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The Transcriptional Regulator, CosR, Controls Compatible Solute Biosynthesis and Transport, Motility and Biofilm Formation in *Vibrio cholerae*

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SUMMARY

Vibrio cholerae inhabits aquatic environments and colonizes the human digestive tract to cause the disease cholera. In these environments, *V. cholerae* copes with fluctuations in salinity and osmolarity by producing and transporting small, organic, highly soluble molecules called compatible solutes, which counteract extracellular osmotic pressure. Currently, it is unclear how *V. cholerae* regulates the expression of genes important for the biosynthesis or transport of compatible solutes in response to changing salinity or osmolarity conditions. Through a genome-wide transcriptional analysis of the salinity response of *V. cholerae*, we identified a transcriptional regulator we name CosR for compatible solute regulator. The expression of *cosR* is regulated by ionic strength and not osmolarity. A transcriptome analysis of a $\Delta cosR$ mutant revealed that CosR represses genes involved in ectoine biosynthesis and compatible solute transport in a salinity-dependent manner. When grown in salinities similar to estuarine environments, CosR activates biofilm formation and represses motility independently of its function as an ectoine regulator. This is the first study to characterize a compatible solute regulator in *V. cholerae* and couples the regulation of osmotic tolerance with biofilm formation and motility.

INTRODUCTION

All microorganisms must cope with variable environments and one important condition that microbial cells must adapt to is osmolarity. Many bacteria adapt to increased osmolarity by importing or producing compatible solutes to counteract osmotic pressure (Wood, 1999). Compatible solutes are small, highly soluble organic molecules that act to stabilize intracellular levels of water and turgor pressure without disturbing cellular function (Brown, 1976). Compatible solutes can be divided into various groups including carbohydrates, polyols, heterosides, amino acids and amino acid derivatives (da Costa et al., 1998; Sleator and Hill, 2002). Common amino acids used as compatible solutes are proline and glutamate. The amino acid derivatives glycine betaine and ectoine are also widely used by bacteria to counteract high osmolarity (Lucht and Bremer, 1994; Pflughoeft et al., 2003; Kapfhammer et al., 2005; Wood, 2007).

The halo-tolerant bacterium *V. cholerae* causes the disease cholera and thrives in aquatic environments. Salinity is a significant factor affecting the growth and distribution of *V. cholerae* in the environment (Singleton et al., 1982a; Miller et al., 1984; Louis et al., 2003; Huq et al., 2005), and cholera epidemics correlate with increased salinity in riverine and estuarine habitats (Faruque et al., 1998; Lobitz et al., 2000; Pascual et al., 2002). *V. cholerae*

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growth and survival is optimal in the range of salinities present in estuarine environments (Singleton et al., 1982a; Louis et al., 2003), although *V. cholerae* is also capable of growth in both fresh and seawater salinities (Singleton et al., 1982b; Alsina and Blanch, 1994; Vital et al., 2007). During infection, *V. cholerae* must adapt to a range of salinities or osmolarities as it passes through the human digestive tract and is released back into the environment (Barua and Burrows, 1974; Gupta and Chowdhury, 1997). Indeed, osmolarity is one factor hypothesized to induce virulence factor production during *V. cholerae* infection. Additionally, virulence factor gene expression and production is modulated by various salinity concentrations (Tamplin and Colwell, 1986; Miller and Mekalanos, 1988; Shikuma and Yildiz, 2009). Thus, salinity is a significant factor *V. cholerae* must cope with as a facultative human pathogen.

To survive estuarine conditions and passage through the human digestive tract where salinity concentrations often vary, *V. cholerae* synthesizes and imports compatible solutes. *V. cholerae* possesses ectoine biosynthesis genes including *ectA*, *ectB*, *ectC* and a putative aspartokinase (VCA0822) that are similar to genes in the ectoine biosynthesis pathway determined for several moderate halophiles (Peters et al., 1990; Louis and Galinski, 1997; Canovas et al., 1998; Ono et al., 1999; Kuhlmann and Bremer, 2002; Pflughoeft et al., 2003). In *V. cholerae*, Δ *ectA* mutants exhibit a growth defect in defined media lacking compensatory compatible solutes glutamate, proline, and aspartate (Pflughoeft et al., 2003). *V. cholerae* also possesses homologs of *opuD* from *Bacillus* species and *putP* from *Vibrio vulnificus*, both of which were shown to transport proline and glycine betaine and facilitate growth (Kaphammer et al., 2005). Compatible solutes are therefore important for *V. cholerae* to grow and persist in environments of variable osmolarities.

Another factor that facilitates *V. cholerae* survival and transmission is biofilm formation. Through a genome-wide transcriptome analysis, we showed previously that salinity affects the expression of biofilm genes in *V. cholerae* (Shikuma and Yildiz, 2009). Furthermore, salinity modulates *V. cholerae* surface attachment and biofilm architecture (Shikuma and Yildiz, 2009). In microcosm experiments, the attachment to zooplankton enhances *V. cholerae* survival (Huq et al., 1983) and biofilm growth improves resistance to protozoan grazing (Matz et al., 2005). Furthermore, mutants defective in biofilm formation are attenuated in the colonization of an infant mouse colonization model (Fong et al., 2010) and *V. cholerae* biofilm aggregates are more infectious than their free-living counterparts (Colwell et al., 2003; Faruque et al., 2006).

Although compatible solute transport and biosynthesis are important for *V. cholerae* survival as a facultative human pathogen, it remains unclear how *V. cholerae* regulates compatible solute genes in response to salinity or osmolarity. In this work, we identify and characterize a previously undescribed MarR-type transcriptional regulator, which we call CosR for Compatible Solute Regulator. *cosR* expression is controlled specifically by ionic strength and not osmolarity. Whole genome transcriptome profiles revealed that CosR represses the expression of the compatible solute transporter, *opuD* and the ectoine biosynthesis genes. Furthermore, CosR represses motility and activates biofilm formation independently of its regulatory role in ectoine biosynthesis. *V. cholerae* therefore utilizes CosR to adapt to environments of varying salinity and for activating biofilm growth in the marine environment.

RESULTS AND DISCUSSION

Identification of CosR

Salinity is an important factor in *V. cholerae* environmental occurrence and incidence of infection, and we previously described the *V. cholerae* transcriptional response to salinity

(Shikuma and Yildiz, 2009). To expand our insight into the mechanisms by which *V. cholerae* responds to salinity, we searched for transcriptional regulators whose expression correlated with salinity concentration. Expression of one such regulator, encoded by VC1278, increased in a salinity-dependent manner (Fig. 1A) (Shikuma and Yildiz, 2009). We named this gene *cosR* (compatible solute regulator) because our results indicate that CosR regulates genes involved in compatible solute production and transport. *cosR* expression was lowest in cells grown in 0 M NaCl, and increased in 0.2 M NaCl (2.4-fold) and 0.5 M NaCl (6.2-fold).

The expression of *cosR* correlated with NaCl concentration; however, *cosR* expression could be dependent on NaCl concentration, ionic concentration or medium osmolarity. To determine whether *cosR* expression would change in response to the concentration of other solutes in the medium, we cultured wild-type *V. cholerae* cells in NaCl, KCl, or the non-metabolizable osmolyte, lactose, and compared the levels of *cosR* mRNA using quantitative PCR (qPCR). Lactose was used previously to increase the osmolarity of the medium without altering ionic strength to observe the differential production of virulence factors (TcpA and cholera toxin) in *V. cholerae* (Miller and Mekalanos, 1988). In agreement with microarray data, *cosR* levels increased in cells grown in 0.2 M NaCl (3.0-fold) and 0.5 M NaCl (4.6-fold) when compared to 0 M NaCl (Fig. 1B). Similarly, levels of *cosR* increased as KCl concentrations increased from 0 M KCl to 0.2 M KCl (2.7-fold) and 0.5 M KCl (4.1-fold). In contrast, cells grown in media containing 0.4 M lactose exhibited similar levels of *cosR* expression when compared to 0 M NaCl. The osmolarity of 0.2 M NaCl or KCl in solution is equivalent to 0.4 M lactose. Expression of *cosR* is therefore dependent on the ionic strength of the medium, and appears not to be regulated by medium osmolarity.

CosR is predicted to be a MarR-type (multiple antibiotic resistance) transcriptional regulator and homologs exist in other related *Vibrio* species (Fig S1). Moreover, the genomes of other *Vibrios* also encode similar osmoadaptation genes (Naughton et al., 2009), leaving open the possibility that this regulator might perform similar functions in related bacteria. A recent study identified another MarR-type regulator, EctR1, from the halotolerant obligate methanotroph, *Methylobacterium alcaliphilum*, which was also shown to regulate ectoine biosynthesis genes (Mustakhimov et al., 2010). EctR1 exhibits a 51% sequence identity to CosR (Fig S1) and both EctR1 and CosR repress ectoine biosynthesis genes in response to NaCl concentration (see below). While *ectR1* is divergently transcribed from the *ectABC* and aspartokinase genes of *M. alcaliphilum*, *cosR* is divergently transcribed from *opuD* encoding a compatible solute transporter in *V. cholerae* (Fig 1C). Furthermore, the *ectABC* and aspartokinase operon is located on chromosome-II of *V. cholerae*, while *cosR* resides on chromosome-I.

CosR represses compatible solute transport and biosynthesis genes

To determine the function of CosR, we generated a strain with an in-frame deletion of *cosR*. Growth of wild type and $\Delta cosR$ strains were similar when grown in LB with 0.2 M NaCl or 0.5 M NaCl (Fig S2). To identify the CosR regulon, we compared transcriptional profiles of the $\Delta cosR$ strain to that of the wild-type strain when grown in LB media supplemented with 0.2 M NaCl or 0.5 M NaCl. Thirty eight genes were differentially regulated 2-fold between $\Delta cosR$ and wild type (Table 1). One of these genes was previously identified to encode a compatible solute transporter, OpuD (Kapfhammer et al., 2005). *opuD* expression in the mutant was 6.2- and 1.9-fold upregulated in 0.2 M NaCl and 0.5 M NaCl, respectively, when compared to the wild type (Table 1). In the sequenced genome of *V. cholerae* strain N16961 (Heidelberg et al., 2000), *cosR* and *opuD* are divergently transcribed and have a 357 bp intergenic region between them. Genes in the ectoine biosynthesis operon, consisting of *ectA*, *ectB*, *ectC* and VCA0822 (Pflughoeft et al., 2003), were also upregulated in the *cosR* mutant in both 0.2 M and 0.5 M NaCl, indicating that CosR is a repressor of genes involved

in production/transport of compatible solutes (Table 1). All genes in the ectoine biosynthesis operon were upregulated in the mutant compared to the wild type in both 0.2 M and 0.5 M NaCl treatments. However, we did observe variation in the magnitude of expression between genes predicted to be the same operon, which we suspect could be due to inherent variability in microarray data collection (Beckman et al., 2004; Morey et al., 2006). Altogether, these results suggest that CosR is a repressor of compatible solute transport and biosynthesis genes.

It is noteworthy that CosR did not solely regulate genes involved in compatible solute production or transport (Table 1). Other genes regulated by CosR included the porin gene, *ompT* (which was 2.2-fold downregulated in the mutant in 0.2 M NaCl) and the transcriptional regulator *cpxR* (which was 2.0-fold upregulated in the mutant in 0.5 M NaCl). The Cpx two-component system is well studied in *E. coli* and medium osmolarity induced by NaCl or sucrose was shown to stimulate this regulon (Prigent-Combaret et al., 2001). In contrast, the *V. cholerae* Cpx system is specifically activated by chloride ions and not osmolarity (Slamti and Waldor, 2009). We show that NaCl and KCl regulate *cosR* expression and CosR regulates *cpxR* (Table 1). We therefore speculate that the Cpx system and CosR might regulate each other's expression.

To substantiate the transcriptional profiling results of the $\Delta cosR$ mutant, qPCR was conducted using RNA isolated from wild type and $\Delta cosR$ strains grown in 0.2 M NaCl and 0.5 M NaCl. In both salinities, the *ectA* and VCA0822 genes had greater message levels in the $\Delta cosR$ mutant when compared to wild type (Fig. 2A and 2B). De-repression of the *ectA* operon was most apparent in the $\Delta cosR$ mutant grown in 0.2 M NaCl, where ectoine biosynthesis genes are normally repressed. The difference in *ectA* and VCA0822 expression between $\Delta cosR$ and wild type strains as quantified by qPCR were not as pronounced as those observed by microarray analyses. We suspect that differences in quantification and normalization methods factor into differences we observed in gene expression. Nonetheless, we do observe similar trends in gene expression with both methods. Altogether, these results corroborate microarray expression profile results of the $\Delta cosR$ mutant, where *cosR* represses the ectoine biosynthesis operon.

To support the gene expression results, we quantified the cellular levels of ectoine in wild type, $\Delta cosR$ and $\Delta ectA$ strains using high-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS). In cells grown in M9 minimal media supplemented with 0.2 M NaCl, ectoine levels were 3.5-fold greater in a $\Delta cosR$ mutant when compared to wild type cells, while ectoine levels were undetectable in a $\Delta ectA$ strain (Fig 2C). These results indicate that CosR represses the biosynthesis of ectoine in *V. cholerae*.

CosR complements a $\Delta cosR$ mutation

To verify that a *cosR* deletion caused derepression of the *ect* operon, the $\Delta cosR$ mutant was complemented *in trans* with a plasmid containing the putative *cosR* promoter and *cosR* gene. Introduction of *cosR* to the $\Delta cosR$ mutant *in trans* decreased the expression of *ectA* and VCA0822 by 2.0- and 1.9-fold, respectively, compared to the $\Delta cosR$ strain with the vector alone (Fig 3). These results indicate that the addition of *cosR in trans* is able to partially complement the expression levels of the *ectA* operon in a $\Delta cosR$ mutant. The activity of some transcriptional regulators is known to be modulated by small molecule ligands. Since CosR regulates compatible solute production in *V. cholerae*, we hypothesized that CosR activity might be modulated by ectoine, proline or glycine betaine, however we observed no difference in expression of *opuD* upon addition or removal of these compatible solutes (Fig S3).

One puzzling aspect of CosR regulation is that *cosR* and the compatible solute genes are upregulated in high salinity, yet CosR is a repressor of the compatible solute genes. We speculate that CosR is involved in a negative autoregulatory loop, which facilitates rapid response times and reduces variation in gene expression (Fig 6) (Yosef and Regev, 2011). This regulatory system makes sense in the context of the *V. cholerae* lifestyle, as rapid response times are likely important to counteract salinity or osmotic shifts in estuarine environments due to tide and storm surge, or during passage through the human digestive tract. The mechanism by which CosR controls compatible solute biosynthesis/transport genes appears to be complex and we are currently working to elucidate this regulatory pathway.

CosR activates biofilm formation in artificial seawater

V. cholerae depends on its ability to form biofilms for its lifestyle as a facultative human pathogen and normal inhabitant of aquatic environments. We therefore asked whether CosR is important for *V. cholerae* to form biofilms. Wild type and $\Delta cosR$ strains were inoculated into once-through flow-cell chambers containing either LB supplemented with 0.2 M NaCl or 50% artificial seawater media (ASW), which mimics conditions found in estuarine environments. In ASW, wild-type *V. cholerae* formed robust biofilms after 72 h, while the $\Delta cosR$ mutant was severely defective in biofilm growth as observed by Confocal Laser Scanning Microscopy (CLSM) (Fig 4A). These results were confirmed using image analysis software to quantify biofilm properties (Table 2). The growth rate of wild type and $\Delta cosR$ was similar in 50% ASW liquid cultures (Fig S2), suggesting that the biofilm defect of the $\Delta cosR$ mutant was not due to differences in growth in this medium. In contrast to ASW-grown biofilms, wild type and $\Delta cosR$ strains did not exhibit differences in biofilm formation when grown with LB medium in flow-cell chambers (Fig 4B, Table 2). These results suggest that CosR activates biofilm formation when grown in ASW.

Since CosR is a repressor of ectoine biosynthesis and a $\Delta cosR$ mutant overproduces ectoine, we asked whether $\Delta ectA$ or $\Delta cosR\Delta ectA$ strains also exhibited biofilm formation phenotypes. When grown in ASW, the $\Delta ectA$ strain was defective in biofilm formation when compared to wild type (Fig 4 and Table 2). Moreover, $\Delta cosR\Delta ectA$ biofilms were more defective in biofilm formation when compared to wild type, $\Delta cosR$ or $\Delta ectA$ strains (Fig 4 and Table 2), suggesting that mutations in $\Delta cosR$ and $\Delta ectA$ additively affect biofilm formation in *V. cholerae* in ASW. Growth of $\Delta ectA$ and $\Delta cosR\Delta ectA$ were similar to wild type in ASW liquid cultures (Fig S2), suggesting that biofilm defects were not due to differential growth of the mutant strains. When grown in a flow cell in LB medium, $\Delta ectA$ and $\Delta cosR\Delta ectA$ strains did not show biofilm defects (Fig 4B, Table 2). These results suggest that the positive effect of CosR on biofilm formation in ASW is independent of ectoine production. Furthermore, the presence of ectoine in the cell, at normal wild type levels or elevated levels in a $\Delta cosR$ mutant, appears to be sufficient to activate biofilm formation. In contrast, above results suggest that the lack of ectoine in a $\Delta ectA$ mutant affects biofilm formation. In agreement with our findings, Kapfhammer et al. (2005) showed previously that ectoine or another compatible solute, glycine betaine, could stimulate *vps* expression and biofilm formation in *V. cholerae*. Although no genes known to be directly involved in biofilm formation were differentially regulated between $\Delta cosR$ and wild-type grown in LB medium (Table 1), CosR might regulate genes important for biofilm formation in cells grown in ASW under flow cell conditions, or regulate biofilm formation through a post-transcriptional mechanism independently of its regulatory effect on ectoine production.

Biofilm formation is a key factor in both the environmental persistence and transmission of *V. cholerae*. We previously showed that salinity modulates biofilm architecture in *V. cholerae* (Shikuma and Yildiz, 2009), and we show here that CosR is important for *V. cholerae* to form biofilms in artificial seawater microcosms. CosR might therefore play a

role in both the environmental survival and transmission throughout the lifecycle of *V. cholerae*.

CosR negatively regulates motility

Since biofilm production and motility are often inversely regulated and motility is important for environmental survival and infectivity of the human host (Richardson, 1991; Gardel and Mekalanos, 1996; Silva et al., 2006), we tested the motility phenotype of $\Delta cosR$ on LB soft agar motility plates containing a range of NaCl concentrations. Wild type showed the highest motility zone diameter at a median salinity of 0.2 M NaCl (Fig 5A). Furthermore, the motility zone of $\Delta cosR$ was greater than that of wild type in salinities between 0.1 and 0.3 M NaCl, while $\Delta cosR$ motility was similar to wild type in low (0 M) or high (0.5 M NaCl) salinities (Fig 5A). When grown at 0.2 M NaCl, the motility phenotype of $\Delta cosR$ could be complemented by *cosR* in trans (Fig 5B), confirming that *cosR* represses motility. Although we observed a decrease in motility diameter for both wild type and $\Delta cosR$ strains when ampicillin was included in the growth medium for plasmid maintenance, there was still a significant difference in motility between wild type and $\Delta cosR$ strains when each contained the empty vector. We then hypothesized that the motility phenotype of a $\Delta cosR$ mutant was due to the overproduction of ectoine, and therefore additionally tested the motility phenotype of $\Delta ectA$ and $\Delta cosR\Delta ectA$ strains in LB containing 0.2 M NaCl. The $\Delta ectA$ motility zone diameter was no different than wild type, and $\Delta cosR\Delta ectA$ motility zone diameter was no different than the $\Delta cosR$ mutant (Fig 5C). These results indicate that CosR is a salinity-dependent repressor of motility and this phenotype is not due to the overproduction of ectoine.

In some microbial pathogens, genes important for compatible solute transport are thought to facilitate infection by mediating stress encountered in the infection process (Sleator and Hill, 2002). In pathogenic *Escherichia coli* and *Staphylococcus aureus*, mutations in compatible solute transporters were shown to reduce colonization and infection in model virulence assays (Culham et al., 1998; Bayer et al., 1999). Deletions of multiple compatible solute transporters in *Listeria monocytogenes* attenuated virulence in a murine infection model (Wemekamp-Kamphuis et al., 2002). It is likely that *V. cholerae* also uses CosR for the regulation of compatible solute transport and biosynthesis in response to various osmotic stresses during its infectious lifecycle. Furthermore, this study implicates CosR as a link between compatible solute production/transport and the regulation of biofilm formation and motility.

While *cosR* expression is activated in response to ionic strength, the expression of *oscR*, encoding another regulator important for the salinity response of *V. cholerae*, is inversely correlated with medium osmolarity (Fig 6) (Shikuma and Yildiz, 2009). *V. cholerae* can therefore distinguish medium ionic strength from osmolarity to differentially activate these regulators. Furthermore, *cosR* and *oscR* are inversely expressed as salinity increases. Given the importance of salinity and osmolarity in the lifestyle of *V. cholerae*, it makes sense that this bacterium would have the means for differentiating between these two signals. CosR also activates biofilm formation and represses motility in salinities between 0.1 and 0.3 M NaCl. This regulation contrasts with OscR, which represses biofilm formation and activates motility under low osmolarity conditions (Shikuma and Yildiz, 2009). *V. cholerae* therefore modulates its behavior in different salinity/osmolarity conditions by differentially activating CosR and OscR.

Conclusion

V. cholerae depends on its ability to sense and respond to various salinities and osmolarities as an aquatic bacterium and human gastrointestinal pathogen. CosR represses compatible

solute gene expression and production in a salinity dependent manner (Fig 6). This work is the first account of a key mechanism by which *V. cholerae* senses and responds to various salinities through the production of compatible solutes for environmental survival. Biofilm formation and motility are important for the environmental survival, transmission and infectivity of *V. cholerae*, and we also show that CosR is important for these phenotypes. CosR is therefore important for *V. cholerae* to cope with osmotic stress and is likely important for *V. cholerae* survival as a facultative human pathogen.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions

The bacterial strains and plasmids used in this study are listed in Table S1. All *V. cholerae* and *E. coli* strains were grown aerobically, at 30°C and 37°C, respectively, unless otherwise noted. Luria Bertani (LB) growth medium (1% Tryptone, 0.5% Yeast Extract) pH 7.5 was supplemented with 0.2 or 0.5 M NaCl, 0.2 or 0.5 M KCl or 0.2 or 0.5 M D-lactose. D-lactose was used as a non-metabolizable osmolyte (Shikuma and Yildiz, 2009). LB-agar and soft agar plates contained 1.5% and 0.3% (wt/vol) granulated agar (Difco), respectively. Concentrations of antibiotics used were as follows: ampicillin (100 µg/ml), rifampicin (100 µg/ml), and gentamicin (30 µg/ml). M9 minimal medium was adapted from Gerhardt et al. (1994) and consisted of 42 mM Na₂HPO₄, 22 mM KH₂PO₄, 19 mM NH₄Cl, 1 mM MgSO₄, 0.1 mM CaCl₂, 1X MEM Vitamin solution (Mediatech), 0.2% DL-lactate and 0.2 M NaCl. Artificial seawater was described previously (Shikuma and Yildiz, 2009), which was adapted from (Reichelt and Baumann, 1973; Lupp et al., 2002), and contained 300 mM NaCl, 50 mM MgSO₄, 10 mM KCl, 10 mM CaCl₂ and 0.333 mM K₂HPO₄ and these components were used at 50% concentrations to replicate estuarine conditions. Tryptone (100 mg/L) and yeast extract (50 mg/L) were used as carbon and nitrogen sources (Singleton et al., 1982a; Mourino-Perez et al., 2003; Shikuma and Yildiz, 2009).

DNA manipulations

Oligonucleotides used in the present study were purchased from Bioneer Corporation and are listed in Table S2. Restriction enzymes, DNA modification enzymes and Phusion High-Fidelity DNA polymerase were purchased from New England Biolabs. DNA sequencing was carried out by Sequetech Corporation.

Generation of in-frame deletion, chromosomal fusion and point mutation strains

In-frame deletion strains were generated according to previously published protocols (Miller and Mekalanos, 1988; Fullner and Mekalanos, 1999; Fong and Yildiz, 2007; Shikuma and Yildiz, 2009). All strains were verified by PCR. Plasmid sequences were verified by DNA sequencing by Sequetech Corporation.

β-galactosidase assays

β-galactosidase assays were performed and Miller units calculated as described previously (Shikuma et al., 2009) which is similar to the procedure described by Miller (Miller, 1972). The assays were repeated with three biological replicates and eight technical replicates.

RNA isolation

V. cholerae cells were grown aerobically overnight in LB supplemented with 0.2 M NaCl. Cultures were diluted 1:200 in fresh LB media containing concentrations of NaCl as indicated and grown aerobically at 30°C with shaking at 200 rpm to an OD₆₀₀ of 0.3 to 0.4, diluted 1:200 once more into fresh media containing the same NaCl concentration and harvested at an OD₆₀₀ of 0.3 to 0.4. Two milliliter aliquots of cultures were collected by

centrifugation for 2 min at room temperature. Cell pellets were immediately resuspended in 1 ml Trizol reagent (Invitrogen) and stored at -80°C . Total RNA was isolated from the cell pellets according to the manufacturer's instructions (Invitrogen). Contaminating DNA was removed by incubating RNA with RNase-free DNase I (Ambion), and an RNeasy Mini kit (QIAGEN) was used to clean up RNA after DNase digestion.

Gene expression profiling

Microarrays used in this study were composed of spotted 70-mer oligos representing the open reading frames present in the *V. cholerae* strain N16961 genome and were printed at UCSC (Beyhan et al., 2006b). Whole-genome expression analyses were performed as described previously (Beyhan et al., 2006a; Shikuma and Yildiz, 2009). Differentially regulated genes were determined using three biological replicates and two technical replicates for each treatment (6 data points for each spot) using a 2-fold difference in gene expression and 3% false discovery rate (FDR) as cut-off values.

Quantitative PCR (qPCR)

qPCR was performed as described previously (Shikuma and Yildiz, 2009). Briefly, cDNA was produced from 0.25 to 1 μg of each RNA sample using an iScript cDNA Synthesis Kit (Bio-Rad). The product was diluted with water (1:4), and 4 μl was used as a template with 12 pmol of each primer in a subsequent PCR reaction using Expand High Fidelity PCR System (Roche). PCR reaction conditions were as follows: 94°C for 2 min, then 25 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec, and a final 72°C for 2 min. The amplified products were analyzed on a 1.5% agarose gel and quantified using ImageQuant software (Molecular Dynamics). Intensities of each DNA band were normalized to the corresponding *gyrA* band. At least three biological replicates were conducted for each treatment tested and reactions lacking reverse transcriptase were used as negative controls.

Determination of cellular ectoine levels

Cell cultures were grown in 25 ml M9 minimal media supplemented with 0.2 M NaCl shaking at 200 rpm at 30°C for 20 h. Extraction of ectoine from cells was performed using a modified Bligh and Dyer technique (Bligh and Dyer, 1959; Kuhlmann and Bremer, 2002). Briefly, 1 ml of cell cultures was pelleted by centrifugation at 16,100 $\times g$ for 15 min at room temperature. Replicate samples were kept for protein quantification using a BCA Protein Assay Kit (Pierce). 400 μl of extraction mixture (methanol/chloroform/water 10:5:4, by vol) was added to samples and vigorously shaken for 45 min. 130 μl chloroform and 130 μl water were added followed by vigorous shaking for 30 min. Phase separation was performed by centrifugation at 16,100 $\times g$ for 30 min at room temperature. 300 μl of aqueous phase was recovered, lyophilized, and resuspended in 100 μl of HPLC grade water.

Samples were analyzed by high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) on a Thermo-Electron Finnigan LTQ mass spectrometer (Thermo, San Jose, CA) coupled with a surveyor HPLC (Thermo, San Jose, CA). Twenty μl of each sample was injected to a Nucleodur NH2 column (4.6 mm $\times$ 150 mm, Macherey-Nagel). The gradient was started at 5% acetonitrile with 0.1 % formic acid and a linear gradient to 80% acetonitrile was achieved in 10 min at a flow rate of 400 $\mu\text{l}/\text{min}$ at 25°C . Ectoine (143.2 m/z) was detected by selected ion-monitoring analyses (SRM) ($m/z = 141.7$ to 144.7) in positive mode and identified by the fragmentation pattern in the second MS (MS/MS). The electrospray voltage was set to 5.0 kV, and a data-dependent selection was monitored to select the most abundant three ions from SRM Scan for MS/MS analysis. The collision-induced dissociation (CID) at normalized collision energy of 35% was used for MS/MS. The retention time and standard curve were determined using commercially available ectoine (Sigma) with concentrations of 5, 10, 30, 70, 100, 300, 700, 1000 and 1200

μM. Ectoine levels were normalized to total protein per ml of culture. Quantification was performed with at least 3 biological replicates.

Motility assays

LB motility plates (0.3% agar) were used to determine the motility of bacterial strains (Wolfe and Berg, 1989). Single colonies were stabbed into LB motility plates containing the indicated NaCl concentrations and antibiotics, where appropriate, and incubated at 30°C for 16 h. The migration zone diameter was then measured for each strain analyzed. Quantification was performed with at least 4 biological replicates and repeated independently at least twice.

Flowcell biofilms

V. cholerae biofilms in flow chambers were produced as described previously (Heydorn et al., 2000a; Beyhan et al., 2007). Briefly, GFP-tagged *V. cholerae* strains were grown overnight in 25 ml of 50% artificial seawater (ASW) at 25°C or 0.2 M NaCl LB at 30°C and 200 rpm. Sterilized flow cell chambers were inoculated with 350 μl of cell suspension in 50% artificial seawater at an OD₆₀₀ of 0.08 to 0.1 or 350 μl of cell suspension diluted with 0.2 M NaCl LB to an OD₆₀₀ of 0.02. The chambers were then inverted and the cells were allowed to attach without flow for 1 h. Flow was then started at a rate of 4.5 ml/h. Flow cell experiments were conducted at room temperature and images were acquired at the timepoints indicated. Confocal images of biofilms were captured with a LSM 5 PASCAL laser scanning microscope (Zeiss) at 488 nm excitation and 543 nm emission wavelengths. Flow cell experiments were conducted with at least two biological replicates. Three dimensional images of biofilms were reconstructed using Imaris software (Bitplane) and quantified using COMSTAT (Heydorn et al., 2000b).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

- Alsina M, Blanch AR. A set of keys for biochemical identification of environmental *Vibrio* species. J Appl Bacteriol. 1994; 76:79–85. [PubMed: 8144409]
- Barua, D.; Burrows, W., editors. Cholera. W. B. Saunders Company; Philadelphia, London, Toronto: 1974.
- Bayer AS, Coulter SN, Stover CK, Schwan WR. Impact of the high-affinity proline permease gene (putP) on the virulence of *Staphylococcus aureus* in experimental endocarditis. Infect Immun. 1999; 67:740–744. [PubMed: 9916085]
- Beckman KB, Lee KY, Golden T, Melov S. Gene expression profiling in mitochondrial disease: assessment of microarray accuracy by high-throughput Q-PCR. Mitochondrion. 2004; 4:453–470. [PubMed: 16120406]
- Beyhan S, Tischler AD, Camilli A, Yildiz FH. Transcriptome and phenotypic responses of *Vibrio cholerae* to increased cyclic di-GMP level. J Bacteriol. 2006a; 188:3600–3613. [PubMed: 16672614]

- Beyhan S, Tischler AD, Camilli A, Yildiz FH. Differences in gene expression between the classical and El Tor biotypes of *Vibrio cholerae* O1. *Infect Immun*. 2006b; 74:3633–3642. [PubMed: 16714595]
- Beyhan S, Bilecen K, Salama SR, Casper-Lindley C, Yildiz FH. Regulation of rugosity and biofilm formation in *Vibrio cholerae*: comparison of VpsT and VpsR regulons and epistasis analysis of vpsT, vpsR, and hapR. *J Bacteriol*. 2007; 189:388–402. [PubMed: 17071756]
- Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. *Can J Biochem Physiol*. 1959; 37:911–917. [PubMed: 13671378]
- Brown AD. Microbial water stress. *Bacteriol Rev*. 1976; 40:803–846. [PubMed: 1008746]
- Canovas D, Vargas C, Calderon MI, Ventosa A, Nieto JJ. Characterization of the genes for the biosynthesis of the compatible solute ectoine in the moderately halophilic bacterium *Halomonas elongata* DSM 3043. *Syst Appl Microbiol*. 1998; 21:487–497. [PubMed: 9924816]
- Colwell RR, Huq A, Islam MS, Aziz KMA, Yunus M, Khan NH, et al. Reduction of cholera in Bangladeshi villages by simple filtration. *Proc Natl Acad Sci*. 2003; 100:1051–1055. [PubMed: 12529505]
- Culham DE, Dalgado C, Gyles CL, Mamelak D, MacLellan S, Wood JM. Osmoregulatory transporter ProP influences colonization of the urinary tract by *Escherichia coli*. *Microbiology*. 1998; 144:91–102. [PubMed: 9467901]
- da Costa MS, Santos H, Galinski EA. An overview of the role and diversity of compatible solutes in Bacteria and Archaea. *Adv Biochem Eng Biotechnol*. 1998; 61:117–153. [PubMed: 9670799]
- Faruque SM, Albert MJ, Mekalanos JJ. Epidemiology, genetics, and ecology of toxigenic *Vibrio cholerae*. *Microbiol Mol Biol Rev*. 1998; 62:1301–1314. [PubMed: 9841673]
- Faruque SM, Biswas K, Udden SM, Ahmad QS, Sack DA, Nair GB, Mekalanos JJ. Transmissibility of cholera: in vivo-formed biofilms and their relationship to infectivity and persistence in the environment. *Proc Natl Acad Sci U S A*. 2006; 103:6350–6355. [PubMed: 16601099]
- Fong JC, Yildiz FH. The *rbmBCDEF* gene cluster modulates development of rugose colony morphology and biofilm formation in *Vibrio cholerae*. *J Bacteriol*. 2007; 189:2319–2330. [PubMed: 17220218]
- Fong JC, Syed KA, Klose KE, Yildiz FH. Role of *Vibrio* polysaccharide (*vps*) genes in VPS production, biofilm formation and *Vibrio cholerae* pathogenesis. *Microbiology*. 2010; 156:2757–2769. [PubMed: 20466768]
- Fullner KJ, Mekalanos JJ. Genetic characterization of a new type IV-A pilus gene cluster found in both classical and El Tor biotypes of *Vibrio cholerae*. *Infect Immun*. 1999; 67:1393–1404. [PubMed: 10024587]
- Gardel CL, Mekalanos JJ. Alterations in *Vibrio cholerae* motility phenotypes correlate with changes in virulence factor expression. *Infect Immun*. 1996; 64:2246–2255. [PubMed: 8675334]
- Gerhardt, P.; Murray, RGE.; Wood, WA.; Kreig, NR. *Methods for Genral and Molecular Bacteriology*. American Society for Microbiology; Washington, DC: 1994.
- Gupta S, Chowdhury R. Bile affects production of virulence factors and motility of *Vibrio cholerae*. *Infect Immun*. 1997; 65:1131–1134. [PubMed: 9038330]
- Heidelberg JF, Eisen JA, Nelson WC, Clayton RA, Gwinn ML, Dodson RJ, et al. DNA sequence of both chromosomes of the cholera pathogen *Vibrio cholerae*. *Nature*. 2000; 406:477–483. [PubMed: 10952301]
- Heydorn A, Ersboll BK, Hentzer M, Parsek MR, Givskov M, Molin S. Experimental reproducibility in flow-chamber biofilms. *Microbiology*. 2000a; 146(Pt 10):2409–2415. [PubMed: 11021917]
- Heydorn A, Nielsen AT, Hentzer M, Sternberg C, Givskov M, Ersboll BK, Molin S. Quantification of biofilm structures by the novel computer program COMSTAT. *Microbiology*. 2000b; 146(Pt 10): 2395–2407. [PubMed: 11021916]
- Huq A, Small EB, West PA, Huq MI, Rahman R, Colwell RR. Ecological relationships between *Vibrio cholerae* and planktonic crustacean copepods. *Appl Environ Microbiol*. 1983; 45:275–283. [PubMed: 6337551]
- Huq A, Sack RB, Nizam A, Longini IM, Nair GB, Ali A, et al. Critical factors influencing the occurrence of *Vibrio cholerae* in the environment of Bangladesh. *Appl Environ Microbiol*. 2005; 71:4645–4654. [PubMed: 16085859]

- Kapfhammer D, Karatan E, Pflughoeft KJ, Watnick PI. Role for glycine betaine transport in *Vibrio cholerae* osmoadaptation and biofilm formation within microbial communities. *Appl Environ Microbiol.* 2005; 71:3840–3847. [PubMed: 16000796]
- Kuhlmann AU, Bremer E. Osmotically regulated synthesis of the compatible solute ectoine in *Bacillus pasteurii* and related *Bacillus* spp. *Appl Environ Microbiol.* 2002; 68:772–783. [PubMed: 11823218]
- Lobitz B, Beck L, Huq A, Wood B, Fuchs G, Faruque AS, Colwell R. Climate and infectious disease: use of remote sensing for detection of *Vibrio cholerae* by indirect measurement. *Proc Natl Acad Sci U S A.* 2000; 97:1438–1443. [PubMed: 10677480]
- Louis P, Galinski EA. Characterization of genes for the biosynthesis of the compatible solute ectoine from *Marinococcus halophilus* and osmoregulated expression in *Escherichia coli*. *Microbiology.* 1997; 143(Pt 4):1141–1149. [PubMed: 9141677]
- Louis VR, Russek-Cohen E, Choopun N, Rivera IN, Gangle B, Jiang SC, et al. Predictability of *Vibrio cholerae* in Chesapeake Bay. *Appl Environ Microbiol.* 2003; 69:2773–2785. [PubMed: 12732548]
- Lucht JM, Bremer E. Adaptation of *Escherichia coli* to high osmolarity environments: osmoregulation of the high-affinity glycine betaine transport system proU. *FEMS Microbiol Rev.* 1994; 14:3–20. [PubMed: 8011357]
- Lupp C, Hancock RE, Ruby EG. The *Vibrio fischeri* sapABCDF locus is required for normal growth, both in culture and in symbiosis. *Arch Microbiol.* 2002; 179:57–65. [PubMed: 12471505]
- Matz C, McDougald D, Moreno AM, Yung PY, Yildiz FH, Kjelleberg S. Biofilm formation and phenotypic variation enhance predation-driven persistence of *Vibrio cholerae*. *Proc Natl Acad Sci U S A.* 2005; 102:16819–16824. [PubMed: 16267135]
- Miller CJ, Drasar BS, Feachem RG. Response of toxigenic *Vibrio cholerae* O1 to physico-chemical stresses in aquatic environments. *J Hyg (Lond).* 1984; 93:475–495. [PubMed: 6512251]
- Miller, JH. Assay of β -galactosidase. In: Miller, JH., editor. *Experiments in molecular genetics*. Cold Spring Harbor Laboratory; Cold Spring Harbor, NY: 1972. p. 352–355.
- Miller VL, Mekalanos JJ. 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.* 1988; 170:2575–2583. [PubMed: 2836362]
- Morey JS, Ryan JC, Van Dolah FM. Microarray validation: factors influencing correlation between oligonucleotide microarrays and real-time PCR. *Biol Proced Online.* 2006; 8:175–193. [PubMed: 17242735]
- Mourino-Perez RR, Worden AZ, Azam F. Growth of *Vibrio cholerae* O1 in red tide waters off California. *Appl Environ Microbiol.* 2003; 69:6923–6931. [PubMed: 14602656]
- Mustakhimov, Reshetnikov AS, Glukhov AS, Khmelenina VN, Kalyuzhnaya MG, Trotsenko YA. Identification and characterization of EctR1, a new transcriptional regulator of the ectoine biosynthesis genes in the halotolerant methanotroph *Methylobacterium alcaliphilum* 20Z. *J Bacteriol.* 2010; 192:410–417. [PubMed: 19897647]
- Naughton LM, Blumberman SL, Carlberg M, Boyd EF. Osmoadaptation among *Vibrio* species and unique genomic features and physiological responses of *Vibrio parahaemolyticus*. *Appl Environ Microbiol.* 2009; 75:2802–2810. [PubMed: 19286794]
- Ono H, Sawada K, Khunajakr N, Tao T, Yamamoto M, Hiramoto M, et al. Characterization of biosynthetic enzymes for ectoine as a compatible solute in a moderately halophilic eubacterium, *Halomonas elongata*. *J Bacteriol.* 1999; 181:91–99. [PubMed: 9864317]
- Pascual M, Bouma MJ, Dobson AP. Cholera and climate: revisiting the quantitative evidence. *Microbes Infect.* 2002; 4:237–245. [PubMed: 11880057]
- Peters R, Galinski EA, Truper HG. The biosynthesis of ectoine. *FEMS Microbiol Lett.* 1990; 71:157–162.
- Pflughoeft KJ, Kierek K, Watnick PI. Role of ectoine in *Vibrio cholerae* osmoadaptation. *Appl Environ Microbiol.* 2003; 69:5919–5927. [PubMed: 14532045]
- Prigent-Combaret C, Brombacher E, Vidal O, Ambert A, Lejeune P, Landini P, Dorel C. Complex regulatory network controls initial adhesion and biofilm formation in *Escherichia coli* via regulation of the csgD gene. *J Bacteriol.* 2001; 183:7213–7223. [PubMed: 11717281]

- Reichelt JL, Baumann P. Taxonomy of the marine, luminous bacteria. *Arch Microbiol.* 1973; 94:283–330.
- Richardson K. Roles of motility and flagellar structure in pathogenicity of *Vibrio cholerae*: analysis of motility mutants in three animal models. *Infect Immun.* 1991; 59:2727–2736. [PubMed: 1855990]
- Shikuma NJ, Yildiz FH. Identification and characterization of OscR, a transcriptional regulator involved in osmolarity adaptation in *Vibrio cholerae*. *J Bacteriol.* 2009; 191:4082–4096. [PubMed: 19329635]
- Shikuma NJ, Fong JC, Odell LS, Perchuk BS, Laub MT, Yildiz FH. Overexpression of VpsS, a hybrid sensor kinase, enhances biofilm formation in *Vibrio cholerae*. *J Bacteriol.* 2009; 191:5147–5158. [PubMed: 19525342]
- Silva AJ, Leitch GJ, Camilli A, Benitez JA. Contribution of hemagglutinin/protease and motility to the pathogenesis of El Tor biotype cholera. *Infect Immun.* 2006; 74:2072–2079. [PubMed: 16552036]
- Singleton FL, Attwell RW, Jangi MS, Colwell RR. Influence of salinity and organic nutrient concentration on survival and growth of *Vibrio cholerae* in aquatic microcosms. *Appl Environ Microbiol.* 1982a; 43:1080–1085. [PubMed: 6896621]
- Singleton FL, Attwell R, Jangi S, Colwell RR. Effects of temperature and salinity on *Vibrio cholerae* growth. *Appl Environ Microbiol.* 1982b; 44:1047–1058. [PubMed: 6295276]
- Slamti L, Waldor MK. Genetic analysis of activation of the *Vibrio cholerae* Cpx pathway. *J Bacteriol.* 2009; 191:5044–5056. [PubMed: 19542291]
- Sleator RD, Hill C. Bacterial osmoadaptation: the role of osmolytes in bacterial stress and virulence. *FEMS Microbiol Rev.* 2002; 26:49–71. [PubMed: 12007642]
- Tamplin ML, Colwell RR. Effects of microcosm salinity and organic substrate concentration on production of *Vibrio cholerae* enterotoxin. *Appl Environ Microbiol.* 1986; 52:297–301. [PubMed: 3752994]
- Vital M, Fuchslin HP, Hammes F, Egli T. Growth of *Vibrio cholerae* O1 Ogawa Eltor in freshwater. *Microbiology.* 2007; 153:1993–2001. [PubMed: 17600045]
- Wemekamp-Kamphuis HH, Wouters JA, Sleator RD, Gahan CG, Hill C, Abee T. Multiple deletions of the osmolyte transporters BetL, Gbu, and OpuC of *Listeria monocytogenes* affect virulence and growth at high osmolarity. *Appl Environ Microbiol.* 2002; 68:4710–4716. [PubMed: 12324311]
- Wolfe AJ, Berg HC. Migration of bacteria in semisolid agar. *Proc Natl Acad Sci U S A.* 1989; 86:6973–6977. [PubMed: 2674941]
- Wood JM. Osmosensing by bacteria: signals and membrane-based sensors. *Microbiol Mol Biol Rev.* 1999; 63:230–262. [PubMed: 10066837]
- Wood JM. Bacterial osmosensing transporters. *Methods Enzymol.* 2007; 428:77–107. [PubMed: 17875413]
- Yosef N, Regev A. Impulse control: temporal dynamics in gene transcription. *Cell.* 2011; 144:886–896. [PubMed: 21414481]

Fig 1. *cosR* expression is regulated by salinity

(A) Relative *cosR* expression in wild type *V. cholerae* grown at the indicated NaCl concentration relative to cells grown at 0 M NaCl in LB media identified through transcriptome analysis. Expression was not significantly different between 0 M and 0.1 M NaCl treatments (Shikuma and Yildiz, 2009). (B) Quantitative PCR of *cosR* expression in total RNA isolated from cells grown in LB supplemented with NaCl, KCl or lactose at the indicated concentrations. Reactions without reverse transcriptase (RT) were included as a negative control. Graph depicts an average of three biological replicates of *cosR/gyrA* message levels and error bars indicate standard deviations, p value determined by student's t test (* $p < 0.05$). (C) Arrangement of genes encoding the transcriptional regulator, CosR, the compatible solute transporter OpuD and proteins required for ectoine biosynthesis, EctA, EctB, EctC and aspartokinase (VCA0822), in *V. cholerae*.

Fig 2. CosR represses compatible solute biosynthesis/transporter genes

Quantitative PCR analysis of (A) *ectA/gyrA* and (B) *VCA0822/gyrA* message levels from wild type and $\Delta cosR$ mutant cells grown in 0.2 M or 0.5 M NaCl in LB. Reactions without reverse transcriptase (RT) were included as a negative control. Bars represent the average of three biological replicates and error bars indicate standard deviations, p value determined by student's t test (*p < 0.01). (C) Quantification of ectoine levels in wild type, $\Delta cosR$ and $\Delta ectA$ strains grown in M9 minimal media supplemented with 0.2 M NaCl. Ectoine was quantified using high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) as described in the materials and methods and normalized to total protein levels. Bars are an average of 3 biological replicates and error bars indicate standard deviations, p value determined by student's t test (*p < 0.001). Not detected (ND).

Fig 3. Complementation of $\Delta cosR$

Quantitative PCR analysis of (A) *ectA/gyrA* and (B) *VCA0822/gyrA* message levels from wild type and $\Delta cosR$ mutant grown in 0.2 M NaCl. Strains contained the pACYC177 plasmid (vector) or pACYC177 harboring the putative *cosR* promoter and coding region (*pcosR*). Reactions without reverse transcriptase (RT) were included as a negative control. Graphs are an average of three biological replicates and error bars indicate standard deviations, p value determined by student's t test (* $p < 0.05$).

Fig 4. CosR activates biofilm formation in seawater microcosms

Representative CLSM micrographs showing horizontal (xy) and vertical (xz) projections of biofilm structures formed by wild type, $\Delta cosR$, $\Delta ectA$ and $\Delta cosR \Delta ectA$ *V. cholerae* in 50% artificial seawater (A) or LB supplemented with 0.2 M NaCl (B). Strains were grown in a once-through flow cell system at room temperature and images were acquired at the indicated timepoints. Scale bar is 50 μm .

Fig 5. CosR represses motility in a salinity-dependent manner

(A) Graph of the motility zone diameter of wild type and $\Delta cosR$ *V. cholerae* strains inoculated in semisolid LB agar plates containing the indicated NaCl concentration after 16 h growth at 30°C. Bars indicate the average of 4 biological replicates and error bars indicate standard deviations, p value determined by student's t test (*p < 0.0005). (B) Graph of the motility zone diameter of wild type or $\Delta cosR$ *V. cholerae* containing an empty pACYC177 plasmid (Vector) or pACYC177 containing the putative promoter and *cosR* coding region (*pcosR*). Strains were inoculated into semisolid LB agar containing 0.2 M NaCl and 100 μ g/ml ampicillin and the motility diameter was measured after 16 h growth at 30°C. Bars indicate the average of 4 biological replicates and error bars indicate standard deviations, p value determined by student's t test (*p < 0.0005). (C) Graph of the motility zone diameter of wild type, $\Delta cosR$, $\Delta ectA$ and $\Delta cosR\Delta ectA$ *V. cholerae* strains inoculated in semisolid LB agar plates containing 0.2 M NaCl after 16 h growth at 30°C. Bars indicate the average of 4 biological replicates and error bars indicate standard deviations, p value determined by student's t test (*p < 0.0005).

Fig 6. Model of the *V. cholerae* salinity/osmolarity response

The expression of *cosR*, encoding a MarR-type transcriptional regulator, is activated by ionic strength. CosR represses expression of the genes encoding the compatible solute transporter, OpuD, and the ectoine biosynthesis proteins. Salinity activates the same set of genes and opposes CosR regulation. CosR additionally activates biofilm formation and represses motility independently of its role as an ectoine regulator. The expression of *oscR*, encoding an IclR-type transcriptional regulator, is upregulated in low salinity/osmolarity conditions (Shikuma and Yildiz, 2009). OscR represses biofilm formation through the repression of the *vps* genes and activates motility.

Table 1

Genes differentially expressed in the *cosR* mutant versus wild type grown to exponential phase in 0.2 M NaCl and 0.5 M NaCl-containing LB.

ORF	Gene	Function	ΔcosR 0.2M / Wt 0.2M	ΔcosR 0.5M / Wt 0.5M
Amino Acid Biosynthesis				
VC0162	<i>ilvC</i>	Ketol-acid reductoisomerase		2.02
VC2746	<i>glnA</i>	Glutamate-ammonia ligase	2.11	
Biosynthesis of Cofactors, Prosthetic Groups and Carriers				
VCA0712	<i>pncA</i>	Pyrazinamidase/nicotinamidase		2.27
Cell Envelope				
VC2633		Fimbrial assembly protein PilN, putative		3.43
Ectoine Biosynthesis				
VCA0822	<i>ask</i>	Aspartokinase putative	73.61	34.67
VCA0823	<i>ectC</i>	Ectoine synthase	85.37	33.86
VCA0824	<i>ectB</i>	Diaminobutyrate pyruvate aminotransferase	6.48	44.71
VCA0825	<i>ectA</i>	L-2, 4-diaminobutyric acid acetyltransferase	42.69	95.24
Energy Metabolism				
VC1690		Alpha-1, 6-galactosidase putative	0.37	
VC1827	<i>manA-2</i>	Mannose-6-phosphate isomerase	0.47	
VCA0828	<i>phhA</i>	Phenylalanine-4-hydroxylase		4.82
Hypothetical Proteins				
VC0316		Hypothetical protein		2.25
VC1020		Hypothetical protein		2.43
VC1389		Hypothetical protein		2.20
VC1586		Hypothetical protein	0.41	
VC1657		Hypothetical protein	2.18	
VC1696		Hypothetical protein	0.40	
VC1764		Hypothetical protein		0.48
VC1787		Hypothetical protein		0.21
VC1808		Hypothetical protein	0.45	
VC2151		Hypothetical protein		2.05
VCA0284		Hypothetical protein		0.45
VCA0326		Hypothetical protein		0.38
VCA0467		Hypothetical protein	2.17	
VCA0839		Hypothetical protein		0.30
VCA0849		Hypothetical protein		2.90
VCA0973		Hypothetical protein		0.36
VC1645		Conserved hypothetical protein	0.47	
VC2473		Conserved hypothetical protein	0.50	
Regulatory Functions				

ORF	Gene	Function	$\Delta cosR$ 0.2M / Wt 0.2M	$\Delta cosR$ 0.5M / Wt 0.5M
VC2692	<i>cpxR</i>	Transcriptional regulator CpxR		2.01
Transport and Binding Proteins				
VC0173		peptide ABC transporter permease protein		3.45
VC1092	<i>oppB</i>	oligopeptide ABC transporter permease protein		2.07
VC1279	<i>opuD</i>	transporter BCCT family	6.17	
VC1854	<i>ompT</i>	ompT protein	0.45	
VC1929	<i>dctP-2</i>	C4-dicarboxylate-binding periplasmic protein		3.08
VCA0516	<i>fruA-2</i>	PTS system fructose-specific IIBC component		0.40
VCA0745	<i>glpF</i>	Glycerol uptake facilitator protein	0.48	

Differentially expressed genes were determined using SAM software, with criteria of a 2-fold change in gene expression and an FDR of 3%.

COMSTAT analysis of biofilm structures formed by wild-type, $\Delta cosR$, $\Delta ectA$ and $\Delta cosR\Delta ectA$ strains in a once-through flow cell system in 50% artificial seawater (ASW) or LB media supplemented with 0.2 M NaCl (LB).

Table 2

Media	Time (h)	Strain	Total biomass ($\mu\text{m}^3/\mu\text{m}^2$)	Thickness (μm)	
				Average	Maximum
ASW	24	Wild type	6.80 (1.51)	7.04 (1.70)	16.28 (7.32)
		$\Delta cosR$	3.24 (1.06)	3.52 (1.39)	8.30 (0.70)
		$\Delta ectA$	6.10 (0.83)	5.89 (0.73)	10.56 (4.35)
		$\Delta cosR \Delta ectA$	1.87 (0.72)	1.87 (0.76)	5.28 (1.11)
ASW	48	Wild type	8.60 (2.45)	9.19 (2.81)	21.21 (2.47)
		$\Delta cosR$	4.65 (1.90)	4.78 (1.89)	16.72 (2.90)
		$\Delta ectA$	5.59 (2.24)	6.29 (2.96)	18.23 (3.51)
		$\Delta cosR \Delta ectA$	0.93 (0.14)	0.98 (0.21)	8.95 (3.17)
ASW	72	Wild type	19.27 (3.33)	21.52 (4.61)	33.34 (4.89)
		$\Delta cosR$	8.47 (2.58)	8.71 (3.31)	20.11 (3.71)
		$\Delta ectA$	9.39 (2.95)	10.42 (3.95)	22.00 (6.05)
		$\Delta cosR \Delta ectA$	4.19 (1.20)	4.75 (1.25)	14.52 (4.71)
LB	24	Wild type	10.6 (1.1)	9.7 (1.1)	16.4 (1.2)
		$\Delta cosR$	8.7 (1.3)	8.0 (1.1)	14.1 (1.9)
		$\Delta ectA$	10.0 (1.2)	9.2 (1.2)	15.5 (0.7)
		$\Delta cosR \Delta ectA$	9.6 (0.6)	8.9 (0.6)	15.6 (1.7)
LB	48	Wild type	19.9 (2.3)	19.1 (2.4)	29.1 (3.4)
		$\Delta cosR$	15.8 (0.8)	15.0 (0.8)	26.1 (3.5)
		$\Delta ectA$	18.5 (1.8)	18.1 (1.9)	28.4 (3.7)
		$\Delta cosR \Delta ectA$	17.0 (1.4)	16.4 (1.7)	26.7 (1.9)

Values presented are averages of data from at least six z-series image stacks. The numbers in parentheses indicate standard deviations.