Cell Envelope Perturbation Induces Oxidative Stress and Changes in Iron Homeostasis in *Vibrio cholerae*[▽][†]

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The *Vibrio cholerae* type II secretion (T2S) machinery is a multiprotein complex that spans the cell envelope. When the T2S system is inactivated, cholera toxin and other exoproteins accumulate in the periplasmic compartment. Additionally, loss of secretion via the T2S system leads to a reduced growth rate, compromised outer membrane integrity, and induction of the extracytoplasmic stress factor RpoE (A. E. Sikora, S. R. Lybarger, and M. Sandkvist, J. Bacteriol. 189:8484–8495, 2007). In this study, gene expression profiling reveals that inactivation of the T2S system alters the expression of genes encoding cell envelope components and proteins involved in central metabolism, chemotaxis, motility, oxidative stress, and iron storage and acquisition. Consistent with the gene expression data, molecular and biochemical analyses indicate that the T2S mutants suffer from internal oxidative stress and increased levels of intracellular ferrous iron. By using a *tolA* mutant of *V. cholerae* that shares a similar compromised membrane phenotype but maintains a functional T2S machinery, we show that the formation of radical oxygen species, induction of oxidative stress, and changes in iron physiology are likely general responses to cell envelope damage and are not unique to T2S mutants. Finally, we demonstrate that disruption of the *V. cholerae* cell envelope by chemical treatment with polymyxin B similarly results in induction of the RpoE-mediated stress response, increased sensitivity to oxidants, and a change in iron metabolism. We propose that many types of extracytoplasmic stresses, caused either by genetic alterations of outer membrane constituents or by chemical or physical damage to the cell envelope, induce common signaling pathways that ultimately lead to internal oxidative stress and misregulation of iron homeostasis.

Vibrio cholerae, a rod-shaped, highly motile, gram-negative bacterium, is the causative agent of the life threatening diarrheal disease cholera (59). The type II secretion (T2S) system plays an important role in the pathogenesis of *V. cholerae* by secreting cholera toxin (63), which is largely responsible for the symptoms of the disease (33). The T2S system is widespread and well conserved in gram-negative bacteria inhabiting a variety of ecological niches and likely contributes to environmental survival as well as to virulence (11, 21). In *V. cholerae*, secretion via the T2S machinery is supported by a transenvelope complex of 12 Eps proteins (EpsC to EpsN) and the type 4 prepilin peptidase PilD (VcpD) (25, 44, 63). Transport of exoproteins by the T2S system occurs via a two-step process. The first step, which is either Sec or Tat dependent, requires recognition of the N-terminal signal peptide of the exoproteins and translocation through the inner membrane to the periplasm. Then the folded proteins engage the T2S machinery and are subsequently exported across the outer membrane to the extracellular milieu (23, 29).

Besides periplasmic accumulation of exoproteins, additional phenotypes of T2S mutants are reported for an increasing number of species, possibly indicating involvement of the T2S system in other important cellular processes. For example, alterations in outer membrane protein composition have been described for T2S mutants of *V. cholerae*, *Aeromonas hydrophila*, marine *Vibrio* sp. strain 60, and *Shewanella oneidensis* (30, 32, 63, 64). The levels of outer membrane porins OmpU, OmpT, and OmpS are decreased in T2S mutants of *V. cholerae* (63, 65), and likewise, disruption of T2S genes in *A. hydrophila* leads to diminished quantities of OmpF and OmpS (30). Similarly, the amounts of the c-type cytochromes MtrC and OmcA in the outer membranes of *S. oneidensis* T2S mutants are reduced (64). Furthermore, we have shown that inactivation of the T2S system in *V. cholerae* results in a reduced growth rate, compromised outer membrane integrity, and, as a consequence, induction of RpoE activity. In addition, our studies showed that *V. cholerae* T2S mutants are unable to survive the passage through the infant mouse gastrointestinal tract (65). Growth defects at low temperatures under laboratory conditions as well as in tap water and amoebae were also observed for T2S mutants of *Legionella pneumophila* (68).

Interestingly, differential abundance of proteins involved in phosphate metabolism and iron uptake has been revealed by proteomic analysis of culture supernatants isolated from wild-type and T2S mutant strains of *Pseudoalteromonas tunicata* (22). Based on these results, it has been suggested that the T2S system might be involved in iron acquisition. Similarly, certain T2S mutants of *Erwinia chrysanthemi* exhibit defects indicative of changes in iron homeostasis (17). It has also been noted that

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype[a]	Reference or source[b]
Strains		
V. cholerae		
N16961	Wild-type El Tor O1 biotype; Sm[r]	Laboratory collection
NΔ*eps*	N16961 Δ*epsCDEFGHIJKLMN*; Cm[r]	65
NΔ*epsD*	N16961 Δ*epsD*; Kan[r]	This study
N*tolA*	N16961 *tolA*::pDH149; Amp[r]	This study
E. coli		
MC1061	F⁻ *araD139* Δ(*ara-leu*)*7697* Δ(*lac*)*X74 rpsL hsdR2 mcrA mcrB1*	9
S17.1	F⁻ *recA pro hsdR* RP4-2; Tc[r]::Mu Kan[r]::Tn7	66
SY327 λpir	Δ(*lac-pro*) *argE*(Am) *recA56 rpoB* λpir; Rif[r]	51
MM294 (pRK2013)	Donor of transfer function for triparental conjugation	48
EDL 933 (O157:H7)	Wild type	MSU collection
EΔ*etpM*	O157:H7 *etpM*::*kan*; Kan[r]	This study
K-12 BW25113	Wild type	2
ECK0728	*tolA*::*kan*; Kan[r]	2
Plasmids		
pPCR-Script	Cloning vector; Amp[r]	Stratagene
pMMB198	*etxB* under *ptac* control in pMMB66EH; Cm[r]	62
pEps	15-kb XbaI-SphI fragment containing entire *eps* operon (from *epsC* to *epsN*) in pMMB190; Amp[r]	65
pMMB-*epsD*	*epsD* cloned into pMMB67; Amp[r]	42
pMMB207	Cloning vector, pMMB66; Cm[r]	53
pDH149	Internal fragment of *tolA* (bp 79-530) cloned into suicide vector pGP704; Amp[r]	28
pAES106	*rpoEp₂*::*lux* pBBR-*lux*; Cm[r]	65
pAES107	*viuPp*::*lux* pBBR-*lux*; Cm[r]	This study
pAES108	*hutAp*::*lux* pBBR-*lux*; Cm[r]	This study
pMMB-*tolA*	*V. cholerae tolA* cloned into pMMB207; Cm[r]	This study

[a] Sm[r], streptomycin resistance; Amp[r], ampicillin resistance; Cm[r], chloramphenicol resistance; Kan[r], kanamycin resistance; Tc[r], tetracycline resistance; Rif[r], rifampin resistance.

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the level of aconitate hydratase, an iron-sulfur cluster-containing enzyme, is reduced in *L. pneumophila* T2S mutants (16).

In this study, in an attempt to explain the phenotypes associated with loss of T2S, we performed microarray gene expression profiling of wild-type and T2S-deficient strains. Our data revealed that inactivation of the T2S machinery results in a metabolic feedback loop leading to oxidative stress and changes in iron metabolism. By analyzing another *V. cholerae* mutant that shares a similar cell envelope phenotype while remaining competent for T2S, we show that the changes in iron homeostasis and oxidative stress are linked to cell envelope damage and extracytoplasmic stress.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. All bacterial strains and plasmids used in this study are listed in Table 1. Bacterial cultures were propagated at 37°C in Luria-Bertani (LB) medium (Fisher Chemicals, Fairlawn, NJ), supplemented as specified in the text. Antibiotics (Sigma-Aldrich, St. Louis, MO) were used at the following concentrations: chloramphenicol, 4 or 10 μg/ml for chromosomal or plasmid expression, respectively, in *V. cholerae* and 30 μg/ml in *Escherichia coli*; carbenicillin, 50 or 100 μg/ml for chromosomal or plasmid expression, respectively; kanamycin, 50 μg/ml; polymyxin B sulfate, 100 U/ml.

RNA isolation, cDNA preparation, hybridization, and data analysis. Overnight-grown cultures of wild-type and isogenic NΔ*eps* strain of *V. cholerae* N16961 were diluted to an optical density at 600 nm (OD_{600}) of 0.03 and grown with aeration in LB medium in three biological replicates. The growth was monitored over time, and 2-ml aliquots of cells were harvested 1 h following entry into stationary phase, when the strains reached an OD_{600} of 4. The cell pellets were immediately resuspended in 1 ml of TRIzol reagent (Invitrogen) and stored at −80°C. Total cellular RNA was subsequently isolated as described

previously (6). The microarrays used in this study are composed of 70-mer oligonucleotides representing the open reading frames present in the *V. cholerae* N16961 genome and were printed at the University of California, Santa Cruz. Whole-genome expression analysis was performed using a common reference RNA, which was isolated from wild-type cells grown in LB medium as described above. RNA isolated from test and reference samples was used in cDNA synthesis and microarray hybridization, and scanning was performed as described previously using three biological and two technical replicates (6). Normalized signal ratios were obtained with LOWESS print-tip normalization using Bioconductor packages (26) in the R environment. Differentially regulated genes were determined with the SAM (significance analysis of microarrays) program (76) using ≥2.0-fold differences in gene expression and a ≤1% false discovery rate (FDR) as cutoff values.

Genetic techniques. Chromosomal DNA isolated from *V. cholerae* N16961 was used as a template for PCR. PCR was carried out with PfuUltra DNA polymerase (Stratagene, La Jolla, CA). Primers were synthesized by IDT Technologies, Inc. (Coralville, IA). The resulting PCR amplification products were purified, subcloned into pPCR-Script Amp SK(+) (Stratagene), and then cloned into the appropriate vector as indicated. Plasmids and suicide vectors were introduced into *V. cholerae* strains by conjugation with *E. coli* S17.1 or by triparental conjugation as described previously (65).

Construction of mutant strains. An *epsD* deletion mutant of *V. cholerae* N16961 was constructed as described previously (42). The *epsD* mutant was complemented with plasmid pMMB-*epsD* in the presence of 10 μM isopropyl-β-D-thiogalactopyranoside (IPTG). A *tolA* insertion mutant of *V. cholerae* N16961 was constructed as described previously (28) using suicide vector pDH149, kindly supplied by Matthew Waldor, Brigham and Women's Hospital, Harvard University. For complementation analysis, the full-length *tolA* gene was amplified using forward primer 5′-GAGCTCTCCTAAAGTAGGGCTACTCACG-3′ and reverse primer 5′-CTGCAGATAACTAGCTCCAATGCTGC-3′ and was cloned into SacI-PstI-digested pMMB207 (53). Expression of the *tolA* gene was induced with 10 μM IPTG. An *etpM* mutant of *E. coli* O157:H7 strain EDL 933, EΔ*etpM*, was constructed by inserting a kanamycin resistance cassette into the *etpM* gene

using the method of Datsenko and Wanner (15). The following primers were used for the construction of the mutant: etpM01 (5′-CCTTACTGGTTGTT GTTTTTGTCTATTACGTCgtgtaggctggagctgcttc) and etpM02 (5′-TACATC AAGCATTAATCTGTAAATTACGACATCAcatatgaatatcctccttag) (lowercase sequences indicate homology to the kanamycin resistance cassette sequence in the plasmid vector) (15). Mass spectroscopy analysis (M. E. Scott and M. Sandkvist, unpublished data) confirmed that the mutant did not secrete the T2S-dependent metalloprotease StcE (36).

Promoter fusion studies and bioluminescence assays. The fragment of chromosomal DNA containing the *hutA* upstream region (220 bp) was PCR amplified as described previously (49) with forward primer 5′-ACTAGTTCTCTCCTATT GATAGTTCACACCGC-3′ and reverse primer 5′-GGATCCGCAATTTCCAT GTTGAGTAAAACGC-3′. The *viuP* upstream region (196 bp) was amplified by using the following primer pair: forward, 5′-ACTAGTGCGGTGTTCGGTGG CT-3′; reverse, 5′-GGATCCCACTGAACAGGCAACCCAAT-3′. The PCR fragments were cloned into SpeI-BamHI-digested pBBR*lux* (40) upstream of promoterless *luxCDABE* to yield the transcriptional fusions *viuP*p::*lux* and *hutA*p::*lux* in pAES107 and pAES108, respectively. Overnight cultures of NΔ*epsD*, N*tolA*, and isogenic wild-type *V. cholerae* N16961 carrying pAES106 (65), pAES107, or pAES108 were diluted (1:100) into fresh LB medium alone or supplemented with an iron chelator (2,2′-dipyridyl at a final concentration of 100 or 150 μM for the T2S or N*tolA* mutants, respectively) or polymyxin B sulfate (100, 200, and 400 U/ml) as indicated in the text. The reporter activities were determined as described previously (65) when the cultures reached the mid-log phase of growth (OD_{600}, about 0.5) and 1 h following entry into stationary phase (at an OD_{600} of 4). The data represent means and standard deviations of the means (SEMs) from three to nine experiments.

Sensitivities to H_2O_2 and streptonigrin. Bacterial cultures were grown aerobically in LB medium. One hundred microliters of mid-log-phase cultures (OD_{600}, approximately 0.5) was uniformly distributed on LB agar plates that were either left unsupplemented or supplemented with the iron chelator 2,2′-dipyridyl (100 and 150 μM; Sigma-Aldrich) or with polymyxin B sulfate (100, 200, and 400 U/ml) as indicated in the text. Sterile filter paper discs (diameter, 7 mm) were placed on the surface of the agar and impregnated with 5 μl of H_2O_2 (44, 88, 176, 440, and 880 mM; Fisher) or streptonigrin (10, 20, 40, 60, 80, and 100 μg/ml; Sigma-Aldrich) for *V. cholerae* and 5 μl of 8.8 M H_2O_2 or 100, 200, 400, 600, 800, or 1,000 μg/ml of streptonigrin for *E. coli*. The zone of growth inhibition around the discs was recorded after 24 h of incubation at 37°C. Means and SEMs for at least three independent experiments are presented.

Detection of intracellular formation of reactive oxygen species (ROS). The level of intracellular oxidation was measured in vivo by using the molecular probe 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA; Cayman Chemical, Ann Arbor, MI) (10, 13). Bacterial cultures were grown in LB medium to the mid-log phase of growth (OD_{600}, approximately 0.5), and the probe was added to a final concentration of 10 μM. The cells were incubated in the dark at 37°C for 1 h, subsequently harvested, washed with 50 mM Tris-HCl buffer (pH 8.0), and disrupted by sonication in the same buffer. Wild-type cells treated with 3 mM H_2O_2 were used as a positive control in each experiment. The total protein concentration in each sample was measured using a modified Bradford assay (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. The fluorescence intensity of cell extracts was measured using the excitation and emission wavelengths of 485 and 530 nm, respectively, in a multidetection microplate reader (Synergy HT; Bio-Tek Instruments, Winooski, VT). Each sample was read in technical triplicate. The emission values were normalized by the protein concentration. Means of four independent experiments and corresponding SEMs are presented.

SDS-PAGE, silver staining, and Western blotting. Samples of whole-cell lysates, culture supernatants, or periplasmic extracts were prepared and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining or Western blotting with anti-EtxB antibodies as described previously (65).

RESULTS AND DISCUSSION

Global analysis of *V. cholerae* gene expression in response to inactivation of the T2S system. Inactivation of the T2S system, by removal of either single *eps* genes or the entire *eps* operon, results not only in the loss of extracellular secretion but also in cell envelope perturbation, upregulation of the σ^E-mediated stress response, and a reduced growth rate (63, 65). To further investigate the cellular response to inactivation of the T2S system, we performed a global transcriptome analysis of NΔ*eps*, in
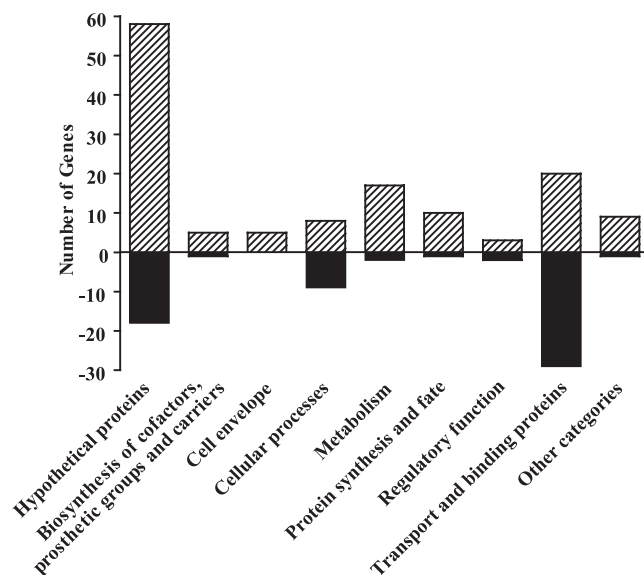

FIG. 1. Functional categories of genes differentially expressed in the NΔ*eps* mutant compared to the wild-type strain. The numbers of genes whose expression is differentially expressed in NΔ*eps* mutant compared to wild-type cells are presented according to the functions assigned by The Institute for Genomic Research. Positive and negative numbers stand for the numbers of genes whose expression is induced and repressed, respectively.

which the entire *eps* operon has been removed (65), and the parental wild-type strain, *V. cholerae* N16961, grown with aeration in LB medium. Because T2S mutants display a reduced growth rate in logarithmically growing cultures, we compared the gene expression profiles at the early stationary phase of growth, when both strains reached a final OD_{600} of 4.0. The gene expression data were analyzed by applying SAM software (76) using a ≥2.0-fold change in gene expression and a ≤1% FDR as cutoffs. Our analysis identified 198 differentially expressed genes, with 63 genes downregulated and 135 genes upregulated in the T2S-deficient strain relative to the wild-type strain (see Tables S1 and S2 in the supplemental material for a complete list of these genes). The differentially expressed genes belong to several functional groups, as shown in Fig. 1. Inactivation of the T2S machinery resulted in changes in the expression of genes coding for many members of the σ^E regulon, some of which are outer membrane constituents, and genes encoding proteins participating in chemotaxis/motility, biofilm formation, transport of amino acids and carbohydrates, central metabolic processes, iron physiology, and stress responses that involve heat shock proteins, chaperones, and proteases. Below we discuss a select subset of differentially expressed genes.

Iron homeostasis is altered in T2S mutants. Remarkably, among the differentially expressed genes, the largest cluster consisted of genes that are known to be regulated by the Fe^{2+}-Fur complex in *V. cholerae* (50). Fur is the major sensor of iron availability, which represses the expression of genes involved in iron acquisition when the level of free iron in the cell is high (1, 50). About 60% (38 out of 63) of the genes downregulated in response to the *eps* mutation overlapped with those that are negatively regulated by Fur and iron

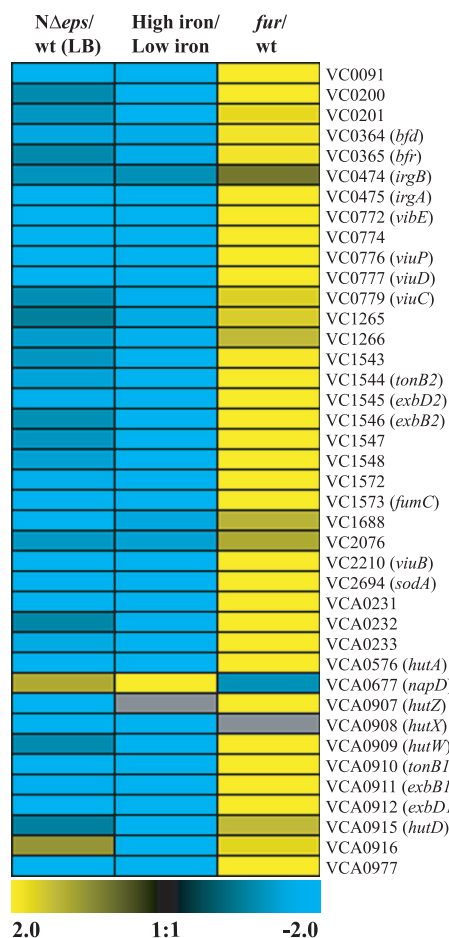| NΔeps/ wt (LB)	High iron/ Low iron	fur/ wt	
			VC0091
			VC0200
			VC0201
			VC0364 (bfd)
			VC0365 (bfr)
			VC0474 (irgB)
			VC0475 (irgA)
			VC0772 (vibE)
			VC0774
			VC0776 (viuP)
			VC0777 (viuD)
			VC0779 (viuC)
			VC1265
			VC1266
			VC1543
			VC1544 (tonB2)
			VC1545 (exbD2)
			VC1546 (exbB2)
			VC1547
			VC1548
			VC1572
			VC1573 (fumC)
			VC1688
			VC2076
			VC2210 (viuB)
			VC2694 (sodA)
			VCA0231
			VCA0232
			VCA0233
			VCA0576 (hutA)
			VCA0677 (napD)
			VCA0907 (hutZ)
			VCA0908 (hutX)
			VCA0909 (hutW)
			VCA0910 (tonB1)
			VCA0911 (exbB1)
			VCA0912 (exbD1)
			VCA0915 (hutD)
			VCA0916
			VCA0977

2.0   1:1   -2.0

FIG. 2. Global transcriptome analysis reveals that iron homeostasis is affected in the T2S mutant of *V. cholerae*. Genes that were differentially expressed in the NΔeps mutant compared to the wild-type (wt) strain grown in LB medium were identified by SAM analysis using a ≥2.0-fold change in gene expression and a ≤1% FDR as criteria. Expression profiles of genes that are regulated by iron and Fur and that are differentially expressed in the T2S-deficient strain are presented as a heat map using a $\log_2$-based color scale (yellow, induced; blue, repressed). A list of genes regulated by iron and Fur was obtained from previously published gene expression data (50).

(Fig. 2). Among these, genes encoding enterobactin receptors (*irgA*, *vctA*), heme transport proteins (*hutA*, *hutD*, *hutZ*, *hutX*), proteins involved in vibriobactin biosynthesis and transport (*viuC*, *viuD*, *viuB*, *viuP*, *vibE*), and proteins participating in TonB-mediated transport (*exbB1*, *exbB2*, *exbD1*, *exbD2*, *tonB1*, *tonB2*) (81) were the most highly repressed (as much as 13-fold) (Fig. 2; see also Table S1 in the supplemental material). Other Fur-Fe^{2+}-regulated genes of note were those encoding superoxide dismutase (*sodA*), fumarate hydratase (*fumC*), bacterioferritin (*bfr*), and bacterioferritin-associated ferredoxin (*bdf*) (50), which were 5.3-, 7.6-, 2.2-, and 2.7-fold downregulated, respectively (see Table S1 in the supplemental material). Although our microarray analysis did not reveal significant changes (twofold or more) in *fur* transcript levels in the T2S mutant, it is known that Fur is produced constitutively in large amounts under normal growth conditions and that its concentration in the cell varies by less than a factor of 2 even after exposure to oxidative stress (79, 84).

In order to validate the data obtained from our microarray analysis, luciferase transcriptional reporter fusions were constructed by cloning the upstream regions of the *viuP* and *hutA* genes into plasmid pBBR-*lux* as described in Materials and Methods. These genes were selected for the following reasons. *viuP* belongs to a gene cluster, *viuPDGC*, that is preceded by a promoter containing a Fur-binding box (82), and expression of three genes of this operon differed significantly between the T2S mutant and the wild-type strain according to our microarray analysis (expression was 2.2- to 4.9-fold repressed in the mutant [see Table S1 in the supplemental material]). *hutA* was the most strongly downregulated gene in the *eps* mutant (13.1-fold) (see Table S1 in the supplemental material) and has been shown previously to be one of the most responsive Fur-Fe^{2+}-regulated genes (50, 73).

To recapitulate the conditions of our microarray experiments, the activity of each reporter was assessed in wild-type *V. cholerae* N16961 and its T2S mutant, NΔepsD, 1 h following entry into the stationary phase of growth, when the cultures reached an OD_{600} of 4. The activity of the *viuPp* reporter fusion was 7.5-fold lower in the T2S mutant than in the wild-type strain (Fig. 3A). In agreement with previous studies, the overall *hutAp* reporter fusion activity was significantly higher than that for the *viuPp* reporter fusion (Fig. 3B) (50, 73). More
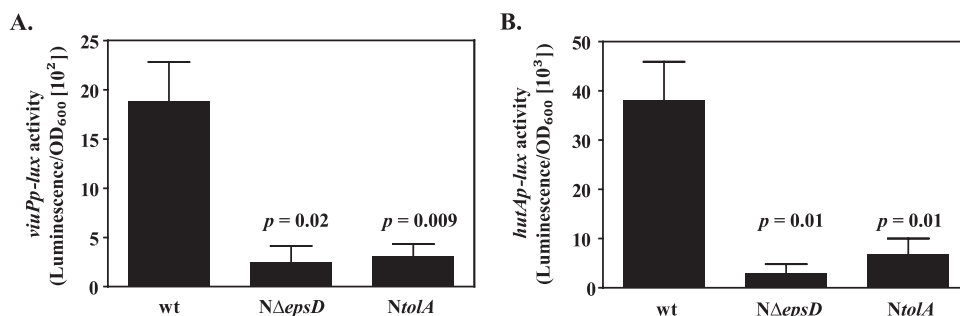
A.

B.

FIG. 3. The reporter activities of Fur- and iron-responsive genes confirm the results of microarray analysis. Wild-type (wt) *V. cholerae* N16961 and its NΔepsD and NtolA mutant strains carrying the luciferase transcriptional fusions *viuPp-lux* (A) and *hutAp-lux* (B) were grown in LB medium, and luminescence was assessed in technical triplicate when the cultures were 1 h into the stationary phase of growth (OD_{600}, 4). The bars show average data derived from four independent experiments; error bars represent the corresponding SEMs. The calculated *P* values displayed above the bars indicate that there were statistically significant differences in reporter activities between the wild-type and mutant strains.

importantly, however, the *hutAp* reporter activity in the NΔ*epsD* strain displayed approximately 13-fold downregulation relative to that in the wild type strain (Fig. 3B). Together, the luciferase transcriptional reporter fusion experiments confirmed the microarray data, in which the expression of the *viuP* and *hutA* genes was 4.9- and 13.1-fold downregulated, respectively (see Table S1 in the supplemental material).

Comparison of our microarray and reporter fusion data with data obtained from transcriptome analysis of iron-depleted *V. cholerae* cells and *fur* mutant cells indicated a possible change in iron homeostasis in the T2S mutants, resulting in an iron buildup in these cells (Fig. 2 and 3). In order to investigate this possibility, we employed the iron-activated antibiotic streptonigrin. This antibiotic exerts its bactericidal effect through interaction with iron, and therefore, an increase in the level of free intracellular ferrous iron correlates with an enhancement in sensitivity to streptonigrin (27, 80, 83). The NΔ*eps* and NΔ*epsD* mutants were much more susceptible to the action of streptonigrin than the wild-type strain (Fig. 4A). At a streptonigrin concentration of 5 μg/ml, the sizes of the inhibition zones displayed by the two T2S mutants were comparable to the size of the zone for wild-type cells that formed around discs immersed with 60 μg/ml streptonigrin (Fig. 4A). The sensitivity to streptonigrin was complemented and restored to the wild-type level by ectopic expression of *epsCDEFGHIJKLMN* and *epsD* in mutant strains NΔ*eps* and NΔ*epsD*, respectively (Fig. 4A). Moreover, we observed that addition of the iron-specific chelator 2,2′-dipyridyl to the medium of the mutant strains restored resistance to streptonigrin almost to the wild-type level (Fig. 4B). These results, along with the microarray and reporter fusion data, further supported the suggestion that the level of intracellular iron is likely higher in the T2S mutants than in the wild-type strain.

Oxidative stress is induced in T2S mutants. Although iron is crucial for the structure and function of many proteins, excess iron can be deleterious to the cells, because it rapidly reacts with ROS, which are a natural consequence of aerobic metabolism, and generates highly toxic hydroxyl radicals via the Fenton reaction (1, 24). Therefore, tight control of iron metabolism is an integral part of the antioxidant defense response (84). Fur provides protection against oxidative damage by inducing the expression of several genes encoding proteins that confer resistance to oxidative stress (74, 75). Our microarray results revealed that the NΔ*eps* mutant displayed significant upregulation (as much as sevenfold) of genes coding for antioxidant proteins, such as catalase (KatB), alkylhydroperoxidase (AhpC), and superoxide dismutase (SodB) (31) (see Table S2 in the supplemental material). To test the hypothesis that *eps* mutants suffer from internal oxidative stress, we first examined their sensitivities to H_2O_2 by measuring the zone of growth inhibition around H_2O_2-immersed paper discs. The cells of T2S mutants were significantly more sensitive to H_2O_2 treatment than wild-type cells (Fig. 5A).

Next, we aimed to measure the level of ROS in exponentially growing cells by using the oxidative-stress-sensitive fluorescent probe H2DCFDA (10, 13, 20). The level of ROS-generated fluorescence was four- to sevenfold greater in the T2S mutants than in the wild-type strain (Fig. 5B). The finding that the level of ROS is higher in T2S mutants provides additional evidence that under conditions where the T2S machinery is inactivated,
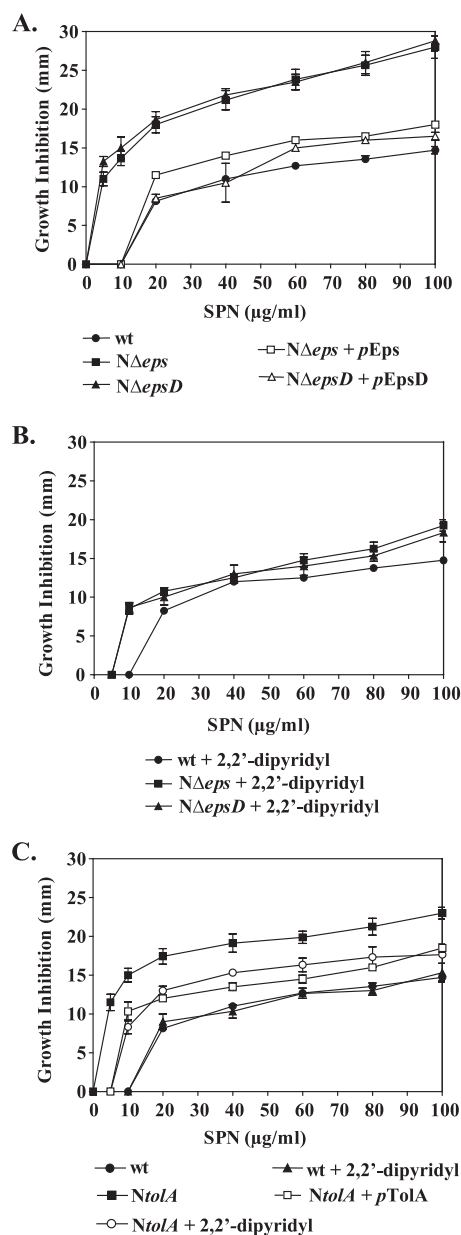

FIG. 4. Susceptibility to streptonigrin (SPN), an indirect measurement of the intracellular free iron content. Wild-type (wt) *V. cholerae* N16961 and T2S mutants (NΔ*eps*, NΔ*epsD*) (A and B) or the N*tolA* mutant (C) were grown in LB medium to mid-log phase (OD$_{600}$, about 0.5), and 100 μl of each culture was spread onto LB agar medium alone (A) or supplemented with the iron-specific chelator 2,2′-dipyridyl to a final concentration of 100 μM (B) or 150 μM (C). Cultures of NΔ*eps*, NΔ*epsD*, and N*tolA* containing plasmid *p*Eps, *p*EpsD, or *p*TolA, respectively, were also spread onto LB agar alone and tested for complementation in the presence of 10 μM IPTG (A and C). Sterile filter paper discs immersed with different concentrations of streptonigrin, as indicated, were placed immediately on the surface of each plate. The diameter of the inhibition zone was scored after 24 h of incubation at 37°C. Each datum point represents the mean and SEM from three to nine separate experiments. In comparison to wild-type *V. cholerae*, both T2S mutants and the N*tolA* strain showed statistically significant increases in susceptibility to streptonigrin at each of the concentrations tested ($P < 0.0001$).
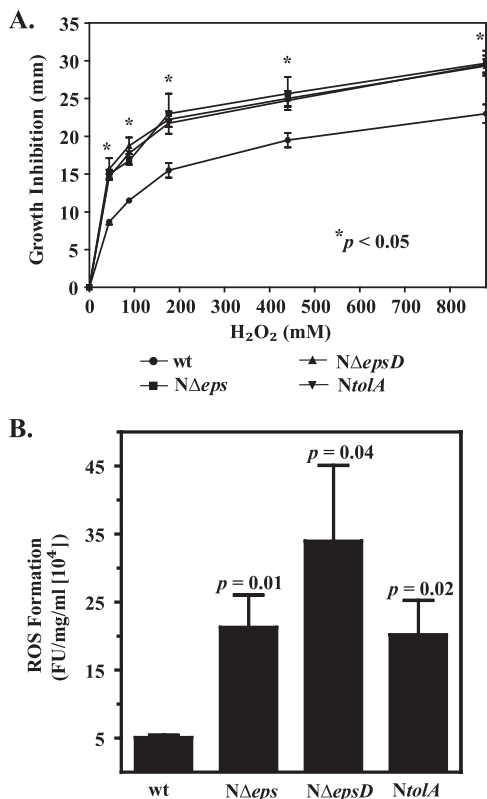
FIG. 5. Sensitivity to hydrogen peroxide and endogenous ROS formation are augmented in T2S- and TolA-deficient strains of *V. cholerae*. (A) Wild-type (wt) *V. cholerae* N16961 and its isogenic T2S-deficient and N*tolA* mutants were grown in LB medium until mid-log phase (OD$_{600}$, about 0.5). Cultures (100 μl) were uniformly distributed on LB agar plates, followed by immediate placing of sterile filter paper discs impregnated with 5 μl of 44, 88, 176, 440, and 880 mM hydrogen peroxide on the surface of the plate. The diameter of the growth inhibition zone was recorded after 24 h of incubation at 37°C. Error bars represent SEMs for four independent experiments, each performed in duplicate. All mutants displayed statistically significantly enhanced susceptibility to hydrogen peroxide relative to that of the parental strain ($P < 0.05$). (B) Intracellular ROS formation was measured in cells growing logarithmically in LB medium and incubated with the oxidative-stress-sensitive probe H2DCFDA as described in Materials and Methods. Intracellular ROS formation was expressed in arbitrary units of fluorescence intensity (FU), and the emission values were normalized to the protein concentration. Means for four independent experiments with corresponding SEMs are presented. The calculated P values shown above each bar indicate statistically significant increases in ROS levels in T2S- and TolA-deficient strains over those in the wild-type strain.
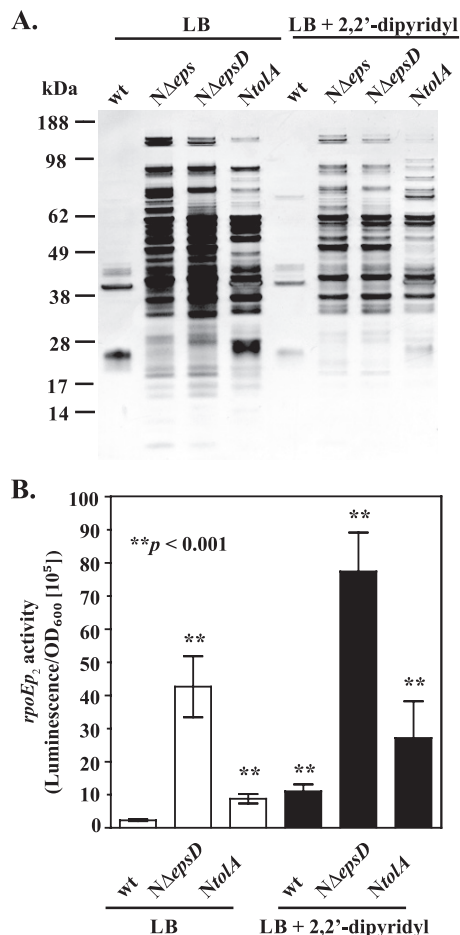

FIG. 6. Outer membrane integrity is compromised regardless of the iron level. (A) Representative protein profiles of the supernatants isolated from stationary-phase cultures of wild-type (wt) *V. cholerae* N16961 and its isogenic NΔ*eps*, NΔ*epsD*, and N*tolA* mutants grown in LB medium without and with the addition of 100 or 150 μM 2,2′-dipyridyl (for the T2S-deficient and N*tolA* mutants, respectively). Samples were loaded onto SDS-PAGE gels by equivalent OD$_{600}$ units, and gels were silver stained. The migration of molecular mass markers is indicated on the left. (B) The expression of the *rpoEp₂-lux* fusion gene was determined in wild-type *V. cholerae* N16961 and its T2S-deficient and N*tolA* mutants grown at 37°C in LB medium alone or supplemented with 100 or 150 μM 2,2′-dipyridyl (for the T2S-deficient and N*tolA* mutants, respectively) to mid-log phase (OD$_{600}$, 0.5). The data are means for three to nine independent experiments (each performed in technical triplicate). Error bars indicate SEMs. The increase in *rpoE* expression was statistically significant for the NΔ*eps*, NΔ*epsD*, and N*tolA* strains regardless of the presence or absence of the iron chelator ($P < 0.001$). Treatment with the iron-specific chelator resulted in the detection of statistically significantly different σ^E activity in the wild-type strain ($P < 0.0001$).

cells are suffering from internal oxidative stress and, as a consequence, display changes in iron homeostasis.

Membrane damage induces oxidative stress. It is well established that accumulation of ROS causes damage to different cellular constituents, including lipids, DNA, and various proteins, particularly iron-sulfur cluster-containing proteins. The oxidative damage of iron-sulfur clusters leads to the release of iron, resulting in changes in iron physiology (31). Therefore, in order to investigate if the alteration in iron homeostasis is a source or a consequence of the compromised membrane integrity of *V. cholerae* T2S mutants, we used SDS-PAGE and silver staining to examine the culture supernatant profiles of

wild-type and T2S-mutants grown in medium supplemented with the iron chelator 2,2′-dipyridyl. In comparison to the situation in the wild-type strain, large amounts of proteins were released to the culture medium when T2S-deficient strains were grown either with or without the iron chelator (Fig. 6A). There was a slight decrease in the amount of proteins leaking out of the mutant strains in the presence of 2,2′-dipyridyl, but the protein profiles remained very similar to those of cultures grown in the absence of the chelator. Leakage of intracellular

proteins to the growth medium is observed in cells with membrane damage and during extracellular stress, when the expression of *rpoE* and the σ^E regulon is induced (47, 65). Therefore, as another indicator of extracellular stress and membrane damage, we used our *rpoEp₂* luciferase reporter fusion (65) to examine *rpoE* expression in the T2S mutant NΔ*epsD* grown in the absence and presence of 2,2′-dipyridyl. Although the presence of the iron-specific chelator rescued the sensitivity to streptonigrin in T2S-deficient cells (Fig. 4B), the expression of *rpoE* remained considerably higher than that in the wild-type strain, indicating that the membrane perturbation persisted regardless of the iron level (Fig. 6B). In fact, expression of *rpoE* was increased in both the wild-type and NΔ*epsD* strains when they were grown with the iron chelator. Furthermore, the reduced growth rate of NΔ*eps* in LB medium was not rescued by the presence of different concentrations of 2,2′-dipyridyl (data not shown). In light of these experiments, we conclude that the changes in iron homeostasis in the T2S mutants of *V. cholerae* most likely occur as a consequence of altered outer membrane integrity. Consistent with this suggestion, in mid-log-phase cultures there was no significant difference in the expression of the Fur-Fe^{2+} regulated *viuP* and *hutA* promoters between wild-type and T2S mutant strains (see Fig. S1 in the supplemental material).

To further test the hypothesis that induction of oxidative stress and misregulation of iron homeostasis are direct consequences of outer membrane damage, we decided to examine a *V. cholerae* mutant that displays outer membrane defects similar to those of *eps* mutants while retaining a functional T2S machinery. Mutations in genes coding for the Tol-Pal system result in loss of outer membrane integrity, which has been evidenced by extracellular release of periplasmic proteins and increased sensitivity to detergents and other membrane-perturbing agents (7, 18, 28, 41, 77). In addition, inactivation of the Tol system renders cells sensitive to high iron concentrations (72). Therefore, to determine if generalized outer membrane damage induces oxidative stress, we constructed a *tolA* mutant of *V. cholerae* N16961 as described previously for strain O395 (28). The newly constructed N*tolA* mutant exhibited a filamentous phenotype (data not shown) and reduced stability of the outer membrane, as has been reported previously for mutations in the *tolA* locus (Fig. 6A) (28). Ectopic expression of *tolA* complemented the defects displayed by N*tolA* mutant cells (data not shown). As with the T2S mutants (65), *rpoE* expression was induced >4-fold in the N*tolA* mutant, as measured by our *rpoEp₂* reporter gene fusion assay (Fig. 6B). Regardless of the outer membrane alterations, the T2S machinery remained functional in the N*tolA* cells (see Fig. S2 in the supplemental material, which shows extracellular secretion of the plasmid-encoded heat-labile enterotoxin EtxB in N*tolA*).

To further explore the role of compromised membrane integrity in the misregulation of iron homeostasis, we first examined the activities of the *viuPp* and *hutAp* reporters in the N*tolA* strain. The luciferase reporter activities were approximately sixfold repressed in the early stationary phase of growth, while no significant changes were detected in mid-log-phase cultures of the N*tolA* mutant (Fig. 3; see also Fig. S1 in the supplemental material). Next, we tested the sensitivity of the N*tolA* mutant to streptonigrin in the absence and presence of the iron chelator. Like the *eps* mutants, cells with an inac-

tivated *tolA* gene displayed increased sensitivity to streptonigrin, which was partially rescued by the presence of 150 μM 2,2′-dipyridyl (Fig. 4C). In addition, plasmid-expressed *tolA* complemented the N*tolA* mutant and restored streptonigrin sensitivity to the wild-type level (Fig. 4C). Together, these experiments suggested that the level of unincorporated iron was also elevated in the N*tolA* strain. Furthermore, the N*tolA* mutant cells showed statistically significantly higher sensitivity to H$_2$O$_2$ (Fig. 5A) and an approximately fivefold greater level of ROS formation than wild-type cells (Fig. 5B). As with T2S mutants, the compromised membrane integrity of the N*tolA* mutant was not suppressed in the presence of 2,2′-dipyridyl, as evidenced by SDS-PAGE and silver staining of supernatants isolated from cultures grown either with or without the iron chelator (Fig. 6A). In agreement with these results, expression of *rpoE* remained induced in N*tolA* regardless of the presence or absence of 2,2′-dipyridyl (Fig. 6B). These data support our hypothesis that alterations in the cell envelope contribute to the induction of internal oxidative stress and the increase in the labile iron pool. Moreover, to show that an alteration in iron homeostasis is a response generally observed with cell envelope damage and is not unique to *V. cholerae*, we also employed an existing *tolA* mutant of the *E. coli* laboratory strain K-12 BW25113 (2). As expected, there was an increase in protein quantity in the culture supernatant of the *tolA* mutant as revealed by SDS-PAGE and silver staining, indicating perturbed outer membrane integrity (see Fig. S3A in the supplemental material), and, in agreement with our hypothesis, the *tolA* mutant displayed increased susceptibility to the iron-activated antibiotic streptonigrin (see Fig. S3B in the supplemental material).

Resolving the role of the T2S system in iron homeostasis was of particular interest, since it has been shown recently that inactivation of the T2S machinery in the marine bacterium *P. tunicata* results in alterations in the levels of two putative TonB receptors and an ABC transporter involved in iron uptake (22). Moreover, it was reported that certain T2S mutants of *E. chrysanthemi* lack a periplasmic FbpA-like iron binding protein and display increased susceptibility to oxidants and streptonigrin in the stationary phase of growth (17). Our comparative analysis of different T2S and N*tolA* mutants exhibiting similarly altered outer membrane integrity, however, suggests that changes in iron homeostasis as well as in oxidative stress are not unique to T2S mutants but rather occur in response to cell envelope perturbation. To investigate the relationship between T2S and iron physiology further, we decided to analyze a T2S mutant of another species, *E. coli*, which does not display apparently altered outer membrane integrity (Fig. 7 A). We found that the susceptibilities to H$_2$O$_2$ and streptonigrin displayed by the T2S mutant of *E. coli* O157:H7 strain EDL 933, EΔ*etpM*, were the same as those of the wild-type strain, indicating that a mutation in the T2S system of *E. coli* O157 does not affect iron homeostasis (Fig. 7B and C). These results add another line of evidence that the T2S system may not be directly involved in iron homeostasis and that the internal oxidative stress and altered iron metabolism observed in T2S mutants of some gram-negative species, including *V. cholerae*, are a consequence of cell envelope perturbation. The reason why T2S mutants of certain species have altered cell envelopes, however, is not clear at this moment, but the alteration may be
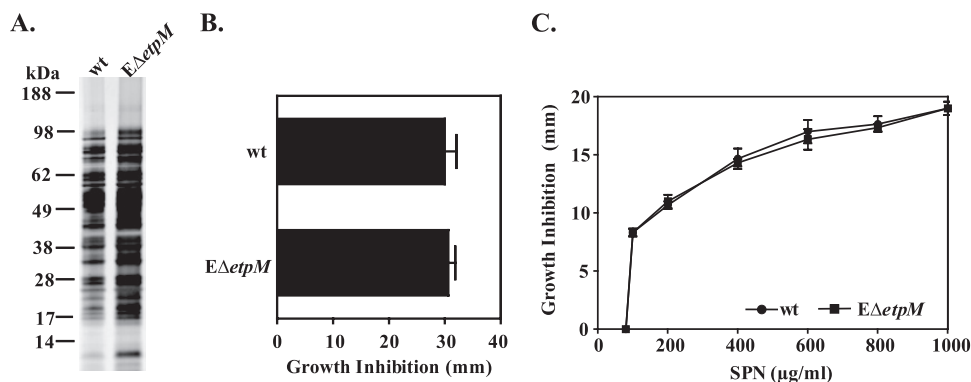
FIG. 7. Iron homeostasis and oxidative stress are not affected in a T2S mutant of *E. coli* O157:H7. (A) Protein profiles of filtered culture supernatants of the *E. coli* O157:H7 parental wild-type (wt) strain and its E∆*etpM* mutant grown in LB medium at 37°C to stationary phase (16 h), revealed by SDS-PAGE and silver staining. Samples were matched by equivalent OD_{600} units. The migration of molecular mass markers is indicated. (B and C) The susceptibilities of the wild-type and E∆*etpM* mutant strains to hydrogen peroxide (B) and streptonigrin (SPN) (C) were tested by uniformly distributing 100 μl of mid-log-phase cultures (OD_{600}, about 0.5) on LB agar plates and then immediately placing sterile filter paper discs impregnated with either 8.8 M hydrogen peroxide or different concentrations of streptonigrin, as indicated, on the surfaces of the plates. The diameter of the inhibition zone was recorded after 24 h of incubation at 37°C. Means and corresponding SEMs for three independent experiments are reported.

the result of uncontrolled activity of periplasmically accumulated T2S substrates, such as proteases and/or chitinases.

It is known that physical or chemical insults to the cell envelope, such as an elevated temperature, pressure, or treatment with antibiotics and antimicrobial peptides, compromise the integrity of the cell envelope and induces several stress regulons, including RpoE, Cpx, RpoS, and PhoP (3, 35, 46, 71). We hypothesized that, like mutations in the *eps* and *tolA* genes, chemical perturbation of the *V. cholerae* cell envelope would induce extracytoplasmic as well as oxidative stress and cause misregulation of iron homeostasis. To test this hypothesis, we first examined *rpoEp₂* reporter activity in wild-type *V. cholerae* N16961 treated with various concentrations of polymyxin B sulfate. We used 100, 200, and 400 U/ml of polymyxin B sulfate, based on our earlier study, which showed that 100 U/ml does not affect the viability of wild-type cells, while treatment with 400 or 800 U/ml results in 26% or 50% inhibition of growth, respectively (65). Wild-type *V. cholerae* cells responded to polymyxin B treatment with >10-fold upregulation of *rpoEp₂* activity at all concentrations tested (Fig. 8A), indicating membrane perturbation. These results are consistent with a previously reported increase in *rpoE* transcription after treatment with sublethal doses of antimicrobial peptides (46). Next, we tested if challenging wild-type cells with polymyxin B affects their sensitivity to H_2O_2. Like T2S and N*tolA* mutants, wild-type *V. cholerae* treated with increasing concentrations of polymyxin B became significantly more vulnerable to H_2O_2 ($P < 0.05$) (Fig. 8B). In addition, streptonigrin-mediated growth inhibition of wild-type cells was significantly increased in the presence of polymyxin B ($P < 0.01$) (Fig. 8C), indicating that chemical perturbation of the cell envelope results in changes in iron metabolism.

Concluding remarks. Our transcriptome profiling and phenotypic analysis demonstrate that inactivation of the T2S system has severe consequences on iron homeostasis and the overall oxidative status of *V. cholerae* cells. In contrast to previous suggestions for other gram-negative bacteria (17, 22), we show that the T2S system is not directly engaged in iron acquisition in *V. cholerae*.

Instead, misregulation of iron homeostasis in *V. cholerae* T2S mutants most likely occurs as a secondary effect related to alterations in the extracytoplasmic compartment.

The complexity of the phenotypes associated with mutations that perturb cell envelope homeostasis suggests that several stress pathways may be induced. Our experimental data are consistent with this suggestion and indicate that at least two global regulators, RpoE and Fur, respond to membrane perturbation caused either by mutations in the T2S system or Tol pathway in *V. cholerae* or by chemical treatment of *V. cholerae* cells with polymyxin B. Since a loss in membrane stability and increased sensitivity to various membrane-permeating agents are also common phenotypes of mutants deficient in other cell envelope constituents and biogenesis systems, such as major lipoprotein (Lpp), lipopolysaccharide synthesis and assembly (Rfa/Waa), the twin-arginine translocation system, and the membrane-derived oligosaccharides (MDO) biogenesis system (37, 38, 69, 70), we propose that these mutants may also suffer from extracytoplasmic stress and increased ROS formation, which cause an imbalance in the labile iron pool. The extracytoplasmic stress may be of various types and may involve signal transduction pathways such as RpoE, CpxAR, BaeRS, Rcs (phosphorelays), or PSP (phage shock response) (43, 57, 58, 60, 61). In fact, activation of both the RpoE and Rcs phosphorelay systems has been observed in *rfa* mutants as well as in MDO-, SurA-, and Tol-deficient strains (8, 12, 19, 43, 52, 78), and induction of oxidative stress has been reported for *waaL* mutants (5). In addition, inactivation of SurA, a periplasmic chaperone required for outer membrane protein biogenesis (67), results in increased sensitivity to iron (72), again suggesting that cell envelope perturbation leads to oxidative stress.

Further support for our model that damage to the cell envelope causes extracytoplasmic stress followed by induction of oxidative stress and alteration in iron homeostasis comes from recent studies of the mechanism of bactericidal antibiotic-mediated cell death. Treatment with penicillin and aminoglycoside antibiotics triggers ROS formation and rapid cell death via a mechanism that appears to involve cross talk between the
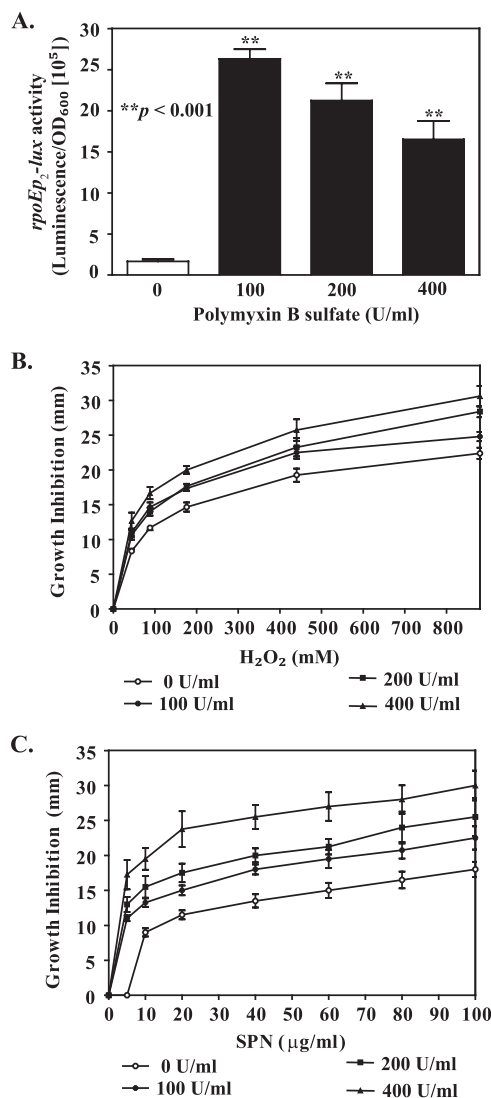
FIG. 8. Extracytoplasmic and oxidative stress responses are triggered in wild-type *V. cholerae* cells following treatment with polymyxin B sulfate. (A) *rpoE* activity was monitored for wild-type *V. cholerae* N16961 grown at 37°C to mid-log phase (OD_{600}, 0.5) in LB medium supplemented with increasing concentrations of polymyxin B sulfate (0, 100, 200, and 400 U/ml). Bars show means and SEMs from four independent experiments. *P* values indicate that the elevation in the envelope stress response in polymyxin B-treated cultures is statistically significant. (B and C) Wild-type *V. cholerae* N16961 was grown in LB medium until mid-log phase (OD_{600}, 0.5), and 100 μl of the culture was spread onto LB agar supplemented with different concentrations of polymyxin B sulfate (0, 100, 200, and 400 U/ml). Sterile filter paper discs immersed with various concentrations of hydrogen peroxide (B) or streptonigrin (SPN) (C) were immediately placed on the surfaces of the plates. The diameter of the growth inhibition zone was scored after 24 h of incubation at 37°C. Each datum point represents the mean and SEMs obtained from five independent experiments (*P* < 0.05).

extracytoplasmic stress regulator Cpx and the redox-responsive Arc system (34, 35). Furthermore, the finding that CpxAR represses the expression of the *efeUOB* operon and activates the transcription of *ftnB*, genes that encode proteins involved in iron transport and storage, respectively, provides additional

support for our model (55). In addition, induced expression of *rpoE* and oxidative stress genes has been observed upon exposure of bacterial cells to high temperatures (4, 39, 56), possibly as a result of disruption of electron transport and an increase in ROS formation (56). Finally, an increasing number of studies reveal functional integration and perhaps cross talk between different global regulons, suggesting that bacteria might respond to various cues by triggering common protective stress responses (14, 45, 54). Taken together, many types of insults to the cell envelope, caused either by physical or chemical damage, antibiotic treatment, or genetic modifications, induce a variety of signaling pathways that ultimately lead to common downstream consequences resulting in internal oxidative stress and changes in iron homeostasis.

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