amidase of *Azotobacter vinelandii* is linked to alginate production because the enzyme's action enables the bacterium to recycle its cell wall (116).

A BLAST search revealed that the amino acid sequence of the *V. cholerae* AmiB is very similar to those of N-acetylmuramyl-L-alanine amidases found in a wide variety of species, including *P. aeruginosa*, *Salmonella enterica* Typhi, *E. coli* O157:H7 and *Yersinia pestis*. The AmiB of *V. cholerae* strain N16961 is predicted to be a 59-kDa protein that is unusually rich in serine (9.5%), proline (6%) and threonine (6%). Such a composition is common in protein domains associated with the cell wall of Gram-positive bacteria (42), and it is similar to a putative peptidoglycan hydrolase (encoded by *acmB*) of *Lactococcus lactis* (69). In *V. cholerae*, as in *E. coli* and *Y. pestis*, *amiB* is located immediately upstream of *mutL*, which has a role in DNA mismatch repair (157, 122). A computer analysis (using PSORT) predicted that the *V. cholerae* AmiB has a cleavable N-terminal signal sequence, and an analysis (using Tmpred) strongly predicted that the

enzyme has two transmembrane domains (score 2363), one at the N-terminal end (amino acids 10-29) - which could also represent an N-terminal signal sequence - and another at the C-terminal end (amino acids 446-465). One would expect TMpred to predict a transmembrane region at the N-terminal end if a *sec*-dependent signal sequence was also predicted. The *V. cholerae* AmiB was also predicted to contain a LysM (lysin motif) domain at its C-terminal end, and this domain has been found in enzymes involved with cell wall degradation (14). Interestingly, the *V. cholerae* AmiB also contains an Arg-Gly-Asp (RGD) motif that is often associated with a surface binding domain for various mammalian adhesion proteins.

Since AmiB is associated with septation in other species, such as *E. coli* (59, 60), I determined whether the *V. cholerae* AmiB mutant's cellular morphology and septation are altered. Examination of the cells revealed obvious differences between the AmiB mutant's and the wild-type strain's morphology and arrangement of cells (Fig. 7). Many cells of the AmiB mutant were altered in shape and some were dramatically increased in size (length and width). In addition, the AmiB mutant had a higher percentage of cells in chains, which suggests that its cell division or septation is affected. On the other hand, the growth rates of the wild-type and the AmiB mutant were similar, which suggests that the observed differences in cell structure and arrangement are not due to an alteration in the mutant's growth rate. Although further studies utilizing electron microscopy are required to characterize the AmiB mutant's cellular structure in more detail, the current results suggest that there is a link between cell structure, division or septation, and the rugose phenotype of *V. cholerae*. Thus, my findings provide evidence for a new putative

function for a prokaryotic amidase, namely its importance for the S to R switch and for biofilm formation.

Another class of mutants of particular interest to me was those with defects in the VC0653 locus, which encodes a putative, GGDEF domain- and EAL domain-containing protein I have designated RocS (designated a "PdeA-like" protein in the database). Three *rocS* mutants were isolated by three independent conjugations. The results of my studies with the mutants suggest that *V. cholerae* RocS has an important role in the bacterium's ability to produce rEPS and biofilms. In addition, motility assays revealed that the RocS mutant's motility was consistently <50% of the parental strain's motility (Fig. 6). Thus, the wild-type locus also appears to be required for optimal functioning of *V. cholerae*'s flagella. In conclusion, my results suggest that *V. cholerae*'s RocS (and c-di-GMP, see below) regulates the expression of several phenotypes, including those with putative roles in the bacterium's virulence and environmental persistence.

Interestingly, GGDEF domains are present in all proteins known to be involved in the regulation of cellulose (β-1,4-glucan) synthesis (10). Cellulose production by *Acetobacter xylinum*, *Rhizobium leguminosarum* bv. *Trifolii*, and *Agrobacterium tumefaciens* is modulated by the opposing effects of two enzymes: diguanylate cyclase (Dgc) and c-di-GMP diesterase (PdeA), each of which controls the cellular level of the novel signaling molecule c-di-GMP (6, 137, 138, 100). Dgc acts as a positive regulator by catalyzing the formation of c-di-GMP, which specifically activates cellulose production; PdeA cleaves c-di-GMP and, thus, negatively regulates cellulose production. In addition, c-di-GMP has been predicted (137) to be a reversible, allosteric activator (effector) of cellulose biosynthesis. Furthermore, genetic complementation studies (10),

using genes from various species encoding proteins with GGDEF domains as the only element in common, suggest that the domain has a role in Dgc activity and is important for modulating the cellular level of c-di-GMP. In that regard, I found that *V. cholerae*'s GGDEF domain-containing RocS protein plays a key role in the switch to the rugose phenotype (production of the rEPS) and biofilm formation.

A BLAST search of the *V. cholerae* RocS revealed that it is highly conserved and has significant homology to putative proteins found in a wide variety of other species; e.g., *P. aeruginosa* strain 0575 (42% identity; 5e-92), *Bacillus anthracis* strain 5593 (37% identity; 6e-90), *Ralstonia solanacearum* strain 0588 (36% identity; 4e-88), and an *A. xylinum* Dgc (40% identity; 9e-82). Although *dgc* and *pdeA* share some homology and have similar domain architecture, my observation that the *rocS* mutant is unable to produce an rEPS-like material is more consistent with a *dgc* mutant that is unable to produce cellulose. Also, *V. cholerae*'s RocS has a slightly higher similarity to *A. xylinum*'s Dgc than it does to its PdeA (data not shown). Recent reports have identified "RocS" homologs in (i) *P. aeruginosa*, which appear to be essential for biofilm formation (24), and (ii) *V. parahaemolyticus*, which regulate capsular polysaccharide production (53). In addition, the autoaggregation phenotype (which is typical of the rugose phenotype) of *Y. pestis* requires the expression of the GGDEF domain-containing protein HmsT (77). Although homologous regulatory (GGDEF domain-containing) proteins have been found in several species and have been associated with wrinkled colonies, EPS production and biofilm formation, their role(s) in the regulation of those phenomena has not been well studied, in part due to the lack of available reagents. There is growing evidence to support the idea that GGDEF domain-containing proteins possess nucleotide

cyclase activity (10, 138, 123, 152) and are widespread among bacteria (30, 44) . The widespread occurrence of these protein homologs in prokaryotes suggests that they represent an important, common regulatory system for EPS production, the rugose phenotype of *V. cholerae*, biofilm formation, and presumably other phenotypes. Future studies will investigate the potential role of RocS, its homologs, and the signal molecule c-di-GMP in the regulation of various phenotypes.

The extracellular matrix (ECM) serves as a scaffold to stabilize the structure of various tissues, and ECM-degrading metalloproteinases (e.g., collagenases, elastases, and other proteases) are potential virulence factors for several bacterial species that damage the basement membranes of eukaryotic cells. For example, studies of proteolytic enzymes produced by various *Vibrio* spp. have reported (85) that their collagenases are virulence factors. In that regard, other studies have found that collagenase activity is rare in *V. vulnificus* (111, 136) but that elastase activity is common among clinical and environmental strains of *V. cholerae* isolated from various sources (52). In addition, previous investigations (97, 118) have claimed that some *V. cholerae* proteases have the potential to elicit tissue injury and disintegration of the ECM by activating human matrix metalloproteases.

The above, previously reported observations prompted me to assess the ability of smooth and rugose variants of epidemic *V. cholerae* O1 strains (including classical and El Tor strains) to produce extracellular collagenase and elastase. I found that (i) the rugose variants yielded less extracellular collagenase activity than did the smooth variants, and (ii) classical strain NCTC 6585 was defective in the production of extracellular elastase. Since *V. cholerae* produces many extracellular enzymes and proteases, several of which

have multiple functions depending on the substrates chosen for study, it is difficult to determine whether a single protein is responsible for the observed differences in collagenase activity between smooth and rugose strains and in elastase activity differences between the biotype. My results suggest that rugosity and the production of extracellular collagenase are inversely linked. Therefore, since several *V. cholerae* proteases have been linked with virulence, my results are consistent with a potential defect in virulence expression in rugose strains compared to smooth strains.

Rugose variants of *V. cholerae* O1 strains have been reported to cause (i) fluid accumulation in the ligated rabbit ileal loop model (Rice 1993), and (ii) clinical diarrhea in human volunteers, which suggests that they are virulent (112). In that regard, my results indicate that extracellular CT production is unaffected in rugose strains and that VpsR does not regulate CT. However, the role(s) - if any - of VpsR and the rugose phenotype in *V. cholerae*'s virulence is not well understood.

My finding that a *vps* (locus VC0920) mutant is slightly less reactogenic in rabbit ileal loops than is its smooth parent suggests that VPS production has a role in the pathogenesis of cholera. However, it is possible that locus VC0920, which encodes a putative glycosyl transferase, has an additional role(s) in virulence. In that regard, previous studies (115, 131) have found that some *V. cholerae* genes are involved in several processes, including LPS production and the expression of the rugose phenotype. I also observed that a *vpsR* mutant was markedly less reactogenic in ileal loops than was the wild-type, parental strain. In addition, I previously reported (131) that the *vpsR* mutant's defect in switching to the rugose phenotype was restored by a *vpsR*-containing plasmid. Thus, my observations suggest that *vpsR* is important for the pathogenesis of

cholera, regulation of VPS biosynthesis, and expression of the rugose phenotype. Perhaps it also regulates other virulence genes and is part of a novel virulence regulon in *V. cholerae*.

The results of the 5-h co-infection assays performed with the infant mouse model suggest that VPS production and the rugose phenotype influence intestinal colonization by *V. cholerae*. To compare the smooth and rugose variants' ability to colonize during the later stages of infection, I performed multiple individual-infection and co-infection assays at 24 h post-inoculation. Initially, I observed a statistically significant difference between the level of colonization by all of the strains when they were singly administered (P = 0.0004; Kruskal-Wallis test) (Table 9 and Fig. 13). In addition, the results showed that the smooth variants colonized ~10-fold better than the rugose variants. Also, the colonizing ability of a *vpsR* mutant (VPS-negative and unable to switch to the rugose phenotype) was significantly lower than that of the wild-type smooth strain and the rugose variant. These results suggest that excessive rEPS production inhibits the bacterium's colonizing ability, and that the inability to produce the rEPS (e.g., by the *vpsR* mutant) results in an even greater defect in the colonization rate. These infant mouse intestinal colonization data support my ileal loop data, and they suggest that the *vpsR* mutant's markedly reduced reactogenicity in the ligated rabbit ileal loop model results from a defect in the mutant's colonizing ability.

I did not observe a significant difference between the colonization rates of the smooth strain and the *vpsR* mutant (P = 0.36; Wilcoxon signed rank test). However, consistent with the results of the 5 h co-infection assays, the smooth strain had a significant 5-fold greater colonization rate than did the rugose variant (P = 0.0034;

Wilcoxon signed rank test). These results are consistent with those of Watnick *et al.* (168), who reported that the colonizing ability of a serogroup O139 flagellar mutant exhibiting the rugose phenotype was significantly lower than that of the wild-type, smooth, parental strain. Also, consistent with the data obtained by the 5 h co-infection assays, the *vpsR* mutant colonized 10-fold better than did the rugose variants in the co-infection assays (P = <0.0009; Wilcoxon signed rank test). Interestingly, although the reason is not understood, the smooth and rugose variants' colonization rates, when they were used to co-infect mice with the *vpsR* mutant, were significantly lower than when the mice were singly-infected with the variants (P = 0.006 [for the smooth variant] and P = 0.012 [for the rugose variant]; Kruskal-Wallis test). Consistent with the previously mentioned results of the 5 h assays, when mice were co-infected with the smooth and rugose variants, the smooth variant's colonizing ability was ca. 10-fold higher than when it was used to co-infect mice with the *vpsR* mutant. Likewise, the *vpsR* mutant colonized 5-fold better during co-infection with the rugose strain than when it was used to singly-infect the mice (P = 0.027; Wilcoxon test). These findings are consistent with the hypothesis that the ability to produce VPS in a carefully regulated manner (i.e., to express the rugose phenotype) is required for efficient intestinal colonization by some strains of *V. cholerae*.

The hypothesis examined during my studies was that VPS, the rugose phenotype, and VpsR have a role in *V. cholerae*'s virulence. The results of my studies with the infant mouse model cannot be explained by the possibility that the rugose variants' colonization rates are lower than those of the smooth strains because of the masking of a "colonization factor" by VPS. If this were the case, the *vpsR* mutant (defective in VPS)

would be expected to colonize as well as the wild-type smooth strains when each of the strains is used to singly-infect the infant mice. In addition, I would not expect the colonization rate of the *vpsR* mutant to be ~five-fold higher in the presence of a rugose variant than it is in the presence of a smooth variant. Thus, my data indicate that VPS production is required for efficient intestinal colonization. In view of the observed effects of VPS overproduction (the rugose phenotype) and VPS underproduction (by the *vpsR* mutant), I predict that there is a regulatory mechanism that controls the kinetics of VPS production and the quantity of VPS produced *in vivo*. Thus, I believe that it would be worthwhile to perform further studies to characterize the regulatory system and signal molecules controlling VPS production and biofilm formation.

In the infant mouse model, controlled VPS production appears to be important for intestinal colonization, and rugose variants appear to promote the virulence of smooth strains. In this model, smooth strains produce VPS in a regulated manner and they, therefore, are expected to colonize better than rugose variants, which produce very large amounts of VPS. If some VPS is required for efficient colonization, a *vpsR* mutant (defective in VPS production) would be expected to colonize significantly less than do wild-type smooth strains and rugose variants. My findings are consistent with this model; i.e., wild-type smooth strains in the presence of a rugose variant colonized mice significantly better than did smooth strains in the presence of the *vpsR* mutant. In addition, the *vpsR* mutant colonized mice significantly better in the presence of rugose cells than it did in the presence of wild-type smooth strains or when inoculated alone. Switching from the smooth to rugose phenotype *in vivo* is not implicit in this model; however, it is possible that some switching occurs *in vivo* at a low frequency, and this

event might further promote colonization by some strains. Therefore, my observation that a rugose variant colonizes the infant mouse model significantly less than does the wild-type smooth strain/variant, but significantly better than does a *vpsR* mutant, suggests that (i) VPS is an important colonization factor for *V. cholerae*, and (ii) rugose variants might, under some conditions, act as "helpers" for intestinal colonization by smooth strains of *V. cholerae*.

CONCLUSION

The overall goal of my study was to investigate the genetics and physiology of the *V. cholerae* rugose phenotype. The specific objectives of my thesis included: (i) developing a culture medium that promotes expression of the rugose phenotype, thus enabling me to compare the frequency of that phenotype's expression by epidemic and non-epidemic *V. cholerae* strains; (ii) identifying genes involved in the switch between the smooth and rugose phenotype; and (iii) determining whether the smooth and rugose variants of *V. cholerae* differ in their virulence.

During my initial studies, I developed a medium (designated alkaline peptone water #3) that promotes, primarily by clinical strains, a very high frequency of rugose production (HFRP). My subsequent findings linked several common themes that have recently been shown to be important for long-term persistence and survival of *V. cholerae*, such as rEPS expression, resistance to environmental stresses, biofilm formation and potentially virulence.

It appears that some *V. cholerae* strains have evolved an efficient mechanism for a high-frequency shift to rEPS expression, which might provide an adaptive advantage in particular niches. In that regard, my findings may explain why some epidemic strains of *V. cholerae* persist for many years in their stressful aquatic reservoirs, and why rugose (and HFRP) *V. cholerae* strains are resistant to various antibacterial treatments. On the basis of my results, I propose that rEPS expression and HFRP are important in the epidemiology and long-term persistence of *V. cholerae*, and that a similar evolutionary selection process may promote the virulence and long-term persistence of other bacterial pathogens in various niches. Understanding the mechanisms that promote the persistence

of *V. cholerae* in the environment is a key factor in advancing our understanding of this important human pathogen and its pathogenic cycle, and it may assist in predicting and controlling cholera epidemics.

During my studies, I exploited the HFRP-promoting conditions to identify and study the genes involved in the molecular switch between the smooth and rugose phenotypes. It appears that some *V. cholerae* strains have evolved an efficient mechanism for a high frequency shift to the rugose phenotype that is associated with rEPS production, increased biofilm formation, and increased cell survival under various stressful conditions. The switching ability that can lead to increased biofilm formation may provide an adaptive advantage in particular environments where strains compete for the same ecological niche and where slight variations in phenotype that promote resistance, increased colonization or cell aggregation are important for adaptation and survival. Thus, my findings provide new insight into the molecular basis underlying the switch between the smooth and rugose phenotypes of *V. cholerae*.

VPS production and switching to the rugose phenotype have been reported (3) to be linked to the type II general extracellular protein secretion pathway that is also involved in the secretion of CT and hemolysin. Although the regulatory pathway involved is not clear, my findings suggest that production of the potential virulence-associated collagenase and elastase activity is inversely linked to the production of VPS and the expression of the rugose phenotype.

VpsR belongs to a family of proteins with homology to signal transduction, two-component regulatory systems required to sense and respond to various environments

(179). My results strongly suggest that VpsR has a role in the pathogenesis of cholera and that, in addition to regulating *vps* genes and VPS production, VpsR regulates additional virulence genes. Until now, the biological role of VPS/the rugose phenotype in *V. cholerae* has not been clearly identified, but I and others have proposed that it enhance the bacterium's environmental survival. Although is possible that VPS and the rugose phenotype have a role in the environment, I propose a new model in which VPS production and the rugose phenotype have a role in pathogenesis. In this model, careful timing and control of VPS production are required for efficient intestinal colonization, and rugose strains act as a 'helper' phenotype that enhances the virulence of some smooth strains. Thus, my findings suggest a new role for Vps, the rugose phenotype and VpsR in epidemic *V. cholerae* strains, which may lead to productive future studies of *V. cholerae*'s rugose phenotype.

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