Biosynthesis of the Osmoprotectant Ectoine, but Not Glycine Betaine, Is Critical for Survival of Osmotically Stressed *Vibrio parahaemolyticus* Cells

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Vibrio parahaemolyticus is a halophile present in marine and estuarine environments, ecosystems characterized by fluctuations in salinity and temperature. One strategy to thrive in such environments is the synthesis and/or uptake of compatible solutes. The *V. parahaemolyticus* genome contains biosynthesis systems for both ectoine and glycine betaine, which are known to act as compatible solutes in other species. We showed that *V. parahaemolyticus* had a 6% NaCl tolerance when grown in M9 minimal medium with 0.4% glucose (M9G) with a >5-h lag phase. By using ^1H nuclear magnetic resonance spectroscopy (^1H -NMR) analysis, we determined that cells synthesized ectoine and glutamate in a NaCl-dependent manner. The most effective compatible solutes as measured by a reduction in lag-phase growth in M9G with 6% NaCl (M9G 6% NaCl) were in the order glycine betaine > choline > proline = glutamate > ectoine. However, *V. parahaemolyticus* could use glutamate or proline as the sole carbon source, but not ectoine or glycine betaine, which suggests that these are bona fide compatible solutes. Expression analysis showed that the *ectA* and *betA* genes were more highly expressed in log-phase cells, and expression of both genes was induced by NaCl up-shock. Under all conditions examined, the *ectA* gene was more highly expressed than the *betA* gene. Analysis of in-frame deletions in *betA* and *ectB* and in a double mutant showed that the *ectB* mutant was defective for growth, and this defect was rescued by the addition of glycine betaine, proline, ectoine, and glutamate, indicating that these compounds are compatible solutes for this species. The presence of both synthesis systems was the predominant distribution pattern among members of the *Vibrionaceae* family, suggesting this is the ancestral state.

Vibrio parahaemolyticus is a halophile that is abundant in the aquatic environment and has been isolated from the water column and sediment and in association with crustaceans, mollusks, fish, and planktonic copepods (1–4). In the marine and estuarine environments, *V. parahaemolyticus* must navigate changing salinities, temperatures, and nutrient limitations and is known to proliferate during the warmer months of the year when the salinity and temperature are elevated (2, 5–9). *Vibrio parahaemolyticus* levels in marine and estuarine waters are linearly dependent on both salinity and water temperature, and in the winter, the bacterium is rarely isolated from the water column and is typically only found in small numbers in sediment (2, 5, 10).

Global climate change has resulted in an overall increase in ocean temperatures as well as the acidification of these waters, which also impacts the distribution of marine species (11–16). In recent years, occurrences of *V. parahaemolyticus* have been documented as far north as Alaska, and this northward migration trend may be attributed to the rise in ocean temperature (8, 9, 17). Interestingly, it was demonstrated that growth in differing NaCl concentrations alters the susceptibility of *V. parahaemolyticus* to other environmental stresses (18). It has been documented that growth of *V. parahaemolyticus* in 3% NaCl compared to 1% NaCl increased survival under both inorganic (HCl) and organic (acetic acid) acid conditions. In addition, at 42°C and –20°C, growth in 1% NaCl compared to 3% NaCl had a detrimental effect (18). It has also been suggested that temperature may play a role in virulence gene regulation (19).

Vibrio parahaemolyticus is of significant medical importance, as it is the leading cause of seafood-associated bacterial gastroenteritis worldwide (20–23). Generally, the bacterium infects the host through the gastrointestinal tract, where it encounters stress

conditions such as low pH, bile salts, antimicrobial peptides, and low salinity as well as challenges from the host immune system. Most infections of *V. parahaemolyticus* occur as a result of consumption of raw or undercooked contaminated seafood.

Bioinformatics analysis has shown that the *V. parahaemolyticus* genome contains two compatible solute biosynthesis gene clusters, ectoine (encoded by *ectABC-aspK*) and glycine betaine (encoded by *betIBA*) biosynthesis gene clusters (24–26). In addition to these synthesis systems, the genome encodes two putative glutamine synthetase genes (VP0121 and VP1781), a glutamate synthetase gene (VPA0765), and a glutamate synthase gene cluster (VP0481–VP0484) as well as a putative bifunctional proline dehydrogenase/pyrroline-5-carboxylate dehydrogenase that can perform the reversible reaction of glutamate to proline. Six putative compatible solute transporters, including four betaine/carnitine/choline transporters (BCCTs) and two ProVWX (also known as ProU) transporters are also contained within the genome (24–26). In many proteobacteria, the response to osmotic stress has two phases. The first phase is the short-term response resulting in the accumulation of K^+ . The second phase is the more long-term strategy, the synthesis and/or accumulation of compatible solutes that can be amassed in high concentrations without disturbing vital cellular functions (27). Compatible solutes include but are

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TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description or relevant characteristic(s)	Reference
<i>V. parahaemolyticus</i> strains		
RIMD2210633	O3:K6 clinical isolate	39
$\Delta betA$	RIMD2210633 $\Delta betA$ (VPA1112)	This study
$\Delta ectB-\Delta betA$	RIMD2210633 $\Delta betA$ (VPA1112) $\Delta ectB$ (VP1721)	This study
$\Delta ectB$	RIMD2210633 $\Delta ectB$ (VP1721)	25
<i>Escherichia coli</i> strains		
DH5 α λpir	λpir $\phi 80dlacZ\Delta M15$ $\Delta(lacZYA-argF)U169$ <i>recA1</i> <i>hsdR17</i> <i>deoR</i> <i>thi-1</i> <i>supE44</i> <i>gyrA96</i> <i>relA1</i>	This study
DH5 α λpir $\Delta betA$	DH5 α λpir containing pDS132 $\Delta betA$	This study
$\beta 2155$ DAP	Donor for bacterial conjugation; <i>thr1004</i> <i>pro</i> <i>thi</i> <i>strA</i> <i>hsdS</i> <i>lacZ</i> $\Delta M15$ (F' <i>lacZ</i> $\Delta M15$ <i>lacTRQJ</i> $\Delta 36$ <i>proA</i> ⁺ <i>proB</i> ⁺) $\Delta dapA$ <i>Erm</i> ^r <i>pirRP4</i> (Km ^r from SM10)	This study
$\beta 2155$ DAP- $\Delta betA$	$\beta 2155$ harboring pDS132 $\Delta betA$	This study
$\beta 2155$ DAP- $\Delta ectB$	$\beta 2155$ harboring pDS132 $\Delta ectB$	This study
Plasmids		
pJET1.2	General cloning vector; Amp ^r	This study
p $\Delta betA$	<i>betA</i> mutant cloned into pJET1.2	
pDS132	Suicide vector for conjugal transfer and integration; R6K <i>γori</i> <i>mobRP4</i> <i>sacB</i> Cm ^r	54
pDS132 $\Delta betA$	pDS132 harboring truncated <i>betA</i> gene	This study
pDS132 $\Delta ectB$	pDS132 harboring truncated <i>ectB</i> gene	25

not limited to trehalose, glycerol, mannitol, free amino acids such as glutamate, glutamine, and proline and their derivatives betaine, glycine betaine, and ectoine (27–33). It has been proposed that most bacteria use the trimethylammonium compound glycine betaine (*N,N,N*-trimethylglycine) as their preferred compatible solute (30, 34). However, one of the most widespread compatible solutes is ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidinecarboxylic acid) (30, 34). Ectoine is the only compatible solute synthesized in *Vibrio cholerae* and *Vibrio fischeri*, and in *V. cholerae*, it was shown to play a role in osmotolerance (35). Previously, we demonstrated using comparative physiological analysis that *V. parahaemolyticus* compared with *Vibrio vulnificus* YJ016, *V. cholerae* N16961, and *V. fischeri* ES114, which all contain fewer compatible solute systems, had a growth advantage under different salinity concentrations and temperatures (25). We showed using one-dimension ¹H nuclear magnetic resonance spectroscopy (¹H-NMR) that at high salinity *V. parahaemolyticus* is capable of *de novo* synthesis of ectoine whereas a $\Delta ectB$ strain is not (25). In a broader context, it has been shown that compatible solutes and solute activities can protect against nonosmotic stresses, for example high-pressure stress can be countered by kosmotropes (36), while some studies have shown that compatible solutes can cross-protect against heat and cold-temperature stress (27, 37, 38).

In this study, we examined the role of compatible solute synthesis in the *V. parahaemolyticus* NaCl tolerance response and determined the compatible solutes synthesized and utilized to greatest effect by this bacterium. It was determined by using ¹H-NMR analysis the major compatible solutes synthesized by *V. parahaemolyticus* when the cells were osmotically challenged. We examined whether *V. parahaemolyticus* could synthesize both ectoine and glycine betaine in the presence of their precursors aspartic acid and choline, respectively. The conditions under which the ectoine and glycine betaine biosynthesis genes are expressed and whether NaCl induces expression were established. Using a molecular genetic approach, constructed deletions in each of the biosynthesis systems was examined for their effect on growth and survival.

MATERIALS AND METHODS

Bacterial strains. Bacterial strains and plasmids used in this work are listed in Table 1. *Vibrio parahaemolyticus* RIMD2210633 serotype O3:K6 (39) and generated mutants were routinely grown at 37°C with aeration in Luria-Bertani (LB) broth (Fisher Scientific, Fair Lawn, NJ) with 3% NaCl (wt/vol), or in M9 minimal medium containing 47.8 mM Na₂HPO₄, 22 mM KH₂PO₄, 18.7 mM NH₄Cl, 8.6 mM NaCl (Sigma-Aldrich, USA) supplemented with 2 mM MgSO₄, 0.1 mM CaCl₂, and 0.4% (wt/vol) glucose as the sole carbon source (M9G) with 3% NaCl (wt/vol) (M9G 3% NaCl). To recreate conditions of elevated osmotic strength, LB and M9G media were prepared with increasing concentrations of NaCl ranging from 1% to 11% NaCl (wt/vol). Compatible solutes or their precursors were added to growth media at the following concentrations: 100 μ M glycine betaine, 100 μ M ectoine, 1,000 μ M choline, 1,000 μ M aspartic acid, 1,000 μ M glutamate, 1,000 μ M glutamine, and 1,000 μ M proline (Sigma-Aldrich, USA). *Escherichia coli* DH5 α λpir used in this study was grown in LB medium containing 1% NaCl (wt/vol) at 37°C under aerobic conditions and *E. coli* $\beta 2155$, an auxotroph for diaminopimelic acid (DAP), was grown at 37°C in LB containing 1% NaCl (LB 1% NaCl) broth supplemented with 0.3 mM DAP. All antibiotics were used at the following concentrations (wt/vol): ampicillin (Amp), 100 μ g/ml; chloramphenicol (Cm), 25 μ g/ml; and streptomycin (Str), 200 μ g/ml.

Growth analysis. Growth analysis of *V. parahaemolyticus* RIMD2210633 and mutants was performed in a 96-well Tecan Sunrise microplate reader (Tecan US Inc., Durham, NC) in LB or M9G medium adjusted to the desired NaCl concentration and in the presence or absence of compatible solute or their precursors. Briefly, precultures of *V. parahaemolyticus* were grown overnight in LB or M9G medium, and a 2% inoculum of stationary-phase cells was used to inoculate fresh medium and grown for 5 h at 37°C with aeration. A 1:40 dilution of this inoculum was subsequently used to inoculate a 96-well microplate filled with 200 μ l/well of medium adjusted to different NaCl concentrations. Bacterial growth was monitored hourly by measuring the optical densities (optical density at 595 nm [OD₅₉₅]) for a period of 24 h or longer. All measurements were done in triplicate using at least two biological replicates. The data obtained were then computed statistically and plotted as the averages of means using Origin 8.5 software program (OriginLab Corporation, MA, USA).

NMR analysis of cellular extracts. Cellular extracts of *V. parahaemolyticus* RIMD2210633 were prepared for nuclear magnetic resonance

TABLE 2 Primer pairs used in this study

Target	Primer ^a	Sequence (5'–3') ^b	Product (bp) ^c
VPA1112	VPA1112-A	TCTAGACCACGTACAGCAAGAGATCT	SOE-AB (379)
	VPA1112-B	cagctgagatctggtacc TTTCATTTTGTGTCTCCTA	
	VPA1112-C	ggtaccagatctcagctg TCTTAATCTTTAAAAACTG	SOE-CD (334)
	VPA1112-D	GAGCTCTCGTTGGCATCCAGTTACC	
	VPA1112FL-F	AACCGTATTTATCGAC	SOE-AD* (713)
	VPA1112FL-R	TTCCAGGTCAGCAAAGCTC	
VPr001	VPr001-RT-F	ACCGCCTGGGGAGTACGGTC	234
	VPr001-RT-R	TTGCGCTCGTTGCGGGACTT	
VPA1112	cbetA-F	AAAGAGGCGGGCTATCCAGAACT	264
	cbetA-R	TTTCTCAAATTCAACGCCGACCGC	
VP1721	cectA-F	CCAATGGCGGTTGTACTGCTGAAA	269
	cectA-R	TCACCGTGAATACACTCGATGCCA	

^a At the end of the primer designations, F stands for forward and R stands for reverse.

^b Lowercase bold letters indicate complementary sequence tags.

^c SOE-AB, product of SOE PCR using VPA1112-A and VPA1112-B primers; SOE-CD, product of SOE PCR using VPA1112-C and VPA1112-D primers; SOE-AD*, product of SOE PCR using VP1112FL-F and VP1112FL-R primers.

spectroscopy (NMR) analysis as previously described (25, 35). In brief, *V. parahaemolyticus* was cultured to log or stationary phase at 37°C in M9G supplemented to a NaCl concentration as indicated. Bacterial cells were then pelleted by centrifugation for 10 min at 1,000 × g, and the cell pellets were washed one time with fresh medium of equal salinity. Cells were then lysed by freeze-thaw cycles three times in dry ice and subsequently resuspended in 750 µl of ethanol. After centrifugation at 4,000 × g, ethanol extracts free of cellular debris were transferred into clean tubes, and ethanol was removed by evaporation in a Savant SpeedVac concentrator (Thermo Scientific, Waltham, MA) for 3 h. The resulting dried pellet material was suspended in 500 µl of deuterium oxide (D₂O) solvent (Cambridge Isotope Laboratories Inc., Andover, MA), and insoluble material was removed by centrifugation. The suspended organic materials were transferred into a 5-mm NMR tube (Wilmad LabGlass, Vineland, NJ), and ¹H-NMR spectral data were obtained by running samples in a Bruker Avance 400 NMR spectrometer at 400 MHz. Acquired ¹H-NMR spectra were processed and analyzed by ACD/NMR Processor Academic Edition software version 12.01 (ACD/Labs, Canada).

RNA isolation and cDNA synthesis. Wild-type *V. parahaemolyticus* RIMD2210633 was grown overnight at 37°C with aeration in LB or M9G medium containing 3% NaCl (wt/vol). A 2% aliquot of the overnight culture was then used to inoculate fresh LB 3% NaCl (wt/vol) or M9G 3% NaCl (wt/vol) and allowed to grow for 4 h (exponential/log phase) or 10 h (stationary phase). Cells grown in LB 3% NaCl (wt/vol) or M9G 3% NaCl (wt/vol) medium to either log or stationary phase were then subjected to osmotic up-shock conditions in 6% NaCl (wt/vol) for 30 min at 37°C. For all conditions examined, total RNA was isolated by adding 2 volumes of RNeasy lysis reagent (Qiagen kit; Valencia, CA) to the cell culture according to the manufacturer's instructions. Isolated RNA was quantified by Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA) and examined by gel electrophoresis on 0.8% agarose to assess quality. RNA was treated with a DNase kit (Turbo DNase; Invitrogen, Carlsbad, CA) to remove any genomic DNA contaminant per the manufacturer's protocol. The first-strand cDNA synthesis reaction was initiated with 500 ng of purified RNA as a template in a reaction primed with 200 ng of random hexamers, according to the manufacturer's protocol (Superscript II reverse transcriptase kit; Invitrogen).

Gene expression analysis. Transcriptional analysis was performed to assess the expression levels of ectoine and glycine betaine synthesis genes of *V. parahaemolyticus* RIMD2210633 in response to both high NaCl concentrations and different growth phases (log- and stationary-phase cells).

Reverse transcriptase PCR (RT-PCR) gene-specific primers were designed to amplify a 250- to 270-bp region of *ectA* and *betA* (Table 2). RT-PCR assays were then performed on cDNA diluted to 1:25 and 1:125. To ensure equal loading of the cDNA template in the RT-PCR and to correct for sampling errors, the expression level of each gene was compared to the level of the 16S rRNA (VPr001) control. Following RT-PCR amplification, the expression levels of the genes tested were assayed by running the samples on 1.8% agarose gel. RT-PCR cycling conditions used in these experiments were as follows: 3 min at 95°C; 29 cycles, with 1 cycle consisting of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C; and 5 min at 72°C. To quantify expression levels of *ectA* and *betA* after NaCl up-shock, quantitative PCR (qPCR) was performed. qPCR analysis was performed in a 20-µl reaction mixture to assess the fold change in expression levels of *ectA* and *betA* transcripts after salt up-shock in log- and stationary-phase cultures of *V. parahaemolyticus*. In brief, diluted cDNA template was mixed with HotStart-IT SYBR green qPCR master mix (USB, Santa Clara, CA) in a 96-well plate, and qPCR analysis was performed using an Applied Biosystems 7500 fast real-time PCR system (Foster City, CA). The following cycling conditions were used for the real-time PCR assay: 2 min at 95°C, followed by 40 cycles, with 1 cycle consisting of 10 s at 95°C and 30 s at 60°C. At the completion of the assay, the generated threshold cycle (*C_T*) mean values were normalized across the samples with the 16S rRNA control, and the gene expression levels relative to culture grown in 3% NaCl for 4 h were calculated by using the $\Delta\Delta C_{T_T}$ method (40). Two technical replicates and at least two biological replicates were performed for all assays. The significance of the different treatments was statistically computed using an unpaired Student's *t* test.

Mutant construction. A mutant harboring an in-frame nonpolar deletion in the choline dehydrogenase gene (*betA*) of *V. parahaemolyticus* was constructed by splicing by overlap extension (SOE) PCR and homologous recombination (41). The 1,746-bp gene sequence for choline dehydrogenase encoded by *vpa1112* (*betA*) located within chromosome II of *V. parahaemolyticus* was retrieved from the NCBI GenBank (reference sequence NC_004605.1). SOE PCR primers along with screening primers were then designed and analyzed using Primer Quest from Integrated DNA Technologies (IDT, USA) (Table 2). The VPA1112-A primer was designed to include an XbaI restriction site at the 5' end, while the VPA1112-B primer was modified at the 5' end by the addition of a 18-nucleotide tag. The VPA1112-C primer was modified at the 5' end to include a complementary sequence of the 18-nucleotide tag. Finally, the VPA1112-D primer was designed to include a SacI restriction site at the 5'

end (Table 2). For SOE PCR amplification, *V. parahaemolyticus* RIMD2210633 genomic DNA was used as a template. Two rounds of PCR amplifications (5 min at 95°C; 29 cycles, with 1 cycle consisting of 30 s at 94°C, 30 s at 51°C, and 2 min at 72°C; 10 min at 72°C) were performed first by using primer pairs VPA1112-A/VPA1112-B and VPA1112-C/VPA1112-D, generating products AB of 379 bp and CD of 334 bp. In the subsequent PCR amplification, primer pair VPA1112-A/VPA1112-D was used to generate a final product (betA-AD*) of 713 bp in size containing restriction sites for XbaI and SacI at the 5' and 3' ends, respectively. The purified betA-AD* DNA fragment was subcloned into the pJET1.2 vector (Fermentas, Glen Burnie, MD). The resulting recombinant plasmid p Δ betA was then transformed into *E. coli* DH5 α λ pir strain and cloned into a suicide vector, pDS132 (37). The resulting recombinant plasmid pDS132 Δ betA was subsequently used to transform *E. coli* β 2155 λ pir DAP auxotroph strain, which was conjugated into *V. parahaemolyticus* using a contact-dependent biparental mating by cross-streaking the two strains on LB agar with 1% NaCl and 0.3 mM DAP. Following a series of selections on chloramphenicol and sucrose plates, the recombinant clones that underwent double crossover (Δ betA) were selected for the phenotype SacB⁺ Cm^s and confirmed by colony PCR and DNA sequencing. Similarly, a Δ ectB Δ betA strain, devoid of both *betA* and *ectB* genes, was constructed by conjugating *E. coli* β 2155 λ pir pDS132 Δ ectB with *V. parahaemolyticus* *betA* mutant strain using the same protocol. The generated Δ ectB Δ betA strain was confirmed by PCR and DNA sequencing.

Phylogenetic tree construction. Detection of homologous protein sequences from compatible solute biosynthesis pathways was performed using EctA (VP1722) and BetA (VPA1112) sequences as probes in searches performed with the BLASTP program at the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov/BLAST). Sequences with minimum E values of 0.0001 without filtering were identified. We constructed a phylogenetic tree based on three housekeeping genes of all species of the *Vibrionaceae* family whose genome sequence is complete. The three housekeeping genes used were RNA polymerase subunit beta (*rpoB*), malate dehydrogenase (*mdh*), both present on chromosome I, and dihydrorotase (*pyrC*) present on chromosome II. Phylogenetic analysis was performed using complete concatenated sequences aligned by Clustal W 2.0, and the neighbor-joining (NJ) method was used for tree construction as implemented in MEGA5 (42). The bootstrap values for NJ trees were obtained after 1,000 generations, and MEGA5 tree viewer was used to visualize the trees and calculate confidence values (42). The locus tags for each of the genes examined are as follows: for *rpoB*, VSAL_12866, VHA_000316, VAS14_19156, V12G01_00020, VAA_00351, VIBR0546_19107, VIBC2010_11541, VC0328, VIC_000047, VcyCZ_010100012493, VF_2414, VFA_003646, VME_32530, VIBHAR_00225, VII00023_15976, VII_003403, VINI7043_00277, VordA3_010100006710, ZP_VIA_004104, VPMS16_1850, VP2922, VrotD_010100000953, VIS19158_14047, VISI1226_14532, AND4_18793, VEJY3_14765, VEA_002173, MED222_00572, VIBRN418_07985, VCJ_000034, VS_2963, VT1337_16523, and VV3159; for *mdh*, VSAL_10359, VHA_002058, VAS14_08310, V12G01_12048, VAA_01685, VIBR0546_19297, VIBC2010_15622, VC_0432, VIC_004828, VcyCZ_010100009988, VF_0276, VFA_000332, VME_16640, VIBHAR_00795, VII00023_17031, VII_003296, VINI7043_19588, VordA3_010100015387, VIA_003995, VPMS16_415, VP0325, VrotD_010100003320, VIS19158_04331, VISI1226_19579, AND4_18426, VEJY3_01590, VEx25_0219, MED222_17215, VIBRN418_11585, VCJ_000156, VS_0358, VT1337_16653, and VV0467; and for *pyrC*, VSAL_110468, VHA_002563, VAS14_0095, V12G01_05941, VAA_00972, VIBR0546_14415, VIBC2010_09342, VCA0925, VIC_003248, VcyCZ_010100004852, VF_A0412, VFA_003087, VME_35630, VIBHAR_05227, VII00023_05282, VII_000508, VINI7043_04290, VordA3_010100001797, VIA_000879, VPMS16_875, VPA0408, VrotD_010100021328, VIS19158_18031, VISI1226_03785, AND4_06254, VEJY3_22466, VEA_001337, MED222_08928, VIBRN418_08787, VCJ_003244, VS_110272, VT1337_19727, and VVA0407.

RESULTS

***Vibrio parahaemolyticus* has a higher salt tolerance in nutrient-rich media.** To determine the role of nutrient availability in salt stress tolerance of *V. parahaemolyticus*, growth was analyzed in the presence of increasing salinity (3% to 11% NaCl) in LB medium at 37°C with aeration (Fig. 1A). It was found that under these conditions *V. parahaemolyticus* can grow in up to 10.5% NaCl (wt/vol). Next, we examined the NaCl tolerance range of *V. parahaemolyticus* in M9G containing 3 to 9% NaCl (wt/vol) at 37°C with aeration (Fig. 1B). Under these conditions in M9G, where compatible solutes and their precursors were not exogenously present, *V. parahaemolyticus* could grow at an upper maximum of 6% NaCl but with an extended lag phase of 5 h (Fig. 1B). This extended lag phase was reduced to 2.5 h or less when ectoine or glycine betaine or their precursors aspartic acid or choline were exogenously supplied (Fig. 1C). These data showed that *V. parahaemolyticus* cells had a broad salt stress tolerance range when grown in complex media compared with defined media. In addition, these data suggested that *V. parahaemolyticus* can synthesize both ectoine and glycine betaine, since the addition of their precursors to the media reduced the lag phase in M9G 6% NaCl.

***Vibrio parahaemolyticus* synthesizes glutamate and ectoine in response to NaCl stress.** The major compatible solutes synthesized by stationary-phase *V. parahaemolyticus* cells were investigated by proton NMR (¹H-NMR) at 400 MHz in the presence of deuterium oxide (D₂O) as the solvent. From cells grown in M9G 1% NaCl, no known major compatible solutes were found to be present (Fig. 2A). Protons corresponding to alanine and other organic compounds were noted, which are likely metabolic products produced in these stationary-phase cells (Fig. 2). In M9G 3% or 6% NaCl, protons peaks corresponding to ectoine and glutamate, as illustrated by their chemical shift values expressed in ppm, were identified (Fig. 2B and C). It also appears that in M9G 6% NaCl, the intensity and peak size increase for ectoine and decrease for glutamate, suggesting that ectoine is produced in a NaCl-dependent manner. Also, an examination of exponential-phase *V. parahaemolyticus* cells was performed by ¹H-NMR, and a similar pattern was found for all three conditions (data not shown).

We determined whether the reduction in the lag phase seen when cells were grown in the presence of choline or aspartic acid was due to conversion of these two precursors to glycine betaine or ectoine, respectively, and not the result of these compounds being used as carbon and energy sources (Fig. 1B). To examine this, *V. parahaemolyticus* was cultured in M9G 6% NaCl supplemented with 1 mM choline or aspartic acid, and the presence of glycine betaine and ectoine was evaluated by ¹H-NMR. It was found that both glycine betaine and ectoine were synthesized in the presence of their respective precursors (data not shown). To address the question of whether *V. parahaemolyticus* can simultaneously synthesize both ectoine and glycine betaine, *V. parahaemolyticus* cells were grown in M9G 6% NaCl supplemented with 1 mM choline and 1 mM aspartic acid. By NMR analysis, it was shown that *V. parahaemolyticus* was able to synthesize both compatible solutes; however, the normalized intensities of the proton peaks corresponding to glycine betaine were much larger than those of the ectoine peaks, suggesting that more glycine betaine was produced than ectoine (Fig. 3).

FIG 1 Growth of *V. parahaemolyticus* RIMD2210633 in LB medium containing 3 to 11% NaCl (A), M9 medium with 0.4% glucose (M9G) containing 3 to 9% NaCl (wt/vol) (B), and M9G with 6% NaCl (M9G 6% NaCl) (C) in the absence or presence of compatible solutes (CS) or their precursors. Symbols: in panel A, open squares, 3% NaCl; closed squares, 6% NaCl; closed stars, 9% NaCl; closed triangles, 10.5% NaCl; open triangles, 11% NaCl; in panel B, open squares, 3% NaCl; open circles, 5.5% NaCl; closed squares, 6% NaCl; filled stars, 9% NaCl; in panel C, closed squares, 6% NaCl; closed triangles, glycine betaine added; closed circles, choline added; open circles, aspartic acid added; open triangles, ectoine added. Values represent the mean optical densities at 595 nm from triplicate technical replicates and at least two biological replicates, and error bars show the standard deviations and may be obscured by symbols.

To examine whether compatible solutes or their precursors can be used as the sole carbon and energy sources, *V. parahaemolyticus* cells were grown in M9 1% NaCl medium supplemented with glycine betaine, choline, ectoine, aspartic acid, proline, glutamate, or glutamine as the sole carbon source. We found that glutamate, aspartic acid, and proline could be used as the sole carbon sources. After an 18-h lag phase, *V. parahaemolyticus* grew poorly in M9 1% NaCl medium supplemented with glutamine. There was no detectable growth from cultures containing choline, ectoine, or glycine betaine as the carbon source. This indicates that ectoine and glycine betaine are bona fide compatible solutes and that choline is used solely as a precursor for glycine betaine synthesis (Fig. 4).

Ectoine synthesis gene *ectA* is highly expressed in *V. parahaemolyticus* cells during salt stress. To determine whether there is differential expression of the ectoine and glycine betaine synthesis genes, we examined expression of *ectA* and *betA* genes from cells grown under different salinity conditions. First, we examined expression from log- and stationary-phase cells grown in LB 3% NaCl (Fig. 5A). Reverse transcriptase PCR (RT-PCR) and quantitative PCR (qPCR) were utilized to determine the transcript levels of *ectA* and *betA*. It was found that both genes in LB were more highly expressed in log-phase cells than in stationary-phase cells (Fig. 5A). Then, expression of these genes was examined under the same conditions after a 30 min up-shock in 6% NaCl. The *ectA* gene showed increased expression in log- and stationary-phase cells after NaCl up-shock. The *betA* gene also showed increased expression after NaCl up-shock in log-phase cells, but not in stationary-phase cells (Fig. 5A).

Next the expression pattern of *ectA* and *betA* was examined in log- and stationary-phase cells grown in M9G 3% NaCl (Fig. 5B). It was found that in defined media, the expression level of *ectA* was induced in log-phase cells compared to stationary-phase cells, whereas *betA* showed low levels of expression in log-phase and stationary-phase cells. Similarly, we found differential expression under the same conditions after a 30-min up-shock in 6% NaCl. Both *ectA* and *betA* showed increased expression after the NaCl up-shock during log-phase growth with *ectA* showing the higher level of expression. In stationary-phase cells after the 30 min up-shock in 6% NaCl only, the *ectA* gene was expressed (Fig. 5B). Using qPCR, we quantified these expression patterns and demonstrated that the *ectA* gene was statistically more highly expressed under all conditions examined (Fig. 5C and D). For example, *ectA* showed approximately 200-fold and 60-fold change in expression after NaCl up-shock in complex and defined media, respectively (Fig. 5C and D), whereas under the same conditions, *betA* showed a 14-fold and 1.75-fold change. Together, these results demonstrated that the expression of both genes was growth phase dependent and induced by NaCl (Fig. 5C and D).

Ectoine synthesis is essential for *V. parahaemolyticus* growth in high-salt, low-nutrient stress conditions. We determined whether synthesis of both ectoine and glycine betaine was critical for *V. parahaemolyticus* growth under osmotic stress. To achieve this, growth of a $\Delta betA$ strain defective in glycine betaine synthesis, a $\Delta ectB$ strain defective in ectoine synthesis, and a $\Delta ectB-\Delta betA$ strain defective in both glycine betaine and ectoine synthesis were examined. Growth of the wild-type strain was compared with that of the *betA* mutant in M9G 6% NaCl medium, and both strains showed comparable growth (Fig. 6A). However,

FIG 2 400-MHz ^1H -NMR spectroscopy of *V. parahaemolyticus* RIMD2210633. The spectral peaks were recorded for cells grown in M9G 1% NaCl (A), M9G 3% NaCl (B), and M9G 6% NaCl (C). The chemical environments of each type of proton or chemical shifts (δ) are expressed in ppm in the figure. The spectral peaks corresponding to the different compounds are labeled with the names of the compounds.

when the $\Delta betA$ strain was cultured in the presence of the precursor choline, no growth occurred, indicating that choline was toxic to the cells, and further examination of these cultures determined that choline was bacteriostatic. To investigate whether this bacteriostatic effect was due to internal accumulation of choline, NMR analysis was performed. Cells were grown to exponential phase in

M9G 6% NaCl, pelleted, and resuspended in M9G 6% NaCl supplemented with 1 mM choline for 1 h, and then NMR analysis was performed. Our results indicated that choline had accumulated internally in the *betA* mutant cells, but no choline was present in wild-type cells grown under the same conditions (data not shown). In M9G 6% NaCl supplemented with glycine

FIG 3 400-MHz ^1H -NMR spectroscopy of *V. parahaemolyticus* RIMD2210633 grown in the presence of compatible solute precursors. The spectral peaks were recorded for *V. parahaemolyticus* grown in M9G 6% NaCl supplemented with aspartic acid and choline. The chemical environments of each type of proton or chemical shifts (δ) are expressed in ppm in the figure. The spectral peaks corresponding to the different compounds detected are labeled with the names of the compounds.

FIG 4 Growth of *V. parahaemolyticus* RIMD2210633 in M9 1% NaCl supplemented with 20 mM compatible solutes or their precursors as the sole carbon sources. Symbols: closed squares, glucose; open squares, glutamate; closed diamonds, aspartic acid; open triangles, proline; open inverted triangles, glutamine. Some of the following symbols that represent no growth may be obscured; closed circles, no glucose; closed triangles, choline; closed inverted triangles, glycine betaine; open circles, ectoine.

betaine, the *betA* mutant grew better than the wild-type strain, which was demonstrated by a reduction in the lag phase from ~5 h to ≤1 h (Fig. 6A). In the same medium supplemented with either ectoine or aspartic acid, the $\Delta betA$ strain also had a reduced lag phase but not to the same extent as when the medium was supplemented with glycine betaine (Fig. 6A). Overall, these data suggest that the betaine synthesis system is not essential for growth in high-salt, low-nutrient media. However, glycine betaine is a more effective compatible solute than ectoine in *V. parahaemolyticus*.

Next, the role of the ectoine synthesis system in the growth of *V. parahaemolyticus* under high-salt and low-nutrient conditions was investigated. A deletion mutation was constructed previously in the *ectB* gene, which knocked out the synthesis system (15). This deletion mutant grew similar to the wild-type strain in M9G 3% NaCl, demonstrating that there is no overall growth defect (data not shown). However, it was found that, unlike the *betA* mutant, the $\Delta ectB$ strain could not grow in M9G 6% NaCl, suggesting that ectoine synthesis is essential (Fig. 6B). We investigated the importance of both synthesis systems by examination of growth of the $\Delta ectB$ - $\Delta betA$ strain under the same growth conditions described above. The double mutant grew similar to the wild type in M9G 3% NaCl but showed no growth in M9G plus 6% NaCl (Fig. 6C). The ability of different compatible solutes (glycine betaine, ectoine, proline, glutamate, and glutamine) and their precursors (aspartic acid and choline) to rescue growth of the double mutant in M9G 6% NaCl was tested (Fig. 6C). It was found that in the presence of glycine betaine (open circles), proline (closed squares), ectoine (open squares), or glutamate (closed triangles), the double mutant strain grew (Fig. 6C). The compatible solutes demonstrated the following effectiveness as determined by lag phase time reduction and overall final OD: glycine betaine > proline > ectoine > glutamate. In the presence of ectoine, there was a slight defect in the $\Delta ectB$ - $\Delta betA$ strain, since the mutant did not show the same reduction in the lag phase as the $\Delta ectB$ strain (Fig. 6C). In summary, under the conditions ex-

amined herein, our data show that the ectoine synthesis system is essential for growth under osmotic stress conditions and ectoine, glycine betaine, and proline are effective compatible solutes used by this species.

The presence of both ectoine and glycine betaine biosynthesis systems is widely distributed among *Vibrio* spp. Of the 33 species of the family *Vibrionaceae* whose complete genome sequences are available in the NCBI genome database, we identified the ectoine cluster alone in 7 species, the glycine betaine cluster alone in 5 species, both gene clusters in 20 species, and neither gene cluster in 1 species (Fig. 7). Depending on the species, the ectoine synthesis system could be present in either chromosome I or II, although for most species it was present in chromosome II. Only in *Vibrio parahaemolyticus*, *Vibrio alginolyticus*, and *Vibrio harveyi* was it contained in chromosome I where it was always adjacent to a BCCT transporter. The ectoine genes were present among all strains of a given species with the exception of *V. harveyi* HY01 and two strains of *Vibrio splendidus*. In *V. harveyi* HY01, the genes appear to be deleted, as we found partial sequence of *aspK* and a homologue of the BCCT transporter encoded by VP1723 in the expected location on the genome as is present in *V. parahaemolyticus*. The two strains of *V. splendidus* have incomplete genome sequences so we cannot speculate on their absence. In cases where it could be determined from the genome sequence, the glycine betaine gene cluster was always present on chromosome II. The genes were present in all representatives of the species. We reconstructed the evolutionary history based on three housekeeping genes, *rpoB*, *mdh*, and *pyrC*, of the 33 species examined. We mapped the distribution of ectoine and betaine biosynthesis gene clusters onto this tree. The predominant distribution is the presence of both systems in most species. This is true for the most divergent species within the group analyzed, *Photobacterium angustum* and *Grimontia hollisae*, which strongly suggests that both systems are ancestral (Fig. 7). The presence of only the ectoine system in both *V. cholerae* and *Vibrio mimicus* but the presence of both synthesis systems in their close relative *Vibrio furnissii* suggests that deletion of the betaine synthesis system occurred in the last common ancestor to give rise to these species. A similar evolutionary scenario can be proposed for the presence of only the ectoine system in *Vibrio ichthyenteri*, *Vibrio* sp. strain N418, and *Vibrio scophthalmi*. It is of interest to note that *V. fischeri* also contains only the ectoine system, but its closest relative on the tree, *Vibrio salmonicida*, contains neither the ectoine nor betaine synthesis system. This is the only species examined within this family that contained neither system. Interrogation of the genome sequence of *V. salmonicida* did uncover a potential proline synthesis system, ProAB and ProI, which would allow the conversion of glutamate to proline for use as a compatible solute.

DISCUSSION

Synthesis and accumulation of compatible solutes are widely used by bacteria as a strategy to relieve the growth constraints imposed by increased osmolarity (27, 30, 34, 43, 44). This study showed that the addition of exogenous compatible solutes and their precursors to M9G 6% NaCl resulted in reduction of lag-phase growth, indicating that *V. parahaemolyticus* is able to transport, accumulate, and synthesize compatible solutes (Fig. 1). Using ¹H-NMR analysis, we showed that ectoine and glutamate were synthesized in M9G 6% NaCl. The role of glutamate in osmotic stress

FIG 5 Expression analysis of *ectA* and *betA* genes. (A and B) RT-PCR of *ectA* and *betA* from *V. parahaemolyticus* cells grown to log or stationary phase in LB (A) or M9G (B) and subjected to 30 min up-shock in 6% NaCl. The cDNA templates were diluted 1:25 and 1:125 as indicated by triangles. The 16S rRNA control is shown for each sample. (C) qPCR of *ectA* and *betA* from cells grown to log or stationary phase in LB and subjected to 30 min up-shock in 6% NaCl. (D) qPCR of *ectA* and *betA* from cells grown to log or stationary phase in M9G and subjected to 30 min up-shock in 6% NaCl. Bars represent the expression of the indicated genes normalized to 16S rRNA and are relative to those of log-phase cells. Error bars indicate standard deviations. P values were calculated using an unpaired Student's t test with a 95% confidence interval. Asterisks denote significant differences as follows: *, $P < 0.05$; **, $P < 0.02$.

has been described, for example in *E. coli* it acts as a K^+ counter anion during the early stage of NaCl stress (45, 46). The moderate halophile *Halobacillus halophilus*, under moderate salinities accumulates glutamate and glutamine to adjust turgor (47). In this organism, one of the major roles of glutamate is to induce proline synthesis at high-salinity conditions (48). How-

ever, in *V. parahaemolyticus*, the accumulation of glutamate is not sufficient for long-term survival, since the *ectB* mutant cannot grow in defined media under high-NaCl stress, but the *betA* mutant can, indicating that ectoine is the main compatible solute synthesized *de novo*. We examined the NMR profile of wild-type cells grown in the presence of equal concentrations of the precu-

FIG 6 Growth of $\Delta betA$ (A), $\Delta ectB$ (B), and $\Delta betA \Delta ectB$ (C) strains in M9G 6% NaCl in the presence or absence of compatible solute or their precursors. Symbols: in panel A, closed squares, wild type; open squares, $\Delta betA$ strain; closed circles, $\Delta betA$ strain with choline added; open circles, $\Delta betA$ mutant with ectoine added; closed triangles, $\Delta betA$ strain with glycine betaine added; open triangles, $\Delta betA$ strain with aspartic acid added; in panel B, closed squares, wild type; open squares, $\Delta ectB$ strain; closed circles, $\Delta ectB$ strain with choline added; open circles, $\Delta ectB$ strain with aspartic acid added; closed triangles, $\Delta ectB$ strain with glycine betaine added; open triangles, $\Delta ectB$ strain with ectoine added; in panel C, closed squares, $\Delta betA \Delta ectB$ strain with proline added; open squares, $\Delta betA \Delta ectB$ strain with ectoine added; open circles, $\Delta betA \Delta ectB$ strain with glycine betaine added; closed circles, $\Delta betA \Delta ectB$ strain; closed triangles, $\Delta betA \Delta ectB$ strain with glutamate added; closed inverted triangles, $\Delta betA \Delta ectB$ strain with choline added; diamonds, $\Delta betA \Delta ectB$ strain with aspartic acid added. Values represent the mean optical densities at 595 nm from triplicate technical replicates and at least two biological replicates, and error bars show the standard deviations.

sors aspartic acid and choline and found that both ectoine and glycine betaine can be synthesized simultaneously. We demonstrated that ectoine and glycine betaine are bona fide compatible solutes that along with choline cannot be used as the sole carbon and energy sources, whereas glutamate, aspartic acid, proline, and to a much lesser extent glutamine can be used as the sole carbon sources. The use of choline and glycine betaine as the sole carbon sources in *Pseudomonas aeruginosa* has been documented and is facilitated in this species by the presence of high-affinity choline and glycine betaine transporters (49–51). This species can also use these substrates as effective compatible solute but cannot synthesize them *de novo*.

V. parahaemolyticus can synthesize ectoine and can synthesize glycine betaine from choline and does so quite effectively but cannot use these compounds as the sole carbon sources. However, as our data have shown, *V. parahaemolyticus* can use proline as the sole carbon source and an effective compatible solute, but it cannot synthesize it *de novo*. Since *V. parahaemolyticus* contains at least six putative compatible solute transporters, uptake of proline may be an important osmotic tolerance strategy for this organism, which needs to be examined further (18, 25, 52).

We showed that both *ectA* and *betA* are constitutively expressed in LB and M9 media, and both are more highly expressed in log-phase cells than in stationary-phase cells. Although we did find that the NaCl up-shock induces expression of both genes under all conditions examined, it was found that the *ectA* gene was always more highly expressed than *betA*. Together with the growth assay and NMR analyses, these data demonstrated that ectoine synthesis is critical for growth under osmotic stress conditions.

The contribution of both *V. parahaemolyticus* synthesis systems to high-salt stress survival was examined via the use of in-frame single and double deletion mutants. These data indicated that the glycine betaine synthesis system was not essential for growth under osmotic stress conditions. We found that the $\Delta ectB$ strain did not grow under high-NaCl stress conditions, indicating that this system is essential. To determine whether there was any cumulative effect in deleting both *ectB* and *betA*, we examined the double mutant and found that the addition of glycine betaine restored growth similar to wild-type levels. We also found that the addition of proline was highly effective in rescuing the double mutant, indicating that this is a powerful osmotic tolerance solute in this species. Glutamate could rescue the double mutant somewhat, but the data suggest that this solute is likely not an important compatible solute for this organism. Overall, these data indicate that glycine betaine, proline, ectoine, and to a much lesser extent glutamate can act as compatible solutes in *V. parahaemolyticus*.

Previously, it was suggested that the ability to synthesize ectoine is specific for halophilic bacteria and the results of our analysis suggest that it is essential for moderate halophile survival (27, 53). In our analysis of the distribution of *EctA* and *BetA* among the family *Vibrionaceae*, we found that of 33 species examined, 20 species contained both systems with nearly 70% containing the ectoine system. Of 284 species of *Gammaproteobacteria* species examined, we found 43% contained the ectoine synthesis genes, indicating that its prevalence is much higher in *Vibrio* species. We did find that most species examined did encode both systems, including *P. angustum* and *G. hollisae*, the two most divergent

FIG 7 Phylogenetic tree based on the concatenated sequences of three housekeeping genes, *rpoB*, *mdh*, and *pyrC*, for 33 species from the family *Vibrionaceae*. Almost all the species shown in the tree are *Vibrio* species. The evolutionary history was inferred using the neighbor-joining method (55). The bootstrap consensus tree inferred from 1,000 replicates is taken to represent the evolutionary history of the taxa analyzed. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Jukes-Cantor method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA5 (42). E, ectoine cluster; B, betaine cluster; B1 and E1, chromosome 1; B2 and E2, chromosome 2.

species, which suggests that both systems are ancestral and important for these diverse marine organisms.

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