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Identification and Functional Analysis of the *putAP* Genes Encoding *Vibrio vulnificus* Proline Dehydrogenase and Proline Permease

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Abstract The pathogenic marine bacterium *Vibrio vulnificus* is the causative agent of food-borne diseases such as life-threatening septicemia. To better understand this organism's strategies to survive osmotic stress, a mutant that was more sensitive to high osmolarity was screened from a library of mutants constructed by a random transposon mutagenesis. By a transposon-tagging method, *putAP* genes encoding a proline dehydrogenase and a proline permease were identified and cloned from *V. vulnificus*. The amino acid sequences deduced from nucleotide sequences of *putAP* from *V. vulnificus* were 38 to 59% similar to those of PutA and PutP reported from other Enterobacteriaceae. Functions of *putAP* genes were assessed by the construction of mutants, whose *putAP* genes were inactivated by allelic exchanges. When proline as the sole carbon or nitrogen source was used, the *putA* mutant was not able to grow to the substantial level, revealing the proline dehydrogenase is the only enzyme for metabolic conversion of proline into other amino acids. Although the growth rate of the *putP* mutant on proline as the sole carbon or nitrogen source was significantly reduced, the mutant still grew. This indicated that at least one more proline permease is produced by *V. vulnificus*. The *putP* mutant decreased approximately 2-$\log_{10}$ CFU/ml after a hyperosmotic challenge, while the parent strain decreased approximately 1-$\log_{10}$ CFU/ml. This result suggests that the gene product of *putP* contributes to the osmotic tolerance of *V. vulnificus*.

Key words: *V. vulnificus*, *putAP*, osmotolerance

Bacteria have evolved with elaborate protection systems to allow survival and/or growth during exposure to environmental changes. Change in the external osmolarity is one of the most common environmental stresses that bacteria routinely encounter [4]. Bacteria respond to external osmolarity increases by actively modulating the pool of osmotically active solutes in their cytoplasm, thereby preventing the loss of water and resulting in enhanced osmotolerance [2, 4]. Bacteria have developed two fundamentally different strategies for maintaining outwardly directed turgor pressure under hyperosmotic surroundings. One is found mainly in Archeabacteria and extremely halotolerant bacteria whose permanent habitats are hypersaline environments [8]. This group of bacteria accumulates large amounts of ion, such as K^+ and Cl^-.

The other is a more versatile and flexible osmostress response and is adopted by bacteria that inhabit environments of moderate salinity or by bacteria whose habitats are subject to osmotic challenges accidentally and periodically, but not permanently. This group of bacteria accumulates large amounts of organic osmolytes, known as compatible solutes or osmoprotectants, in their cytoplasm. Although a variety of novel organic osmolytes have been identified as osmoprotectants, proline, glycine betaine, ectoine, and trehalose are probably the most widely used compatible solutes in the bacteria [22]. Important roles of proline as an osmoprotectant have been most extensively studied with *Escherichia coli*, *Salmonella typhimurium*, and other Enterobacteriaceae [4, 5].

Vibrio vulnificus is an opportunistic Gram-negative pathogen that commonly contaminates raw oysters, and is the causative agent of food-borne diseases such as gastroenteritis and life-threatening septicemia [1]. Like many other food-borne pathogenic bacteria, *V. vulnificus* occurs in various environments with different osmotic strength and has to cope with ever changing osmolarity in their growth environments. However, until now, a definitive analysis of

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the responsive adaptation of the pathogen to changes in osmolarity has not been made, and the molecular mechanisms by which the bacterium can survive in hyperosmotic environments have not yet been characterized. This lack of information on the mechanisms of osmotolerance makes it difficult to understand how the pathogen survives osmotic stress imposed not only by natural ecosystems but also by the present-day control practices, such as adding salt or sugar to suppress its growth in raw seafood.

Accordingly, as an effort to identify the genes involved in osmotolerance, a library of *V. vulnificus* mutants was constructed using the transposon mini-Tn5 *lacZ*1 [6], and a mutant that was more sensitive to hyperosmolarity was screened in the present study. By a transposon-tagging method, *putAP* genes encoding a proline dehydrogenase and a proline permease were identified and cloned from *V. vulnificus*. The nucleotide and deduced amino acid sequences of the *putAP* genes were analyzed. *V. vulnificus* null mutants whose *putAP* genes were separately disrupted were also constructed, and the physiological characteristics of the mutants were compared to that of the parental wild-type.

MATERIALS AND METHODS

Strains, Plasmids, and Culture Media

The strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains used for plasmid DNA replication or conjugational transfer of plasmids were grown in Luria-Bertani (LB) broth or on LB broth containing 1.5% (w/v) agar. Unless noted otherwise, the *V. vulnificus* strains were grown in LB medium supplemented with 2.0% (w/v) NaCl (LBS).

Table 1. Plasmids and bacterial strains used in this study.

Strain or plasmid	Relevant characteristics[a]	Reference or source
Bacterial strains		
V. vulnificus		
ATCC29307	Clinical isolate; virulent	Laboratory collection
MO6-24/O	Clinical isolate; virulent	Laboratory collection
HJK991	MO6-24/O with *putA*::mini Tn5 *lacZ*1	This study
HJK001	ATCC29307 with *putA::nptI*; Km^r	This study
HJK002	ATCC29307 with *putP::nptI*; Km^r	This study
E. coli		
DH5α	*supE44 Δ lacU169 (ϕ80 lacZ* ΔM15*) hsdR17 recA1 endA1 gyrA96* thi-1 relAI	Laboratory collection
SY327λpir	*Δ(lac pro) argE*(Am) *recA56 gyrA rpoB λpir*; host for π-requiring plasmids; conjugal donor	[16]
SM10λpir	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu *λpir*; km^r; host for π-requiring plasmids; conjugal donor	[16]
Plasmids		
mini-Tn5 *lacZ*1	R6Kγ*ori*; Suicide vector; *oriT* of RP4; Ap^r	[6]
PGEM7Zf(+)	Ap^r	Promega corp.
pUC18	Ap^r	Laboratory collection
pLAFR3	IncP *ori*; cosmid vector; Tc^r	[25]
pUC4K	pUC4 with *nptI*; Ap^r, Km^r	[17]
PCVD442	R6K γ*ori*; *sacB*; suicide vector; *oriT* of RP4; Ap^r	[9]
pRK415	IncP *ori*; broad-host-range vector; *oriT* of RP4; Tc^r	[11]
pHJK9913	Cosmid library containing *putAP*; Tc^r	This study
pHJK002	8.3-kb *SalI* fragment containing *putAP* cloned into pUC18; Ap^r	This study
pHJK0022	pGEM-7Zf(+) with *putA*; Ap^r	
pHJK0024	pGEM-7Zf(+) with *putP*; Ap^r	
pHJK0041	pHJK0022 with *putA::nptI*; Ap^r, Km^r	This study
PHJK0043	pCVD442 with *putA::nptI*; Ap^r, Km^r	This study
pHJK0045	pRK415 with *putA*; Tc^r	This study
pHJK0055	pHJK0024 with *putP::nptI*; Ap^r, Km^r	This study
pHJK0057	pCVD442 with *putP::nptI*; Ap^r, Km^r	This study
pHJK0059	pRK415 with *putP*; Tc^r	This study

[a] Ap^r, ampicillin resistant; Km^r, kanamycin resistant; Tc^r, tetracycline resistant.

Table 2. Oligonucleotides used in this study.

Oligonucleotide	Oligonucleotide sequence, 5'→3'[a]	Location[b]	Use
PTn-1	TTCTTCACGAGGCAGACCTCAGCGC	Tn5	Amplification of Tn5 flanking region
PTn-2	CCGCACTTGTGTATAAGAGTCAG	Tn5	Amplification of Tn5 flanking region
BB-P1	GGCCACGCGTCGACTAGTCANNNNNNNNNNACGCCC	Arbitrary	Amplification of Tn5 flanking region
BB-P2	GGCCACGCGTCGACTAGTCA	Arbitrary	Amplification of Tn5 flanking region
PUTA004	CATGAATTCCTCCTTATGCGTTCTCAGTC	putA	Amplification of *putA* gene
PUTA005	ACTCTAGATTCGGGGATGAGATTGAGGAAA	putA	Amplification of *putP* gene
PUTP004	GTGGATCCAAGATTACTCTAGCTCAACCA	putP	Amplification of *putP* gene
PUTP005	GCGCTGCAGTCTAATGAGGACTATCTAATGA	putP	Amplification of *putP* gene

[a]Regions of oligonucleotides not complementary to corresponding genes are underlined.
[b]To where the nucleotides are hybridized.

Cloning and Sequence Analysis of the *V. vulnificus putAP* Genes

A mutant, strain HJK991, that was more sensitive to hyperosmotic stress (8% NaCl in LB, w/v) was screened from a library of *V. vulnificus* mutants generated by a random transposon mutagenesis using a mini-Tn5 *lacZ*1 [6]. A DNA segment flanking the transposon insertion was amplified by PCR as described previously [26]. In the first PCR, the arbitrary primer BB-P1 and the Tn5-specific primer PTn-1 were used (Table 2). For the second PCR, the first PCR product and a pair of primers, a second arbitrary primer BB-P2 and another Tn5-specific primer PTn-2 (Table 2), were used as the template and primers, respectively. Since the deduced amino acid sequence of the resulting PCR product, a 337-bp DNA fragment, revealed 45% identity with that of *E. coli putA*, the DNA was labeled with [α-^{32}P]dCTP and named Put-P. To clone the full genes of the *putAP* operon, a cosmid library of *V. vulnificus* ATCC29307 constructed using pLAFR3 [23, 25] was screened using Put-P as a probe. A colony showing a positive signal was isolated, and the cosmid DNA was purified and named pHJK9913 (Fig. 1A). An 8.3-kb band from the cosmid DNA digested with *Sal*I was purified and ligated into pUC18 (New England Biolabs) to result in pHJK002 as shown in Fig. 1A. The nucleotide sequence of the 8.3-kb DNA fragment in pHJK002 was determined by primer walking (Korea Basic Science Institute, Kwang-Ju, Korea).

Generation of the *put::nptI* Mutant

The *putA* gene in pHJK0022 that was constructed by ligation of a 2.7-kb *Sac*I fragment of pHJK002 with pGEM7zf(+) was inactivated *in vitro* by insertion of *nptI* encoding for aminoglycoside 3'-phosphotransferase and conferring resistance to kanamycin [17]. The 1.2-kb DNA fragment carrying *nptI* was isolated from pUC4K (Pharmacia, Piscataway, NJ, U.S.A.) and inserted into a *Pst*I site present within the open reading frame (ORF) of *putA*. The 3.9-kb *putA::nptI* cartridge from the resulting construct (pHJK0041) was liberated by digestion with *Sac*I and ligated with *Sac*I-digested pCVD442 [9], forming pHJK0043 (Fig. 1B).

To inactivate the *putP*, a 2.1-kb *Sph*I-*Nru*I DNA fragment carrying whole *putP* was isolated from pHJK002 and ligated with pGEM7zf(+) to yield pHJK0024. The

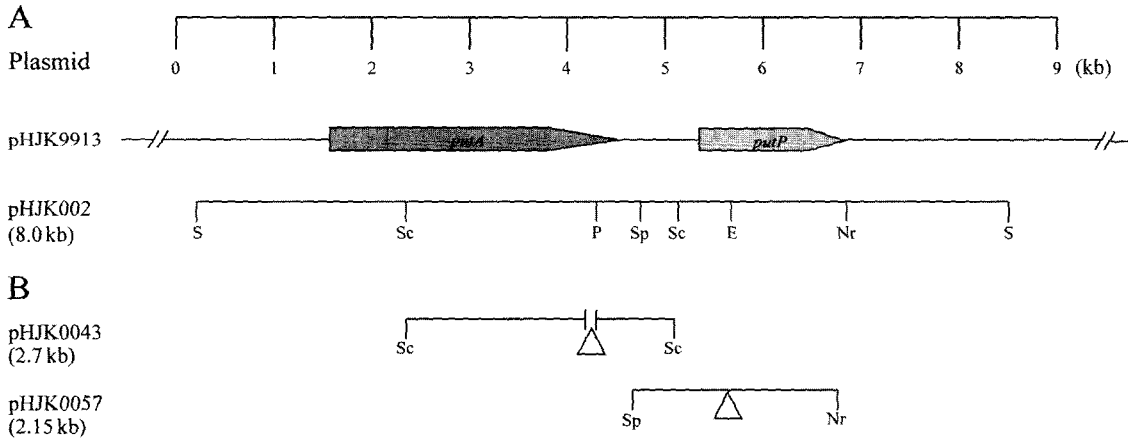

Fig. 1. Physical map of the *put* genes on the *V. vulnificus* ATCC29307 chromosome and plasmids used in this study.
(A) Plasmid pHJK002 was used to determine the nucleotide sequence of *putAP*. The open boxes and thick lines represent the coding regions of *putAP* genes and chromosomal DNA, respectively. (B) Depicted are regions cloned in each of the plasmid used for the construction of the *put::nptI* mutants. The insertion positions *nptI* cassette are indicated by open triangles. Abbreviations; E, *Eco*RI; Nr, *Nru*I; P, *Pst*I; S, *Sal*I; Sc, *Sac*I; Sp, *Sph*I.

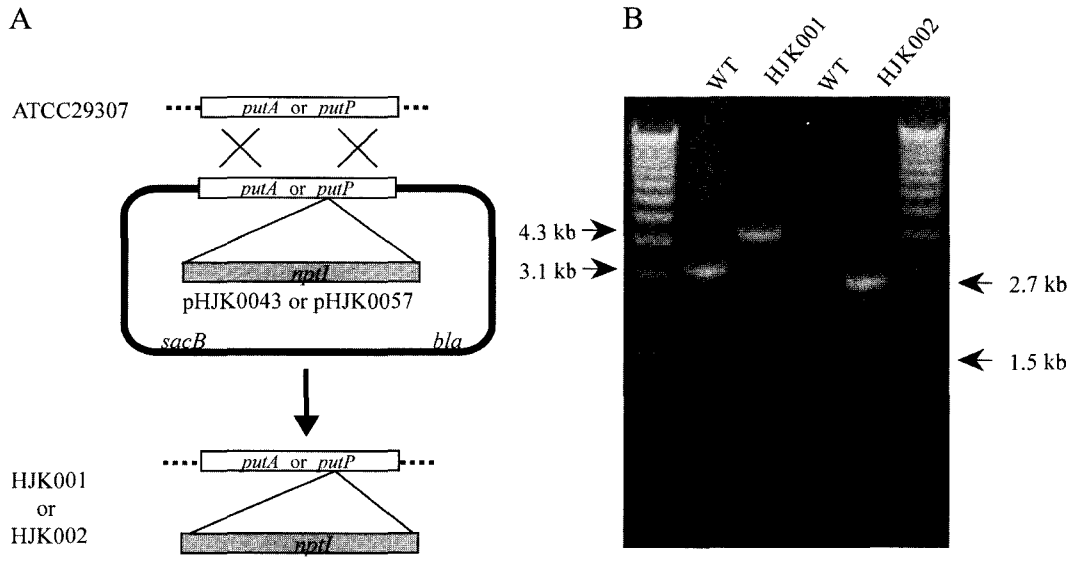

Fig. 2. Allelic exchange procedure and construction of *put::nptI* isogenic mutants.
(A) Double homologous recombinations between strain ATCC29307 and plasmids pHJK0043 or pJHK0057 lead to an interruption of respective *put* genes and resulted in construction of mutants HJK001 or HJK002, respectively. Dashed lines represent the bacterial chromosome; a full line, the plasmid DNA; open boxes, the target *put* genes; shaded boxes, the *nptI* gene; large X's represent genetic crossing over. Abbreviations; *sacB*, levansucrase gene; *bla*, β-lactamase gene. (B) PCR analysis of ATCC29307 and isogenic mutants generated by allelic exchange. Molecular size markers (1-kb ladder, GIBCO-BRL, Gaithersburg, MD, U.S.A.) appear in the end lanes of the gel.

putP in pHJK0024 was inactivated by inserting the *nptI* fragment into the *Eco*RI site of *putP* ORF, resulting in pHJK0055. The *putP::nptI* cartridge was isolated after digestion of pHJK0055 with *Sph*I and *Nru*I, and ligated with pCVD442 to construct pHJK0057 (Fig. 1B).

E. coli SM10 λ*pir*, *tra* (containing pHJK0043 or pHJK0057) were used as a conjugal donor to generate the *put::nptI* mutants of *V. vulnificus* ATCC29307 by homologous recombination (Fig. 2A). The conjugation and isolation of the transconjugants were conducted using the methods previously described [10], and a double crossover, in which each wild-type *put* gene was replaced with the *put::nptI* allele, was confirmed by PCR as shown in Fig. 2B. The *V. vulnificus* mutants chosen for further analysis were named HJK001 for the *putA* mutant and HJK002 for the *putP* mutant, respectively.

Complementation of the *put::nptI* Mutants

The ORF and upstream region of the *putA* and *putP* were amplified by PCR using the following pairs of primers: PUTA004 and PUTA005 for the *putA*, and PUTP004 and PUTP005 for the *putP*, as shown in Table 2. The amplified *putA* was digested with *Xba*I and *Eco*RI and then ligated with pRK415 [11] digested with the same enzymes, to result in pHJK0045. In a similar way, pHJK0059 was constructed by subcloning the amplified *putP* digested with *Pst*I and *Bam*HI into pRK415 linearized with the same enzymes. Since the broad host range vector pRK415 has an IncP1 origin and RP4 *oriT*, the resulting plasmids were mobilizable into *V. vulnificus* by conjugation.

Measurement of Proline Dehydrogenase Activity

For measurements of proline dehydrogenase activity, cultures of *V. vulnificus* strains in LBS were incubated at 30°C under aeration. Samples of 1.5 ml were removed at the indicated times, and bacterial cells were harvested and washed by centrifugation. Bacterial cell pellets were resuspended in 1 ml of 0.1 M cacodylate buffer (pH 6.6), mixed with 5 ml of toluene and shaken for 10 min at 37°C. Minor modifications of procedures described previously [7, 18] were used for determination of proline dehydrogenase activity. The assay was initiated by adding 1 ml of 1 M proline and 200 μl of 0.05 M *o*-aminobenzaldehyde (predissolved in 20% ethanol) to 1 ml cell suspension as an enzyme source. After incubation for 45 min at 37°C with shaking, the reaction was stopped by the addition of 200 μl of 20% (w/v) trichloroacetic acid. The reaction mixture was clarified by centrifugation, and absorbance of supernatant was measured at 443 nm. A unit of the enzyme activity was defined by the method of Ostrovsky *et al.* [18]. Averages and standard errors of the mean (SEM) were calculated from at least three independent determinations.

Assay for Proline Transport

Transport of proline into the bacterial cell was assayed as described elsewhere [19, 27] with minor modifications. Cultures of *V. vulnificus* strains were grown in M9 broth with 0.4% glucose as a carbon source to late-log phase (OD_{600} of 1.0). Cells were harvested from 15-ml cultures by centrifugation, washed twice with the growth medium. Cells were resuspended in 2 ml of the same medium

to give a concentration of approximately 0.1 mg total cellular protein/ml as measured by the method of Bradford [3]. For transport assay, the reactions were initiated by adding 0.03 ml cell suspension to 1 ml reaction mixture containing 10 μM L-[U-^{14}C]proline (275 mCi/mmol) and 10 mM nonradioactive proline. Cell suspension and reaction mixture were prewarmed at 37°C for 5 min. After incubation at 37°C with shaking, the reactions were terminated at the indicated times by rapid filtration through membrane filters (type HA, 0.45 μm pore size, Millipore Corp., Bedford, MA, U.S.A.). The cells were quickly washed with 2 ml of M9 broth containing 50 mM $MgCl_2$ and 0.5 M sucrose. The filters were then dried in scintillation vials. The uptake of radioactive proline was quantified by adding 6 ml of the scintillation cocktail (RIALUMA, LUMAC. LSC, Netherlands) and counting samples with liquid scintillation counter (WALLAC 1409, Wallac OY, Torku, Finland).

Measurement of Cell Growth with Proline as the Sole Carbon or Nitrogen Source

Wild-type *V. vulnificus* ATCC29307 and its isogenic *put* mutants, inoculated with an initial cell density of approximately 0.005 (OD_{600}), were grown at 30°C under aeration in 250-ml Erlenmeyer flasks. To measure the growth of strains with proline as a sole carbon source, M9 media [24], in which glucose was deleted and proline (a final concentration of 20 mM) was supplemented as a sole carbon source, was used. In a similar way, NH_4Cl of M9 media was replaced with proline (a final concentration of 20 mM) to measure growth on proline as a sole nitrogen source.

Hyperosmotic Challenges

For comparison of tolerance to high osmolarity, the survivals of parental, wild-type *V. vulnificus* ATCC29307 and its *putP* mutant, HJK002, were measured in LBS plate supplemented with 0.5 M sucrose as a source of osmotic stress. Cultures were grown to late exponential phase in M9 with 1 mM proline. Inocula then removed were used to spread in triplicate on LBS plate and LBS plate with 0.5 M sucrose at the same time. Following incubation at 30°C overnight, the percentage of survivors on LBS plate with 0.5 M sucrose was calculated by using the CFU/ml as appeared on LBS as 100%.

Nucleotide Sequence Accession Number

The nucleotide sequence of *putAP* genes of *V. vulnificus* ATCC29307 was deposited under accession number AF 454004 in the GenBank.

RESULTS

Sequence Analysis of the *V. vulnificus putAP* Genes

The nucleotide sequence of the 8.3-kb DNA fragment in pHJK002 revealed two coding regions consisting of 3,132 and 1,494 nucleotides, and they were 789-bp apart from each other (Fig. 1A). A database search for amino acid sequences similar to those deduced from the coding regions revealed two other PutAP proteins from *E. coli* and *Salmonella typhimurium* strains, with high levels of identity. This information suggested that the coding regions are homologs of *putAP* genes reported from other Enterobacteriaceae, and led us to name the coding regions as *putAP* of *V. vulnificus*.

The amino acid sequence deduced from the *putA* coding sequence revealed a protein, a proline dehydrogenase composed of 1,043 amino acids with a theoretical molecular mass of 115,088 Da and a pI of 5.76. The amino acid sequence of the *V. vulnificus* PutA were 46% to 59% identical to those of the PutA from *E. coli*, *S. typhimurium*, and *Pseudomonas aeruginosa*, respectively (data not shown). The amino acid sequence deduced from the *putP* nucleotide sequence revealed a proline permease composed of 497 amino acids with a theoretical molecular mass of 53,140 Da and a pI of 6.98. The amino acid sequence of the *V. vulnificus* proline permease, PutP, was 38% and 39% identical to those of the PutP of *E. coli* and *S. typhimurium*, respectively (data not shown). All these informations suggested that the *putAP* genes encode the proline dehydrogenase and proline permease of *V. vulnificus* ATCC29307.

Construction and Confirmation of the *V. vulnificus putA* Mutant and *putP* Mutant

To examine the function of the gene product of *putAP* genes, the chromosomal *putA* and *putP* of wild-type *V. vulnificus* were replaced with *putA*::*nptI* and *putP*::*nptI* by homologous recombination events, leading to the generation of HJK001 and HJK002, respectively. The insertional disruption of each *put* gene in the mutants was confirmed by PCR. PCR analysis of genomic DNA from ATCC29307 with primers PUTA004 and PUTA0005 produced a 3.1-kb fragment (Fig. 2B), whereas genomic DNA from the HJK001 resulted in an amplified DNA fragment approximately 4.3 kb in length. The 4.3-kb fragment is in agreement with the projected size of the DNA fragment containing wild-type *putA* (3.1 kb) and the *nptI* genes (1.2 kb). Similarly, the construction of HJK002 was confirmed by PCR using primers PUTP004 and PUTP005 as shown in Fig. 2B.

Proline Dehydrogenase Activity of *V. vulnificus* ATCC29307 and its Isogenic *putA* Mutant

Proline dehydrogenase activity of strain ATCC29307 appeared at the beginning of growth and reached a maximum during the stationary phase (Fig. 3A). Therefore, cultures of ATCC29307 and the *putA* mutant, grown for 10 h (approximately OD_{600} 2.0), were used to compare proline dehydrogenase activities. The disruption of *putA* in HJK001 resulted in the complete loss of proline dehydrogenase

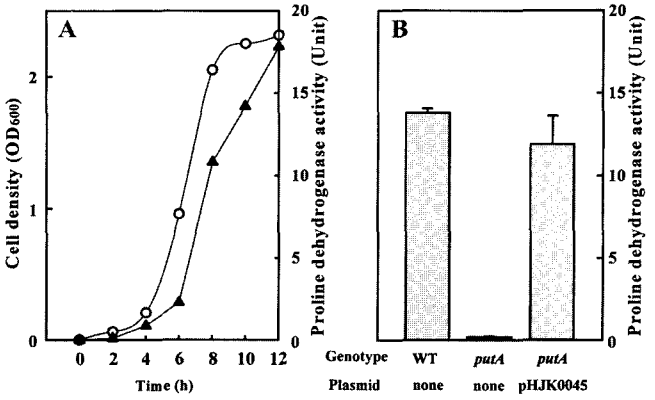

Fig. 3. Proline dehydrogenase activities of *V. vulnificus* strains.
(A) Wild-type ATCC29307 strain was grown in LBS and samples removed at the indicated times were analyzed for proline dehydrogenase activity (○) and cell density (◆). (B) Proline dehydrogenase activities were determined from samples removed at OD_{600} 2.0 from cultures of wild-type and the *putA* isogenic mutant. Complementation of the mutant with a functional *putA* (pHJK0045) is also presented as indicated. Proline dehydrogenase activities were calculated as described in Materials and Methods. Error bars represent SEM.

activity (Fig. 3B). We examined if reintroduction of recombinant *putA* could complement the lack of proline dehydrogenase activity of HJK001 cells, and found that the proline dehydrogenase activity of the HJK0011 (pHJK0045) was restored to a level comparable to the wild-type level of ATCC29307 (Fig. 3B). Therefore, the decreased proline dehydrogenase activity of HJK001 resulted from inactivation of functional *putA* rather than any polar effects on any genes downstream of *putA*. These data demonstrated that the *putA* gene encoded the proline dehydrogenase activity of *V. vulnificus*. The observation that the level of proline dehydrogenase activity in the mutant was undetectable revealed the existence of only one proline dehydrogenase produced by *V. vulnificus* ATCC29307.

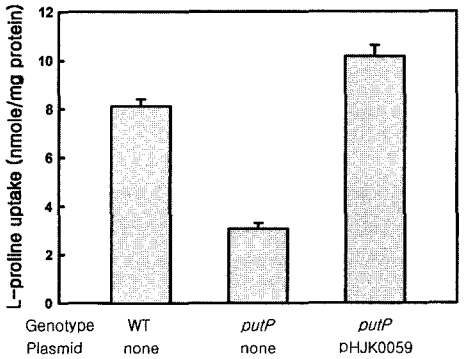

Fig. 4. Effects of mutation in the *putP* gene on proline permease activity.
Proline uptake by wild-type and its isogenic *putP* mutant. Transport measurements were performed after 2 h by the filtration method as described in Materials and Methods.

Effects of Mutations in the *putP* Gene on Proline Permease Activity

To test whether the mutation of *putP* was functionally impaired for proline transport, proline uptake was compared in wild-type and its isogenic *putP* mutant (Fig. 4). When compared in the absence of osmotic pressure, the level of proline uptake was lower in the HJK002 strain. While the uptake of proline was present at about 8 nmol/mg protein in the wild-type, the residual level of proline uptake in HJK002 was approximately half of that in the wild-type. The reduced uptake of proline due to the disruption of *putP* demonstrated that PutP acted as a major, if not only, proline transporter.

Effects of *put* Mutations on the Growth of *V. vulnificus* Using Proline as a Sole Carbon or Nitrogen Source

When proline was used as a sole carbon source, the growth of the *put* mutants was comparable to that of parent strain. Twenty mM proline as a sole carbon source supported growth of wild-type and stationary-phase cells, when LBS was used (Fig. 5A). In contrast to this, HJK001 that is deficient of functional PutA, was not able to grow at all, revealing conversion of proline into glutamate is essential for growth of *V. vulnificus* with proline as a sole carbon source. Growth of the HJK002, the *putP* mutant, on proline as a sole carbon source was significantly reduced with a longer lag phase, suggesting that the gene product of *putP* was responsible for major uptake of proline. However, it is noteworthy that the mutant still exhibited growth, indicating the existence of at lease one more proline permease produced by *V. vulnificus*, as observed in other enteric bacteria.

In contrast to this, the growth of either wild-type or its isogenic *put* mutants was not evident when proline was used as a sole nitrogen source (data not shown). Therefore,

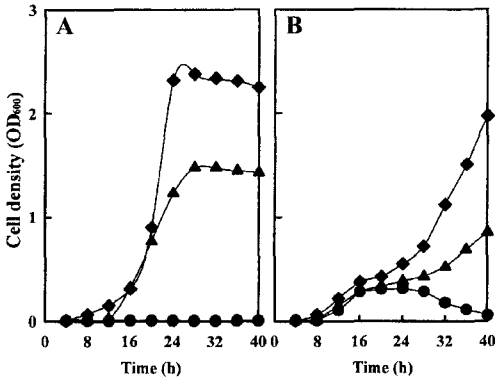

Fig. 5. Growth kinetics of *V. vulnificus* strains in defined medium using proline as a sole nitrogen source.
Cultures of ATCC29307 (◆), HJK001 (●), and HJK002 (▲) were grown in M9 media supplemented with 20 mM proline as a sole carbon source (A) and nitrogen source (B). Details are described in Materials and Methods.

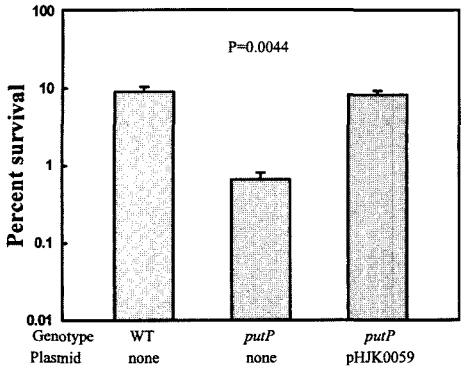

Fig. 6. Hyperosmolarity tolerance of ATCC29307 and isogenic *putP* mutant.

Cultures, grown in M9 with 1 mM proline, were used for acid challenge as described in Materials and Methods. The percent of survivors was calculated using the CFU/ml of strains that survived on LBS plate as 100%. Survivors of the mutants complemented with a functional *putP* (pHJK0059) are also presented as indicated. The statistical significance of the difference among the *V. vulnificus* strains was evaluated with Student's unpaired t test (SAS software, SAS Institute Inc., Cary, NC, U.S.A.). Significance was accepted at $p<0.05$. All bars represent the mean number of survivors from three independent trials, and error bars represent the SEM.

M9, in which NH_4Cl was replaced with proline as a sole nitrogen source and supplemented with yeast extract, (0.01%, w/v) was used to compare their growth rates (Fig. 5B). Although the growth of HJK002 was still evident, its growth rate was seriously damaged, compared to that of wild-type, again suggesting that the PutP protein was the primary system for proline uptake in *V. vulnificus*. As expected, HJK001, in which functional proline dehydrogenase was lacking, could not grow to a substantial level. This indicates that proline dehydrogenase is physiologically important for metabolic conversion of proline into other amino acids.

Osmotic Tolerance of the *putP* Mutant

The survival of the *putP* mutant was compared to that of wild-type in high-osmotic-strength medium. The survival of the parent strain (ATCC29307) was significantly greater ($p<0.05$) than that of the *putP* mutant (HJK002), when the cells were challenged in LBS adjusted by 0.5 M sucrose (Fig. 6). The parent strain decreased ca. 1.0 log CFU/ml (90%), while the HJK002 strain decreased ca. 2.0 $\log_{10}$ CFU/ml (99%) after the hyperosmotic challenge. This indicated that the *putP* mutant was more sensitive to high-osmotic stress than the wild-type, and the PutP played an important role in the osmotic tolerance of *V. vulnificus*. In contrast to the results with HJK002 cells, the survival of HJK001 was not significantly different from that of the parental wild-type (data not shown).

Complementation of *putP::nptI* in HJK002 with a functional *putP* (pHJK0059) restored osmotic tolerance to a level comparable to that of the parent strain (Fig. 6); however, introduction of a control plasmid (pRK415) into HJK002 did not significantly affect survival during the hyperosmotic challenge (data not shown).

DISCUSSION

Many bacteria can utilize proline as a sole source of carbon, nitrogen, and energy. In enteric bacteria, proline is catabolized via divergently transcribed products of *putA* and *putP* genes [12]. Although the enzymes are quite conserved among the different microorganisms, the genetic organization and regulatory mechanisms that control the expression of the genes are highly divergent. Proline is converted to glutamate by the action of two enzymes: proline dehydrogenase and Δ-pyroline-5-carboxylate dehydrogenase. In enteric bacteria, *putA* encodes an enzyme for both steps of proline utilization [12, 15].

In enteric bacteria, proline is taken up by several transport systems (proline permeases) that differ in their affinity for proline [28]. The *putP* gene encodes a high-affinity sodium-proline symporter that transports proline independently of medium osmolarity. The PutP permease is the primary proline transport protein, since *putP* mutants cannot grow on proline as the sole carbon and nitrogen source [13, 21]. The *proP* gene encodes a transport system with a low affinity for proline and glycine-betaine [2]. The *proU* encodes ProU, which transports proline with a low affinity and glycine-betaine with a high affinity [2]. Both ProP and ProU permeases are induced by high osmolarity but not by proline [4, 28]. Although the ProP and ProU transport systems can transport proline, the rate of proline transport by these systems is too low to allow growth on proline as the sole carbon or nitrogen source.

Our data show that the *V. vulnificus* strain with a null mutation in the *putP* gene still exhibited growth, even at lower level, on proline as the sole carbon or nitrogen source (Fig. 5), revealing the existence of at least one more proline uptake system active enough to support growth. The growth of the *V. vulnificus putP* mutant can be also explained if the ProP or ProU (or their homologs) have higher affinity for proline and allow *V. vulnificus* to utilize proline as the sole carbon or nitrogen source. However, additional experiments are in need for a definitive answer, since either ProP or ProU have not yet been identified in *V. vulnificus*.

It has been proposed that PutP transports proline for use as a carbon and nitrogen source rather than as an osmoprotectant in *E. coli* and *Salmonella* spp. [20, 28]. However, osmotic challenge experiments using wild-type and its isogenic *putP* mutant indicated that PutP played a

physiologically significant role in proline uptake for osmoprotective purpose (Fig. 6). Consistent with this, PutP of *V. vulnificus* was related with the sequence of the OpuE protein from *Bacillus subtilis*, and their similarity is at the same level as that observed between PutP proteins of other enteric bacteria (data not shown). OpuE is a high-affinity transporter for proline as observed in PutP, and is unrelated to ProP. However, it does mediate proline uptake for osmoprotective purpose and its transcription is upregulated in response to increase in medium osmolarity [27]. Therefore, it is not surprising that PutP of *V. vulnificus* is involved in the acquisition of proline for osmoprotective purpose.

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