

Compromised Outer Membrane Integrity in *Vibrio cholerae* Type II Secretion Mutants[∇]

Aleksandra E. Sikora, Suzanne R. Lybarger, and Maria Sandkvist*

Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, Michigan

Received 16 April 2007/Accepted 11 September 2007

The type II secretion (T2S) system of *Vibrio cholerae* is a multiprotein complex that spans the cell envelope and secretes proteins important for pathogenesis as well as survival in different environments. Here we report that, in addition to the loss of extracellular secretion, removal or inhibition of expression of the T2S genes, *epsC-N*, results in growth defects and a broad range of alterations in the outer membrane that interfere with its barrier function. Specifically, the sensitivity to membrane-perturbing agents such as bile salts and the antimicrobial peptide polymyxin B is increased, and periplasmic constituents leak out into the culture medium. As a consequence, the σ^E stress response is induced. Furthermore, due to the defects caused by inactivation of the T2S system, the Δeps deletion mutant of *V. cholerae* strain N16961 is incapable of surviving the passage through the infant mouse gastrointestinal tract. The growth defect and leaky outer membrane phenotypes are suppressed when the culture medium is supplemented with 5% glucose or sucrose, although the *eps* mutants remain sensitive to membrane-damaging agents. This suggests that the sugars do not restore the integrity of the outer membrane in the *eps* mutant strains per se but may provide osmoprotective functions.

Gram-negative bacteria possess highly sophisticated and organized cell envelopes that consist of inner and outer membranes separated by the periplasmic compartment and the peptidoglycan layer. The outer membrane is made up of a lipopolysaccharide (LPS)-phospholipid asymmetric bilayer and functions as a barrier preventing entry of toxic substances, including antibiotics, dyes, and detergents (60, 72). At the same time, the outer membrane allows nutrient acquisition and transport of molecules in and out of the cell. Several dedicated transport systems have evolved for this purpose, and at least six pathways are required for extracellular protein secretion alone (43, 67). One such system is the type II secretion (T2S) system, which has been identified in a wide variety of Proteobacteria, including many pathogens (for reviews, see references 12 and 74). Many of the proteins secreted by the T2S pathway, such as proteases, lipases, cellulases, pectinases, phospholipases, lipases, and toxins, contribute to virulence. These secreted proteins are synthesized with signal peptides and are first transported into the periplasmic compartment by Sec- or Tat-dependent processes and then cross the outer membrane through the T2S machinery (66, 89).

In recent years, much attention has been paid to the interactions between individual components and the mechanism by which the multiprotein T2S complex is assembled. The present model for T2S machines includes a secretion pore in the outer membrane, a multiprotein subcomplex localized in the cytoplasmic membrane, a pseudopilus that spans the periplasmic compartment, and an ATPase in the cytoplasm (38). Besides the interactions within the T2S complex, it appears that T2S components also interact with other cell envelope constituents.

For example, it has been shown that an alteration in the LPS structure affects the function of the T2S system in *Pseudomonas aeruginosa* (6, 53). In addition, interaction with peptidoglycan is a prerequisite for the ExeAB complex to support assembly of the outer membrane protein ExeD in the *Aeromonas hydrophila* T2S system (34). It also has been demonstrated that T2S mutants of *A. hydrophila*, *V. cholerae*, and *Vibrio* sp. strain 60 possess altered outer membrane protein profiles in addition to defects in extracellular secretion (33, 35, 37, 77). The amounts of outer membrane proteins, including OmpU, OmpT, and the LamB homolog OmpS, are considerably diminished in *eps* mutants of *V. cholerae* (64, 77). Similarly, the levels of OmpS and OmpF porins are reduced in *exe* mutants of *A. hydrophila* (33). The reason for these outer membrane defects is not known but might suggest a role for the T2S machinery in outer membrane biogenesis (33, 37, 77). The outer membrane alterations might be responsible for the slow-growth phenotype noted for T2S mutants of *V. cholerae* (39, 64, 77). Although no outer membrane changes have been reported for T2S mutants of *Legionella pneumophila*, they also display reduced growth rates under certain growth conditions (82).

In this study, we have taken a closer look at the effect of inactivation of the T2S machinery on outer membrane integrity in *V. cholerae*. We show that removal or inactivation of the Eps system results in a compromised outer membrane evidenced by several phenotypes, including extracellular release of periplasmic content and sensitivity to bile salts and polymyxin B. We also show that *V. cholerae* lacking a functional Eps system is unable to survive the passage through the infant mouse intestine and is rapidly cleared.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All strains used in this study are listed in Table 1. Strains were cultured in Luria-Bertani (LB) broth at 37°C, supplemented as specified in the text.

V. cholerae TRH7000 and its mutant derivatives were cultured in the presence of thymine (100 $\mu\text{g/ml}$) (29). Lipid agar was used to determine the export of

* Corresponding author. Mailing address: University of Michigan Medical School, Department of Microbiology and Immunology, 1150 West Medical Center Drive, 6741 Medical Science Building II, Ann Arbor, MI 48109-0620. Phone: (734) 764-3552. Fax: (734) 764-3562. E-mail: mariasan@umich.edu.

[∇] Published ahead of print on 21 September 2007.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype ^a	Reference or source
Strains		
<i>V. cholerae</i>		
O395	Classical O1 biotype, Sm ^r	Laboratory collection
Mut3	O395 <i>epsE::lacZ kan Sm^r Kan^r</i>	77
JJM43	O395 Δ <i>toxR43</i> Δ <i>ctxA1</i> Sm ^r	27
Mut4	O395 Δ <i>toxR43</i> Δ <i>ctxA1</i> <i>epsE::lacZ kan Sm^r</i>	77
O395 Δ <i>rpoE</i>	O395 Δ <i>rpoE</i> Sm ^r	49
TRH7000	El Tor O1 biotype, <i>thy Hg^r Δ(ctxA-ctxB)</i>	29
Mut1	TRH7000 <i>epsE::lacZ kan Kan^r</i>	75
Mut6	TRH7000 <i>epsF::kan Kan^r</i>	75
Mut5	TRH7000 <i>epsG::kan Kan^r</i>	77
Mut 8	TRH7000 <i>epsL::kan Kan^r</i>	75
PU3	TRH7000 <i>epsM::Tn5 Kan^r</i>	62
TRH Δ <i>epsC</i>	TRH7000 Δ <i>epsC</i> Kan ^r	S. R. Lybarger et al. (unpublished data)
TRH Δ <i>epsL</i>	TRH7000 Δ <i>epsL</i> Kan ^r	This study
P _{BAD} :: <i>eps</i>	TRH7000 P _{BAD} :: <i>eps</i>	This study
TRH Δ <i>eps</i>	TRH7000 Δ <i>epsC-N</i> Cm ^r	This study
N16961	Wild-type El Tor O1 biotype, Sm ^r	Laboratory collection
N Δ <i>eps</i>	N16961 Δ <i>epsC-N</i> Cm ^r	This study
569B	Classical O1 biotype Sm ^r	Laboratory collection
M14	569B <i>epsE-A321V</i>	32
Bah2	El Tor O1 biotype, E7946, Δ <i>attRS</i>	87
Bah2 <i>epsD::kan</i>	Bah2 <i>epsD::kan</i>	14, 15
<i>E. coli</i>		
MC1061	F ⁻ <i>araD139</i> Δ (<i>ara-leu</i>)7697 Δ (<i>lac</i>)X74 <i>rpsL hsdR2 mcrA mcrB1</i>	11
S17.1	F ⁻ <i>recA pro hsdR RP4-2 Tc^r::Mu Tn^r::Tn7</i>	81
SY327 λ pir	Δ (<i>lac-pro</i>) <i>argE</i> (Am) <i>recA56 rpoB</i> λ pir Rif ^r	55
MM294 (pRK2013)	Donor of transfer function for triparental conjugation	52
Plasmids		
pPCR-Script	Cloning vector, Amp ^r	Stratagene
pBAD33	Cloning vector, <i>araC</i> -P _{BAD} Cm ^r	25
pJN105	Cloning vector, <i>ori</i> pBBR1 <i>araC</i> -P _{BAD} <i>mob</i> Gm ^r	59
pK18 <i>mobsacB</i>	<i>ori</i> -pMB1 <i>oriT</i> (RP4) <i>sacB lacZα Kan^r</i>	79
pMMB-GFP	pMMB66- <i>gfp</i> coding sequence, Amp ^r	80
pMMB68	<i>etxB</i> under P _{TAC} control in pMMB66EH, Amp ^r	76
pMMB190	Cloning vector, <i>pTACplacUV5</i> Amp ^r	57
pCH40	<i>pbla::phoA</i> Tet ^r	31
pMS43	<i>epsE-K270A</i> under P _{TAC} control in pMMB207, Cm ^r	14, 75
pCVD442	<i>ori R6K mobRP4 sacB</i> Amp ^r	17
pUC18K	Cloning vector, <i>aphA-3</i> Amp ^r Kan ^r	51
pBBR <i>lux</i>	<i>ori</i> pBBR1, promoterless <i>luxCDABE</i> Cm ^r	46
pAES100	1,078-bp EcoRI-XbaI fragment upstream of <i>epsC</i> in pK18 <i>mobsacB</i> , Kan ^r	This study
pAES101	1,075-bp fragment downstream of <i>epsN</i> cloned into XbaI- and SphI-digested pAES100, Kan ^r	This study
pAES102	923-bp fragment containing Cm ^r cassette cloned into XbaI site of pAES101, Kan ^r Cm ^r	This study
pAES103	1,070-bp fragment upstream of <i>epsC</i> cloned into SphI-ClaI site of pJN105, Gm ^r	This study
pAES104	1,079-bp fragment of full-length, promoterless <i>epsC</i> cloned into EcoRI- and XbaI-cut pAES103, Gm ^r	This study
pAES105	SphI-XbaI 3,479-bp fragment containing upstream region of <i>epsC</i> , <i>araC</i> , and <i>pBAD::epsC</i> from pAES104 in pK18 <i>mobsacB</i> , Kan ^r	This study
pAES106	<i>rpoEP2</i> cloned into pBBR <i>lux</i> , Cm ^r	This study
pMMB131	pMMB68 gene fusion between <i>etxB</i> signal sequence and <i>phoA</i> under P _{TAC} control, Amp ^r	This study
pEps	15-kb XbaI-SphI fragment containing entire <i>eps</i> operon (from <i>epsC</i> to <i>epsN</i>) in pMMB190, Amp ^r	This study

^a Amp^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Gm^r, gentamicin resistance; Hg^r, mercury resistance; Kan^r, kanamycin resistance; Rif^r, rifampin resistance; Tet^r, tetracycline resistance.

lipase on solid agar. The medium was prepared as described previously (40) with modified minimal medium (47.75 mM Na₂HPO₄, 22 mM KH₂PO₄, 8.5 mM NaCl, 18.69 mM NH₄Cl, 0.1% Tween 20, 0.2 mM CaCl₂) containing olive oil as the only source of carbon.

Antibiotics (Sigma-Aldrich, St. Louis, MO) were used at the following concentrations: chloramphenicol, 4 or 10 μ g/ml for chromosomal or plasmid expres-

sion, respectively, for *V. cholerae* and 30 μ g/ml for *Escherichia coli*; carbenicillin, 100 μ g/ml; kanamycin, 50 μ g/ml; streptomycin, 100 μ g/ml; and polymyxin B sulfate, 100 U/ml.

Construction of Δ *eps*, P_{BAD}::*eps*, and Δ *epsL* strains. Chromosomal DNA isolated from *V. cholerae* TRH7000 or N16961 was used as the template for PCR where indicated. PCRs were carried out with PfuUltra DNA polymerase (Strat-

agene, La Jolla, CA). Primers were synthesized by IDT Technologies, Inc. (Coraville, IA). All PCR products were first subcloned into pPCR-Script Amp SK(+)(Stratagene) and then processed further.

The Δeps strains were generated by PCR amplification of the upstream region of the *epsC* gene with primers 5'-GAATCTTGCTCGAAATCTTCAGCTG-3' (forward [Fwd]) and 5'-TCTAGACGTTAAATGTCGACTTGC-3' (reverse [Rev]). The resulting 1,078-bp fragment was digested with EcoRI and XbaI and ligated into pK18*mobsacB* (79) that had been likewise digested to yield pAES100. The region downstream of the *epsN* gene (the last gene in the *eps* operon) was amplified using primers 5'-TCTAGAATCCCATTTCACG C-3' (Fwd) and 5'-GCATGCTGTTGCTCAGGATCTATAC-3' (Rev). The resulting fragment of 1,075 bp subsequently was digested with XbaI and SphI and ligated into similarly cut pAES100 to yield pAES101. The chloramphenicol cassette was obtained by amplification of the *cat* gene from pBAD33 (25) with primers 5'-TCTAGATAGCACCAGCGCTTAAAG-3' (Fwd) and 5'-TCTAGAGCGCCGAATAAATACCTG-3' (Rev), and the resulting product was digested with XbaI. The 923-bp fragment was ligated into XbaI-digested pAES101 to form pAES102, which then was conjugated from the *E. coli* strain S17.1 into the *V. cholerae* N16961 and TRH7000 strains as described previously (81, 88). Transconjugants in which pAES102 had recombined into the respective *V. cholerae* genomes were selected on LB agar containing chloramphenicol. To select for the second recombination event, independent colonies of TRH7000 and N16961 cointegrants were cultured overnight in LB broth supplemented with chloramphenicol, diluted, and cultured to late log phase and spread on LB agar containing 10% sucrose and no NaCl. Chloramphenicol-resistant colonies were screened on LB agar containing 1% milk for the loss of plasmid-encoded kanamycin resistance and for the loss of protease secretion. Colonies that were kanamycin sensitive, chloramphenicol resistant, and protease negative were designated TRH Δeps and N Δeps for *V. cholerae* strains TRH7000 and N16961, respectively.

The P_{BAD}::*eps* strain was constructed as follows. The upstream region of the *epsC* gene was amplified using primers 5'-GCATGCTGCTCGAAATCTTCA G-3' (Fwd) and 5'-ATCGATACGTTAAATGTCGACTTGC-3' (Rev) and was digested with SphI and ClaI. The 1,070-bp fragment was cloned into similarly digested pJN105 (59) to yield pAES103. Chromosomal DNA containing promoterless *epsC* was amplified using primers 5'-GCGAATTCAGTACAGAAAG GAATAACG-3' (Fwd) and 5'-TCTAGACTAGAGGGGTAGACAGC-3' (Rev) and was digested with EcoRI and XbaI. The 1,079-bp fragment was cloned into EcoRI/XbaI-cut pAES103 to yield pAES104, resulting in the placement of the *epsC* gene behind the P_{BAD} promoter. The 3,479-bp SphI-XbaI fragment containing the upstream region of the *epsC* gene, *araC*, and P_{BAD}::*epsC* was moved from pAES104 and ligated into pK18*mobsacB* (79). The resulting plasmid, pAES105, subsequently conjugated from the *E. coli* strain S17.1 (81, 88), was employed to introduce the mutation onto the *V. cholerae* TRH7000 chromosome. Conjugants were plated on LB agar supplemented with kanamycin and arabinose (0.01%).

To select for the second recombination event, everything was completed as described above with the following modifications. Independent colonies of transconjugants were cultured in LB broth supplemented with arabinose (0.01%) to late log phase and were spread on LB agar containing 10% sucrose, 0.01% arabinose, and no NaCl. All colonies were screened for the loss of kanamycin resistance after being patched on LB agar containing 1% milk with or without 0.01% arabinose. Clones that did secrete protease in the presence, but not in the absence, of arabinose were selected for further analysis.

To generate the TRH $\Delta epsL$ strain, 1-kb regions upstream and downstream of the *epsL* gene were amplified using primer pairs 5'-GAGCTCTTATTGTGA TTCTGCTCCT-3' (Fwd) and 5'-GGTACCAAACCAGCCAAGGGATATC-3' (Rev) as well as 5'-TCTAGAGTTTGTGGTGAAGCCCAAG-3' (Fwd) and 5'-GTCCGACAAGCTAAGTGCCTTCG-3' (Rev) and were cloned into pUC18K (51). This resulted in a sequence containing *aph-3*, which confers kanamycin resistance, flanked by homologous regions for recombination and allelic exchange. The entire fragment was excised using flanking restriction enzyme sites and was cloned into pCVD442 (17). The suicide vector was propagated in SY327 λ pir (55) and was conjugated into TRH7000 with the assistance of MM294/pRK2013 (52). Carbenicillin-resistant transconjugants resulting from the integration of the suicide vector onto the chromosome were isolated. Positive integrants then were grown nonselectively in LB broth and were plated on LB agar without NaCl (supplemented with 10% sucrose) to facilitate the second recombination event. Colonies from the sucrose agar were scored on LB agar containing either kanamycin or carbenicillin and 1% nonfat milk to visualize protease secretion.

Following the selection procedures described above, deletion of the *eps* operon and the *epsL* gene as well as the exchange of the region containing the *eps*

promoter were confirmed by PCR, sequencing (at the University of Michigan DNA Sequencing Core), and Western blotting with antisera against Eps proteins.

Growth curve experiments. Equivalent dilutions (based on the optical density at 600 nm [OD₆₀₀]) of overnight LB broth cultures of wild-type and *eps* mutant *V. cholerae* strains grown with or without different saccharides, including glucose, sucrose, maltose, mannose, lactose, galactose, sorbitol, melibiose, raffinose, xylose, and arabinose (up to 10% final concentration), or osmoprotectants, such as glycine-betaine and proline (up to 500 mM final concentration), or NaCl (up to 350 mM) were used as the inocula for cultures. The growth at 37°C was monitored over time by OD₆₀₀ measurements at 30-min intervals. All experiments were performed in triplicate.

Antimicrobial assays. To determine the lowest concentration of polymyxin B sulfate or bile salts inhibiting CFU to 50% (MIC₅₀), overnight cultures of wild-type and *eps* mutants of *V. cholerae* were diluted 1:100 in LB broth and grown to mid-log phase (OD₆₀₀ of approximately 0.5). To quantify the number of CFU, 10-fold dilutions of cultures were plated using Spiral Tech (Spiral Biotech, Norwood, MA) on a series of LB agar plates containing increasing concentrations of polymyxin B sulfate (0, 25, 50, 100, 200, 400, 600, 800, and 1,000 U/ml; Sigma-Aldrich) or bile salts (0, 0.0001, 0.0005, 0.001, 0.005, 0.01, 0.05, 0.1, 0.2, and 0.4%; Sigma-Aldrich). Colonies were counted using Q-Quant (Spiral Biotech) after growth at 37°C for 18 h. Mutant colonies were counted following 48 h of incubation due to slower growth. All assays were performed at least in triplicate.

Enzymatic assays. For enzymatic assays, wild-type and mutant strains were grown in LB broth supplemented with isopropyl- β -D-thiogalactopyranoside (IPTG) (at a 50 μ M final concentration) to induce the expression of plasmid-encoded green fluorescent protein (GFP), β -lactamase, or alkaline phosphatase (AP). Following growth for 16 h at 37°C (late-stationary-phase cultures; OD₆₀₀ of approximately 4), culture media were separated from the cells by centrifugation (10 min, 8,000 rpm) and passed through 0.22- μ m filters. Periplasmic extracts were obtained by incubating cells in 50 mM Tris, pH 8.0, with 2,000 U/ml of polymyxin B sulfate for 30 min (63). The spheroplasts were pelleted, and the supernatant was saved as a periplasmic extract.

A spectrophotometric assay was used to measure AP activity (22, 70) by incubating culture supernatants and periplasmic extracts with *p*-nitrophenylphosphate (Sigma-Aldrich) at 0.1 mg/ml in 50 mM Tris, pH 8.0, 1 mM MgCl₂ for 15 min at 37°C. The assays were stopped by adding EDTA to a final concentration of 0.05 M, and the absorbance at 405 nm was measured for each sample.

β -Lactamase activity was determined by incubating culture supernatants and periplasmic extracts with nitrocefin (EMD Chemicals, San Diego, CA) at 0.1 mg/ml in 50 mM Tris, pH 8.0, 1 mM MgCl₂ for 5 min at 37°C and measuring the absorbance at 482 nm (28).

GFP fluorescence in culture supernatants and whole-cell extracts was measured by excitation and emission at 485 and 535 nm, respectively (18).

All enzymatic assays were performed in quadruplicate. The percentage of the total amount detected in the supernatants was calculated, and means and corresponding standard errors of the means (SEM) are presented.

To determine the level of protease secretion, filtered supernatants from overnight cultures (16 h; OD₆₀₀ of approximately 4) were assayed in 5 mM HEPES, pH 7.5, and 0.05 mM *N*-tert-butoxy-carbonyl-Gln-Ala-Arg-7-amido-4-methylcoumarin (Sigma-Aldrich) for 10 min at 37°C using the excitation and emission wave lengths of 385 and 440 nm, respectively (10). The assay was performed in triplicate, and means and SEM are presented.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), silver staining, and Western blotting. Samples of whole-cell lysates, culture supernatants, or periplasmic extracts prepared as described above and matched by equivalent OD₆₀₀s were boiled in SDS sample buffer and analyzed on 4 to 12% Bis-Tris polyacrylamide gels (NuPAGE; Invitrogen). The gels subsequently were silver stained using a SilverQuest silver staining kit (Invitrogen) according to the manufacturer's instructions or were transferred to nitrocellulose (BioTraceNT; Pall Corporation, Pensacola, FL) in NuPAGE transfer buffer (Invitrogen). The blots were incubated with polyclonal antisera against *V. cholerae* OmpU (1:30,000; kind gift of K. Klose) or monoclonal anti-EtxB (1:30,000 [76]). Horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (1:10,000; Bio-Rad, Hercules, CA) and rabbit anti-mouse immunoglobulin G (1:3,000; Bio-Rad) were used as the secondary antisera, and the blots were developed with the SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL) or ECL plus Western blotting detection reagent (GE Healthcare, Buckinghamshire, United Kingdom).

Construction of *rpoEP2-lux* reporter fusion and monitoring of bioluminescence. A transcriptional *rpoEP2-lux* reporter was constructed based on an earlier study (44). Briefly, the *rpoEP2* promoter region of *V. cholerae* N16961 chromo-

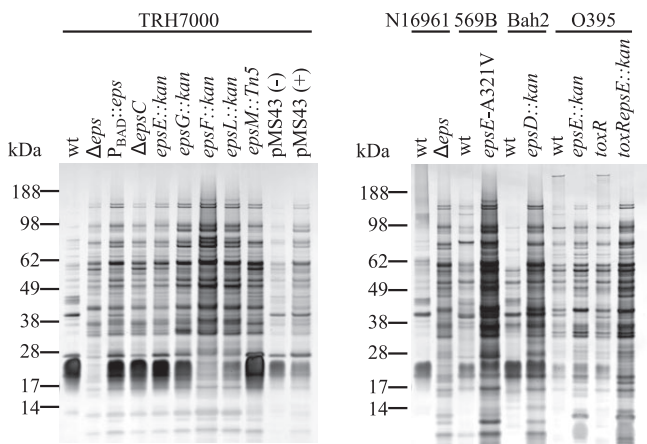


FIG. 1. Increased protein content in the culture supernatant of *V. cholerae* *eps* mutants. Wild-type (Wt) and mutant *V. cholerae* strains (with mutated alleles indicated in italics) were grown in LB broth at 37°C to stationary phase (16 h). Culture supernatants were separated from cells by centrifugation, matched by equivalent OD₆₀₀ units, and analyzed by SDS-PAGE and silver staining. The El Tor biotype is represented by *V. cholerae* strains TRH7000, N16961, and Bah2. Strains 569B and O395 are representatives of the classical biotype. Plasmid pMS43-encoded EpsE-K270A (a dominant-negative mutant protein) was expressed in wild-type TRH7000 by growth in the absence (-) or presence (+) of 10 μM IPTG. The migration of molecular mass markers is indicated.

somal DNA was amplified using primers 5'-GGATCCAGATATGGGTACTC ATTATG-3' (Fwd) and 5'-ACTAGTCTGCCTTGATAAGGTTTTGAGTAA A-3' (Rev). The amplified 142-bp fragment was cloned into BamHI-SpeI-digested pBRRlux containing promoterless *luxCDABE*, kindly supplied by Brian Hammer and Bonnie Bassler (46), to yield pAES106. The *rpoEP2::lux* fusion was verified by sequencing. Wild-type, P_{BAD}::*eps*, Δ*epsL*, and Δ*epsL*+pEpsL strains of *V. cholerae* TRH7000 containing pAES106 were cultured in LB broth, supplemented with arabinose where indicated, to mid-log phase (OD₆₀₀ of approximately 0.5) or stationary phase (OD₆₀₀ of approximately 4). Serial dilutions of the cell cultures were transferred to 96-well white opaque microtiter plates (Lumitrac), and bioluminescence was measured using a multidetection microplate reader (SynergyHT; Bio-Tek Instruments, Winoosh, VT). Bioluminescence was standardized for cell density by dividing the arbitrary light units by the absorbance at 600 nm. The data represent average values and corresponding SEM of three independent experiments (in which each experiment was performed in three technical replicates).

Infant mouse studies. Overnight cultures of wild-type and NΔ*eps* strains of *V. cholerae* N16961 were administered into anesthetized 4- to 5-day-old suckling CD-1 mice (3) in a peroral inoculum of 10⁶ and 10⁷ to 10⁸ cells, respectively, in accordance with University of Michigan Committee on Use and Care of Animals guidelines. Mice were separated from their mothers and were kept at 30°C for 24 h, at which time they were euthanized, their intestines were isolated and homogenized in 5 ml of LB broth, and the number of CFU was determined by plating dilutions on LB agar with streptomycin.

Statistical analysis. A Student's *t* test was applied for all statistical analyses, and values were considered significant at *P* < 0.05.

RESULTS

Alterations in the Eps system cause a leaky outer membrane. Inactivation of the *V. cholerae* T2S system results in growth defects (39, 64, 77) and an aberrant outer membrane protein profile (64, 77). In order to address the consequences of the outer membrane alterations in *V. cholerae* and to test the hypothesis that the integrity of the outer membrane is compromised by T2S disruption, we analyzed the protein content of the culture supernatant isolated from late-stationary-phase

cultures of wild-type and *eps* mutant strains grown in LB broth at 37°C by SDS-PAGE and silver staining (Fig. 1). Wild-type and mutant strains displayed very different extracellular protein profiles, and the numbers of protein species as well as their amounts were dramatically increased in the culture supernatant of the *eps* mutants. This unusual phenomenon, generally not expected of secretion mutants, was observed regardless of biotype (classical or El Tor), ToxR (virulence gene regulator) status, or specific *eps* mutant genotype. Similar observations also were made when a plasmid-encoded dominant-negative mutant of EpsE, EpsE-K270A, was expressed in the wild-type strain TRH7000. In addition to blocking toxin secretion (14, 75), overproduction of the mutant EpsE-K270A protein following addition of IPTG resulted in increased leakage of proteins to the culture supernatant of the wild-type strain as shown in Fig. 1.

In order to determine whether the outer membrane defects were due to the loss of T2S and not simply due to uncontrolled activities of the remaining Eps proteins, additional mutants were constructed. The mutations were introduced into TRH7000, a nontoxicogenic *V. cholerae* strain in which most *eps* mutations have been constructed (77), and N16961, a virulent strain that can be administered into infant mice for colonization studies (3, 21). The chromosomal region encoding the 12 Eps proteins was replaced with a chloramphenicol cassette by homologous recombination in *V. cholerae* strains TRH7000 and N16961, resulting in strains designated TRHΔ*eps* and NΔ*eps*, respectively. Additionally, we generated a strain of *V. cholerae* TRH7000, designated P_{BAD}::*eps*, in which the chromosomal region containing the native *eps* promoter was replaced with the arabinose-inducible promoter (P_{BAD}). As expected, complete removal of the *eps* genes or growth of the P_{BAD}::*eps* mutant in the absence of arabinose prevented extracellular secretion of several proteins (Fig. 2). There was a sixfold reduction in the level of extracellular protease of the mutants, as determined by the rate of *N*-tert-butoxy-carbonyl-Gln-Ala-Arg-7-amido-4-methyl-coumarin hydrolysis (Fig. 2A). The ability to secrete lipase was tested on lipid agar medium (40). Wild-type *V. cholerae* grows on this medium by secreting a lipase that is capable of hydrolyzing an emulsion of olive oil and uses the hydrolyzed products as a sole carbon and energy source. In contrast, TRHΔ*eps* was not capable of growth on this medium, likely due to its inability to secrete the lipase (Fig. 2B). Finally, to determine the level of toxin secretion in the mutant strains, culture supernatants and periplasmic extracts isolated from wild-type and mutant TRH7000 cells that express EtxB, the B subunit of the *E. coli* heat-labile enterotoxin (which is secreted with the same mechanism as that for cholera toxin [30]), were analyzed by SDS-PAGE and immunoblotting with monoclonal anti-EtxB (Fig. 2C). While EtxB was present in the supernatants of the wild-type strain and the P_{BAD}::*eps* mutant grown in the presence of 0.01% arabinose, EtxB accumulated in the periplasm of the TRHΔ*eps* mutant and the P_{BAD}::*eps* strain grown in the absence of arabinose. The secretion of protease, lipase, and EtxB was restored in the TRHΔ*eps* mutant in the presence of a plasmid, pEps, that carries the entire *eps* operon or by adding arabinose to a final concentration of 0.01% to the growth medium of the P_{BAD}::*eps* strain (Fig. 2A to C).

Similar to the single-gene-deletion mutants, a small-colony

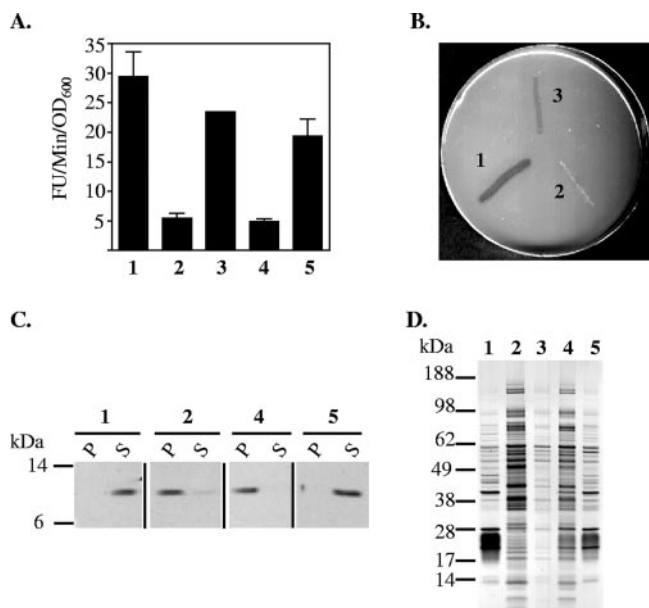


FIG. 2. Inverse correlation between T2S and passive leakage. Wild-type and *eps* mutant strains of *V. cholerae* TRH7000 were analyzed for the secretion of protease, lipase, and toxin, as well as for the release of cellular proteins to the extracellular environment. (A) Culture supernatants were obtained from stationary-phase cultures as described in Materials and Methods and were tested for the presence of extracellular protease using the proteolytic substrate *N*-tert-butoxy-carbonyl-Gln-Ala-Arg-7-amido-4-methyl-coumarin. The rate of hydrolysis (relative fluorescence units [FU]/min/OD₆₀₀ unit) is presented as the means of three independent experiments \pm SEM. There was a statistically significant difference between the protease activity of wild-type and *eps* mutant strains ($P < 0.005$). (B) Lipase secretion was determined by growth on lipid agar containing olive oil as the only source of carbon. (C) The distribution of the *E. coli* heat-labile enterotoxin B subunit in culture supernatants (S) and periplasmic extracts (P) was determined by SDS-PAGE and immunoblotting using monoclonal anti-EtxB. (D) Culture supernatants were obtained from stationary-phase cultures, matched by equivalent OD₆₀₀ units, and analyzed by SDS-PAGE and silver staining for total protein content. Lane 1, wild type; lane 2, TRH Δ *eps*; lane 3, TRH Δ *eps*+pEps; lane 4, P_{BAD}::*eps*; lane 5, P_{BAD}::*eps* grown in the presence of arabinose at a 0.01% final concentration.

phenotype also was observed for the Δ *eps* mutants and the P_{BAD}::*eps* strain grown without arabinose regardless of growth temperature (20, 30, and 37°C), and the doubling time in LB broth at 37°C increased from 20 min for the wild-type strain N16961 to 90 min for the N Δ *eps* mutant (see Fig. 6A). The ability to retain intracellular proteins also was compromised in both the TRH Δ *eps* and N Δ *eps* strains, as well as the P_{BAD}::*eps* strain grown in the absence of arabinose, as the protein profile of the growth medium from these mutants contained an increased level of proteins (Fig. 1 and 2D). This suggests that the permeability of the outer membrane is increased when the Eps system is inactivated, while the active secretion of Eps-dependent proteins, including protease, lipase, and toxin, is diminished (Fig. 2). As expected, the leaky outer membrane phenotype could be corrected with the pEps plasmid that encodes all the *eps* genes or by the addition of arabinose to the culture of the P_{BAD}::*eps* strain (Fig. 2D).

The outer membrane integrity of the TRH Δ *eps* and P_{BAD}::*eps* strains was further assessed by specifically determin-

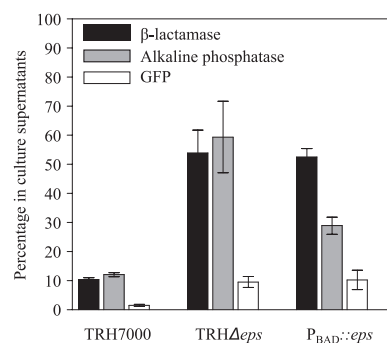


FIG. 3. Inactivation of the Eps pathway results in the leakage of periplasmic proteins. Culture supernatants and periplasmic as well as cytoplasmic extracts from cells present in overnight cultures of wild-type TRH7000, TRH Δ *eps*, and P_{BAD}::*eps* (grown in the absence of arabinose) were assayed for the presence of β -lactamase, AP, or GFP as described in Materials and Methods. The level of each of these proteins in the culture supernatant was expressed as a percentage of the total amount of each protein present in both culture supernatant and cellular extract. The means of four independent experiments \pm SEM are presented. For all proteins tested, $P < 0.02$.

ing the distribution of periplasmic and cytoplasmic protein markers present in the culture supernatant following growth for 16 h (to an approximate OD₆₀₀ of 4). The majority (90%) of plasmid-encoded β -lactamase and AP produced by the wild-type TRH7000 strain remained periplasmic, while 55 to 65% of the total content of these periplasmic enzymes escaped to the culture media from the TRH Δ *eps* and P_{BAD}::*eps* mutants cells (Fig. 3). In addition, there were detectable levels (12% of the total amount) of the cytoplasmic GFP in the culture supernatant of the mutants, suggesting that there is also an increase in cell lysis in the late stationary phase of growth (Fig. 3).

Although *eps* mutants release a greater number of protein species than the wild-type strains, several proteins present in the wild-type culture medium are absent in the supernatant of the *eps* mutants (Fig. 1 and 2D). Some of them likely represent cholera toxin, lipase, and protease, but the 38-, 26-, and 22-kDa species may correspond to yet-to-be identified T2-secreted proteins. To identify these proteins, culture supernatants were acetone precipitated, followed by SDS-PAGE and Coomassie staining (data not shown). The three bands representing proteins with molecular masses corresponding to 38, 26, and 22 kDa present in wild-type but not N Δ *eps* supernatants were excised and subjected to tandem mass spectrometry (at the Michigan Proteome Consortium). These proteins turned out to be outer membrane proteins, not extracellularly secreted proteins, and were identified as OmpU, OmpV (85), and OmpW (36, 58). Their presence in the wild-type culture supernatant likely is due to the release of outer membrane fragments and/or vesicles during growth overnight, as immunoblotting revealed that OmpU could be removed from the culture supernatant by centrifugation at 170,000 \times g (data not shown). The reduced level of OmpU in the culture supernatant of the N Δ *eps* mutant likely reflects its overall reduced intracellular level (see Fig. 7). The lack of OmpV and OmpW in the N Δ *eps* culture supernatant also may be due to their reduced levels in the outer membrane of the N Δ *eps* mutant.

Inactivation of the Eps system results in increased sensitivity to antimicrobial agents. The aberrant outer membrane

TABLE 2. Effect of *eps* mutation on antimicrobial resistance

Strain	MIC ₅₀ ^a	
	Polymyxin B (U/ml)	Bile salts (%)
N16961		
Wild type	800	0.05
NΔ <i>eps</i> ^c	100	0.0005
NΔ <i>eps</i> + <i>pEps</i>	800	0.05
TRH7000		
Wild type	400	ND ^b
TRHΔ <i>eps</i> ^c	100	ND
P _{BAD::eps} ^c	100	ND
TRHΔ <i>eps</i> + <i>pEps</i>	400	ND
P _{BAD::eps} +arabinose	400	ND
TRHΔ <i>epsL</i> ^c	100	ND
TRHΔ <i>epsL</i> + <i>pEpsL</i>	400	ND

^a The assays were conducted at least three times, and all standard deviations were less than 15%.

^b ND, not determined.

^c Colonies were counted after 48 h of incubation at 37°C.

protein profile and the increased outer membrane permeability of the *eps* mutants suggested that they are hypersensitive to membrane-perturbing agents such as antimicrobial peptides and detergents. Polymyxin B, a peptide antibiotic, binds to lipid A and is lethal to many gram-negative bacteria (for a review, see reference 26). To test the hypothesis that the *eps* mutants exhibit an increased susceptibility to polymyxin B, we determined the MIC₅₀ of polymyxin B for wild-type and mutant cells. Specifically, mid-log-phase cultures (at an OD₆₀₀ of ~0.5) of wild-type N16961 and the isogenic NΔ*eps* strain were spread on LB agar supplemented with increasing concentrations of polymyxin B, as described in Materials and Methods. Polymyxin B, at 800 U/ml, reduced the CFU of the wild-type strain by 50%. For comparison, the MIC₅₀ for the NΔ*eps* mutant was as low as 100 U/ml (Table 2). Although OmpU has been implicated in polymyxin B resistance in *V. cholerae* (48), Δ*ompU* strains are capable of selective growth in the presence of this cationic peptide (64). Likewise, *V. cholerae* strain TRH7000 lacks detectable levels of OmpU, yet it is capable of growth at several hundred units of polymyxin B per milliliter (77), and the MIC₅₀ for polymyxin B was only reduced to 400 U/ml (Table 2). Compared to the wild-type cells of TRH7000, both the TRHΔ*eps* and the P_{BAD::eps} strains exhibited a four-fold increase in sensitivity to this cationic peptide (Table 2). Resistance to polymyxin B was restored to wild-type levels in the TRHΔ*eps* and NΔ*eps* mutants with the plasmid carrying the entire *eps* operon or in the P_{BAD::eps} mutant grown in the presence of arabinose (Table 2). Finally, the same level of sensitivity to polymyxin B was observed with single-gene-deletion mutant TRHΔ*epsL* (Table 2), indicating that different types of *eps* mutants display similar phenotypes. The sensitivity to polymyxin B was reversed in TRHΔ*epsL* by expressing plasmid-encoded EpsL.

Bile normally is present in the small intestine, and resistance to this detergent-like agent is important for the survival of enteric pathogens. The global virulence regulator ToxR mediates enhanced bile resistance in *V. cholerae* (65). As TRH7000 produces no detectable ToxR (77), we tested the wild-type strain and NΔ*eps* mutant of N16961 to determine the lowest

concentration of bile salts (sodium cholate/deoxycholate at a 1:1 ratio) that inhibits CFU to 50%. The mutant strain displayed a severe growth defect in the presence of bile salts, and the MIC₅₀ was reduced 100-fold. The resistance to bile salts was restored when the mutant strain was complemented (Table 2).

NΔ*eps* mutant does not survive the mouse gastrointestinal tract. Because the NΔ*eps* mutant was sensitive to an antimicrobial peptide and bile salts, we speculated that it may have difficulties surviving the intestinal environment of the infant mouse, a model for *V. cholerae* infection (3, 21). To test this hypothesis, overnight cultures of the NΔ*eps* mutant and its isogenic wild-type *V. cholerae* strain, N16961, were separately inoculated perorally into 5-day-old mice. Twenty-four hours later, the intestines were excised and homogenized and were plated on LB agar to determine the number of CFU (Fig. 4). The number of CFU for the wild-type strain increased sevenfold following intestinal colonization (from 8.4×10^6 in the inoculum to an average of 5.8×10^7 in five mice in the first experiment and from 1.8×10^6 in the inoculum to an average of 1.16×10^7 in a total of five mice in the second experiment). The opposite trend was observed following inoculation of the NΔ*eps* mutant. In the first experiment, out of four inoculated mice, NΔ*eps* cells were recovered from one mouse only at 143 CFU, whereas 3.2×10^7 CFU were recovered from the inoculum. In the second experiment the input for the mutant strain was increased to 2.2×10^8 , but no culturable bacteria were recovered from any of the five infected animals. The combined results from the two independent experiments conducted for both the wild-type and mutant strains presented in Fig. 4 strongly suggest that the intestinal environment is not conducive to mutant growth, and therefore the NΔ*eps* mutant cells were cleared from the gastrointestinal tract of the inoculated mice.

Induction of *rpoE* promoter activity in T2S mutants. Homeostasis of the bacterial cell envelope is governed by several extracytoplasmic response systems, which are activated by dis-

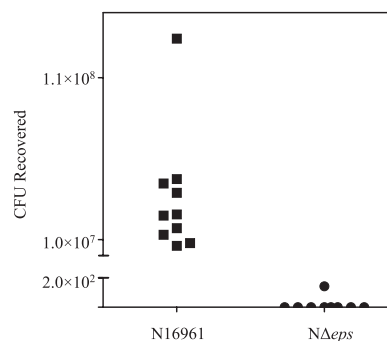


FIG. 4. NΔ*eps* mutant does not survive the passage through the infant mouse gastrointestinal tract. Wild-type and NΔ*eps* mutant strains of *V. cholerae* N16961 were separately inoculated into 4- to 5-day-old suckling mice as described in Materials and Methods. After 24 h postinoculation, the small intestines of the infected animals were removed and homogenized in LB broth, and serial dilutions were plated on LB agar supplemented with streptomycin to determine the number of CFU. The combined results of two independent experiments are presented. Datum points represent the CFU of wild-type N16961 (squares) and NΔ*eps* mutant (circles) recovered from individual mice.

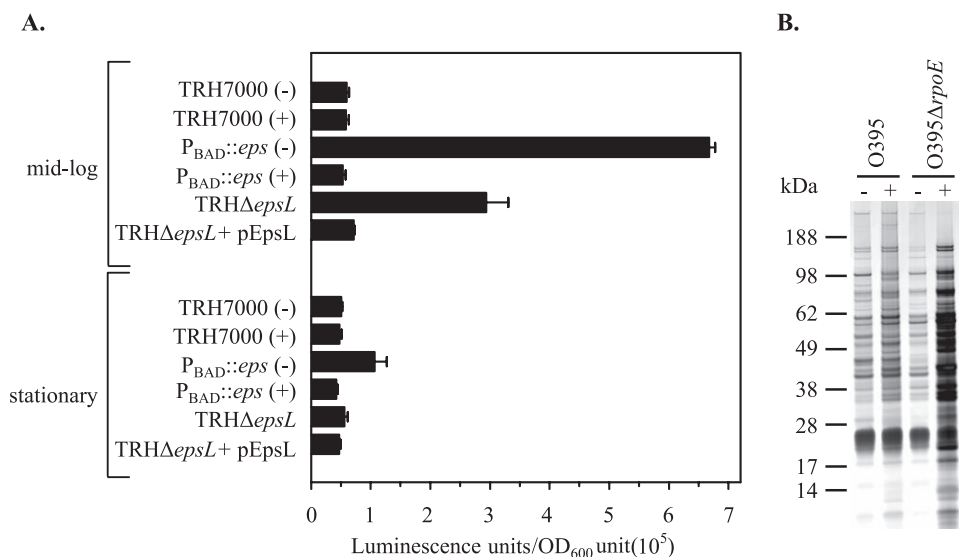


FIG. 5. Increased σ^E activity in *V. cholerae* lacking functional T2S machinery. (A) The expression of the *rpoEP2::lux* fusion gene was determined with wild-type, P_{BAD}::*eps*, Δ*epsL*, and Δ*epsL*+pEpsL strains of *V. cholerae* TRH7000 grown in LB broth at 37°C to mid-log and stationary phase (OD₆₀₀ = 0.5 and OD₆₀₀ = 4, respectively). The increased σ^E activities observed in P_{BAD}::*eps* grown in the absence of arabinose (-) and TRHΔ*epsL* strains could be complemented by the addition of 0.01% arabinose (+) to the growth medium and the expression of plasmid-encoded EpsL, respectively. The results presented are the means and corresponding SEM from three independent experiments. The difference in the induction of the σ^E response between the wild-type and P_{BAD}::*eps* strains grown in the absence of arabinose and that of the TRHΔ*epsL* mutant was statistically significant ($P < 0.004$) in mid-log-phase cultures, while the difference between stationary-phase cultures was not significant ($P > 0.05$). (B) Wild-type and *rpoE* mutant strains of *V. cholerae* O395 containing plasmid pMS43 were grown in LB broth at 37°C to late stationary phase (16 h) in the absence (-) or presence (+) of 10 μM IPTG to induce the expression of the dominant-negative mutant protein EpsE-K270A. Culture supernatants were separated from cells by centrifugation, matched by equivalent OD₆₀₀ units, and analyzed by SDS-PAGE and silver staining.

tinct signals (19, 73). The alternative sigma factor, σ^E (RpoE), is activated in response to misfolded outer membrane proteins and envelope damage and controls the expression of several periplasmic chaperones, proteases, LPS biosynthesis proteins, and outer membrane proteins that ensure proper outer membrane assembly (2, 72). As activation of the σ^E stress response is an indication of outer membrane damage, we reasoned that increased σ^E activity in the *eps* mutants would be another means by which damage to the outer membrane can be demonstrated. Therefore, a luciferase reporter gene fusion was constructed to determine whether *eps* mutants display enhanced σ^E activity. As in *E. coli* (68, 69, 71), the *rpoE* gene of *V. cholerae* is under the control of two promoters, a distal σ^{70} -dependent P1 and P2, located immediately upstream of *rpoE*. The P2 promoter matches the σ^E consensus sequence and is positively regulated by RpoE (44). The P2 promoter region was cloned upstream of the luciferase gene to monitor the promoter activity, and the resulting *rpoEP2::lux* transcriptional fusion plasmid was introduced into wild-type, P_{BAD}::*eps*, Δ*epsL*, and Δ*epsL*+pEpsL strains of *V. cholerae* TRH7000. When mid-log-phase cultures (OD₆₀₀ ~ 0.5) were examined, P_{BAD}::*eps* grown in the absence of arabinose exhibited 11-fold increases in σ^E activity compared to the activity of the wild-type strain (Fig. 5A). The σ^E activity was reduced to the basal level by inducing expression of the *eps* operon with the addition of arabinose to the medium (Fig. 5A). Similar results were observed in the single-gene-deletion mutant TRHΔ*epsL*, in which there was an approximately fivefold greater stimulation of transcription (Fig. 5A). The elevated activity of *rpoE* in the

TRHΔ*epsL* mutant was restored to wild-type levels by ectopic expression of *epsL* (Δ*epsL*+pEpsL) (Fig. 5A). A twofold upregulation of *rpoE* also was observed for the P_{BAD}::*eps* strain grown in the absence of arabinose in stationary-phase cells, but the increase was not statistically significant.

As upregulation of σ^E activity by either overexpression of the *rpoE* gene or inactivation of the gene encoding the anti- σ^E factor RseA also can result in growth defects and compromised outer membrane integrity (50, 61), it was unclear whether inactivation of the *eps* genes causes membrane defects that in turn induce the σ^E stress response or, alternatively, whether the loss of Eps function induces σ^E expression, which in turn causes the outer membrane defects. In a first attempt to distinguish between these possibilities, we determined the effect of inactivating the Eps system on the outer membrane integrity of a *V. cholerae* *rpoE* mutant. This was accomplished by expression of the dominant-negative EpsE mutant, EpsE-K270A, in wild-type and *rpoE* mutant strains of *V. cholerae* O395 (49). If upregulation of *rpoE* is responsible for the increased outer membrane permeability, then expression of EpsE-K270A should not result in the accumulation of periplasmic proteins in the culture supernatant of the *rpoE* mutant. If, on the other hand, the σ^E activity is induced in response to the cell envelope damage caused by inactivation of *eps* genes, then the outer membrane in the *rpoE* mutant also should be compromised. Examination of supernatants isolated from late-stationary-phase cultures revealed that overproduction of EpsE-K270A resulted in increased leakage of intracellular proteins into the growth medium of both wild-type and *rpoE* mutant strains (Fig.

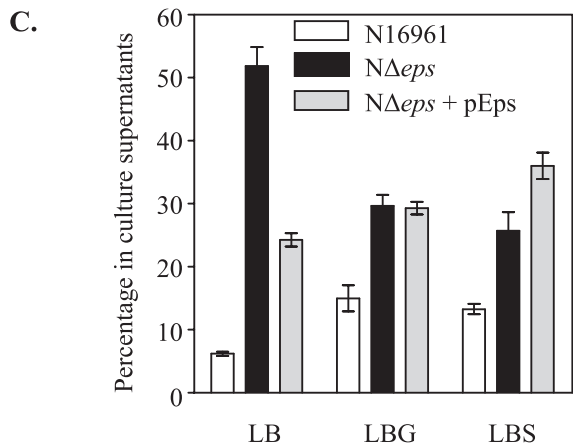
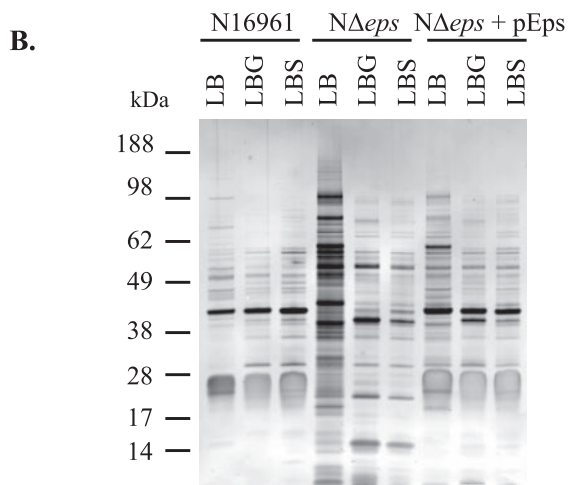
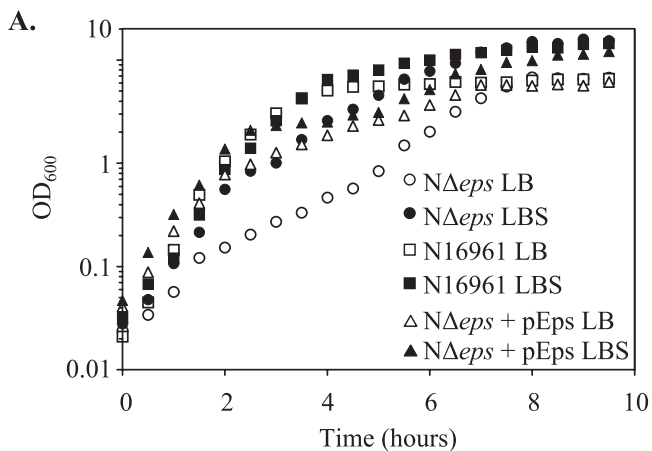


FIG. 6. Sucrose and glucose suppress the growth defect and leaky phenotype of the Δeps mutant strain. (A) Growth of wild-type, NΔeps, and NΔeps+pEps strains of *V. cholerae* N16961 in LB broth or LB broth supplemented with sucrose (LBS) at a 5% final concentration was monitored at OD₆₀₀. (B) Culture supernatants were isolated from wild-type, NΔeps, and NΔeps+pEps strains grown in LB broth, LBG (LB with glucose at a 5% final concentration), or LBS and analyzed by SDS-PAGE and silver staining. (C) Culture supernatants and periplasmic fractions of wild-type, NΔeps, and NΔeps+pEps cells grown in LB broth, LBG, or LBS were assayed for the presence of β-lactamase as described in Materials and Methods. The level of β-lactamase in the culture supernatant was expressed as a percentage of the total amount of the enzyme present in both the culture supernatant and cellular

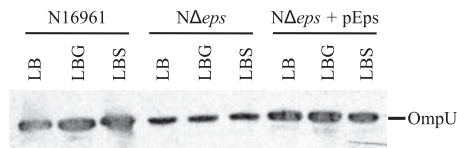


FIG. 7. Reduced level of OmpU in Δeps mutants. Western blot of cellular extracts obtained from cultures of wild-type, NΔeps, and NΔeps+pEps strains of *V. cholerae* N16961 grown in LB broth or in LB broth supplemented with glucose (LBG) or sucrose (LBS) to a 5% final concentration for 16 h at 37°C. Samples were matched by equivalent OD₆₀₀ units, separated by SDS-PAGE, transferred to nitrocellulose, and probed with OmpU antiserum.

5B). Consistent with these findings, similar observations were made when EpsE-K270A was expressed in mutant strains lacking DegS or YeaL (49), in which σ^E remains permanently bound to the anti-σ^E factor RseA and cannot be activated (data not shown). Taken together, these results suggest that the σ^E activity is induced in response to the outer membrane damage caused by inactivation of the *eps* genes.

Suppression of growth defect and leaky outer membrane.

The *eps* mutants are not capable of growth in LB broth lacking NaCl (data not shown), while the wild-type strains grow without salt, albeit slowly, suggesting that *eps* mutants are sensitive to hypoosmotic growth conditions. To test this hypothesis and to determine if the growth defect can be suppressed by the addition of osmoprotectants, we compared the growth of the NΔeps mutant to that of its isogenic wild type, strain N16961, in the absence or presence of two osmolytes, sucrose and glucose. Addition of sucrose (Fig. 6A) or glucose (data not shown) to the growth medium decreased the NΔeps mutant doubling time from 90 to 25 min, comparable to the 20-min generation time of wild-type cells. Titration of the sugars (from 1 to 10% final concentration) revealed that the minimal concentration of glucose and sucrose that suppressed the growth defect in the mutant strain was 5% (data not shown). We next examined the protein profiles of the culture supernatants isolated from the wild-type, NΔeps mutant, and NΔeps+pEps strains following growth in the absence or presence of 5% glucose or sucrose. As mentioned before, a large amount of protein was detected in the culture supernatant of the NΔeps mutant grown in LB broth without glucose and sucrose (Fig. 6B). In contrast, considerably less protein was observed in the culture supernatants isolated from the NΔeps mutant grown in the presence of glucose or sucrose. The results presented in Fig. 6B suggest that these sugars prevent the nonspecific release of periplasmic content into the growth medium. The level of the periplasmic enzyme β-lactamase in the culture supernatant was reduced from 49% in LB broth alone to 29 and 25% in LB broth containing glucose and sucrose, respectively (Fig. 6C). In addition, the level of extracellular GFP was decreased from 6.0% ± 0.1% to 1.3% ± 0.3% and 0.7% ± 0.3% (means ±

extract. The means of at least three independent experiments and corresponding SEM are presented. There was a statistically significant decrease in leakage of β-lactamase to the culture supernatant of NΔeps grown in LBG or LBS medium compared to the leakage of NΔeps grown in LB medium (*P* < 0.0005).

SEM) when the $N\Delta eps$ mutant was grown in the presence of glucose and sucrose, respectively.

Although a slight enhancement of growth in the presence of bile salts on LB agar supplemented with sucrose was observed equally for the wild-type and $N\Delta eps$ mutant strains, the mutant cells remained extremely sensitive to this detergent-like agent. While bile salts alone at a 0.05% final concentration resulted in 50% inhibition of growth of the wild-type strain (Table 2), the presence of sucrose improved the growth and resulted in only 23% growth inhibition (data not shown). Similarly, the level of growth inhibition by 0.0005% bile salts of the mutant strain in the absence and presence of sucrose were 50 and 12%, respectively. The $N\Delta eps$ mutant still was not capable of growth on higher bile salts concentrations in the presence of sucrose, however, suggesting that the sugars do not restore the integrity of the outer membrane per se. Instead, these saccharides may protect the *eps* mutants, possibly by reestablishing the osmotic balance within the cells, thus preventing the leakage of periplasmic material. Further support for the suggestion that the sugars do not restore the outer membrane to wild-type function is the finding that the level of the outer membrane porin, OmpU, remained low in the $N\Delta eps$ mutant following growth overnight in LB broth supplemented with sucrose or glucose (Fig. 7).

When additional sugars (lactose, galactose, sorbitol, melibiose, raffinose, xylose, and arabinose) or osmoprotectants (glycine-betaine and proline) were tested, only maltose and mannose were found to increase the growth rate of the $N\Delta eps$ mutant and to prevent the release of periplasmic content (data not shown). The reason why the other saccharides tested were not capable of protecting the mutant cells is not known; however, none of these sugars is metabolized by *V. cholerae* (41).

DISCUSSION

Inactivation of the well-conserved T2S system, Eps, in *V. cholerae* results in pleiotropic effects. While the outer membrane is capable of supporting assembly and function of certain complex structures, such as the polar flagellum and the toxin-coregulated pilus (8, 14, 64; also data not shown), other properties of the outer membrane are severely compromised when the Eps system is inactivated. For example, the protein content of the outer membrane in *eps* mutants is altered such that the levels of OmpU, OmpT, OmpS, and perhaps also OmpV and OmpW are reduced compared to wild-type levels (64, 77). Inactivation of the T2S system also increases the sensitivity to agents normally excluded by the outer membrane, such as polymyxin B and bile salts (Table 2). Together, the outer membrane changes likely are responsible for the pronounced growth defect of the *eps* mutants. The outer membrane and secretion defects of the $N\Delta eps$ mutant also contribute to the inability to survive the mouse gastrointestinal tract. This may be due to the loss of secretion of an important colonization factor and/or the increased sensitivity the mutant displays to bile salts and antimicrobial peptides, although increased sensitivity to bile salts by itself does not appear to affect the ability of *V. cholerae* to colonize the mouse infant intestine (64). Previously, *pilD* mutants of *V. cholerae* were shown to be defective in colonization of the infant mouse model. PilD is an enzyme that participates in many pathways, including process-

ing of the pseudopilins of the T2S system as well as the type IV pilin subunits PilA, TcpA, and MshA (21, 47). Hence, the deficiency in colonization of the *pilD* mutants could not be attributed to the loss of function of a single pathway. Here we show that inactivation of the T2S system results in a phenotype similar to that of the *pilD* mutants, suggesting that the colonization defect of the *pilD* mutants is, in part, due to defects brought upon T2S disruption.

One of the consequences of the altered outer membrane in the *eps* mutants is the leakage of periplasmic material. Significant amounts of β -lactamase and AP escape from the periplasmic compartment to the culture medium during growth. Interestingly, while these resident periplasmic enzymes are not retained efficiently by the outer membrane, proteins normally transported by the Eps system do not appear to leak out from the *eps* mutants to the same degree. Using a combination of detection methods including immunoblotting (Fig. 2C) and enzyme-linked immunosorbent assay (13, 64, 77, 78), we and others have shown that neither cholera toxin nor EtxB leak out in appreciable amounts to the culture medium of *eps* mutants. If the extracellular protease and lipase do leak out, they must be inactive, as is the case for the LasB protease, which diffuses out of secretion-defective *P. aeruginosa* LPS mutants in an inactive form (53). The reason for the differential leakage in the *V. cholerae eps* mutants is not known. It is not dependent merely on size, as one of the released proteins, AP, is a dimer of 94 kDa, while a retained protein, the assembled EtxB pentamer, is only 58 kDa. Differences in shape or surface properties may be responsible for the selective release. Alternatively, the retained non-secreted proteins may be trapped through interaction(s) with other cell envelope constituents in the *eps* mutants and therefore cannot diffuse freely into the culture medium.

The leaky phenotype is not due simply to decreased levels of the major porin OmpU or OmpT, as the structural integrity of the outer membrane remains unaffected in mutant strains lacking these porins (48, 64). The leakage could, however, be attributed to the combined loss of several outer membrane proteins. Consistent with this suggestion is the recent finding that inactivation of the *E. coli ompR* gene results in a reduced growth rate, a leaky outer membrane, and increased sensitivity to detergents (50). OmpR is a response regulator that controls the expression of several outer membrane proteins, including OmpF and OmpC (20). Similar pleiotropic effects also are associated with mutations of the Tol-Pal system in species such as *E. coli*, *V. cholerae*, and *Erwinia chrysanthemi* (4, 5, 45, 90). Besides providing a means for colicins and filamentous phages to enter bacterial cells, the Tol-Pal system also has been suggested to maintain the integrity/stability of the outer membrane (24) and to participate in cell division by assisting in the invagination of the outer membrane during septum formation (23). Other cell envelope mutants with phenotypes resembling the *eps* phenotype arise from mutations in genes encoding one of the most abundant outer membrane proteins, Lpp (86, 91), the Tat system (50, 84), or in genes coding for proteins involved in the biosynthesis of phospholipids and LPS (42).

The physiological phenotype of *eps* mutants suggests that the T2S complex plays a role in maintaining the integrity of the cell envelope, perhaps by supporting transport of a subset of outer membrane constituents. A recent transcriptome analysis of *V. cholerae* N16961 indicated that the expression of the *eps* genes

is reduced in an *rpoE* mutant and increased in a mutant strain that produces an excess of active RpoE due to inactivation of *rseA* (16). Although these findings suggest that the *eps* genes are positively regulated by σ^E , further experimentation is needed to verify that σ^E directs the transcription of the *eps* gene cluster. Being part of the σ^E regulon would be a further indication of a role for the Eps system in *V. cholerae* outer membrane biogenesis.

Whatever the role of the Eps system in outer membrane biogenesis, the altered outer membrane architecture of the *eps* mutants may prevent proper insertion and assembly of outer membrane proteins, resulting in their misfolding and periplasmic accumulation (72). This may, in turn, result in induction of the σ^E stress response with a mechanism similar to that described for *E. coli* (reviewed in references 1 and 2). The induction of expression from the P2 promoter of the *rpoE* gene in our *eps* mutants is indicative of increased σ^E activity, consistent with our suggestion that a cell envelope stress response is induced when T2S is prevented. The increased σ^E activity likely upregulates the expression of other σ^E -dependent genes as well, and as some of these genes encode proteases, including the DegP homolog DO (16), which has been shown to eliminate misfolded proteins (56, 83), the reduced levels of some of the outer membrane proteins in the *eps* mutants may be due to proteolytic degradation.

When secretion via the T2S system was blocked by expression of the dominant-negative mutant protein EpsE-K270A, damage to the outer membrane was observed in both wild-type and *rpoE* mutant strains of *V. cholerae*. The *rpoE* mutant may be slightly more sensitive to EpsE-K270A expression than its parental wild-type strain, as more material appeared to leak out to the culture medium of this mutant (Fig. 5B). While this suggests that the σ^E regulon serves to protect the cells to some degree from the damage that occurs when the T2S system is inactivated, quantitative analysis of the level of leakiness has to await the construction of an $\Delta eps \Delta rpoE$ mutant, as the dominant-negative plasmid system used in this study is somewhat unstable. It is possible, however, that such a mutant is not viable.

The release of periplasmic proteins to the growth medium may be a consequence of hypoosmotic swelling of the *eps* mutant cells due to the altered outer membrane architecture. Less of the periplasmic material is released when the *eps* mutants are grown in medium containing sugar at a high concentration, suggesting that swelling no longer occurs. The reason only metabolizable sugars prevent the leakage is not clear, but it may be that the protection by these sugars is mediated within the bacterial cell. Transport of the sugars into the periplasmic compartment or perhaps even the cytoplasm may be a prerequisite for osmoprotection. It is possible that the normal protection against hypoosmotic conditions by osmoregulated periplasmic glucans (membrane-derived oligosaccharides) is compromised in the *eps* mutants (7, 9, 54) but can be compensated for by the import of saccharides such as glucose and sucrose.

ACKNOWLEDGMENTS

We gratefully acknowledge Victor DiRita for help with the mouse infection studies and Tanya L. Johnson for critically reading the manuscript. We thank Karl Klose for OmpU antibodies, Jyl Matson and

Victor DiRita for $\Delta rpoE$, $\Delta degS$, and $\Delta yaeL$ mutant strains of *V. cholerae* Q395, and Bonnie Bassler and Brian Hammer for plasmid pBBRlux.

This work was supported by grant AI49294 from the National Institutes of Health (to M.S.).

REFERENCES

- Ades, S. E. 2004. Control of the alternative sigma factor σ^E in *Escherichia coli*. *Curr. Opin. Microbiol.* 7:157–162.
- Alba, B. M., and C. A. Gross. 2004. Regulation of the *Escherichia coli* sigma-dependent envelope stress response. *Mol. Microbiol.* 52:613–619.
- Angelichio, M. J., J. Spector, M. K. Waldor, and A. Camilli. 1999. *Vibrio cholerae* intestinal population dynamics in the suckling mouse model of infection. *Infect. Immun.* 67:3733–3739.
- Bernadac, A., M. Gavioli, J. C. Lazzaroni, S. Raina, and R. Lloubes. 1998. *Escherichia coli tol-pal* mutants form outer membrane vesicles. *J. Bacteriol.* 180:4872–4878.
- Bina, J. E., and J. J. Mekalanos. 2001. *Vibrio cholerae tolC* is required for bile resistance and colonization. *Infect. Immun.* 69:4681–4685.
- Bitter, W., R. van Bostel, M. Groeneweg, P. S. Carballo, U. Zahringer, J. Tommassen, and M. Koster. 2007. Species-specific functioning of the *Pseudomonas XcpQ* secretin: role for the C-terminal homology domain and lipopolysaccharide. *J. Bacteriol.* 189:2967–2975.
- Bohin, J. P. 2000. Osmoregulated periplasmic glucans in *Proteobacteria*. *FEMS Microbiol. Lett.* 186:11–19.
- Bose, N., S. M. Payne, and R. K. Taylor. 2002. Type 4 pilus biogenesis and type II-mediated protein secretion by *Vibrio cholerae* occur independently of the TonB-facilitated proton motive force. *J. Bacteriol.* 184:2305–2309.
- Bouchart, F., A. Delangle, J. Lemoine, J. P. Bohin, and J. M. Lacroix. 2007. Proteomic analysis of a non-virulent mutant of the phytopathogenic bacterium *Erwinia chrysanthemi* deficient in osmoregulated periplasmic glucans: change in protein expression is not restricted to the envelope, but affects general metabolism. *Microbiology* 153:760–767.
- Camberg, J. L., T. L. Johnson, M. Patrick, J. Abendroth, W. G. Hol, and M. Sandkvist. 2007. Synergistic stimulation of EpsE ATP hydrolysis by EpsL and acidic phospholipids. *EMBO J.* 26:19–27.
- Casadaban, M. J., and S. N. Cohen. 1980. Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. *J. Mol. Biol.* 138:179–207.
- Cianciotto, N. P. 2005. Type II secretion: a protein secretion system for all seasons. *Trends Microbiol.* 13:581–588.
- Connell, T. D., D. J. Metzger, M. Wang, M. G. Jobling, and R. K. Holmes. 1995. Initial studies of the structural signal for extracellular transport of cholera toxin and other proteins recognized by *Vibrio cholerae*. *Infect. Immun.* 63:4091–4098.
- Davis, B. M., E. H. Lawson, M. Sandkvist, A. Ali, S. Sozhamannan, and M. K. Waldor. 2000. Convergence of the secretory pathways for cholera toxin and the filamentous phage, CTXphi. *Science* 288:333–335.
- Davis, B. M., and M. K. Waldor. 2000. CTXphi contains a hybrid genome derived from tandemly integrated elements. *Proc. Natl. Acad. Sci. USA* 97:8572–8577.
- Ding, Y., B. M. Davis, and M. K. Waldor. 2004. Hfq is essential for *Vibrio cholerae* virulence and downregulates sigma expression. *Mol. Microbiol.* 53:345–354.
- Donnenberg, M. S., and J. B. Kaper. 1991. Construction of an *aeae* deletion mutant of enteropathogenic *Escherichia coli* by using a positive-selection suicide vector. *Infect. Immun.* 59:4310–4317.
- Drew, D., D. Sjostrand, J. Nilsson, T. Urbig, C. N. Chin, J. W. de Gier, and G. von Heijne. 2002. Rapid topology mapping of *Escherichia coli* inner-membrane proteins by prediction and PhoA/GFP fusion analysis. *Proc. Natl. Acad. Sci. USA* 99:2690–2695.
- Duguay, A. R., and T. J. Silhavy. 2004. Quality control in the bacterial periplasm. *Biochim. Biophys. Acta* 1694:121–134.
- Forst, S., J. Delgado, G. Ramakrishnan, and M. Inouye. 1988. Regulation of *ompC* and *ompF* expression in *Escherichia coli* in the absence of *envZ*. *J. Bacteriol.* 170:5080–5085.
- Fullner, K. J., and J. J. Mekalanos. 1999. Genetic characterization of a new type IV-A pilus gene cluster found in both classical and El Tor biotypes of *Vibrio cholerae*. *Infect. Immun.* 67:1393–1404.
- Garen, A., and C. Levinthal. 1960. A fine-structure genetic and chemical study of the enzyme alkaline phosphatase of *E. coli*. I. Purification and characterization of alkaline phosphatase. *Biochim. Biophys. Acta* 38:470–483.
- Gerding, M. A., Y. Ogata, N. D. Pecora, H. Niki, and P. A. de Boer. 2007. The trans-envelope Tol-Pal complex is part of the cell division machinery and required for proper outer-membrane invagination during cell constriction in *E. coli*. *Mol. Microbiol.* 63:1008–1025.
- Goemaere, E. L., E. Cascales, and R. Lloubes. 2007. Mutational analyses define helix organization and key residues of a bacterial membrane energy-transducing complex. *J. Mol. Biol.* 366:1424–1436.
- Guzman, L. M., D. Belin, M. J. Carson, and J. Beckwith. 1995. Tight regu-

- lation, modulation, and high-level expression by vectors containing the arabinose P_{BAD} promoter. *J. Bacteriol.* **177**:4121–4130.
26. Hancock, R. E. 1997. Peptide antibiotics. *Lancet* **349**:418–422.
 27. Herrington, D. A., R. H. Hall, G. Losonsky, J. J. Mekalanos, R. K. Taylor, and M. M. Levine. 1988. Toxin, toxin-coregulated pili, and the *toxR* regulon are essential for *Vibrio cholerae* pathogenesis in humans. *J. Exp. Med.* **168**:1487–1492.
 28. Hirst, T. R., and J. Holmgren. 1987. Transient entry of enterotoxin subunits into the periplasm occurs during their secretion from *Vibrio cholerae*. *J. Bacteriol.* **169**:1037–1045.
 29. Hirst, T. R., L. L. Randall, and S. J. Hardy. 1984. Cellular location of heat-labile enterotoxin in *Escherichia coli*. *J. Bacteriol.* **157**:637–642.
 30. Hirst, T. R., J. Sanchez, J. B. Kaper, S. J. Hardy, and J. Holmgren. 1984. Mechanism of toxin secretion by *Vibrio cholerae* investigated in strains harboring plasmids that encode heat-labile enterotoxins of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **81**:7752–7756.
 31. Hoffman, C. S., and A. Wright. 1985. Fusions of secreted proteins to alkaline phosphatase: an approach for studying protein secretion. *Proc. Natl. Acad. Sci. USA* **82**:5107–5111.
 32. Holmes, R. K., M. L. Vasil, and R. A. Finkelstein. 1975. Studies on toxinogenesis in *Vibrio cholerae*. III. Characterization of nontoxigenic mutants in vitro and in experimental animals. *J. Clin. Investig.* **55**:551–560.
 33. Howard, S. P., J. Critch, and A. Bedi. 1993. Isolation and analysis of eight *exE* genes and their involvement in extracellular protein secretion and outer membrane assembly in *Aeromonas hydrophila*. *J. Bacteriol.* **175**:6695–6703.
 34. Howard, S. P., C. Gebhart, G. R. Langen, G. Li, and T. G. Strozen. 2006. Interactions between peptidoglycan and the ExeAB complex during assembly of the type II secretin of *Aeromonas hydrophila*. *Mol. Microbiol.* **59**:1062–1072.
 35. Ichige, A., K. Oishi, and S. Mizushima. 1988. Isolation and characterization of mutants of a marine *Vibrio* strain that are defective in production of extracellular proteins. *J. Bacteriol.* **170**:3537–3542.
 36. Jalajakumari, M. B., and P. A. Manning. 1990. Nucleotide sequence of the gene, *ompW*, encoding a 22kDa immunogenic outer membrane protein of *Vibrio cholerae*. *Nucleic Acids Res.* **18**:2180.
 37. Jiang, B., and S. P. Howard. 1992. The *Aeromonas hydrophila* *exE* gene, required both for protein secretion and normal outer membrane biogenesis, is a member of a general secretion pathway. *Mol. Microbiol.* **6**:1351–1361.
 38. Johnson, T. L., J. Abendroth, W. G. Hol, and M. Sandkvist. 2006. Type II secretion: from structure to function. *FEMS Microbiol. Lett.* **255**:175–186.
 39. Judson, N., and J. J. Mekalanos. 2000. Transposon-based approaches to identify essential bacterial genes. *Trends Microbiol.* **8**:521–526.
 40. Kagami, Y., M. Ratliff, M. Surber, A. Martinez, and D. N. Nunn. 1998. Type II protein secretion by *Pseudomonas aeruginosa*: genetic suppression of a conditional mutation in the pilin-like component XcpT by the cytoplasmic component XcpR. *Mol. Microbiol.* **27**:221–233.
 41. Kaper, J. B., J. G. Morris, Jr., and M. M. Levine. 1995. Cholera. *Clin. Microbiol. Rev.* **8**:48–86.
 42. Kloser, A., M. Laird, M. Deng, and R. Misra. 1998. Modulations in lipid A and phospholipid biosynthesis pathways influence outer membrane protein assembly in *Escherichia coli* K-12. *Mol. Microbiol.* **27**:1003–1008.
 43. Kostakioti, M., C. L. Newman, D. G. Thanassi, and C. Stathopoulos. 2005. Mechanisms of protein export across the bacterial outer membrane. *J. Bacteriol.* **187**:4306–4314.
 44. Kovacikova, G., and K. Skorupski. 2002. The alternative sigma factor σ^E plays an important role in intestinal survival and virulence in *Vibrio cholerae*. *Infect. Immun.* **70**:5355–5362.
 45. Lazzaroni, J. C., N. Fognini-Lefebvre, and R. Portulier. 1989. Cloning of the *excC* and *excD* genes involved in the release of periplasmic proteins by *Escherichia coli* K12. *Mol. Gen. Genet.* **218**:460–464.
 46. Lenz, D. H., K. C. Mok, B. N. Lilley, R. V. Kulkarni, N. S. Wingreen, and B. L. Bassler. 2004. The small RNA chaperone Hfq and multiple small RNAs control quorum sensing in *Vibrio harveyi* and *Vibrio cholerae*. *Cell* **118**:69–82.
 47. Marsh, J. W., and R. K. Taylor. 1998. Identification of the *Vibrio cholerae* type 4 prepilin peptidase required for cholera toxin secretion and pilus formation. *Mol. Microbiol.* **29**:1481–1492.
 48. Mathur, J., and M. K. Waldor. 2004. The *Vibrio cholerae* ToxR-regulated porin OmpU confers resistance to antimicrobial peptides. *Infect. Immun.* **72**:3577–3583.
 49. Matson, J. S., and V. J. DiRita. 2005. Degradation of the membrane-localized virulence activator TpeP by the YaeL protease in *Vibrio cholerae*. *Proc. Natl. Acad. Sci. USA* **102**:16403–16408.
 50. McBroom, A. J., A. P. Johnson, S. Vemulapalli, and M. J. Kuehn. 2006. Outer membrane vesicle production by *Escherichia coli* is independent of membrane instability. *J. Bacteriol.* **188**:5385–5392.
 51. Ménard, R., P. J. Sansonetti, and C. Parsot. 1993. Nonpolar mutagenesis of the *ipa* genes defines IpaB, IpaC, and IpaD as effectors of *Shigella flexneri* entry into epithelial cells. *J. Bacteriol.* **175**:5899–5906.
 52. Meselson, M., and R. Yuan. 1968. DNA restriction enzyme from *E. coli*. *Nature* **217**:1110–1114.
 53. Michel, G., G. Ball, J. B. Goldberg, and A. Lazdunski. 2000. Alteration of the lipopolysaccharide structure affects the functioning of the Xcp secretory system in *Pseudomonas aeruginosa*. *J. Bacteriol.* **182**:696–703.
 54. Miller, K. J., E. P. Kennedy, and V. N. Reinhold. 1986. Osmotic adaptation by gram-negative bacteria: possible role for periplasmic oligosaccharides. *Science* **231**:48–51.
 55. Miller, V. L., and J. J. Mekalanos. 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. *J. Bacteriol.* **170**:2575–2583.
 56. Misra, R., A. Peterson, T. Ferenci, and T. J. Silhavy. 1991. A genetic approach for analyzing the pathway of LamB assembly into the outer membrane of *Escherichia coli*. *J. Biol. Chem.* **266**:13592–13597.
 57. Morales, V. M., A. Backman, and M. Bagdasarian. 1991. A series of wide-host-range low-copy-number vectors that allow direct screening for recombinants. *Gene* **97**:39–47.
 58. Nandi, B., R. K. Nandy, A. Sarkar, and A. C. Ghose. 2005. Structural features, properties and regulation of the outer-membrane protein W (OmpW) of *Vibrio cholerae*. *Microbiology* **151**:2975–2986.
 59. Newman, J. R., and C. Fuqua. 1999. Broad-host-range expression vectors that carry the L-arabinose-inducible *Escherichia coli* *araBAD* promoter and the *araC* regulator. *Gene* **227**:197–203.
 60. Nikaido, H. 2003. Molecular basis of bacterial outer membrane permeability revisited. *Microbiol. Mol. Biol. Rev.* **67**:593–656.
 61. Nitta, T., H. Nagamitsu, M. Murata, H. Izu, and M. Yamada. 2000. Function of the σ^E regulon in dead-cell lysis in stationary-phase *Escherichia coli*. *J. Bacteriol.* **182**:5231–5237.
 62. Overbye, L. J., M. Sandkvist, and M. Bagdasarian. 1993. Genes required for extracellular secretion of enterotoxin are clustered in *Vibrio cholerae*. *Gene* **132**:101–106.
 63. Peek, J. A., and R. K. Taylor. 1992. Characterization of a periplasmic thiol: disulfide interchange protein required for the functional maturation of secreted virulence factors of *Vibrio cholerae*. *Proc. Natl. Acad. Sci. USA* **89**:6210–6214.
 64. Provenzano, D., C. M. Lauriano, and K. E. Klose. 2001. Characterization of the role of the ToxR-modulated outer membrane porins OmpU and OmpT in *Vibrio cholerae* virulence. *J. Bacteriol.* **183**:3652–3662.
 65. Provenzano, D., D. A. Schuhmacher, J. L. Barker, and K. E. Klose. 2000. The virulence regulatory protein ToxR mediates enhanced bile resistance in *Vibrio cholerae* and other pathogenic *Vibrio* species. *Infect. Immun.* **68**:1491–1497.
 66. Pugsley, A. P. 1993. The complete general secretory pathway in gram-negative bacteria. *Microbiol. Rev.* **57**:50–108.
 67. Pukatzki, S., A. T. Ma, D. Sturtevant, B. Krastins, D. Sarracino, W. C. Nelson, J. F. Heidelberg, and J. J. Mekalanos. 2006. Identification of a conserved bacterial protein secretion system in *Vibrio cholerae* using the *Dictyostelium* host model system. *Proc. Natl. Acad. Sci. USA* **103**:1528–1533.
 68. Raina, S., D. Missiakas, and C. Georgopoulos. 1995. The *rpoE* gene encoding the σ^E (σ^{24}) heat shock sigma factor of *Escherichia coli*. *EMBO J.* **14**:1043–1055.
 69. Rouvière, P. E., A. De Las Peñas, J. Mecas, C. Z. Lu, K. E. Rudd, and C. A. Gross. 1995. *rpoE*, the gene encoding the second heat-shock sigma factor, σ^E , in *Escherichia coli*. *EMBO J.* **14**:1032–1042.
 70. Roy, N. K., R. K. Ghosh, and J. Das. 1982. Monomeric alkaline phosphatase of *Vibrio cholerae*. *J. Bacteriol.* **150**:1033–1039.
 71. Rudd, K. E., H. J. Sofia, E. V. Koonin, G. Plunkett III, S. Lazar, and P. E. Rouvière. 1995. A new family of peptidyl-prolyl isomerases. *Trends Biochem. Sci.* **20**:12–14.
 72. Ruiz, N., D. Kahne, and T. J. Silhavy. 2006. Advances in understanding bacterial outer-membrane biogenesis. *Nat. Rev. Microbiol.* **4**:57–66.
 73. Ruiz, N., and T. J. Silhavy. 2005. Sensing external stress: watchdogs of the *Escherichia coli* cell envelope. *Curr. Opin. Microbiol.* **8**:122–126.
 74. Sandkvist, M. 2001. Type II secretion and pathogenesis. *Infect. Immun.* **69**:3523–3535.
 75. Sandkvist, M., M. Bagdasarian, S. P. Howard, and V. J. DiRita. 1995. Interaction between the autokinase EpsE and EpsL in the cytoplasmic membrane is required for extracellular secretion in *Vibrio cholerae*. *EMBO J.* **14**:1664–1673.
 76. Sandkvist, M., T. R. Hirst, and M. Bagdasarian. 1990. Minimal deletion of amino acids from the carboxyl terminus of the B subunit of heat-labile enterotoxin causes defects in its assembly and release from the cytoplasmic membrane of *Escherichia coli*. *J. Biol. Chem.* **265**:15239–15244.
 77. Sandkvist, M., L. O. Michel, L. P. Hough, V. M. Morales, M. Bagdasarian, M. Koomey, and V. J. DiRita. 1997. General secretion pathway (*eps*) genes required for toxin secretion and outer membrane biogenesis in *Vibrio cholerae*. *J. Bacteriol.* **179**:6994–7003.
 78. Sandkvist, M., V. Morales, and M. Bagdasarian. 1993. A protein required for secretion of cholera toxin through the outer membrane of *Vibrio cholerae*. *Gene* **123**:81–86.
 79. Schäfer, A., A. Tauch, W. Jäger, J. Kalinowski, G. Thierbach, and A. Puhler. 1994. Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. *Gene* **145**:69–73.

80. Scott, M. E., Z. Y. Dossani, and M. Sandkvist. 2001. Directed polar secretion of protease from single cells of *Vibrio cholerae* via the type II secretion pathway. *Proc. Natl. Acad. Sci. USA* **98**:13978–13983.
81. Simon, R., U. Priefer, and A. Puhler. 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram-negative bacteria. *Biotechnology* **1**:784–790.
82. Söderberg, M. A., O. Rossier, and N. P. Cianciotto. 2004. The type II protein secretion system of *Legionella pneumophila* promotes growth at low temperatures. *J. Bacteriol.* **186**:3712–3720.
83. Spiess, C., A. Beil, and M. Ehrmann. 1999. A temperature-dependent switch from chaperone to protease in a widely conserved heat shock protein. *Cell* **97**:339–347.
84. Stanley, N. R., K. Findlay, B. C. Berks, and T. Palmer. 2001. *Escherichia coli* strains blocked in Tat-dependent protein export exhibit pleiotropic defects in the cell envelope. *J. Bacteriol.* **183**:139–144.
85. Stevenson, G., D. I. Leavesley, C. A. Lagnado, M. W. Heuzenroeder, and P. A. Manning. 1985. Purification of the 25-kDa *Vibrio cholerae* major outer-membrane protein and the molecular cloning of its gene: *ompV*. *Eur. J. Biochem.* **148**:385–390.
86. Suzuki, H., Y. Nishimura, S. Yasuda, A. Nishimura, M. Yamada, and Y. Hirota. 1978. Murein-lipoprotein of *Escherichia coli*: a protein involved in the stabilization of bacterial cell envelope. *Mol. Gen. Genet.* **167**:1–9.
87. Taylor, D. N., K. P. Killeen, D. C. Hack, J. R. Kenner, T. S. Coster, D. T. Beattie, J. Ezzell, T. Hyman, A. Trofa, M. H. Sjogren, et al. 1994. Development of a live, oral, attenuated vaccine against El Tor cholera. *J. Infect. Dis.* **170**:1518–1523.
88. Taylor, R. K., C. Manoil, and J. J. Mekalanos. 1989. Broad-host-range vectors for delivery of *TnphaA*: use in genetic analysis of secreted virulence determinants of *Vibrio cholerae*. *J. Bacteriol.* **171**:1870–1878.
89. Voulhoux, R., G. Ball, B. Ize, M. L. Vasil, A. Lazdunski, L. F. Wu, and A. Filloux. 2001. Involvement of the twin-arginine translocation system in protein secretion via the type II pathway. *EMBO J.* **20**:6735–6741.
90. Webster, R. E. 1991. The *tol* gene products and the import of macromolecules into *Escherichia coli*. *Mol. Microbiol.* **5**:1005–1011.
91. Yem, D. W., and H. C. Wu. 1978. Physiological characterization of an *Escherichia coli* mutant altered in the structure of murein lipoprotein. *J. Bacteriol.* **133**:1419–1426.