LeuO and H-NS are part of an expanded regulatory network for ectoine biosynthesis expression

Keywords: Ectoine biosynthesis regulation, Vibrio, LeuO, NhaR, H-NS, ToxR
Abstract

Bacteria accumulate compatible solutes, to maintain cellular turgor pressure when exposed to high salinity. In the marine halophile *Vibrio parahaemolyticus*, the compatible solute ectoine is biosynthesized *de novo*, which is energetically more costly than uptake; therefore, tight regulation is required. To uncover novel regulators of the ectoine biosynthesis *ectABC-aspk* operon, a DNA affinity pulldown of proteins interacting with the *ectABC-aspk* regulatory region was performed. Mass spectrometry analysis identified, amongst others, five regulators: LeuO, NhaR, OmpR, TorR, and the nucleoid associated protein H-NS. To determine their role in ectoine biosynthesis, in-frame non-polar deletions were made for each gene and P_{ectA-gfp} promoter reporter assays were performed. P_{ectA-gfp} expression was significantly repressed in the ΔleuO mutant and significantly induced in the ΔnhaR mutant compared to wild type, suggesting positive and negative regulation, respectively. Both ΔompR and ΔtorR mutants also showed induced expression of P_{ectA-gfp}, but not to the same extent as ΔnhaR. The Δhns showed no change compared to wild type. To examine whether H-NS interacts with LeuO or NhaR at the ectoine regulatory region, double deletion mutants were created. In a ΔleuO/Δhns mutant, P_{ectA-gfp} showed reduced expression, but significantly more than ΔleuO suggesting H-NS and LeuO interact to regulate ectoine expression. Whereas ΔnhaR/Δhns had no additional effect as compared to ΔnhaR suggesting NhaR regulation is independent of H-NS. Next, a P_{leuO-gfp} reporter analysis was performed in wild type ΔleuO, Δhns, ΔleuO/Δhns and ΔtoxR, which encodes ToxR a positive regulator of *leuO*. P_{leuO-gfp} showed significantly increased expression in the ΔleuO, Δhns and ΔleuO/Δhns mutants as compared to wild type, indicating both are repressors of *leuO*, and ΔtoxR showed reduced expression. Growth pattern analysis of the ΔleuO,
∆hns, and ∆leuO/∆hns mutants in M9G 6%NaCl all showed growth defects compared to wild type, with ∆leuO/∆hns showing the greatest effect. Overall, the data show that NhaR is a negative regulator and LeuO is a positive regulator of ectoine expression and suggest LeuO is an anti-silencer of H-NS.  

**Importance:** Ectoine is a commercially used compatible solute that acts as a biomolecule stabilizer because of its additional role as a chemical chaperone. The *de novo* biosynthesis of ectoine is carried out by approximately 70% of *Vibrionaceae* species many of which are marine halophiles. This study identified LeuO as a positive regulator of ectoine biosynthesis and also showed that similar to enteric species, LeuO is an anti-silencer of the nucleoid associated protein H-NS. NhaR was a negative regulator of ectoine biosynthesis but growth analysis in high salinity showed that LeuO, NhaR and H-NS are required for optimal growth under these conditions. These data indicate that all three regulators also play a wider role in high NaCl tolerance.  

**Introduction**  
When bacteria are exposed to high salinity conditions, an efflux of water across the osmotic gradient occurs lowering cellular turgor pressure (1-5). The initial short-term response to osmotic up shock is the uptake of potassium (K+), which is accompanied by glutamate accumulation in Gram-negative bacteria to maintain electro neutrality. This is the short-term strategy because high concentrations of K+ have deleterious effects on cellular processes (1, 2, 6, 7). The long term response to increased osmotic stress is characterized by the uptake or biosynthesis of compatible solutes, which are small organic molecules that provide osmotic protection by helping to maintain the turgor pressure of the cell and can be accumulated to extremely high intracellular concentrations (3, 8, 9).
The compatible solute ectoine was initially discovered to provide osmotic protection in anoxygenic phototrophs (10), but subsequent analysis showed that it functions as a compatible solute in numerous Gram-negative and Gram-positive bacteria (reviewed in (3, 8, 11-14), which included many members of the family Vibrionaceae (15-19). The ability to be accumulated to very high intracellular concentrations without interfering with molecular processes is a hallmark of compatible solutes. Additionally, ectoine was shown to act as a chemical chaperone through the stabilization of proteins, thus it is an important compound for commercial uses in medicine and cosmetics to stabilize biomolecules against factors such as heating, freezing, desiccation, and UV radiation (20-23). Therefore, understanding the regulation of the ectoine biosynthetic pathway is important to increase efficient industrial production using natural bacterial producers (24).

Biosynthesis of ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidinecarboxylic acid) is de novo from L-aspartic acid, and performed by the evolutionarily conserved operon ectABC, which encodes the EctA, EctB, and EctC proteins (25-27). In many species an aspartokinase (aspk) is clustered with the ectoine biosynthesis operon, which converts aspartic acid to β-aspartyl phosphate, which is then converted to L-aspartate-β-semialdehyde by aspartate semialdehyde dehydrogenase (Asd). This intermediate from the aspartic acid pathway is incorporated into the ectoine biosynthesis pathway when it is converted to L-2,4-diaminobutyrate by EctB (L-2,4-diaminobutyrate transaminase). EctA (L-2,4-diaminobutyrate Nγ-acetyltransferase) then acetylates this product to form Nγ-acetyldiaminobutyrate. The final step to produce ectoine is a cyclic condensation reaction performed by EctC (L-ectoine synthase) (13, 27).

In some species clustered with the ectoine biosynthesis operon is ectR, a MarR-type regulator shown to repress transcription of the ectoine operon (24, 28-30). Among Vibrionaceae, an ectR
homolog is only present only in _Aliivibrio_ species (31, 32). Previous studies in _V._
_parahaemolyticus_ and _V. cholerae_ showed that _ectABC-aspK_ transcription was repressed in low
salinity by another MarR-type regulator named CosR (17, 31, 33). CosR from _V._
_parahaemolyticus_ showed 31% amino acid identity to _Aliivibrio fischeri_ EctR, with less than
60% query coverage and does not cluster with _ectABC-aspk_, but is located elsewhere in the
genome (31). The quorum sensing master regulators AphA and OpaR were found to activate and
repress expression of _ectABC-aspk_, respectively in _V. parahaemolyticus_ (17). AphA and OpaR
also regulate expression of CosR, creating a tightly controlled feed-forward loop for ectoine
expression across the growth curve (17).

In this study, we set out to identify novel regulators of the ectoine biosynthesis genes by
performing a DNA affinity chromatography pulldown using the regulatory region of the _ectABC-
aspk_ operon. We identified numerous candidates and selected the regulators LeuO, NhaR,
OmpR, TorR, and the nucleoid associated protein (NAP) H-NS to examine further. We
constructed in-frame nonpolar deletions in each gene. To determine the role that these putative
regulators play, reporter assays were used with _gfp_ placed under the control of the regulatory
region of the ectoine biosynthesis genes. P_{ectA-gfp} expression was first examined in wild type and
each single mutant of _ΔleuO, ΔnhaR, ΔompR, ΔtorR_, and _Δhns_. In addition, double mutants
_ΔleuO/Δhns_ and _ΔnhaR/Δhns_ were constructed to determine whether the NAP, H-NS interacted
with either LeuO or NhaR to control ectoine gene expression. To determine the physiological
role of LeuO, NhaR and H-NS, growth pattern analysis was performed under high salinity
conditions.

**Results**
Ectoine is present in cells grown in M9G 3% NaCl and absent in M9G 1% NaCl. To confirm *V. parahaemolyticus* de novo ectoine biosynthesis is a response to increased salinity, $^1$H nuclear magnetic resonance spectroscopy ($^1$H-NMR) was performed. Ethanol extracts of *V. parahaemolyticus* wild type cells grown in minimal medium supplemented with glucose (M9G) with either 1% NaCl or 3% NaCl overnight at 37°C were used to obtain H-NMR spectra (Fig. S1A). Peaks corresponding to the hydrogen atoms of ectoine are labeled and were present in M9G 3% NaCl extracts but were not detectable in M9G 1% NaCl extracts as previously shown (34) (Fig. S1A). In addition to intracellular accumulation of ectoine, we also wanted to demonstrate that *ectABC-aspk* expression is increased in M9G 3% NaCl. A transcriptional reporter assay was performed, where *gfp* was placed under the control of the ectoine regulatory region (P$_{ectA}$-*gfp*). The expression was examined in wild type cells grown in either M9G 1% NaCl or M9G 3% NaCl by measuring the specific fluorescence of P$_{ectA}$-*gfp* as a cumulative readout of ectA transcription (Fig. S1B). The observed level of specific fluorescence of P$_{ectA}$-*gfp* was significantly increased in M9G 3% NaCl (4.0-fold) relative to that of cells grown in M9G 1% NaCl (Fig. S1B).

**Identification of putative regulators of the ectoine biosynthesis operon.** With the knowledge that ectoine is present in cells grown in M9G 3% NaCl, we performed a promoter pulldown under this growth condition. A promoter pulldown was performed using a 5’-biotin tagged DNA fragment of the *ectABC-aspk* regulatory region bound to streptavidin beads. As a negative control DNA probe, the coding region of downstream *ectB* gene was used. To identify novel regulators of ectoine biosynthesis, we grew cells to mid-exponential phase in M9G 3% NaCl and subsequent cell lysate was incubated with DNA probe-coated beads. Bound proteins were then eluted using a stepwise NaCl gradient and separated with SDS-PAGE gel. Bands that were
present in the target lanes, but not present in the ectB negative control lanes were selected for
analysis via mass spectrometry (Fig. S2). The proteins identified with mass spectrometry
analysis were aligned to the V. parahaemolyticus genome and sorted by gene class and ranked by
score (Table 1). Candidate proteins of interest that belonged to the transcriptional regulator gene
class were selected. These candidate proteins are described in Table 1 and include homologs of
regulators NhaR (VP0527), OmpR (VP0154), and TorR (VP1032). NhaR and OmpR had
previously been shown in Escherichia coli to be involved in osmotic stress (35-40). NhaR, a
LysR-type transcriptional regulator, shows 62% amino acid homology to NhaR from E. coli K-
12, OmpR showed 84% amino acid identity and TorR 57% amino acid homology to OmpR and
TorR respectively from E. coli. NhaR, OmpR, and TorR have not been previously examined in
Vibrio species. An additional regulator of interest, LeuO (VP0350) was also examined further. In
V. parahaemolyticus, LeuO is a LysR-type regulator previously described as under the control of
ToxRS and negatively regulated by calcium (41, 42). A homolog of the H-NS (histone-like
nucleoid structuring) protein was also identified in our pulldown. In E. coli and Salmonella
enterica H-NS was shown to be a universal silencer of gene expression (43-48). In addition, an
E. coli study by Shimada and colleagues identified 140 LeuO-binding sites with over 90% of
these sites also showing H-NS binding. Further studies have shown that LeuO plays a significant
role as an antagonist of H-NS gene silencing (43, 49, 50). As a proof of concept for the
pulldown, LeuO and NhaR proteins were purified, and electrophoretic mobility shift assays
(EMSAs) were performed using the regulatory region of ectABC-aspk as a DNA probe. In these
assays both proteins bound to the DNA probe (Fig. S3).

**PectA-gfp reporter shows differential expression in ΔleuO, ΔnhaR, ΔompR and ΔtorR mutants.** To examine the role of leuO, nhaR, ompR, and torR candidate regulators in the control
of the ectoine biosynthesis operon, we performed GFP reporter assays using a reporter plasmid where \textit{gfp} was under the control of the ectoine regulatory region (\(P_{\text{ectA-gfp}}\)). This reporter plasmid was transformed into wild type or in-frame non-polar mutants of \(\Delta\text{leuO}, \Delta\text{nhaR}, \Delta\text{ompR},\) and \(\Delta\text{torR}\). Cultures were grown overnight in M9G 3%NaCl and relative fluorescence intensity (RFU) was measured. Specific fluorescence was calculated by dividing RFU by OD. From this analysis, the relative fluorescence (RFU) measured in the \(\Delta\text{leuO}\) mutant background was 1.7-fold reduced as compared to wild type (\textbf{Fig. 1}). This data suggests that LeuO is an activator of ectoine biosynthesis genes. The relative fluorescence measured in a \(\Delta\text{nhaR}\) background was significantly increased 1.8-fold as compared to wild type, suggesting that NhaR is a repressor of the ectoine operon (\textbf{Fig. 1}). \(P_{\text{ectA-gfp}}\) expression in the \(\Delta\text{ompR}\) (1.3-fold) and \(\Delta\text{torR}\) (1.4-fold) mutants were also increased as compared to wild type, but not to the same level as NhaR, indicating that both OmpR and TorR are negative regulators of the ectoine biosynthesis operon.

**LeuO and H-NS interplay to control \(P_{\text{ectA-gfp}}\).** In \textit{E. coli} and \textit{V. cholerae}, H-NS has been shown to interact with LeuO in both cooperative and antagonistic relationships at different gene loci (43, 51-54). Therefore, we wanted to examine whether H-NS and LeuO interact at the \(ectABC\text{-aspk}\) locus. We constructed an in-frame non-polar \(\Delta\text{hns}\) mutant and a double \(\Delta\text{leuO/\Delta hns}\) mutant to examine H-NS regulation of \(ectABC\text{-aspk}\) in a \(P_{\text{ectA-gfp}}\) reporter assay. In these assays, \(P_{\text{ectA-gfp}}\) in the \(\Delta\text{leuO}\) mutant showed significantly reduced expression, and in the \(\Delta\text{hns}\) mutant expression was similar to wild type (\textbf{Fig. 2}). However, in the \(\Delta\text{leuO/\Delta hns}\) mutant expression was reduced, but not to the same level as the \(\Delta\text{leuO}\) single mutant (\textbf{Fig. 2}). This indicates interplay between both LeuO and H-NS to control ectoine gene expression with LeuO playing an anti-silencer role.
To further investigate the relationship between LeuO and H-NS in *V. parahaemolyticus*, a *gfp*-expressing reporter plasmid under the control of the LeuO regulatory region (P<sub>leuO-gfp</sub>) was constructed. Relative fluorescence and OD<sub>595</sub> were measured after overnight growth in M9G 3%NaCl in wild type, ΔleuO, Δhns, ΔleuO/Δhns, and ΔtoxR. When compared to wild type, P<sub>leuO-gfp</sub> expression in the Δhns mutant showed a 4.0-fold increased relative fluorescence indicating that H-NS is a repressor of *leuO* expression (Fig. 3). P<sub>leuO-gfp</sub> expression was 2.4-fold higher in ΔleuO and 2.8-fold higher in ΔleuO/Δhns when compared to wild type. These data indicate that both H-NS and LeuO are repressors of *leuO* expression and in the absence of both negative regulators other proteins are able to access the regulatory region (Fig. 3). In *V. parahaemolyticus*, ToxR was shown to be a positive regulator of *leuO* expression (41, 55, 56) and this was confirmed in this reporter assay, with ΔtoxRS (1.8-fold) showing significantly less expression of P<sub>leuO-gfp</sub> when compared to wild type (Fig. 3).

**H-NS and LeuO are important for growth in high salinity.** To assess whether the deletions of *leuO* and *hns* have a physiological effect, we examined the ability of *V. parahaemolyticus* wild type, ΔleuO, Δhns, and ΔleuO/Δhns mutants to grow in M9G 1% NaCl, 3% NaCl, or 6% NaCl at 37°C (Fig. 4). In M9G 1%NaCl and 3%NaCl, there were no significant growth defects observed among the strains examined (Fig. S4A, B). However, growth in M9G 6%NaCl showed the wild type and Δhns had a 4 h lag phase, the ΔleuO mutant had a lag phase of 5 h, and ΔleuO/Δhns had a 12 h lag phase (Fig. 4). The double deletion mutant had a final OD<sub>595</sub> of 0.2, which when compared to wild type with final OD of 0.54 was significantly less (Fig. 4). These data demonstrate that both LeuO and H-NS play a significant role in the osmotic stress response and are likely required for salinity tolerance outside of ectoine biosynthesis.
**NhaR is a negative regulator of the ectoine biosynthesis operon.** Next, we considered NhaR and H-NS interplay in the regulation of *ectABC-ask*. To accomplish this, we examined transcription of the ectoine biosynthesis regulatory region in a Δ*nhaR*/Δ*hns* double mutant. P_{ectA-}gfp expression was significantly higher in a Δ*nhaR* mutant (1.8-fold) as compared to wild type (24,856 RFU/OD) confirming our previous results that NhaR is a negative regulator (**Fig. 5**). P_{ectA-}gfp expression in the Δ*hns* mutant (1.0-fold) was similar to wild type. Expression of P_{ectA-}gfp in the Δ*hns*/Δ*nhaR* mutant (1.8-fold) was increased as compared to wild type, but not significantly different from Δ*nhaR*, suggesting that there is no interplay between H-NS and NhaR in ectoine expression.

Next we examined the effect of deleting *nhaR*, *hns*, and *hns/nhaR* on growth of *V. parahaemolyticus*. Growth of the deletion mutants in M9G 1%NaCl and 3% NaCl was similar to wild type (**Fig. S4C, D**). However, Δ*nhaR*, Δ*hns*, and Δ*hns*/Δ*nhaR* mutants grown in M9G 6% NaCl, reached a final OD_{595} of 0.37, 0.36, and 0.38 respectively as compared to wild type OD_{595} of 0.50 (**Fig. 6**). This growth data indicates that there are some defects when grown in increased salinity for Δ*nhaR* but deletion of *hns* does not contribute further to this defect. This supports the expression data that NhaR and H-NS do not interact to control ectoine expression, but the data does indicate that NhaR is important for high NaCl tolerance.

**Discussion**

When bacterial cells are exposed to increased salinity, the osmotic pressure of the cell decreases and water escapes from the cell (1-4). To maintain the appropriate turgor pressure, small organic compounds called compatible solutes are accumulated (3, 8, 9, 32). This accumulation occurs either via uptake or biosynthesis and in *V. parahaemolyticus* at least four transporters for the uptake of compatible solutes and two compatible solute biosynthesis operons are present (19, ...
The biosynthesis of ectoine and not glycine betaine was previously shown to be essential for *V. parahaemolyticus* survival in high salinity (15). However, the biosynthesis of ectoine is costly as it drains intracellular pools of metabolites such as aspartic acid. Therefore, multiple regulators that provide tight control are needed to manage ectoine biosynthesis (58).

In this study, we identified several novel regulators of the ectoine biosynthesis operon (Fig. 7). We found that NhaR acted as a repressor of ectoine biosynthesis. However, the ∆*nhaR* mutant had a growth defect when grown under high NaCl conditions. This could be explained by the fact that NhaR is known to regulate genes related to the osmotic stress response outside of ectoine biosynthesis (59-62). In *E. coli*, *nhaR* is present in an operon with *nhaA* which encodes a Na+/H+ antiporter. NhaR was shown to activate transcription of *nhaA*, which is induced in high salt and alkaline pH and is essential for survival under these conditions. It was also suggested that NhaR is a sensor and transducer of Na+ because the presence of Na+ alters the contact points between NhaR and the *nhaA* regulatory region (63). Therefore, our observed growth defect further suggests regulation by NhaR at other loci important for the response to increased salinity. A homolog of *nhaA* is present in *V. parahaemolyticus*, which could explain the growth defect in high salinity for the *V. parahaemolyticus* ∆*nhaR* mutant.

Our data established that LeuO is a positive regulator of ectoine biosynthesis gene expression. The subsequent analysis of a double ∆*leuO/∆hns* mutant suggests LeuO also likely plays a role as an anti-silencer of H-NS at this locus. In addition, we found that LeuO and H-NS both act as repressors of *leuO* in *V. parahaemolyticus*. LeuO was first identified as a LysR-type regulator and shown to positively regulate the *leuABCD* leucine synthesis operon in *Salmonella enterica* (64, 65). Subsequently, chromatin-immunoprecipitation-on-chip analysis showed 178 LeuO binding sites in *S. enterica*, indicating its role as a global regulator (66). H-NS was also shown to
be a global regulator in this species, silencing the transcription of a large number of horizontally acquired genes. Many of these genes clustered together on the genome within pathogenicity islands, regions that were AT rich and allowed H-NS binding along long sections of DNA (47, 67). A similar phenomenon was show in E. coli horizontally acquired DNA (48). These data led to the idea that H-NS allows for the uptake of horizontally acquired DNA and insertion into the genome in a relatively benign state (45, 47, 67). In V. parahaemolyticus, LeuO was first named CalR as it was regulated by calcium (42). LeuO was subsequently shown to negatively control swarming motility (lafK) and type III secretion system-1 (T3SS-1, exsA) in V. parahaemolyticus (41, 42). LeuO was also shown to be regulated by NaCl and to positively regulate ompU, which encodes an outer membrane porin important for acid stress (41). Additionally, the global regulator ToxR was shown to be essential for LeuO expression (41). Also, in V. parahaemolyticus, LeuO was shown to be an antagonist of H-NS at loci required for type VI secretion system-1 (T6SS-1) expression (68). In V. cholerae, H-NS was shown to have many roles in silencing genes expression such as those required for capsule biosynthesis and virulence factors acquired by horizontal gene transfer (69-72). In this species, LeuO was shown to be required for biofilm formation, and LeuO and H-NS were shown to interact at the vieA locus (53, 68, 73, 74). In addition, in V. vulnificus another important human pathogen, ToxR was shown to activate leuO expression and LeuO was shown to be an auto-repressor with high levels of LeuO binding (73, 74). HNS was subsequently shown to repress leuO by binding to sites that overlap with ToxR and LeuO binding sites (73, 74). In a similar pattern, our data also showed that leuO is an auto-repressor and that H-NS represses leuO, which is likely using a similar regulatory mechanism to that in V. vulnificus (Fig. 7). Furthermore, our growth data showed that deletion of either leuO or hns resulted in defects when grown at high salinity and a double ΔleuO/Δhns
mutant showed a growth defect that was more pronounced than either of the single mutants. The observed defects in high salinity suggests a larger role for both regulators in the osmotic stress response. The more pronounced growth defect observed for ∆leuO/∆hns suggest that these proteins interact at other loci important for responses to increased salinity.

Methods

**Bacterial strains, media and culture conditions.** All strains and plasmids used in this study are listed in Table S2. *V. parahaemolyticus* RIMD2210633, a streptomycin-resistant clinical isolate, was used in this study as the wild-type (WT) strain (75, 76). Unless stated otherwise, *V. parahaemolyticus* was grown in either lysogeny broth (LB; Fisher Scientific, Fair Lawn, NJ) with 3% (wt/vol) NaCl (LB 3%) or M9 minimal media (47.8 mM Na₂HPO₄, 22 mM KH₂PO₄, 18.7 mM NH₄Cl, 8.6 mM NaCl; Sigma Aldrich) supplemented with 2 mM MgSO₄, 0.1 mM CaCl₂, 20 mM glucose as the sole carbon source (M9G) and 3% (wt/vol) NaCl at 37°C. *E. coli* strains were grown in either LB supplemented with 1% (wt/vol) NaCl (LB1%) or M9G supplemented with 1% (wt/vol) NaCl. The strain *E. coli* β2155 λpir strain is a diaminopimelic acid (DAP) auxotrophic strain and was grown with 0.3 mM DAP (77). All strains were grown at 37°C with aeration. Antibiotics were added to growth media at the following concentrations as necessary: ampicillin (Amp), 100 µg/mL; chloramphenicol (Cm), 12.5 µg/mL; tetracycline (Tet), 1 µg/mL; kanamycin (Km), 50 µg/mL.

**Preparation of cellular extracts and proton nuclear magnetic resonance (¹H-NMR).** Wild-type *V. parahaemolyticus* was grown overnight at 37°C in either M9G 1% NaCl or M9G 3%NaCl. Stationary phase cells were then pelleted and washed twice with 1XPBS. Three freeze-thaw cycles were performed with the cell pellets to increase lysis, and the cells were then suspended in 750 µL ethanol. Debris was pelleted by centrifugation, and the ethanol solution was
transferred to a clean tube and evaporated under vacuum. Then deuterium oxide (D₂O) was used to resuspend the pellet, and insoluble material was removed by centrifugation. The solution was then transferred to a 5-mm NMR tube for analysis on a Bruker AVANCE 600NMR spectrometer at a proton frequency of 600.13 MHz with a sweep of 12,376 Hz and a relaxation delay of 5 s. Sixteen scans were co-added for each spectrum.

**DNA-Affinity Pulldown.** DNA-affinity chromatography pulldown was performed as previously described (78-80). The bait DNA primers were designed to amplify the 323-bp regulatory region of *ectA* (VP1722) with a biotin moiety added to the 5’ end. A 327-bp negative-control bait DNA probe was amplified from a coding region of *ectB* (VP1721), which was also labeled with a biotin moiety at the 5’ end. Both probes were amplified using Phusion HF polymerase (New England Biolabs) PCR. Ten reactions were completed for each probe and then pooled and purified using an ethanol extraction technique (81). The pulldown was completed twice, once after growth in non-inducing conditions (LB3%) and once after growth in inducing conditions (M9G3%) in an effort to obtain both positive and negative regulators of the ectoine biosynthesis operon. The wild-type strain was grown overnight in LB3%, cells were pelleted and washed two times with 1XPBS and diluted 1:50 into 500 mL of LB3% or M9G3%. The cultures were then grown to an OD₅₉₅ of 0.5, pelleted and stored overnight at -80°C. The pellet was then suspended in 1.5 mL Fast-Break lysis buffer (Promega, Madison, WI) with 1 mM PMSF, 0.5 mM benzamidine, 0.1 μg/mL lysozyme and incubated for 30 min. at room temperature. The cells were then sonicated on ice at 30% pulse amplitude for 15 seconds with 1 min. rest, and this was repeated 5 times. The cell lysate was then clarified by centrifugation for 30 min. at 4°C. The cell lysate was precleared with streptavidin DynaBeads (Thermo Scientific, Waltham, MA) to remove nonspecific protein-bead interactions.
Streptavidin M280 DynaBeads (ThermoFisher) were then incubated two times with 10 µg of biotinylated probe for 20 min. The precleared cell lysate was then incubated for 30 min. with the bait-coated beads at room temperature with constant rotation in the presence of 100 µg sheared salmon sperm DNA as a non-specific competitor. This was completed twice with washes in between. Protein candidates were then eluted from the bait DNA-bead complex with a stepwise NaCl gradient (100 mM, 200 mM, 300 mM, 500 mM, 170 mM, and 1 M). Next, 6X SDS and 1 mM β-mercaptoethanol (BME) was added to elution samples which were then boiled at 95°C for 5 min. Samples were then separated using a 12% SDS-PAGE and visualized using the Pierce Silver Stain Kit (ThermoFisher). The P_{ectA} bait and ectB negative control were loaded next to each other in order of increasing NaCl concentrations. Bands that were present in the P_{ectA} bait but not the negative control lanes were excised, de-stained, and digested with trypsin following the standard procedure for mass spectrophotometry preparation with C18 ZipTips (Fisher Scientific, Fair Lawn, NJ). The samples were then analyzed with a Q-exactive Orbitrap mass spectrometer with nano-flow electrospray (Thermo). Proteins were subsequently identified using Proteome Discover 2.1 software.

**Protein Purification.** Protein were purified as previous described (82). The full-length genes for leuO (VP0350) and nhaR (VP0527) were cloned into a pET-28a(+) vector with a C-terminal histidine tag, transformed into E. coli Dh5α, purified, and sequenced. These vectors pETleuO and pETnhaR were then transformed into E. coli BL21 (DE3) cells, and expression was induced with 0.5mM IPTG at 0.4 OD_{595} and then cells were grown overnight at 25°C. Cells were then pelleted and resuspended with lysis buffer (50 mM sodium phosphate, 200 mM NaCl, 20 mM imidazole, pH 7.4, 1.0 mM phenylmethanesulfonyl fluoride, 0.5 mM benzamidine) and lysed via sonication. Debris was pelleted, and supernatant applied to an IMAC column packed with HisPur.
Ni-NTA resin (ThermoFisher) that was equilibrated with column buffer (50 mM sodium phosphate, 200 mM NaCl, 20 mM imidazole, pH 7.4). The column was washed with buffer containing increasing concentrations of imidazole (20mM – 100mM) to remove any contaminants. His-tagged proteins were eluted using 500mM imidazole and then dialyzed overnight at 4°C in sodium phosphate buffer to remove any excess salts. Samples of supernatant and each flow through, wash, and elution were analyzed via SDS-PAGE gel to assess protein purity and verify purification.

**Electrophoretic mobility shift assay.** DNA fragments were designed as outlined in Table S3, to encompass the entire region (322 bp) or sections of the ectoine biosynthesis regulatory region (125, 137, 106-bp). The concentration of purified protein (LeuO-His or NhaR-His) was determined using the Bradford Assay, and LeuO (0 to 2.175 uM) or NhaR (0 to 2.175 uM) was incubated for 20 minutes with 30 ng of each DNA fragment in a binding buffer (10 mM Tris [pH 7.4 at 4°C], 150 mM KCl, 0.1 mM dithiothreitol, 0.1 mM EDTA [pH 8.0], 5% polyethylene glycol). The reactions were then loaded (10 µL) on a 6% native acrylamide gel (pre-run 200V for 2 hours at 4°C) and run for 2 hours at 4°C (200V) in 0.5X TBE running buffer. To visualize, gels were stained in an ethidium bromide bath for 15 minutes.

**Mutant strain construction.** In-frame deletion mutants of *nhaR* (VP0527), *torR* (VP1032), and *hns* (VP1133), were designed as previously described via allelic exchange (17). A truncated region of the *nhaR* (15-bp of the 891-bp), *torR* (54-bp of the 714-bp), and *hns* (33-bp of the 408-bp) genes was generated using the primers in Table S3. The truncated products were then ligated with the suicide vector pDS132 using the Gibson assembly protocol and transformed into *E. coli* Dh5α (83, 84). The resulting plasmids pDS∆nhaR, pDS∆torR, and pDS∆hns were purified and transformed into *E. coli* β2155 λpir (diaminopimelic acid auxotroph), followed by conjugation.
and homologous recombination into the *V. parahaemolyticus* RIMD2210633 genome. Single crossover of the plasmids into the genome were selected for by plating onto chloramphenicol (CM) and screened via PCR for a truncated allele. To induce a double-crossover event, the single-cross strain was grown overnight in the absence of CM, leaving behind either the truncated allele or the wild-type allele. The cultures were spread on 10% sucrose plates, and healthy colonies were screened for double-crossover and gene truncation; colonies that contain the plasmid will appear soupy on the plate due to the presence of the *sacB* selectable marker. In-frame deletions were confirmed with sequencing.

An in-frame deletion of *ompR* (VP0154) was created in *V. parahaemolyticus* RIMD2210633 through splicing by overlap extension (SOE) PCR and homologous recombination (85). Primers were designed to create a 692-bp truncated allele of *ompR* using the primer pair listed in Table S3. This truncated product was ligated into the cloning vector pJET1.2 with T4 DNA ligase and transformed into *E. coli* Dh5α λpir. The truncated *ompR* allele was then excised from the pJET∆ompR vector using restriction enzymes (SacI, XbaI) and ligated into the suicide vector pDS132. The pDS∆ompR vector was then transformed into *E. coli* β2155 λpir (DAP auxotroph), and the same procedure as described above was completed. Double mutants ∆leuO/∆hns and ∆hns/∆nhaR were generated by conjugating *E. coli* β2155 λpir + pDSnhaR with *V. parahaemolyticus* ∆leuO and β2155 λpir + pDSnhaR with *V. parahaemolyticus* ∆hns. The same procedure as described above was carried out to achieve these double deletions.

**Transcriptional GFP-reporter assay.** The reporter construct pRUP_{ectA-gfp} was created previously using the pRU1064 vector, which contains a promoterless *gfp* cassette, as well as Tet and Amp resistance genes (17, 86). Using *E. coli* β2155 λpir containing pRUP_{ectA-gfp}, the reporter plasmid was conjugated into each of the *V. parahaemolyticus* mutant strains ∆leuO,
\( \Delta \text{nhaR}, \Delta \text{torR}, \Delta \text{ompR}, \Delta \text{hns}, \Delta \text{leuO}/\Delta \text{hns}, \) and \( \Delta \text{hns}/\Delta \text{nhaR} \). Strains were grown overnight in LB3\% with tetracycline. The cultures were then washed two times with 1XPBS, diluted 1:100 into M9G3\% with tetracycline, and grown for 20 hours. The reporter expression was determined by measuring the relative fluorescence with excitation at 385 nm and emission at 509 nm in black, clear bottomed 96-well microplates on a Tecan Spark microplate reader with Magellan software (Tecan Systems, Inc., San Jose, CA). Specific fluorescence was calculated by dividing the relative fluorescence units (RFU) by the optical density of each well. At least two biological replicates were performed for each experiment. Statistics were completed either with a Student’s T-test or ANOVA followed by Tukey-Kramer Post Hoc test, as designated.

ACKNOWLEDGEMENTS

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Figure Legends

Fig. 1. Expression of a PectA-gfp transcriptional fusion in wild-type (WT) V. parahaemolyticus ΔleuO, ΔnhaR, ΔompR, and ΔtorR mutants. Cultures were grown overnight in M9G 3% NaCl and relative fluorescence intensity (RFU) was measured. Specific fluorescence was calculated by dividing RFU by OD. Mean and standard deviation of three biological replicates are shown. Statistics were calculated using a Student’s t-test; (*, P < 0.05; ***, P < 0.001).

Fig. 2. Expression of a PectA-gfp transcriptional fusion in wild-type (WT) V. parahaemolyticus ΔleuO, Δhns, and ΔleuO/Δhns mutants. Cultures were grown overnight in M9G3% and relative fluorescence intensity (RFU) was measured. Specific fluorescence was calculated by dividing RFU by OD. Mean and standard deviation of three biological replicates are shown. Statistics were calculated using an ANOVA followed by Tukey-Kramer post hoc test; (***, P < 0.001).

Fig. 3. Expression reporter assays of PleuO-gfp transcriptional fusion in V. parahaemolyticus wild type (WT), ΔleuO, Δhns, ΔleuO/Δhns, and ΔtoxRS mutants. Cultures were grown overnight in M9G3% and relative fluorescence intensity (RFU) was measured. Specific fluorescence was calculated by dividing RFU by OD. Mean and standard deviation of two biological replicates are shown. Statistics were calculated using a Student’s t-test; (*, P < 0.05; **, P < 0.005).

Fig. 4. Growth analysis of ΔleuO, Δhns and ΔleuO/Δhns mutants in M9G supplemented with 6%NaCl. Cells were grown to OD 0.5 in M9G1% and then inoculated into M9G M9G 6%NaCl and growth was measured every hour for 24h at 37°C. Mean and standard deviation of two biological replicates are shown. For all growth curves the area under the curve (AUC) was calculated for each strain and a Student’s t-test was performed (*, P < 0.05; **, P < 0.005; ***, P < 0.001).
Fig. 5. Expression of a $P_{ectA-gfp}$ transcriptional fusion in wild type (WT) *V. parahaemolyticus*, $\Delta nhaR$, $\Delta hns$, and $\Delta hns/\Delta nhaR$ mutants. Cultures were grown overnight in M9G3% and relative fluorescence intensity (RFU) was measured. Specific fluorescence was calculated by dividing RFU by OD. Mean and standard deviation of three biological replicates are shown. Statistics were calculated using an ANOVA followed by Tukey-Kramer post hoc test; (***, P < 0.001).

Fig. 6. Growth analysis of $\Delta nhaR$, $\Delta hns$ and $\Delta nhaR/\Delta hns$ mutants in M9G supplemented with 6%NaCl. Cells were grown to OD 0.5 in M9G1% and then inoculated into M9G6% NaCl and growth was measured every hour for 24 h at 37°C. Mean and standard deviation of two biological replicates are shown. For all growth curves the area under the curve (AUC) was calculated for each strain and a Student’s t-test was performed (**, P < 0.005).

Fig. 7. Model of ectoine biosynthesis gene expression in *V. parahaemolyticus*. Solid arrows represent positive regulation while hammer lines represent negative regulation. Previously it was shown that LeuO is required for OmpU expression, an important component of the acid stress response. Our data now show that the LeuO regulon also contains *ectABC* required for high salinity tolerance.

Supplementary data

Fig. S1. Ectoine production and *PectA-gfp* expression induced in M9G3% NaCl. **A.** $^1$H-NMR spectroscopy of *V. parahaemolyticus* wild type (WT) cell lysate grown in M9G 1%NaCl or M9G 3%NaCl. The spectral peaks for ectoine are indicated with arrows, and chemical shifts are expressed in ppm. **B.** Expression of *PectA-gfp* transcriptional fusion in *V. parahaemolyticus* WT. Cultures were grown overnight in M9G 1%NaCl or M9G 3%NaCl and relative fluorescence intensity (RFU) was measured. Specific fluorescence was calculated by dividing RFU by OD.
Mean and standard deviation of two biological replicates are shown. Statistics were calculated using a Student’s t-test; (**, P < 0.005).

**Fig. S2. DNA-affinity pulldown, protein separation with SDS-PAGE.** Proteins that bind to the regulatory region of *ectABC-aspk* were identified with DNA affinity chromatography and mass spectrometry. Shown here are gel pieces that were excised to identify candidate proteins.

**Fig. S3. LeuO and NhaR bind to the *ectABC-ask* regulatory region.** A. Region upstream of *ectABC-aspk* operon (P*ectA*) is presented here both as a large probe (2) and segmented into three smaller probes (1A, 1B, and 1C). B. LeuO binds to probe P*ectA*-2 and to the *leuO* regulatory region. C. NhaR binds to the P*ectA*-2 regulatory region. D. LeuO binds to segmented portions of P*ectA* probes. E. NhaR binds to segmented portions of P*ectA* probes. EMSA’s were performed with 30 ng of P*ectA*-gfp probe and purified LeuO or NhaR protein (0 to 2.175 uM) with DNA: protein molar rations of 1:0, 1:1, 1:10, 1:20, and 1:50.

**Figure S4. Growth analysis of mutants in 1%NaCl and 3%NaCl.** A. Wild type, Δ*leuO*, Δ*hns*, and Δ*leuO*/Δ*hns* in M9G supplemented with 1%NaCl or B. M9G 3%NaCl. C. Wild type, Δ*nhaR*, Δ*hns*, and Δ*hns*/Δ*nhaR* in M9G 1%NaCl or D. M9G 3%NaCl. Cells were grown to OD 0.5 in M9G1% and then inoculated into M9G 1%NaCl or M9G 3%NaCl and growth was measured every hour for 24 h at 37°C. Mean and standard deviation of two biological replicates are shown.
Table 1. Candidate proteins identified in DNA protein pulldown assay.

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**Fig. 1**

The figure shows a bar graph representing the specific fluorescence (RFU/OD) for different genotypes in a PectA-gfp reporter assay. The genotypes compared are WT, ΔleuO, ΔnhaR, ΔompR, and ΔtorR. The graph includes statistical significance marks (* and ***) indicating differences in fluorescence levels between the genotypes.
Fig. 3
Fig. 4
Fig. 5
Fig. 6
Fig. 7