Role For Glycine Betaine Transport In Vibrio Cholerae Osmoadaptation And Biofilm Formation Within Microbial Communities

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Abstract

Vibrio cholerae is a halophilic facultative human pathogen found in marine and estuarine environments. Accumulation of compatible solutes is important for growth of V. cholerae at NaCl concentrations greater than 250 mM. We have identified and characterized two compatible solute transporters, OpuD and PutP, that are involved in uptake of glycine betaine and proline by V. cholerae. V. cholerae does not, however, possess the bet genes, suggesting that it is unable to synthesize glycine betaine. In contrast, many Vibrio species are able to synthesize glycine betaine from choline. It has been shown that many bacteria not only synthesize but also secrete glycine betaine. We hypothesized that sharing of compatible solutes might be a mechanism for cooperativity in microbial communities. In fact, we have demonstrated that, in high-osmolarity medium, V. cholerae growth and biofilm development are enhanced by supplementation with either glycine betaine or spent media from other bacterial species. Thus, we propose that compatible solutes provided by other microorganisms may contribute to survival of V. cholerae in the marine environment through facilitation of osmoadaptation and biofilm development.

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Estuarine ecosystems are positioned at the interface between freshwater systems and the ocean. Consequently, their inhabitants must continually adapt to temporal and spatial fluctuations in salt concentration. Bacteria living in these ecosystems have developed a variety of strategies to cope with osmotic stress. Decreases in external osmotic pressure are overcome by rapid expulsion of a nonrandom assortment of osmotically active molecules through mechanosensitive channels (1, 21). Bacteria adapt to increases in external osmolarity both by importing charged ions from the environment and by importing or synthesizing cytoplasmic solutes. Although charged ions are readily available in seawater, osmoadaptation by import of charged ions alone may increase the ionic strength of the cytoplasm to a point at which optimal functioning of bacterial enzymes is not possible. An alternative strategy is to accumulate compatible solutes, which are small, zwitterionic, highly soluble organic molecules that are thought to stabilize proteins and lead to hydration of the cell (25, 26). However, compatible solutes are not as readily available in seawater as charged ions are, and synthesis of compatible solutes in the face of osmotic stress is costly. The cytoplasm of halophilic or salt-loving bacteria contains enzymes that function best at elevated ionic strength. Thus, they have an absolute requirement for charged ions in their cytoplasm, and the optimum habitats of halophiles are determined by their salt requirements. Halophiles use compatible solutes rather than charged ions to compensate for the high environmental osmolarity only if the optimum environmental salt concentration is exceeded.

Compatible solutes are diverse, including amino acids such as proline and glutamate, amino acid derivatives such as glycine betaine and ectoine, and sugars such as trehalose and sucrose. Uptake of released compatible solutes from the environment is the preferred mechanism for osmoadaptation, and most bacteria are able to import at least one compatible solute. Five uptake systems are known in *Bacillus subtilis*. OpuA, OpuB, and OpuC are ABC-type transporters, whereas OpuD and OpuE are secondary transporters of the BCCT family (*b*etaine *c*arnitine *c*holine *t*ransporter) and the sodium/solute transporter family, respectively (1). OpuD is involved in glycine betaine uptake and is regulated by osmolarity both at the transcriptional level and at the level of transporter activity (5). OpuE is a proline transporter whose transcription is activated by high osmolarity, suggesting a role in osmoadaptation (27, 30). The PutP transporter of *Escherichia coli* is homologous to OpuE. Unlike OpuE, however, uptake of proline via PutP serves only metabolic purposes, while the additional transporters, ProP and ProU, are utilized for proline uptake during osmotic stress (18). A *Vibrio vulnificus* PutP homolog has been identified as well. Transcription of the gene encoding this transporter is activated by NaCl, proline, and the cyclic AMP receptor protein (14).

If compatible solutes are not available in the environment, they may be synthesized either de novo or from precursor molecules. For example, many bacteria possess the *betAB* and *ectABC* genes, which encode enzymes responsible for the oxidation of choline to glycine betaine and the conversion of l-aspartate-β-semialdehyde to ectoine, respectively (6, 11, 12, 17, 24).

V. cholerae is not only the infectious agent of the diarrheal disease cholera but also a halophilic bacterium found in estuarine and coastal waters (15). In fact, increases in estuarine salinity have been associated with peaks in cholera incidence (19). We have previously demonstrated that *V. cholerae* grows best in medium containing 200 mM NaCl when K^+ is present, suggesting

that *V. cholerae* accumulates K^+ in its cytoplasm. When *V. cholerae* is grown in medium containing NaCl concentrations higher than 200 mM, compatible solutes accumulate within its cytoplasm (24). These compatible solutes may be derived from synthesis of ectoine or transport of glycine betaine. We have previously shown that a *V. cholerae* Δ*ectA* mutant lacking one of the genes in the ectoine synthesis gene cluster grows quite poorly in medium containing 500 mM NaCl (24).

In the marine environment, *V. cholerae* has been found in association with the surfaces of plants, algae, phytoplankton, and zooplankton, and plankton blooms in the Bay of Bengal have been associated with cholera outbreaks in Bangladesh, an area endemic for the pathogen (2, 15, 16, 28). Previous studies have shown that *V. cholerae* O139 uses at least two strategies for surface attachment or biofilm formation in the presence of specific environmental activators (7). Monosaccharides induce synthesis of the VPS exopolysaccharide by enzymes that are encoded by the *vps* genes. This leads to *vps*-dependent biofilm development. A different type of biofilm is formed in seawater. Intercellular interactions in this biofilm depend on Ca^{2+} ions, which are thought to bridge the negative charges of the capsule and/or O-antigen (8).

Compatible solutes are released into the environment from the roots of living plants, decaying plants and animals, and osmotically downshocked cells (1, 3). Because compatible solutes would rapidly disperse after release into the aquatic environment, we hypothesize that, within diffusion-limited microbial communities such as biofilms, concentrations of these released compatible solutes would be sufficient to enhance bacterial growth in high-osmolarity environments. Glycine betaine, one of the most widespread compatible solutes, is found in plants, mammals, archea, and bacteria (1, 25, 26). To address our hypothesis, we have identified and characterized two transporters of *V. cholerae* that are involved in the uptake of glycine betaine. We have then shown that the compatible solute glycine betaine is present in the spent media of other *Vibrio* species in concentrations high enough to promote growth of *V. cholerae* in a high-osmolarity minimal medium. In addition to enhancing growth in this medium, glycine betaine also enhances biofilm formation, an effect that can be reproduced by supplementing high-osmolarity media with the spent supernatants of other *Vibrio* species. Finally, we have demonstrated that enhancement of biofilm formation by glycine betaine occurs at the transcriptional level by activation of *vps* gene transcription. We, therefore, hypothesize that glycine betaine may play an important role in establishment and survival of surface-attached microbial communities in estuarine and marine environments.

MATERIALS AND METHODS

Bacterial strains and media. The *V. cholerae* strains used in this study are shown in Table 1. The minimal medium (MM) used for osmoadaptation experiments has been described elsewhere (24). In this medium, a mixture of amino acids provides the sole carbon and nitrogen source. *V. cholerae vps*-dependent biofilms were formed in modified MM (biofilm MM) designed as follows to maximize exopolysaccharide synthesis: (i) half the amino acid concentration was used, (ii) 0.5% glucose was added, and (iii) the medium was supplemented with 17 μM $FeSO_4$ and 3.3 mM potassium phosphate (pH 7). The various media were supplemented with NaCl,

glycine betaine, proline, and choline as specified in the text. All chemicals were purchased from Sigma unless otherwise noted.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
V. cholerae strains		
MO10	Clinical O139 isolate, India 1992; Sm^r	31
PW411	MO10Δ*ectA*	24
PW477	MO10Δ*opuD*	This study
PW478	MO10Δ*putP*	This study
PW653	MO10Δ*putP*Δ*opuD*	This study
PW654	MO10Δ*ectA*Δ*putP*Δ*opuD lacZ*::*gfp*	This study
PW357	MO10 *lacZ*::pAJH2	4
PW647	MO10Δ*opuD lacZ*::pAJH2	This study
V. parahaemolyticus	Clinical isolate	S. Calderwood
V. fluvialis	Clinical isolate	S. Calderwood
V. vulnificus	Clinical isolate	S. Calderwood
Plasmids		
pAJH2	*vpsL* (VC0934) promoter-*lacZ* gene fusion	4
pJZ111	pCVD442::*Plac*::*gfp*::*lacZ*	J. Zhu
pDK1	pWM91::Δ*opuD*	This study
pDK2	pWM91::Δ*putP*	This study

Construction of in-frame deletion mutants. Strains harboring 1,051-bp and 1,132-bp in-frame deletions in the chromosomal loci VC1279 (*opuD*) and VCA1071 (*putP*), respectively, were constructed by double homologous recombination as previously described (4). The primer pairs used to amplify regions flanking the deletions were as follows: (i) 5′ end of *opuD*, PopuD1 (GTT TGG GAT CAG TGC AGG TT) and PopuD2 (TTA CGA GCG GCC GCA TCC ACC TAA CCG AAT TTT GC); (ii) 3′ end of *opuD*, PopuD3 (TGC GGC CGC TCG TAA AGT ATT ACC TCA GGC GGC AAA) and PopuD4 (CCA TGA ACT CAG GTT CGG TT); (iii) 5′ end of *putP*, PputP1 (AAC GGA TCT GCT TAC CTT GC) and PputP2 (TTA CGA GCG GCC GCA CCA GCC ACT CAT ATC GGA TG); and (iv) 3′ end of *putP*, PputP3 (TGC GGC CGC TCG TAA GTG TGA CGA TTG TGG TTT GG) and PputP4 (CTT TTT GAA GGT GGC GTG TT).

Planktonic and biofilm growth assays. For planktonic growth experiments, *V. cholerae* strains were first grown overnight at 27°C in 5 mM NaCl-MM and then diluted 100-fold into wells containing the medium described in the text. Growth experiments were conducted in 96-well plates at 27°C, and cell densities were measured as the optical density at 655 nm (OD_{655}) in a microplate reader (model 680; Bio-Rad). Each growth experiment was conducted in triplicate.

For biofilm growth experiments, cells were grown overnight on LB-agar plates and resuspended in biofilm MM. The cell suspension was used to inoculate borosilicate tubes filled with 300 μl of the indicated growth medium to yield an initial OD_{655} of 0.05. After incubation of the biofilms for 24 h at 27°C, planktonic cells were removed, 300 μl of fresh medium was added to the tubes, and biofilm cells were dislodged by vortexing in the presence of 1-mm borosilicate beads (Biospec). The density of planktonic and biofilm cells was measured in a microplate reader as described above.

Preparation of supernatants. Fifty milliliters of 500 mM NaCl-MM with or without 1 mM choline was introduced into a 250-ml flask and inoculated with a small amount of *V. cholerae*, *Vibrio parahaemolyticus*, *V. vulnificus*, or *Vibrio fluvialis* grown overnight on an LB-agar plate. This preparation was incubated with shaking for 48 h at 27°C. Cells were pelleted by gentle centrifugation, and the resultant supernatants were filter sterilized with 0.22-μm syringe filters (Fisher Scientific). The sterile supernatants were diluted fourfold in MM or biofilm MM as described in the text.

β-Galactosidase activity. To measure transcription of *vpsL*, the relevant *V. cholerae* strain harboring a *vpsL-lacZ* fusion was inoculated into a 50-ml polypropylene tube containing 5 ml of the relevant growth medium to obtain a final OD_{655} of 0.05. Planktonic cells were removed after 24 h of growth at 27°C under static conditions. One milliliter of Z-buffer and borosilicate beads were added to the remaining adherent cells and then cells were dislodged by vortexing (20). The optical densities of both planktonic and biofilm cell suspensions were measured. Planktonic cells were then pelleted by gentle centrifugation and resuspended in 700 μl of Z-buffer. For lysis, cells were subjected to three freeze-thaw cycles (−80°C to 42°C). Cell debris was pelleted at 13,000 rpm. The protein concentration of each lysate was measured in triplicate using the Coomassie Plus protein assay (Pierce). A 100-μl volume of 2-nitrophenyl β-d-galactopyranoside was added to the remaining lysate, and this mixture was incubated overnight at 37°C. Before quantification of β-galactosidase activity by measurement of the OD_{405}, the mixture was spun at high speed to remove particulates. Relative β-galactosidase activity was calculated by division of the measured OD_{405} by the protein concentration and multiplication by 1,000.

Preparation of cell extract and NMR analysis. *V. vulnificus* was incubated in a 2-liter Erlenmeyer flask with shaking at 37°C in 500 ml of MM supplemented with choline. When stationary phase was reached, cells were pelleted gently, and the spent growth medium was removed. Cell pellets were subjected to three freeze-thaw cycles to enhance lysis. Pellets were then 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 (Speedvac DNA A-110; Savant). The resulting pellet was resuspended in 800 μl of D_2O (Aldrich). After removal of insoluble material by centrifugation, the solution was transferred to a 5-mm nuclear magnetic resonance (NMR) tube for analysis. One-dimensional, double-filtered quantum coherence and heteronuclear multiple-quantum coherence experiments were performed on an AMX500 spectrometer (Bruker).

RESULTS

Compatible solute transporters of *V. cholerae*.In order to identify the glycine betaine uptake system of *V. cholerae*, we searched the *V. cholerae* genome for homologs of known glycine betaine transporters. This revealed the presence of a gene at locus VC1279 encoding a protein of 540 amino acids in the BCCT family of secondary transporters, which includes well-characterized members such as OpuD of *Bacillus subtilis* (5) and BetP of *Corynebacterium glutamicum* (10). Based on homology and the studies presented here, we have named this protein OpuD. *V. cholerae* OpuD is most similar to the putative transporters of the related

halophiles *V. parahaemolyticus* (locus VP1905; 76% identical, 85% similar), *Photobacterium profundum* SS9 (locus PBPRA2516; 74% identical, 85% similar), and *V. vulnificus* strains CMCP6 and YJ016 (locus VV12243/VV2103; 74% identical, 84% similar).

To determine the role of *V. cholerae* OpuD in osmoadaptation, a mutant harboring an in-frame deletion within the *opuD* coding sequence was constructed. When grown in 500 mM NaCl-MM alone, growth of the Δ*opuD* mutant was similar to that of wild-type *V. cholerae* (data not shown). However, in the presence of 0.25 mM glycine betaine, the Δ*opuD* mutant exhibited a longer growth delay than wild-type *V. cholerae* (Fig. 1). Whereas wild-type *V. cholerae* displayed no delay in growth upon transfer to 500 mM NaCl-MM supplemented with glycine betaine, growth of the Δ*opuD* mutant began only after a delay of approximately 15 h. We reasoned that if glycine betaine transport were absent in the Δ*opuD* mutant, the growth of the Δ*opuD* mutant in 500 mM NaCl-MM supplemented with glycine betaine should parallel growth of wild-type *V. cholerae* in unsupplemented 500 mm NaCl-MM. In fact, the Δ*opuD* mutant grew slightly more rapidly in the presence of glycine betaine than wild-type *V. cholerae* in unsupplemented 500 mM NaCl-MM (Fig. 1). Thus, we suspected the presence of an additional, minor glycine betaine uptake system.

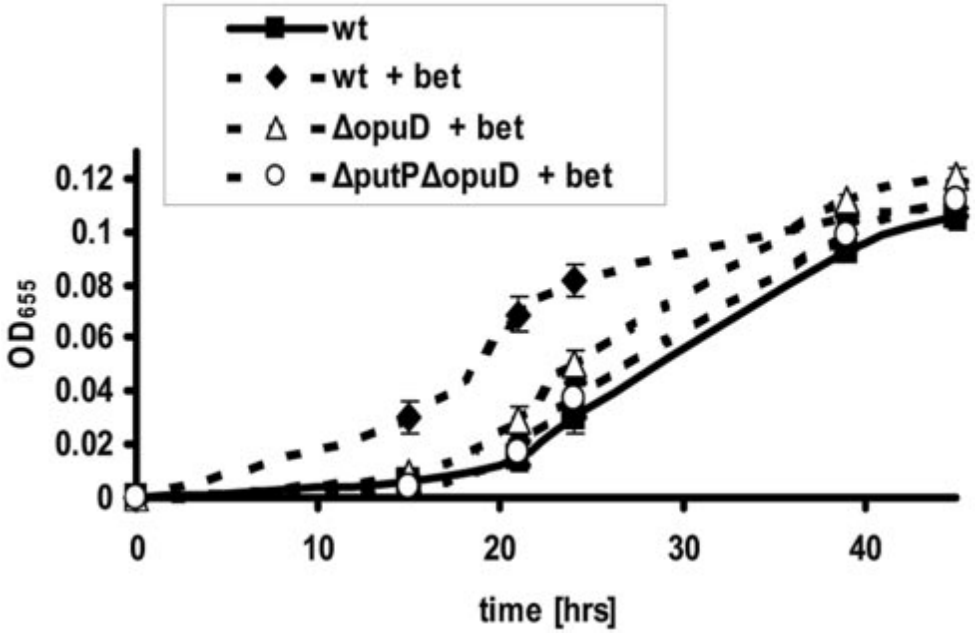

FIG. 1. Growth phenotype of wild-type *V. cholerae* (MO10; wt) and the glycine betaine transporter mutants Δ*opuD* and Δ*putP* Δ*opuD*. Cultures were grown in 500 mM NaCl–MM with or without supplementation with 0.25 mM betaine (bet).

Many transporters involved in bacterial osmoadaptation are able to import both proline and glycine betaine into bacterial cells (1). Therefore, we searched the *V. cholerae* genome for homologs of proline transporters that might also carry out glycine betaine transport in the

absence of *opuD*. A putative protein encoded by locus VCA1071 with 42% identity and 60% similarity to OpuE of *B. subtilis* was identified in a BLAST search. Because this protein is most similar to PutP, a recently characterized proline permease of *Vibrio vulnificus*, we have named the *V. cholerae* protein PutP as well. To assess the role of PutP in glycine betaine uptake, a mutant harboring mutations in both *opuD* and *putP* was constructed, and its growth in 500 mM NaCl-MM supplemented with glycine betaine was assessed. As shown in Fig. 1, growth of the *V. cholerae* Δ*opuD* Δ*putP* mutant in 500 mM NaCl-MM supplemented with glycine betaine was similar to that of wild-type *V. cholerae* in unsupplemented 500 mM NaCl-MM. These results suggest that, under these growth conditions, transport of glycine betaine into *V. cholerae* is primarily carried out by OpuD and PutP.

Because PutP is most homologous to a proline permease, we hypothesized that PutP could transport proline as well as glycine betaine into *V. cholerae*. To assess the role of the PutP permease in proline uptake, we constructed a mutant harboring an in-frame deletion within the *putP* gene and compared its growth in high-osmolarity medium to that of wild-type *V. cholerae*. In 500 mM NaCl-MM, no difference in growth was observed between wild-type *V. cholerae* and the Δ*putP* mutant (data not shown). In the presence of 0.1 mM proline, growth of the Δ*putP* mutant was only modestly slower than that of wild-type *V. cholerae* grown in similar medium and still much greater than wild-type *V. cholerae* grown in 500 mM NaCl-MM alone (Fig. 2). Furthermore, growth of the Δ*putP* mutant was not significantly different from that of wild-type *V. cholerae* at higher proline concentrations (data not shown). This suggested to us that an additional proline transporter was encoded in the *V. cholerae* genome. We hypothesized that this transporter of proline might be OpuD. As shown in Fig. 2, growth of a mutant harboring deletions in *opuD* and *putP* was indistinguishable from that of wild-type *V. cholerae* in 500 mM NaCl-MM. This suggests that in 500 mM NaCl-MM, OpuD and PutP are responsible for most of the proline uptake by *V. cholerae*.

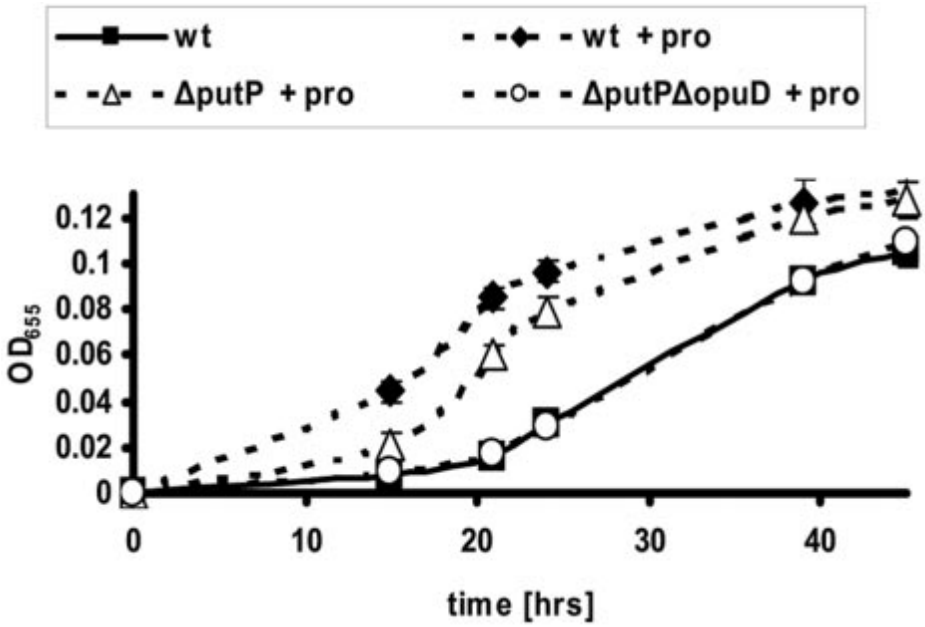

FIG. 2. Growth phenotype of wild-type *V. cholerae* (MO10; wt) and the proline transporter mutants Δ*putP* and Δ*putP* Δ*opuD*. Cultures were grown in 500 mM NaCl–MM with or without 0.1 mM proline (pro).

Other species serve as a source for compatible solutes. We have shown that *V. cholerae* is able to transport glycine betaine. However, the genome of *V. cholerae* does not contain genes homologous to the known glycine betaine synthesis genes, *bet* and *gbs*, suggesting that it lacks the ability to synthesize glycine betaine from choline. Similarly, *V. cholerae* can only synthesize ectoine, while transporters of ectoine have been identified in other halophiles (24, 29). This suggested to us that a microbe might expand its repertoire of osmoadaptive mechanisms by living within a microbial community collectively possessing the ability to synthesize many types of compatible solutes. We first examined the genomes of other *Vibrio* species for homologs of the *bet* genes, which are responsible for glycine betaine synthesis from choline. A BLAST search revealed that the chromosomes of *V. parahaemolyticus* and *V. vulnificus* both encode such homologs. To show that *V. vulnificus* is indeed able to synthesize glycine betaine from choline, a cell extract of *V. vulnificus* grown in 500 mM NaCl-MM supplemented with choline was analyzed by NMR. The NMR spectrum documents the presence of glycine betaine in the cytoplasm of *V. vulnificus* grown in high-osmolarity medium (Fig. 3).

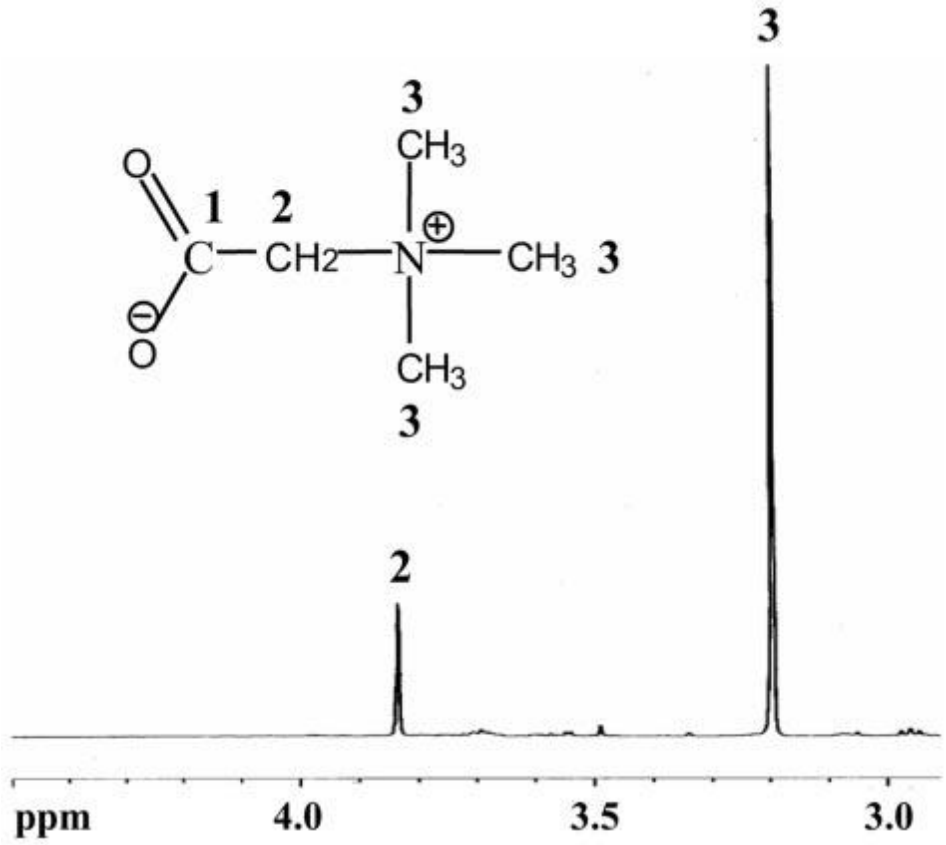

FIG. 3. One-dimensional 1H-NMR spectrum of *V. vulnificus* cell extracts grown in 500 mM NaCl–MM supplemented with 1 mM choline. *V. vulnificus* accumulates the compatible solute glycine betaine within its cytoplasm, which is synthesized from choline. The numbers above the peaks correspond to the carbon atoms to which the hydrogen atoms are attached.

We hypothesized that *V. cholerae* OpuD might import glycine betaine released into the environment by osmoadapted species capable of synthesizing and secreting glycine betaine. We used the following bioassay to determine whether glycine betaine was released into the environment by osmoadapted vibrios grown in the presence of choline. To allow for osmoadaptation, *V. cholerae*, *V. vulnificus*, *V. parahaemolyticus*, and *V. fluvialis* were grown for 48 h in either 500 mM NaCl-MM alone or 500 mM NaCl-MM supplemented with 1 mM choline. The spent media from these cultures were collected, filter sterilized, and mixed in a ratio of 1:3 with fresh 500 mM NaCl-MM. We tested the ability of the resulting media to enhance growth of wild-type *V. cholerae*, a Δ*ectA* mutant, and a Δ*ectA ΔopuD* mutant (Fig. 4). Spent media from cultures of *V. cholerae* grown in 500 mM NaCl-MM both alone and supplemented with choline had no effect on growth of wild-type *V. cholerae* following osmotic shock. This supports our hypothesis that *V. cholerae* is not able to synthesize glycine betaine from choline. Furthermore, spent media from cultures of other *Vibrio* species grown in 500 mM NaCl-MM alone also did not have an effect on growth of wild-type *V. cholerae*. This suggests that these vibrios do not

secrete significant amounts of glycine betaine in the absence of choline. In contrast, spent media from cultures of *V. parahaemolyticus*, *V. fluvialis*, and *V. vulnificus* grown in the presence of choline enhanced growth of wild-type *V. cholerae* and the Δ*ectA* mutant but did not enhance growth of the Δ*ectA* Δ*opuD* mutant, which is defective in glycine betaine uptake. These results suggest that, when grown in high-osmolarity medium supplemented with choline, some *Vibrio* species synthesize glycine betaine from choline and release a portion of it into the environment. This glycine betaine may, in turn, be transported by other bacteria for use in osmoadaptation.

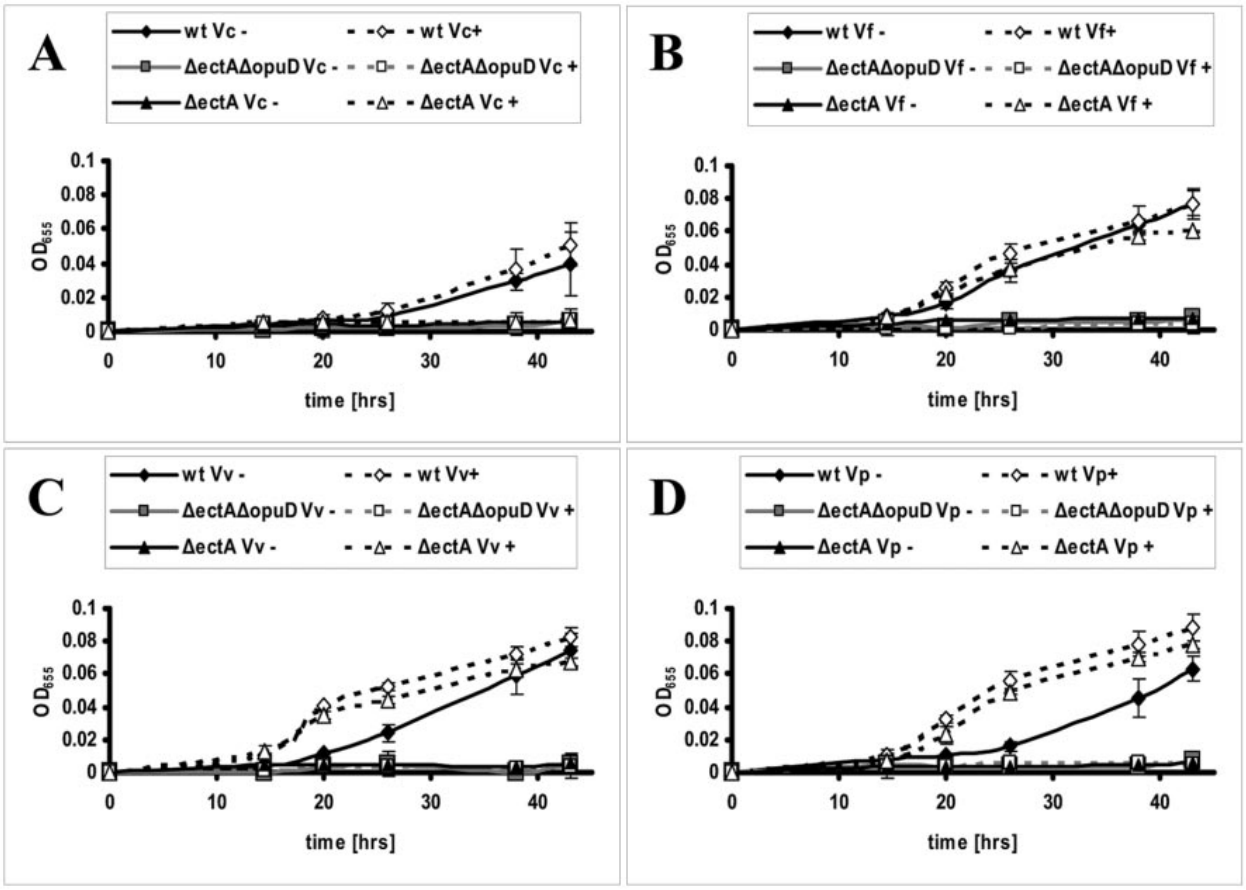

FIG. 4. Growth of wild-type *V. cholerae* (wt) and mutant strains in MM supplemented with supernatants from a number of *Vibrio* species. The supernatants were prepared by growing cultures of (A) *V. cholerae* (Vc), (B) *V. fluvialis* (Vf), (C) *V. vulnificus* (Vv), and (D) *V. parahaemolyticus* (Vp) in 500 mM NaCl–MM with (+) or without (-) 1 mM choline.

Supplementation with glycine betaine increases *V. cholerae* surface attachment in 500 mM NaCl-MM.Our experiments with spent media suggested that compatible solutes released by microorganisms within a microbial community would be available for uptake by other members. We hypothesized that the diffusion-limited environment of the biofilm would provide the ideal milieu for such a process. Thus, we chose to study the influence of osmoadaptation on biofilm

formation. In MM, *V. cholerae* biofilm formation occurs only in the presence of monosaccharides and is dependent on the synthesis of the VPS exopolysaccharide (7). To maximize *vps*-dependent surface attachment, we used biofilm MM consisting of 500 mM NaCl-MM supplemented with glucose, iron, and phosphate as described above. For these experiments, wild-type *V. cholerae* or mutants were harvested from LB-agar plates and incubated in either 5 mM NaCl or 500 mM NaCl-biofilm MM. Growth of planktonic and biofilm cells was measured. Under these conditions, growth of planktonic wild-type *V. cholerae* and Δ*ectA* and Δ*ectA* Δ*opuD* Δ*putP* mutants in 5 mM NaCl-MM and 500 mM NaCl-MM did not differ by more than approximately 10% (Fig. 5). In contrast, when wild-type *V. cholerae* was cultured in 500 mM NaCl-biofilm MM, the OD_{655} of surface-attached cells was 40% less than that measured for cells cultured in 5 mM NaCl-biofilm MM. In 500 mM NaCl-biofilm MM, growth of Δ*ectA* and Δ*ectA* Δ*opuD* Δ*putP* mutant biofilms was even more profoundly affected. However, growth of wild-type *V. cholerae* as well as the Δ*ectA* mutant biofilms in 500 mM NaCl-biofilm MM increased to levels observed for cells grown in 5 mM NaCl-biofilm MM when the medium was supplemented with glycine betaine. Biofilm formation in 500 mM NaCl-biofilm MM by the Δ*ectA* Δ*opuD* Δ*putP* mutant, which neither synthesizes ectoine nor transports glycine betaine, was unaffected by the presence of glycine betaine. Glycine betaine had no effect on surface accumulation of wild-type *V. cholerae* or the Δ*ectA* and Δ*ectA* Δ*opuD* Δ*putP* mutants in 5 mM NaCl-biofilm MM.

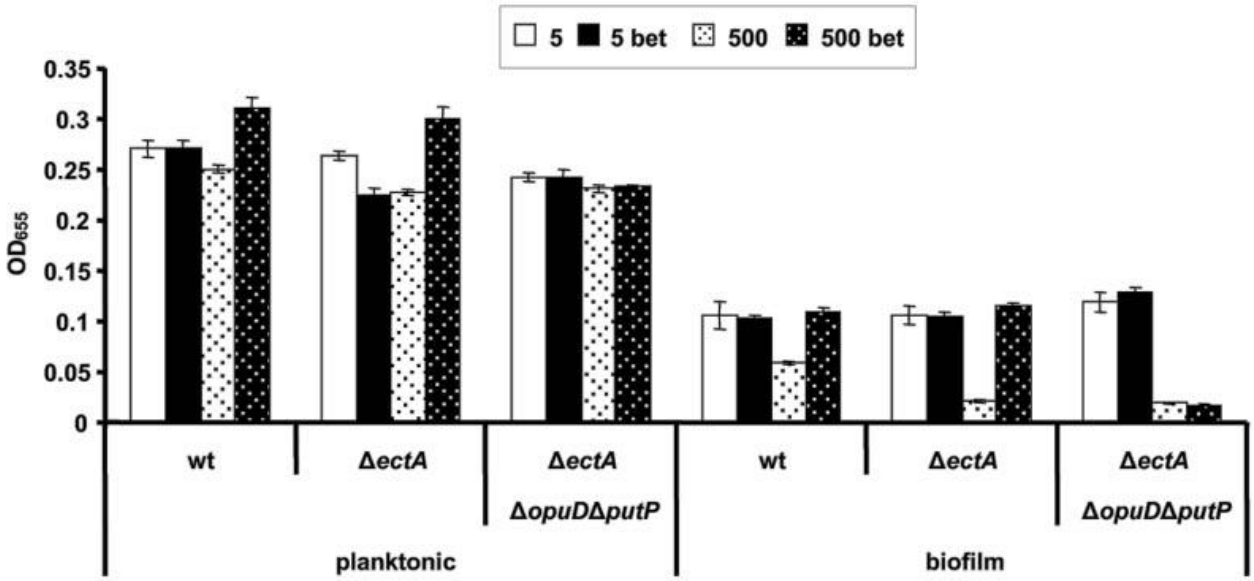

FIG. 5. OD655 of biofilm cells of *V. cholerae* MO10 wild-type (wt) and mutant strains after 24 h in the presence or absence of 0.25 mM betaine (bet) in 5 mM or 500 mM NaCl–biofilm MM.

In 500 mM NaCl-biofilm MM, biofilm formation by wild-type *V. cholerae* as well as a Δ*ectA* mutant increased in the presence of glycine betaine. We have previously identified nucleosides and monosaccharides as environmental signals that increase *V. cholerae* biofilm formation by activating transcription of the *vps* genes that are located in an operon involved in VPS

exopolysaccharide synthesis (4, 7, 32). We questioned whether glycine betaine might also enhance biofilm formation by increasing *vps* gene transcription. We, therefore, measured *vpsL* transcription using a previously constructed strain harboring a chromosome-based reporter fusion of the *vpsL* promoter to the *lacZ* gene (4). Biofilms were formed in 500 mM NaCl-biofilm MM alone or supplemented with 0.25 mM glycine betaine. Supplementation with glycine betaine did, in fact, lead to a large increase in *vpsL* transcription in both planktonic and biofilm-associated wild-type *V. cholerae* (Fig. 6). In contrast, supplementation of 500 mM NaCl-biofilm MM with glycine betaine did not affect *vpsL* transcription in a Δ*opuD* mutant. These results suggest that glycine betaine transport into the cell increases surface association by increasing *vps* gene transcription and, hence, VPS synthesis.

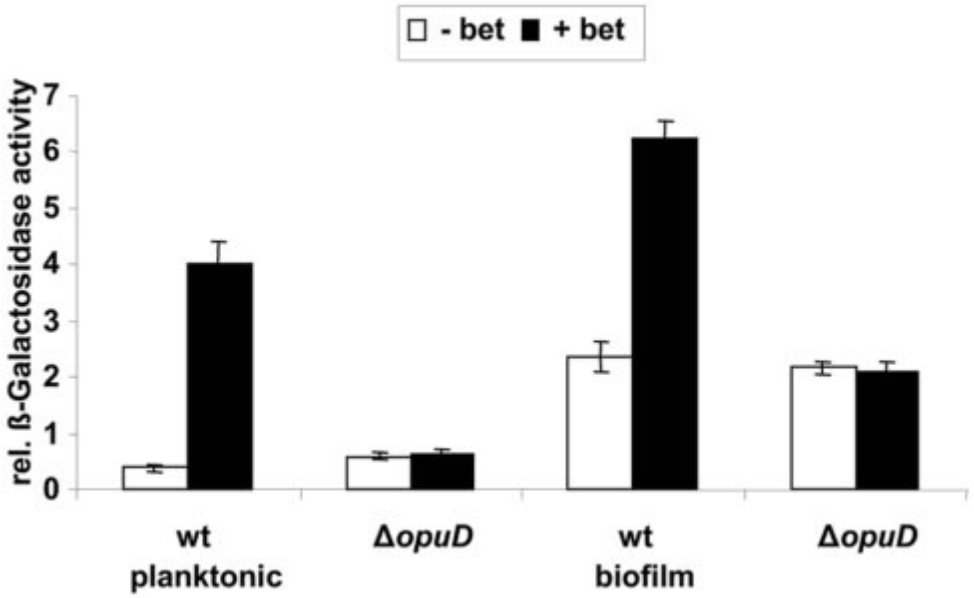

FIG. 6. Relative _-galactosidase activity of a chromosomal *vpsL* promoter-*lacZ* construct in *V. cholerae* planktonic and biofilm cells. Biofilms were formed over 24 h in 500 mM NaCl–biofilm MM with or without 0.25 mM betaine (bet). Strains MO10 *lacZ*::pAJH2 and glycine betaine transporter mutant MO10Δ*opuD* *lacZ*::pAJH2 were used to show the effect of betaine on *vpsL* transcription.

Other bacterial species may enhance *V. cholerae* biofilm formation in high salt.We have shown above that glycine betaine is synthesized from choline by selected *Vibrio* species and can accelerate growth of *V. cholerae* in high-osmolarity medium. Since glycine betaine enhances biofilm formation in 500 mM NaCl-biofilm MM, we hypothesized that spent supernatants of these *Vibrio* species would also increase biofilm formation by *V. cholerae* at high osmolarity. As described above, we mixed spent supernatants with fresh 500 mM NaCl-biofilm MM in a ratio of 1:3 and then allowed wild-type *V. cholerae* and mutants to form biofilms in this medium. As can be seen in Fig. 7, addition of wild-type *V. cholerae* supernatants to 500 mM NaCl-biofilm MM did not affect growth or biofilm formation by the Δ*ectA* or Δ*ectA* Δ*opuD* *V. cholerae* mutants. Addition of supernatants from the other three *Vibrio* species grown in the absence of choline

also showed no effect. In contrast, addition of spent supernatants from other *Vibrio* species grown in 500 mM NaCl-MM supplemented with choline increased both planktonic growth and biofilm formation by the *V. cholerae* Δ*ectA* mutant but had no effect on biofilm formation by the Δ*ectA* Δ*opuD* mutant. These results suggest that glycine betaine in the spent supernatants is responsible for increased planktonic growth and biofilm formation by *V. cholerae*.

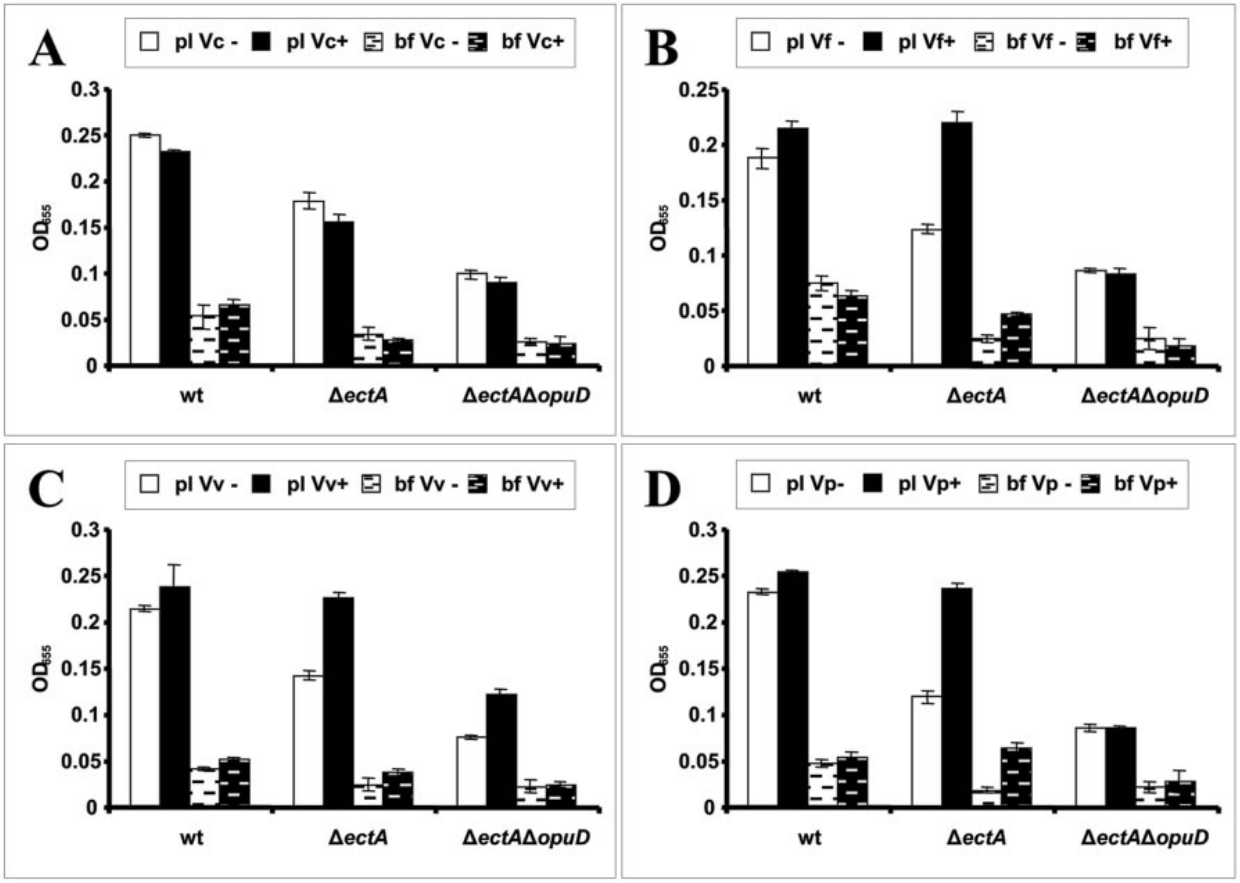

FIG. 7. OD655 of planktonic and biofilm cells of *V. cholerae* wild-type (wt) and mutant strains after 24 h in 500 mM NaCl–biofilm MM supplemented with supernatants of different *Vibrio* species. The supernatants were prepared by growing cultures of (A) *V. cholerae* (Vc), (B) *V. fluvialis* (Vf), (C) *V. vulnificus* (Vv), and (D) *V. parahaemolyticus* (Vp) in 500 mM NaCl–MM with (+) or without (-) 1 mM choline. Sterile supernatants of these cultures were mixed 1:3 with 500 mM NaCl–biofilm MM for this experiment.

DISCUSSION

ABC transporters utilize ATP to power transport, whereas secondary transporters rely on ion gradients across the cell membrane. Because Na^+ gradients across cell membranes are a prerequisite of life in the sea, many secondary transporters of halophilic bacteria utilize Na^+ symport. The search for compatible solute transporters of *V. cholerae* yielded two secondary transporters but no ABC-type transporters. Since *V. cholerae* is a natural inhabitant of estuarine

ecosystems, which are subject to constant fluctuations in salt concentrations, secondary transporters might be the more economical solution to the recurrent need for efflux and uptake of compatible solutes.

Numerous studies have demonstrated the ability of *V. cholerae* to attach to biotic surfaces, and an association with plankton blooms has been established (15). In addition, seawater isolated during phytoplankton blooms supports growth of *V. cholerae* (22). Based on these observations, plankton are hypothesized to provide nutrients to adherent *V. cholerae*. Synthesis of compatible solutes is costly to bacterial cells (23). Thus, not only nutrients but also compatible solutes released from plankton may play an important role in the survival and growth of *V. cholerae* in seawater. Because glycine betaine is one of the most widespread compatible solutes, a high-affinity transporter of glycine betaine is likely to be of great advantage to a bacterial cell in the marine environment. The experiments we have performed with spent bacterial supernatants clearly show that compatible solutes synthesized by other bacteria are released in concentrations high enough to enhance growth of *V. cholerae* in high-osmolarity medium. Although the glycine betaine in our spent supernatants may have been released only from leaky or dead cells, there is evidence that bacteria release glycine betaine into the environment. Using *E. coli bet* mutants, Lamark et al. showed that *E. coli* excretes glycine betaine when grown in high-osmolarity medium supplemented with choline. This glycine betaine is then imported into cells by the ProU and ProP transporters (13). Efflux of glycine betaine has also been observed in *Salmonella enterica* serovar Typhimurium and was suggested to be a means of regulating the intracellular glycine betaine pool (9). In a microbial community such as a biofilm, it is likely that any bacterium possessing a high-affinity glycine betaine uptake system would benefit from this type of glycine betaine efflux.

We have previously identified nucleosides and monosaccharides as environmental signals that induce *V. cholerae* biofilm development (4, 7). We have now shown that glycine betaine not only increases growth of *V. cholerae* at high salt concentrations, but also enhances surface attachment through induction of *vps* gene transcription. Thus, glycine betaine is an additional environmental signal that induces biofilm development.

In most aquatic environments, microbes are found in association with surfaces. Because microbes synthesize and excrete glycine betaine, we hypothesize that concentrations of compatible solutes are increased in and around the biofilms formed on biotic surfaces. Furthermore, the presence of glycine betaine increases *V. cholerae* surface attachment at high salt concentrations, suggesting that *V. cholerae* favors the surface-attached state during times of osmotic stress. Because a microbial community such as a biofilm is composed of microbes with diverse synthetic capabilities, we propose that microbes expand their repertoire of responses to osmotic stress by joining the biofilm community. Thus, surface attachment in response to glycine betaine may be a beneficial adaptive response.

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