

Osmoadaptation among *Vibrio* Species and Unique Genomic Features and Physiological Responses of *Vibrio parahaemolyticus*^{∇†}

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Vibrio parahaemolyticus is a moderately halophilic bacterium found in estuarine and marine coastal ecosystems worldwide. Although the ability of *V. parahaemolyticus* to grow and proliferate in fluctuating saline environments is well known, the underlying molecular mechanisms of osmoadaptation are unknown. We performed an in silico analysis of *V. parahaemolyticus* strain RIMD2210633 for genes homologous to osmotic stress response genes in other bacteria. We uncovered two putative compatible solute synthesis systems (encoded by *ectABC* and *betABI*) and six putative compatible solute transporters (encoded by four *bct* loci and two *proVWX* loci). An ectoine synthesis system clustered with a betaine/carnitine/choline transporter and a ProU transporter (encoded by homologues of *proVWX* from *Escherichia coli*), and a betaine synthesis system clustered with a ProU transporter (encoded by homologues of *proVXW* from *Pseudomonas syringae*). This is at least double the number present in *V. cholerae*, *V. fischeri*, or *V. vulnificus*. Six additional *Vibrio* species contain both *ectABC* and *betABI*, i.e., *V. alginolyticus* 12G01, *V. angustum*, *V. harveyi* BAA-1116, *V. splendidus* LGP32, *Vibrio* sp. strain MED222, and *Vibrio* sp. strain Ex25. *V. harveyi* HY01 and *V. splendidus* 12B01 only encoded the betaine system. In addition, *V. alginolyticus* had a compendium of systems identical to that found in *V. parahaemolyticus*. Comparative physiological analysis of RIMD2210633 with *V. vulnificus* YJ016, *V. cholerae* N16961, and *V. fischeri* ES114 grown at different salinities and temperatures demonstrated that *V. parahaemolyticus* had a growth advantage under all of the conditions examined. We demonstrate, by one-dimensional nuclear magnetic resonance analysis, that *V. parahaemolyticus* is capable of de novo synthesis of ectoine at high salinity whereas a Δ *ectB* knockout strain is not. We constructed a single-knockout mutation in *proU1*, but no growth defect was noted, indicating transporter system redundancy. We complemented *E. coli* MKH13, a compatible solute transporter-negative strain, with *bct2* and demonstrated uptake of betaine at high salt concentrations.

Vibrio parahaemolyticus is a moderate halophile prevalent in all of the coastal waters around the world, particularly in the warmer summer months (17). *V. parahaemolyticus* is found associated with zooplankton and phytoplankton and is present in sea sediment (18–20). *V. parahaemolyticus* is a pathogen of fish and humans and is the leading cause of seafood-associated bacterial gastroenteritis worldwide. Fish and shellfish, particularly oysters, are implicated as the major vectors for infection (5, 7, 27). Numerous outbreaks of *V. parahaemolyticus* infection in the Pacific Northwest have resulted in severe economic losses and closures in the seafood industry (27). A number of environmental factors affect the occurrence and distribution of *V. parahaemolyticus*, such as temperature, salinity, oxygen availability, plankton, and tidal flushing (8–10, 18–20). Because all of the *V. parahaemolyticus* strains inhabit marine, brackish, and estuarine waters, fluctuations in temporal and persistent salinity pose a constant challenge to the adaptive response of the organism.

In most bacteria, the response to osmotic upshock has two phases (3, 11, 31, 32, 40, 43). The immediate and short-term

response to hyperosmotic and high-salinity changes is the accumulation of K⁺. This is the primary strategy for many extremophiles living in high-salinity environments (37). Because high K⁺ concentrations are detrimental to most cells, a more long-term strategy to deal with osmotic upshock is required (3, 11, 31, 32, 40, 43). The second strategy, and the one more widely used among halophiles and for salt adaptation in general among bacteria, actinomycetes, algae, fungi, and yeasts, is the synthesis and/or accumulation of organic osmotic solutes (Fig. 1) (3, 11, 31, 32). These are known as compatible solutes or osmolytes since they are amassed in high concentrations without disturbing vital cellular functions (6). Osmolytes include sugars such as trehalose, free amino acids such as proline and glutamate, and their derivatives betaine, glycine betaine, and ectoine, as well as a number of esters and amines (6, 11, 34–36, 40).

The majority of bacteria utilize the trimethylammonium compound glycine betaine (*N,N,N*-trimethylglycine) as their preferred compatible solute (23, 24, 26, 29, 40, 43). *Escherichia coli*, which can grow at a maximum NaCl concentration of 0.5 M, can convert choline to betaine by using enzymes encoded by *betABI*, and choline is transported into the cell by the high-affinity BetT system, as well as by a low-affinity ProU transporter encoded by *proVWX* (11). One of the most widespread compatible solutes is ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidinocarboxylic acid) (23, 24, 26, 29, 40, 43, 44). The

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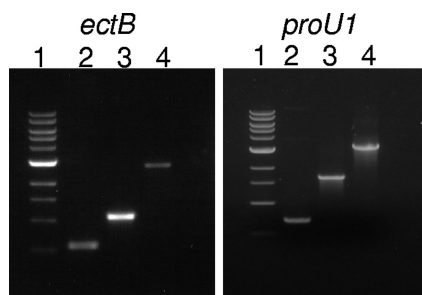


FIG. 1. PCR confirmation of truncated alleles and double-crossover events in deletion mutagenesis of the *ectB* and *proU1* genes of *V. parahaemolyticus* RIMD2210633. *ectB*: lane 1, 1-kb DNA ladder; lane 2, 533-bp *ectAD* product generated via SOE PCR; lane 3, 1.04-kb truncated *ectB* (double crossover); lane 4, 2.73-kb wild type. *proU1*: lane 1, 1-kb DNA ladder; lane 2, 428 bp; lane 3, 1.64 kb (double crossover); lane 4, 3.18-kb wild type.

pathway for ectoine synthesis has been determined for several moderate halophiles, and in all cases the products of the *ectABC* genes are required (15, 41, 42). Ectoine was shown to play a role in osmotolerance in *V. cholerae*; when Pflughoeft et al. exposed a Δ *ectA* mutant strain to high osmolarity, they observed a pronounced growth delay compared to the wild-type strain (33). In *E. coli*, which lacks an ectoine synthesis system, the ProP (encoded by *proP*) and ProU transporters were shown to take up a wide variety of osmoprotectants, including ectoine (22). ProU shows a preference for glycine betaine and proline betaine in *E. coli* and is highly upregulated in high-osmolarity medium (12).

In this study, we first examined the genome of *V. parahaemolyticus* RIMD2210633 and identified homologues of *ectABC* and *betABI*, as well as homologues of four betaine/carnitine/choline transporters (BCCTs) and two ProU compatible solute transporters, triple the number of systems identified in *V. cholerae* and double the number present in *V. vulnificus* and *V. fischeri*. Six additional *Vibrio* species encode both *ectABC* and *betABI*, i.e., *V. alginolyticus* 12G01, *V. angustum*, *V. harveyi* BAA-1116, *V. splendidus* LGP32, *Vibrio* sp. strain MED222, and *Vibrio* sp. strain Ex25. *V. alginolyticus* 12G01 had the same number and arrangement of compatible solute systems as *V. parahaemolyticus*. Comparative growth analysis experiments demonstrated that at high salinity and at high or low temperatures, *V. parahaemolyticus* had a growth advantage over *V. cholerae*, *V. vulnificus*, and *V. fischeri*. We show that the *ectABC* gene cluster in *V. parahaemolyticus* is required for de novo ectoine synthesis but that there is functional redundancy due to the large number of compatible solute transporters available.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The bacterial strains and plasmids used in this study are described in Table 1. *V. parahaemolyticus* RIMD2210633 was cultured in Luria-Bertani medium (LB) with a final NaCl content of 3% (pH 7.5) and grown at 37°C. *E. coli* strain DH5 α - λ *pir* was grown in LB broth at 37°C. The auxotrophic *E. coli* DAP strain was supplied with 0.3 mM diaminopimelic acid for growth in LB broth. Chloramphenicol was added at a concentration of 25 μ g/ml. The minimal medium used in growth studies was the previously described osmolarity medium (OM) (21, 33). The OM was adjusted by addition of NaCl at 200 mM (1%) for overnight preculture and 500 mM (3%), 1,000 mM (6%), or 1,500 mM (9%) for growth analysis over 24 h.

Genomic analysis and construction of Δ *ectB* and Δ *proU1* deletion mutants. Homology searches were conducted with the BLAST (basic local alignment search tool) function (1). Genomic DNA was isolated with the Gnome DNA extraction kit in accordance with manufacturer's instructions. Plasmid DNA was

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
<i>V. parahaemolyticus</i> RIMD2210633	O3:K6 clinical isolate	25
Δ <i>ectB</i>	RIMD2210633 <i>ectB</i> mutant (VP1721)	This study
Δ <i>proU1</i>	RIMD2210633 <i>proU1</i> mutant (VP1726-VP1728)	This study
<i>V. vulnificus</i> YJO16	Clinical isolate	4
<i>V. cholerae</i> N16961	O1 El Tor biotype	13
<i>V. fischeri</i> ES114	Isolate from <i>Euprymna scolopes</i>	38
<i>E. coli</i> MKH13	Δ <i>proP2</i> Δ <i>proU608</i> Δ <i>putPA101</i>	12
DH5 α - λ <i>pir</i>	λ <i>pir</i> ϕ 80/ <i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>recA1</i> <i>hsdR17</i> <i>deoR</i> <i>thi-1</i> <i>supE44</i> <i>gyrA96</i> <i>relA1</i>	
DH5 α - λ <i>pir</i> Δ <i>ectB</i>	DH5 α - λ <i>pir</i> harboring pDS132 Δ <i>ectB</i>	This study
DH5 α - λ <i>pir</i> Δ <i>proU1</i>	DH5 α - λ <i>pir</i> harboring pDS132 Δ <i>proU1</i>	This study
β 2155 DAP	Donor for bacterial conjugation; <i>thr1004</i> <i>pro</i> <i>thi</i> <i>strA</i> <i>hsdS</i> <i>lacZ</i> Δ M15 (<i>F'</i> <i>lacZ</i> Δ M15 <i>lacTRQJ</i> Δ 36 <i>proA</i> ⁺ <i>proB</i> ⁺) Δ <i>dadA</i> <i>Erm</i> ^r <i>pirRP4</i> (Km ^r from SM10)	
β 2155 DAP- Δ <i>ectB</i>	β 2155 harboring pDS132 Δ <i>ectB</i>	This study
β 2155 DAP- Δ <i>proU1</i>	β 2155 harboring pDS132 Δ <i>proU1</i>	This study
Plasmids pDS132	R6K γ <i>ori</i> <i>mobRP4</i> <i>sacB</i> Cm ^r ; suicide vector for conjugal transfer and integration	
pBBR1MCS		
pDS132 Δ <i>ectB</i>	pDS132 containing truncated <i>ectABC</i> region	This study
pDS132 Δ <i>proU1</i>	pDS132 containing truncated <i>proVWX</i> system	This study
pVP1723	pBBR1MCS containing <i>bcct2</i>	This study

TABLE 2. Primers used in this study

Primer	Sequence 5'-3'	T _a ^b (°C)	%GC	Product size (bp)
SOEAect	CTT AGC TTT TAG TCA ACG AGC	56	43	
SOEBect	GAA GGA ACG GAA ACG CAC ATC	59	52	246 (SOEAect)
SOECect	UGAT GTG CGT TTC CGT TCC TTCU CCG TTA CCT CTG AAT	47	50	
SOEDect	TGA TCA CAT CAG CAC CTT GG	55	48	287 (SOECect)
SOEApro	CAT GGA TCC CAT ACT GGA AG	55	50	
SOEBpro	CGT GGC GAG CAG TCA CAT C	57	58	409 (SOEApro)
SOECpro	UGAT GTG ACT GCT CGC CAC GCUC GTA TTG GGT AAG TGG	47	60	
SOEDpro	CGT GCG CTT CGA TGA CTG	53	60	328 (SOECpro)
SOEFLectF	CTT GCC ATT GAT CGT CAC G	49	53	
SOEFLectR	CAC TGC ATT CTG ACT CAT G	50	47	2,733 (wild type)
SOEFLproF	CA CTT CTG CAT GAA TGG C	50	53	
SOEFLproR	GCT CGA TAA CTT GGC TCC	51	56	4,168 (wild type)

^a Overhangs complementary to SOEB primers are underlined.

^b T_a, annealing temperature.

isolated with a QIAprep Spin miniprep kit (Qiagen, Hilden, Germany). Splicing by overlap extension (SOE) PCR and allelic exchange were used in the creation of intragenic in-frame mutations in *ectB* (VP1721) and *proU1* (VP1726 to VP1728) in *V. parahaemolyticus* RIMD2210633, and the sizes of the regions removed from the genes are 1,670 and 2,504 bp, respectively, as described previously (28). Primer pairs SOEAect/SOEBect and SOECect/SOEDect were used to amplify a 246-bp and a 287-bp PCR product, respectively, with *V. parahaemolyticus* RIMD2210633 as the template (Table 2). The resulting PCR products were purified and ligated together with T4 DNA ligase (New England BioLabs, Beverly, MA). The resulting product was PCR amplified with primer pair SOEAect/SOEDect to generate a 533-bp product named *ectAD* (Fig. 1, lane 2). The *ectAD* product was restricted with the XbaI and SacI enzymes (New England BioLabs), as was the suicide vector pDS132, and they were ligated together with T4 DNA ligase (New England BioLabs) and designated pDS132Δ*ectB*. pDS132Δ*ectB* was transformed into *E. coli* DH5α-λpir via electroporation and then plasmid purified and transformed into auxotrophic *E. coli* strain β2155 DAP. Transformed cells were designated β2155 DAP-Δ*ectB*. Next, conjugal transfer was performed by cross-streaking each *E. coli* β2155 DAP-Δ*ectB* donor strain with *V. parahaemolyticus* RIMD2210633 initially on LB agar with a final salt content of 250 mM (1.5%) NaCl supplemented with 0.3 mM diaminopimelic acid. Growth from these plates was then transferred to LB containing 25 μg/ml chloramphenicol and 200 μg/ml streptomycin. In order to cure exconjugate colonies of the integrated pDS132 plasmid, overnight cultures were serially diluted to 10⁻⁹ cells and plated on LB plates containing 10% sucrose. Double-crossover deletion mutant Δ*ectB* isolates were screened for by PCR analysis with SOEFLectF and SOEFLectR and confirmed by sequencing (Table 1). The Δ*ectB* mutant was designated *V. parahaemolyticus* strain Δ*ectB*. Deletion mutant Δ*proU1*, containing a deletion in the ProU1 transporter system, was constructed in the same manner with SOE primer pairs described in Table 2. PCR analysis with primer pair SOEFLproF/SOEFLproR was used to confirm integration of the nonfunctional gene copies in their respective mutant strains (Fig. 1 and Table 2). All of the strains were sequenced at MWG to verify the mutated alleles.

Growth analysis. Precultures of either wild-type or mutant bacteria were grown to stationary phase (i.e., 1 × 10⁹ CFU/ml) at 37°C in OM supplemented with 200 mM (1%) NaCl. A 2% inoculum of these overnight cultures was diluted in a 1:100 ratio in 5 ml of fresh OM containing various concentrations of NaCl and organic supplements as indicated. A 200-μl volume of the inoculated medium was then added to a 96-well microtiter plate and incubated at 37°C. All of the assays were done in triplicate, and all of the experiments were performed three times. Optical densities were measured at various time points with a microplate reader. SigmaPlot software was used to construct graphs based on the data obtained. Statistical analysis was carried out with the Student *t* test. For comparative growth analysis of *V. parahaemolyticus* RIMD2210633, *V. vulnificus* YJO16, *V. cholerae* N16961, and *V. fischeri* ES114, the bacteria were cultured routinely overnight and diluted in a 1:100 ratio in 5 ml fresh LB containing 3%, 6%, or 9% NaCl. Growth was analyzed over a 24-h period at 42°C, 37°C, 30°C,

or 20°C at all of these NaCl concentrations. Graphs were constructed with SigmaPlot software based on the data obtained.

Preparation of cell extracts and nuclear magnetic resonance (NMR) analysis. The *V. parahaemolyticus* wild-type and Δ*ectB* mutant strains were incubated with shaking at 37°C in OM supplemented with either 200 mM or 1,000 mM NaCl as previously described (12). Briefly, stationary phase cells were pelleted at 1,000 × *g* for 10 min and the spent growth medium was removed. Three freeze-thaw cycles were performed on cell pellets to increase cell lysis, and then they were resuspended in 750 μl of ethanol. Debris was removed by centrifugation, and the ethanol extract was transferred to a clean tube. The ethanol was subsequently removed from the extracted material by evaporation under vacuum. The pellet was resuspended in 500 μl of D₂O (Aldrich), and insoluble material was removed by centrifugation. The solution was transferred to a 5-mm NMR tube for analysis, which was carried out on a Bruker AVANCE 600 NMR spectrometer operating at a proton frequency of 600.13 MHz (12). A standard proton NMR experiment, with a sweep width of 12,376 Hz and a relaxation delay of 5 s, was used to obtain NMR spectra at 298 K. Sixteen scans were coadded for each spectrum represented here.

Complementation of transporter-deficient *E. coli* MKH13 with a putative BCCT (VP1723) from *V. parahaemolyticus*. *V. parahaemolyticus* RIMD2210633 genomic DNA was isolated with a GNOME DNA isolation kit (MP Biomedicals) according to the manufacturer's instructions. Primers bect2F (TCT AGA AAC TTG TGC TTG GTG ATG TG) and bect2R (GAG CTC ACG GCA CAC TTT CGC ATG), used for PCR amplification of VP1723 from the genomic DNA used as the template, were designed to include 87 bp upstream of the start codon and 47 bp downstream of the stop codon, incorporating an XbaI site on the 5' end and a SacI site on the 3' end. The resulting 1,808-bp product was cloned into pCR2.1 with a TOPO-TA cloning kit (Invitrogen), sequenced, and subsequently subcloned into plasmid pBBR1MCS following XbaI/SacI digestion of both the vector and the insert, resulting in the construct pVP1723. Electrocompetent MKH13 was generated according to a standard protocol (39) and transformed with pVP1723, and transformants were selected for chloramphenicol (25 μg/ml) resistance on LB. Cm^r MKH13 was subsequently screened for the presence of VP1723 by PCR.

RESULTS AND DISCUSSION

Comparative genome analysis. *V. parahaemolyticus* is a moderately halophilic bacterium that can grow at 0.1 M to 1.5 M NaCl concentrations; however, the underlying molecular mechanisms of osmoadaptation are unknown. We examined the *V. parahaemolyticus* RIMD2210633 genome sequence for homologues of specialized systems previously shown to play a role in the osmotic stress response in bacteria. In silico genome analysis of RIMD2210633 revealed the presence of two puta-

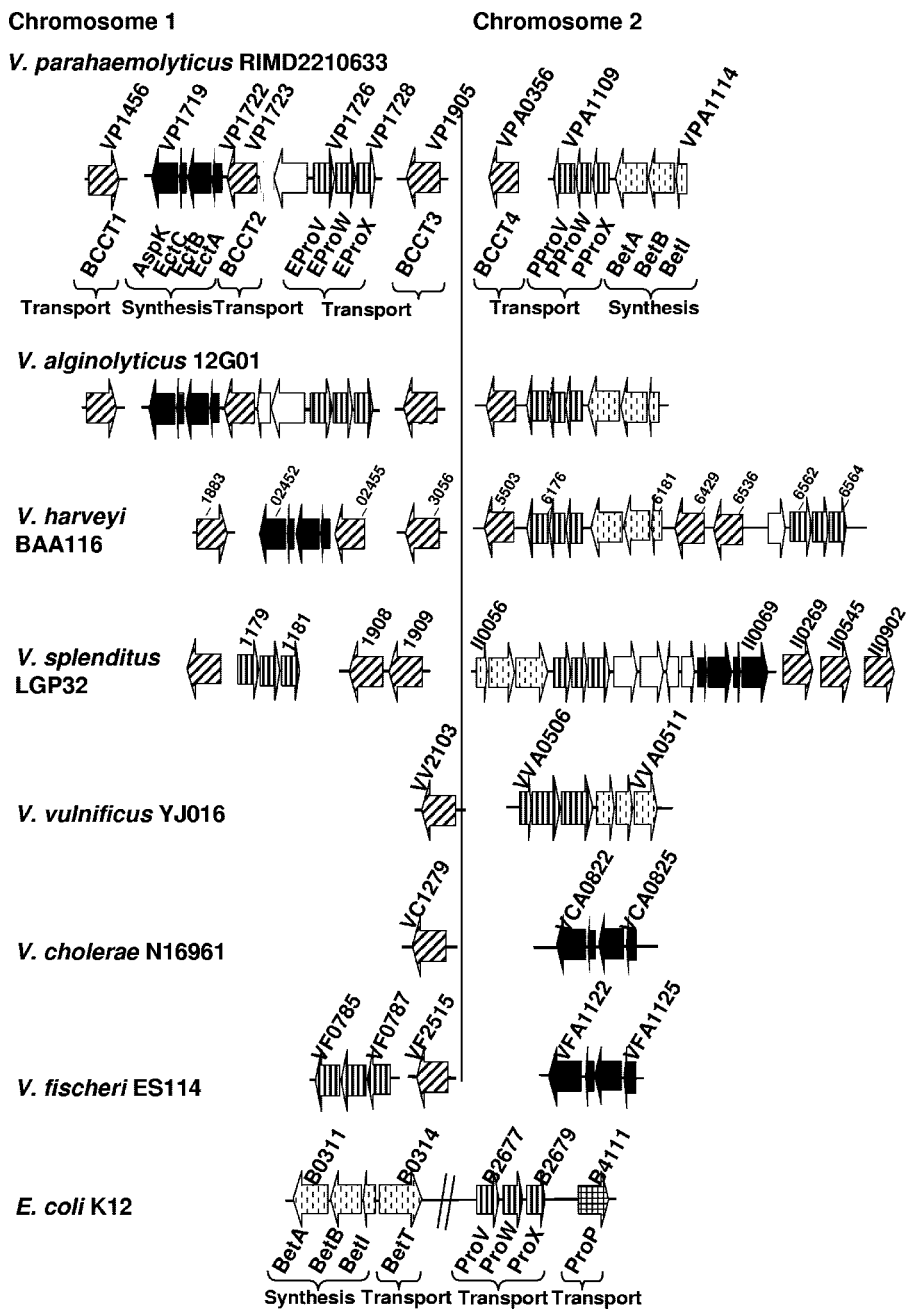


FIG. 2. Distribution of osmolyte transport and synthesis systems among seven *Vibrio* species and *E. coli* K-12. Arrows indicate the direction of transcription. Transporters belonging to the BCCT family are represented by diagonal stripes. Solid black arrows represent putative genes involved in ectoine synthesis. Open arrows indicate hypothetical proteins. Vertical stripes represent genes homologous to that which encodes the ProU (ProVWX) transporter. Dashed lines indicate genes involved in glycine betaine synthesis (*betABI*). The grid arrow represents the ProP transporter of *E. coli* K-12.

tive compatible solute synthesis systems (ectoine and betaine) and six putative compatible solute transporters (Fig. 2). On chromosome 1, two of the compatible solute transporters, a ProU1 transporter (*proVWX*) and BCCT2 (*bctt*), were clustered with the ectoine synthesis system (*ectABC*). The *ectABC* genes were homologous to those described in *V. cholerae* N16961, and the *proVWX* genes were homologous to those described in *E. coli* K-12. On chromosome 2, the betaine synthesis system (encoded by *betABI*) clustered with another

ProU2 transporter encoded by *proVWX* that is homologous to the *Pseudomonas syringae* sequence (Fig. 2). Three additional BCCTs were identified, open reading frames VP1456, VP1905, and VPA0356, herein referred to as BCCT1, BCCT3, and BCCT4, respectively (Fig. 2). Molecular analysis of 42 *V. parahaemolyticus* isolates found that the *ectABC* and *betABI* gene clusters are present in all of the strains tested (data not shown). Bioinformatic analysis of all of the available *Vibrio* genome sequences in the database revealed an interesting distribution

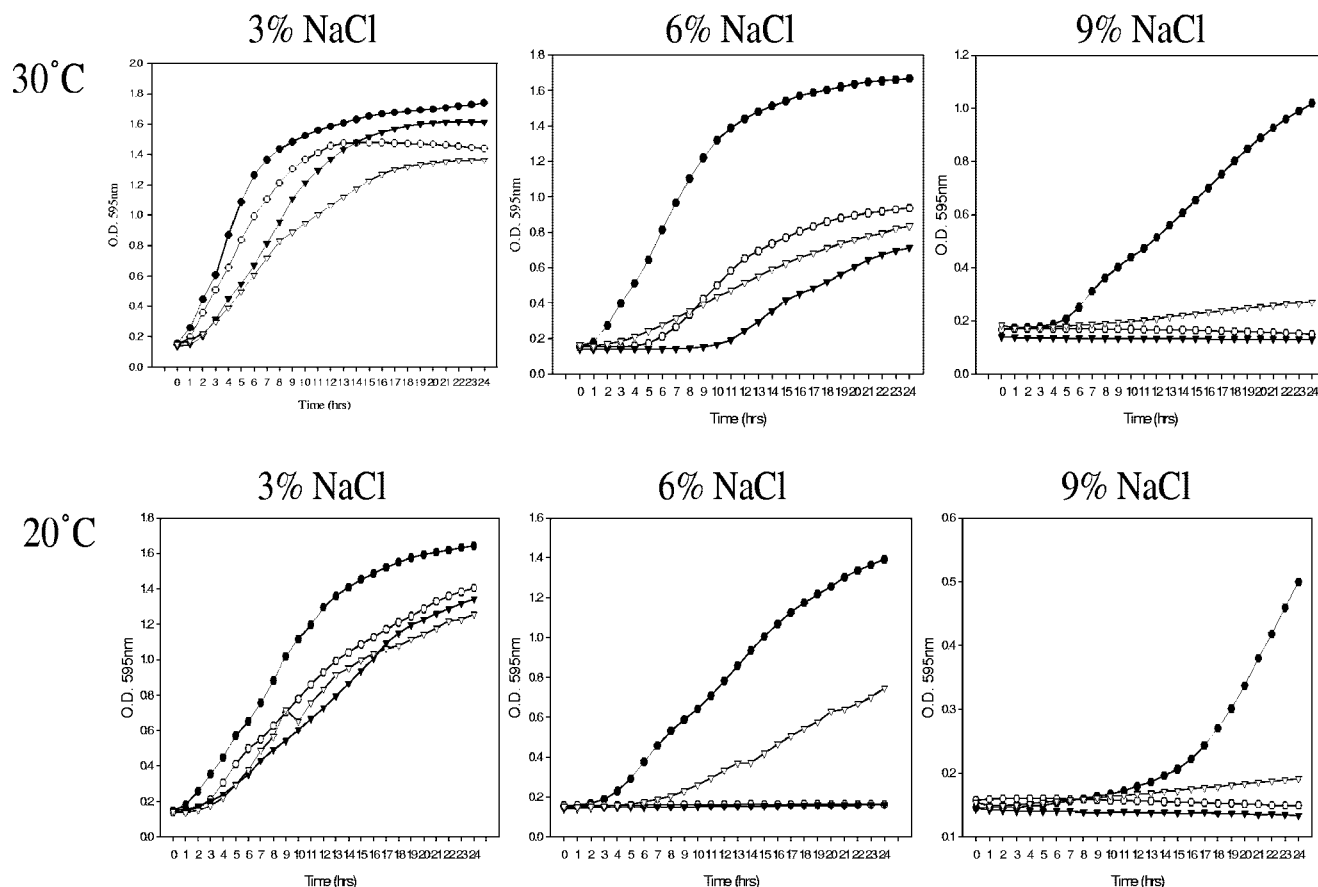


FIG. 3. Analysis of *Vibrio* species growth in LB containing 3% (500 mM), 6% (1,000 mM), or 9% (1,500 mM) NaCl at 30°C and 20°C. Solid circles represent *V. parahaemolyticus* RIMD2210633, open circles represent *V. vulnificus* YJ016, solid triangles represent *V. cholerae* N16961, and open triangles represent *V. fischeri* ES114. O.D., optical density.

of systems. Among the 36 *Vibrionaceae* genomes available, representing 15 different species, we found that *betABI* is present in 12 species, *V. alginolyticus* 12G01, *V. angustum* S14, *V. campbellii* AND4, *Vibrio* sp. strain Ex25, *Vibrio* sp. strain

MED222, *V. harveyi*, *V. parahaemolyticus*, *V. splendidus*, *V. shilonii* AK1, *V. vulnificus*, *Photobacterium profundum*, and *Photobacterium* sp., and absent from 3 species, *Alivibrio salmonicida*, *V. cholerae*, and *V. fischeri*. The *ectABC* cluster is

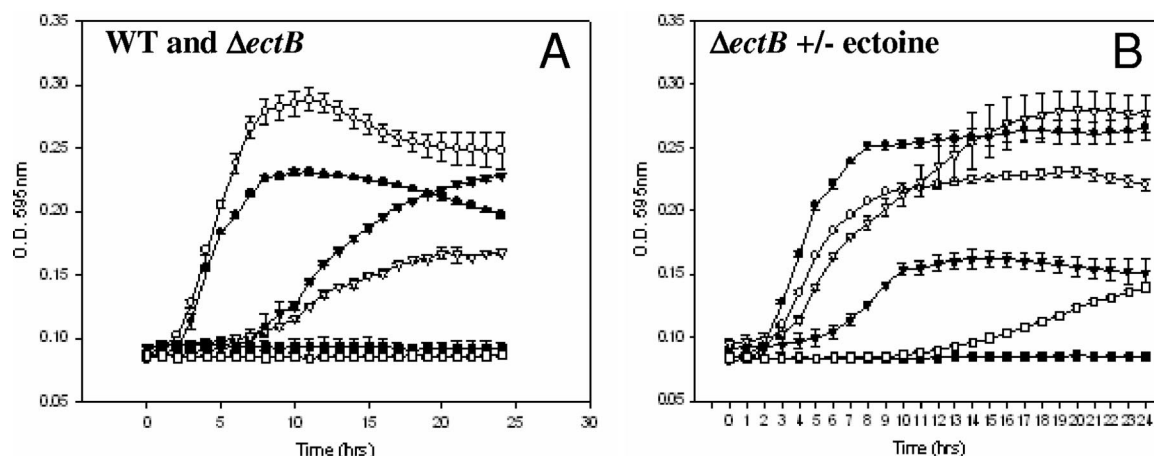


FIG. 4. (A) Growth analysis of wild-type (WT) (solid symbols) and Δ *ectB* mutant (open symbols) *V. parahaemolyticus* following transfer from OM containing 200 mM (1%) NaCl to OM containing 500 mM (3%) NaCl (circles), 1,000 mM (6%) NaCl (triangles), or 1,500 mM (9%) NaCl (squares). (B) Growth of the Δ *ectB* mutant in OM following transfer from OM containing 200 mM (1%) NaCl to OM containing 500 mM (3%) NaCl (solid circles), 1,000 mM (6%) NaCl (triangles), or 1,500 mM (9%) NaCl (squares) without supplementation (solid symbols) or with supplementation (open symbols) with 500 μ M ectoine. O.D., optical density.

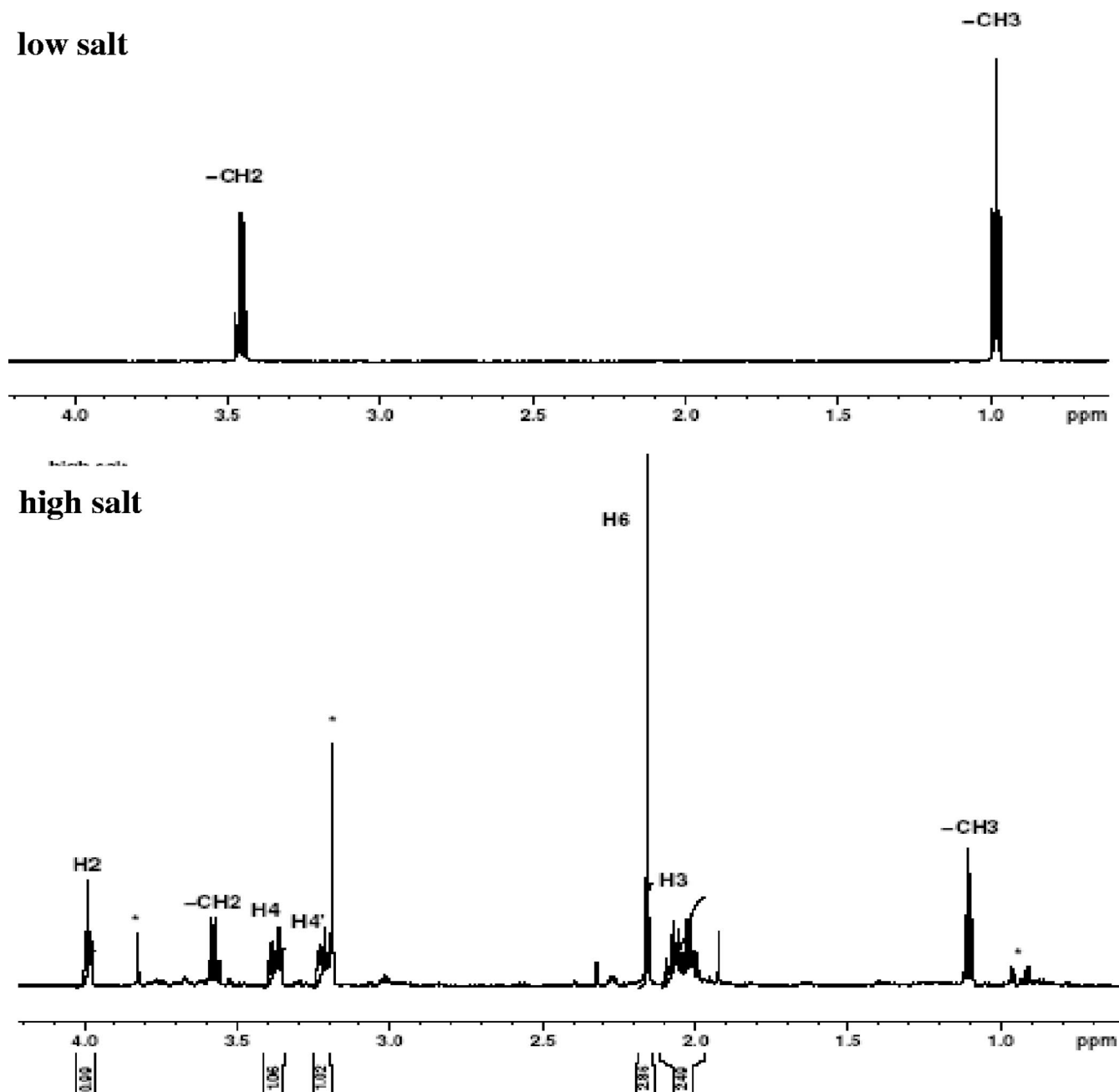


FIG. 5. One-dimensional ^1H -NMR spectra of *V. parahaemolyticus* grown in low (200 mM) and high (1,000 mM) NaCl concentrations. The two-dimensional double-filtered quantum coherence and heteronuclear multiple-quantum coherence spectra of these extracts were also measured and compared with the spectrum of purified ectoine (data not shown). This comparison supported the spectral assignments shown.

present in 10 species and absent from 5 species, *A. salmonicida*, *V. campbellii*, *V. shilonii*, *V. vulnificus*, and *Photobacterium* sp. strain SKA34, and can be absent from some strains within a species. Only *A. salmonicida* and two *V. cholerae* strains, V51 and RC385, appear to lack both synthesis systems. We identified seven species that encode both putative osmolyte synthesis systems, *V. alginolyticus* 12G01, *V. angustum*, *Vibrio* sp. strain Ex25, *Vibrio* sp. strain MED222, *V. harveyi* BAA-1116, *V. splendidus* LGP32, and *P. profundum* 3CTK. *V. harveyi* HY01, *V. splendidus* 12B01, *P. profundum* SS9, and *Photobacterium* sp. strain Ska34 only encode the betaine system.

In *V. alginolyticus* 12G01, a close relative of *V. parahaemolyticus*, the same number and distribution of compatible solute transport and synthesis systems were present (Fig. 2). In *V. harveyi* BA116, homologues of all of the *V. parahaemolyticus* systems are present; however, the two ProU transporters are on chromosome 2 and adjacent to one of them is an integrase. In *V. splendidus* LGP32, three BCCTs and one ProU transporter are present on chromosome 1. On chromosome 2, both *ectABC* and *betABI* are present, along with ProU and three BCCTs (Fig. 2). Analysis of the genomes of *V. cholerae* N16961, *V. vulnificus* YJ016, and *V. fischeri* ES114 revealed

that *V. parahaemolyticus* contains at least double the number of osmotolerance systems present in these isolates (Fig. 2). Overall, our data suggest that *ectABC* is the most variable in its distribution and location on the genome, whereas *betABI* is always found on chromosome 2 and associated with a ProU transporter, which may be the ancestral state in *Vibrio*.

Comparative growth analysis of *Vibrio* species. In order to assess whether the carriage of such a large number of systems gives *V. parahaemolyticus* a growth advantage, we examined the ability of strain RIMD2210633 to grow under increasing NaCl concentrations and at different temperatures compared to *V. vulnificus*, *V. cholerae*, and *V. fischeri* under the same conditions. First, we examined the growth of all of the strains in LB broth containing 3%, 6%, or 9% NaCl at 30°C. *V. parahaemolyticus* exhibited the best growth of all of the strains at this temperature under all of the salinity conditions examined (Fig. 3). In LB containing 3% NaCl, *V. cholerae* displayed a 1-h delay in growth while both *V. vulnificus* and *V. fischeri* exhibited a 2-h delay in growth. At 6% NaCl, an extended growth delay was observed in all three species; a 4-h lag in growth was observed in *V. fischeri*, and a 4-h lag was observed in *V. vulnificus*, while *V. cholerae* displayed the longest lag in growth at 9 h. *V. parahaemolyticus* was the only organism capable of growth in LB containing 9% NaCl (Fig. 3). Growth analysis was also performed at 37°C under identical conditions, and similar patterns of growth were observed in all of the strains, with the exception of *V. fischeri*, which cannot grow at this temperature (data not shown). Growth was examined at 20°C under the same salinity conditions as above, and *V. parahaemolyticus* again displayed the best growth of all of the species examined at 3% and 6% NaCl (Fig. 3). Similar data were obtained for growth at 42°C in 3% and 6% NaCl, again with the exception of *V. fischeri*, which cannot grow at this temperature (data not shown). The comparative physiological data presented here highlight the growth advantage *V. parahaemolyticus* maintains under conditions of elevated salinity at high and low temperatures compared to the other *Vibrio* species, which contain a limited number of compatible solute systems. Most notable is the pronounced advantage *V. parahaemolyticus* displays at the highest osmolarity examined. We could speculate from our data that the multisystem osmotolerance strategy available to *V. parahaemolyticus* allows it to survive in more extreme environments than its close relatives (Fig. 2 and 3). The finding that *V. parahaemolyticus* also survives better under temperature stress at high salinity suggests that the compatible solute systems may also function to cross-protect against other stresses. This may be particularly important in the human host, where the bacterium must survive the acid, bile, and hydrogen peroxide damage encountered in this niche.

Growth analysis of wild-type *V. parahaemolyticus* and the Δ ectB mutant in OM. To determine further the role of the compatible solute synthesis systems in osmotolerance in *V. parahaemolyticus*, we constructed a knockout mutation in the *ectB* gene by SOE PCR and allelic exchange (Fig. 1). The wild-type RIMD2210633 and Δ ectB mutant strains were grown in LB broth containing 500 mM, 1,000 mM, or 1,500 mM, and the two strains showed similar growth patterns (data not shown). This finding suggests that components of LB broth may provide substrates for alternative osmotolerance pathways

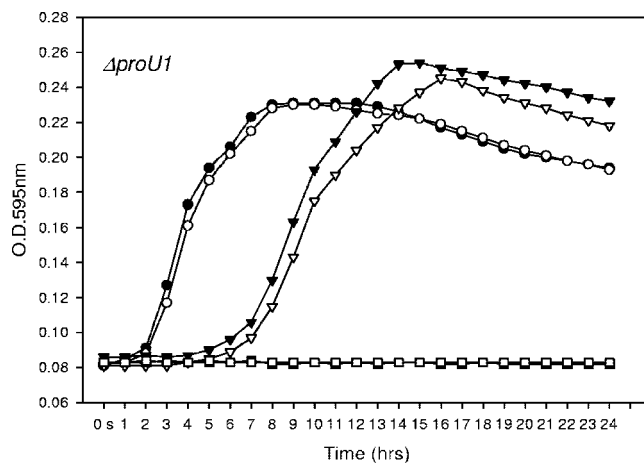


FIG. 6. Growth analysis of wild-type (solid symbols) and Δ proU1 mutant (open symbols) *V. parahaemolyticus* following transfer from OM containing 200 mM (1%) NaCl to OM containing 500 mM (3%) NaCl (circles), 1,000 mM (6%) NaCl (triangles), or 1,500 mM (9%) NaCl (squares). O.D., optical density.

in *V. parahaemolyticus* similar to what was noted in *V. cholerae* (33). Therefore, we used the defined OM, which limits the number of osmotolerance pathways available (33). Our wild-type strain and the Δ ectB mutant strain were first grown on OM supplemented with 200 mM NaCl and then transferred to OM supplemented with 500 mM, 1,000 mM, or 1,500 mM NaCl (Fig. 4A). In OM plus 500 mM NaCl, the wild-type and mutant strains showed similar growth patterns; however, in 1,000 mM NaCl, the mutant had an extended lag phase and only reached an optical density of 0.15 and no growth of either strain was found at 1,500 mM NaCl (Fig. 4A). Thus, it appears that the Δ ectB mutant has a growth defect at high NaCl concentrations. Next, we examined the growth of the Δ ectB mutant in OM supplemented with ectoine at 500 mM, 1,000 mM, or 1,500 mM NaCl (Fig. 4 B). We found that the presence of ectoine in the medium restored the growth of the Δ ectB mutant in OM containing 1,000 mM NaCl. In addition, after an 11-h lag phase, growth occurred in 1,500 mM NaCl, suggesting that ectoine is used as a compatible solute under these conditions. In contrast, the Δ ectB mutant in OM supplemented with aspartic acid, the precursor for ectoine synthesis, had no effect on growth (data not shown).

Growth in high-osmolarity medium induces ectoine synthesis. Having observed growth of the wild type in OM supplemented with 1,000 mM NaCl, we suspected that ectoine might be synthesized de novo by the cell in response to increased external osmolarity. To test this, we made ethanol extracts of *V. parahaemolyticus* cells grown in OM supplemented with 200 mM or 1,000 mM NaCl and determined the one-dimensional H-NMR spectra of these extracts (Fig. 5). Peaks corresponding to the various hydrogen atoms of ectoine are labeled in Fig. 5. Comparative analysis clearly indicates that ectoine-specific peaks are missing from the spectrum of the cell extract derived from *V. parahaemolyticus* grown in OM supplemented with a low NaCl concentration (200 mM) but present in cells grown at a NaCl high concentration (1,000 mM) (Fig. 5). To demonstrate further that *ectABC* is required for ectoine synthesis, we determined the one-dimensional H-NMR spectra of extracts

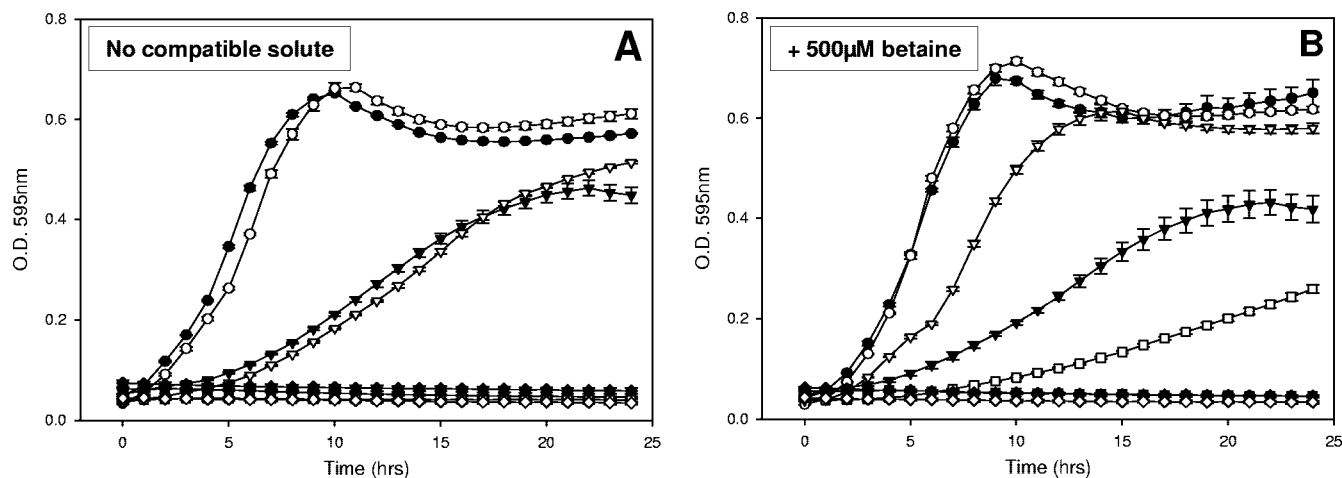


FIG. 7. Growth of *E. coli* MKH13 (MKH13/pBBR1MCS, empty symbols) or MC1 (MKH13/pVP1723, filled symbols) in M9 minimal medium supplemented with NaCl to either a 1% (circle), a 3% (triangle), a 6% (square), or a 9% (diamond) final concentration in the absence (A) or presence (B) of 500 μ M glycine betaine. O.D., optical density.

from our Δ ectB mutant grown at low and high NaCl concentrations. From this analysis, we found no ectoine-specific peaks produced, which was as expected since the organism can no longer synthesize ectoine de novo (see Fig. S1 in the supplemental material).

Growth analysis of wild-type *V. parahaemolyticus* and the Δ proU mutant in OM. To begin to determine the role of multiple compatible transporters in *V. parahaemolyticus*, we constructed a knockout mutation in *proU1* that clustered with *ectABC* (Fig. 2). Using SOE PCR and allelic exchange, we created a Δ proU1 mutant (Fig. 1). Our wild-type and Δ proU1 mutant strains were first grown on OM supplemented with 200 mM NaCl and then transferred to OM supplemented with 500 mM, 1,000 mM, or 1,500 mM NaCl. The Δ proU1 mutant did not show a growth defect compared to the wild type (Fig. 6). When grown on OM (1,000 mM NaCl) supplemented with ectoine or aspartic acid, the Δ proU1 mutant had shortened lag times (data not shown). This result suggests that both of these substrates are used as compatible solutes. Neither ectoine nor aspartic acid had an effect on the growth of the mutant in OM containing 500 mM NaCl, similar to the result obtained with the Δ ectB mutant under the same conditions. This result indicates that *V. parahaemolyticus* primarily uses these substrates as osmolytes and not as a carbon source. Overall, our results indicate that under the conditions we examined, the presence of multiple systems within the genome of *V. parahaemolyticus* results in redundancy. We suggest that under low-salinity conditions, *V. parahaemolyticus* may use one set of transporters, whereas under high-salinity conditions, it may switch its osmotolerance strategy to a different set of transporters. In addition, *V. parahaemolyticus* may contain a large number of synthesis and transporter systems that are required for cross-protection against other stresses.

Complementation of *E. coli* MKH13 with *bcct2* from *V. parahaemolyticus*. To determine the role of the putative transporter BCCT2, we cloned the *bcct2* gene (open reading frame VP1723) into pBBR1MCS and transformed this plasmid into the compatible transport-deficient strain *E. coli* MKH13, resulting in strain *E. coli* MC1 (12). We examined the growth of

MC1 in 0.5 M NaCl unsupplemented or supplemented with betaine, choline, glutamate, or proline. We found growth in M9 medium supplemented with 6% NaCl only in the presence of betaine, suggesting that BCCT2 has specificity for this substrate, similar to other BCCTs (Fig. 7).

Conclusions. The emergence of the highly virulent *V. parahaemolyticus* O3:K6 serovar in 1996 with a subsequent increase in the reported cases of *V. parahaemolyticus* has renewed interest in this organism's ecology and evolution (25, 30). *V. parahaemolyticus* O3:K6 now has a global distribution and was recovered as far north as Alaska, indicating dissemination to new geographic climes and the organism's ability to survive more extreme fluctuations in environmental conditions (9, 27). Genomic comparisons have identified a large number of regions unique to *V. parahaemolyticus*, many of these predominantly associated with pathogenic isolates only (2, 14, 16). Through a combination of bioinformatic, molecular genetic, physiological, and biochemical analyses, we have highlighted the role of osmolyte synthesis and transport systems in osmotolerance. The presence of both ectoine and betaine synthesis systems suggests that the ability to synthesize osmolytes de novo is an important survival strategy for this organism and for *Vibrio* species in general. The presence of redundant systems suggests that *V. parahaemolyticus* may use different osmotolerance strategies under different conditions, a possibility which needs to be examined further. In addition, the presence of so many compatible solute synthesis and transport systems in the cell may be important for cross-protection against other stresses.

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