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Characterization of the *Vibrio vulnificus putAP* Operon, Encoding Proline Dehydrogenase and Proline Permease, and Its Differential Expression in Response to Osmotic Stress

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The *Vibrio vulnificus putAP* genes encoding a proline dehydrogenase and a proline permease are transcribed in the same direction. Proline dehydrogenase activity and the level of *putA* transcript were determined to reach a maximum in exponential phase and were then repressed when growth slowed down. Northern blotting and primer extension analyses revealed that transcription of *putAP* genes results in two different transcripts, transcript A (*putA* transcript) and transcript AP (*putAP* transcript). Expression of *putAP* genes was directed by two promoters, promoter P_{putA} and promoter P_{putAP}. A *crp* null mutation decreased proline dehydrogenase activity and the level of the *put* transcripts, indicating that transcription of *putAP* is under the positive control of cyclic AMP receptor protein. Proline dehydrogenase and the level of both *put* transcripts were increased by proline but repressed by glutamate. In contrast, the level of transcript A, not transcript AP, increased when proline dehydrogenase was induced by NaCl. Since P_{putA} activity, not P_{putAP} activity, was increased by NaCl, it is apparent that transcript A and transcript AP are transcribed through P_{putA} and P_{putAP}, respectively. Cells challenged with NaCl and various hyperosmotic stresses accumulated higher levels of glutamate than control cells, indicating that glutamate is a compatible solute in *V. vulnificus*.

Change in the external osmolarity is one of the most common environmental stresses that bacteria routinely encounter (10). The main strategy that members of the family *Enterobacteriaceae* use to adapt to high osmolarity is the accumulation of organic solutes, known as compatible solutes or osmoprotectants (6, 10, 12, 14). It has been well demonstrated that the osmolytes do not interfere with macromolecules and allow the maintenance of vital cellular functions. Hence, the osmolytes can be accumulated to high levels to prevent the loss of water caused by high external osmolarity and consequently contribute to the maintenance of the outwardly directed turgor pressure required for growth. Although a variety of novel organic osmolytes as osmoprotectants have been identified, glycine betaine, ectoine, proline, glutamate, and trehalose are probably the most widely used compatible solutes in bacteria (6, 10).

Vibrio vulnificus is an opportunistic gram-negative bacterial pathogen that commonly contaminates raw oysters and is the causative agent of food-borne diseases, such as gastroenteritis and life-threatening septicemia. Mortality from septicemia is very high (>50%), and death may occur within 1 to 2 days after the first signs of illness (2, 20). Like many other food-borne pathogenic bacteria, *V. vulnificus* occurs in various environments having different osmotic strengths; it naturally inhabits coastal seawaters, contaminates shellfish, survives current control practices (such as adding salt or sugar to suppress its growth), and colonizes in the human body. These facts indicate that *V. vulnificus* has to cope with ever-changing osmolarity in its growth environments. However, only a few studies have addressed the molecular mechanisms whereby the bacterium can survive in hyperosmotic environments (19).

In a previous report, a mutant that was sensitive to high osmolarity was screened from a library of mutants constructed by a random transposon mutagenesis (19). We identified and cloned *putAP* genes encoding a proline dehydrogenase and a proline permease of *V. vulnificus* by a transposon-tagging method (19). Functions of *putAP* genes were assessed by the construction of mutants, and the gene products of *putAP* appeared to contribute to the osmotic tolerance of *V. vulnificus*. However, until now, no definitive analysis of the regulatory mechanisms by which the bacterium can modulate the expression of the *putAP* genes has been reported. Neither the promoter(s) of the *putAP* genes nor *trans*-acting protein(s) regulating *putAP* expression has been identified previously. Furthermore, the question of whether the *putAP* genes are transcribed as a single operon or two separate transcriptional units has not been addressed previously.

Therefore, in an effort to elucidate the regulatory mechanisms of *putAP* expression at a molecular level, we examined the influence of the growth phase on proline dehydrogenase synthesis. We demonstrated that the transcription of the *putAP* genes is dependent on the growth phase, initiated at two different sites, and results in two different transcripts. The effects of a *crp* null mutation on proline dehydrogenase activity and the cellular level of the *put* transcripts were also examined. Transcription of the *putAP* operon has been analyzed under different conditions, and we showed that *putAP* transcription is induced in the presence of proline and hyperosmotic stresses but repressed in the presence of glutamate. Finally, the possible role of glutamate as a compatible solute in *V. vulnificus* was also examined.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Reference or source
Strains		
V. vulnificus		
ATCC 29307	Clinical isolate; virulent	Laboratory collection
KC74	ATCC 29307 with *crp*::*nptI*; Km^r	15
HJK001	ATCC 29307 with *putA*::*nptI*; Km^r	19
HJK002	ATCC 29307 with *putP*::*nptI*; Km^r	
E. coli		
DH5α	*supE44* Δ*lacU169* (ϕ80 *lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Laboratory collection
SM10λ*pir*	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu λ*pir;* Km^r; host for π-requiring plasmids; conjugal donor	Laboratory collection
Plasmids		
pRK415	IncP *ori;* broad-host-range vector; *oriT* of RP4; Tc^r	17
pHJK002	8.3-kb *Sal*I fragment containing *putAP* cloned into pUC18; Ap^r	19
pKC0004	pRK415 with *crp;* Tc^r	15

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

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% (wt/vol) agar. Unless noted otherwise, *V. vulnificus* strains were grown in LB medium supplemented with 2.0% (wt/vol) NaCl (LBS). When appropriate, antibiotics were added to media at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; and tetracycline, 10 μg/ml.

General genetic methods. Procedures for the isolation of plasmid DNA and genomic DNA and transformation were performed as described by Sambrook and Russell (32). Restriction and DNA-modifying enzymes were used as recommended by the manufacturer (New England Biolabs, Beverly, Mass.). DNA fragments were purified from agarose gels using the Geneclean II kit (Bio 101, Inc., Vista, Calif.). Primary DNA cloning and manipulation were conducted in *E. coli* DH5α, and restriction mapping was performed to confirm that transformants contained the appropriate plasmids. PCR amplification of DNA was performed using a GeneAmp PCR system 2400 (Perkin-Elmer, Norwalk, Conn.) by standard protocols.

Measurement of cell growth and proline dehydrogenase activity. Cultures of *V. vulnificus* strains were grown at 30°C with aeration. Samples (5 ml) were removed at regular intervals for determination of cell densities, proline dehydrogenase activity, *put* transcript levels, and cellular protein concentrations. Growth was monitored by measuring the optical density at 600 nm (OD_{600}) of the culture. Cultures with an OD_{600} of >0.8 were diluted prior to measurement. The proline dehydrogenase activity was determined as previously described (19). One unit of enzyme activity was defined by the method of Ostrovsky et al. (29). Protein concentrations were determined by the method of Bradford (5), with bovine serum albumin as the standard. Averages and standard errors of the mean (SEM) were calculated from at least three independent determinations.

RNA purification and analysis of the *put* transcripts. Total cellular RNAs from *V. vulnificus* strains were isolated using a Trizol reagent kit in accordance with the manufacturer's protocol (GIBCO-BRL, Gaithersburg, Md). RNA was treated with RNase-free DNase I (Sigma, St. Louis, Mo.) to remove contaminating genomic DNA.

A series of reactions was performed according to standard procedures (32) with 20 μg of total RNA for either Northern dot blot analysis or Northern blot analysis. RNA was transferred to a nylon membrane and hybridized as previously described (9, 15). Two DNA probes, PUTAP and PUTPP, were labeled with [α-^{32}P]dCTP using the Prime-a-gene labeling system (Promega, Madison, Wis.) and used for hybridization (9, 16). The PUTAP probe was prepared by labeling the 920-bp DNA fragment in the *putA* gene and amplified by PCR using primers PUTA004 and PUTA005 (Table 2). A 1.1-kb DNA fragment, containing the coding region of *putP*, was amplified by PCR using primers PUTP004 and PUTP005 (Table 2) and then labeled for the PUTPP probe. The blots were visualized and quantified using a phosphorimaging analyzer (BAS1500 model; Fuji Photo Film Co., Ltd, Tokyo, Japan) and the Image Gauge (version 3.12) program.

Primer extension analysis. Primer extension experiments were performed with SuperScript II RNase H$^-$ reverse transcriptase (GIBCO-BRL) by the method of Sambrook and Russell (32). A 27-base oligonucleotide (PUTA0012 [Table 2]) complementary to the open reading frame of *putA* was used as the primer. The primer was end labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. The cDNA products were purified and resolved on a sequencing gel along sequencing ladders generated with the same primer used for primer extension. The nucleotide sequence of pHJK002 (Table 1) was determined by the dideoxy-chain termination method with Top DNA polymerase (Bioneer, Seoul, Korea) in accordance with the manufacturer's protocols. The bands were visualized and quantified as described above for Northern analysis.

Effects of proline and glutamate and hyperosmotic stresses on the expression of *putAP*. To examine the effects of proline and glutamate on the expression of *putAP*, cultures of *V. vulnificus* ATCC 29307 were grown in M9 broth (32) supplemented with various levels of proline or glutamate. Similarly, when the effects of various osmolytes on the expression of *putAP* were analyzed, M9-N broth in which NaCl was omitted but 200 mM proline was added was used. The inocula were from late-exponential-phase cultures in M9 broth. The cultures were adjusted to an initial pH of 7.0 with 100 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid [TES]. The *put* transcript, total protein, and glutamate levels and proline dehydrogenase activity of the cultures removed at an OD_{600} of 0.8 were analyzed and compared.

TABLE 2. Oligonucleotides used in this study

Oligonucleotide	Oligonucleotide sequence (5′→3′)[a]	Location[b]	Use
PUTA004	CATGAATTCCTCCTTATGCGTTCTCAGTC	*putA*	Amplification of *putA* gene
PUTA005	ACTCTAGATTCGGGGATGAGATTGAGGAAA	*putA*	Amplification of *putA* gene
PUTA0012	GAGATCAATGCCCATAGCTTATCGAGC	*putA*	Identification of transcription start sites
PUTP004	GTGGATCCAAGATTACTCTAGCTCAACCA	*putP*	Amplification of *putP* gene
PUTP005	GCGCTGCAGTCTAATGAGGACTATCTAATGA	*putP*	Amplification of *putP* gene

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

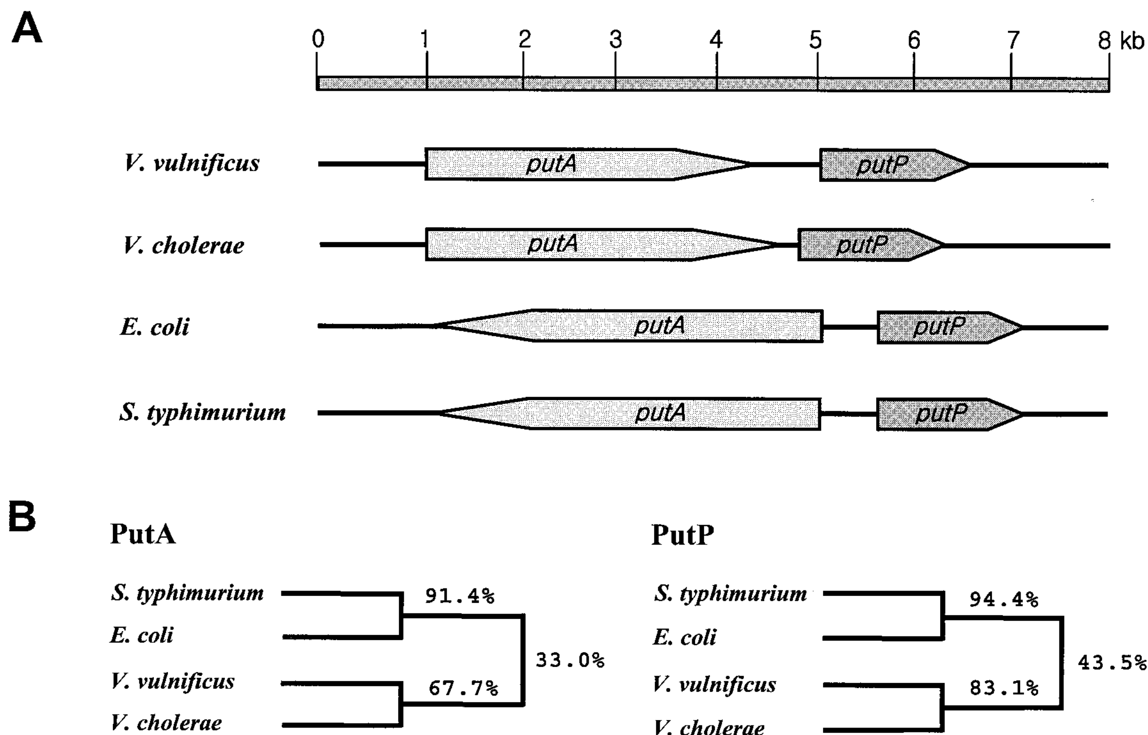

FIG. 1. Genetic organization of the *putAP* operons of different microorganisms and amino acid sequence relatedness of their gene products. The microorganisms were *V. vulnificus*, *V. cholerae*, *E. coli*, and *S. enterica* serotype Typhimurium. (A) The coding regions of *putAP* genes (shaded boxes) and chromosomal DNA (thick lines) are shown. The direction of transcription is indicated by the arrows. (B) Dendrograms showing relatedness of PutA and PutP were derived using the CLUSTALW alignment program (http//www.ebi.ac.uk/clustalw) and based on the amino acid sequences in the GenBank database (National Center for Biotechnology Information database).

Glutamate determinations. Cellular levels of glutamate were determined essentially as described previously (3). Cells recovered from 5 ml of culture broth were mixed with 5 ml of boiling distilled water and vortexed vigorously to extract glutamate. The cells were spun down, and glutamate in the supernatant was quantified spectrophotometrically by the method of Witt (35). The means of the results from at least three independent experiments were used.

RESULTS

Organization and sequence relatedness of the *V. vulnificus putAP* genes. A 8.3-kb DNA fragment from *V. vulnificus* ATCC 29307, which carries the *putAP* genes, was cloned in pHJK002 (19) (Table 1). The nucleotide sequence (GenBank accession number AF454004) revealed that the two coding regions consisting of *putA* and *putP* are transcribed in the same direction (Fig. 1A). Alignment of the translated *putAP* sequences to those of PutAP of *E. coli* and *Salmonella enterica* serovar Typhimurium revealed about 38% amino acid identity (Fig. 1B) (http//www.ebi.ac.uk/clustalw). However, compared with the PutA proteins of *E. coli* and *S. enterica* serovar Typhimurium, the *V. vulnificus* PutA protein was missing approximately 70 and 200 amino acid residues at the N- and C-terminal regions, respectively. Moreover, the *putAP* genes of *E. coli* and *S. enterica* serovar Typhimurium, which are well characterized at the molecular level, are transcribed divergently. Further searches for amino acid sequences similar to those of *V. vulnificus* PutAP identified two uncharacterized coding regions from the *Vibrio cholerae* genome sequence database with higher levels of identity (Fig. 1B). These two coding regions, the presumed *V. cholerae putAP* genes, are organized in the same orientation as in *V. vulnificus putAP* (Fig. 1B). The difference in genetic organization indicates that the regulatory mechanisms for the expression of *V. vulnificus putAP* genes would be different from those for *putAP* genes in *E. coli* and *S. enterica* serovar Typhimurium.

Expression of the *V. vulnificus putA* gene as a function of growth. A number of the genes that contribute to stress tolerance in bacteria are induced at the onset of stationary phase. In order to examine whether the expression of *putA* is influenced by growth phase, the proline dehydrogenase activity of *V. vulnificus* ATCC 29307 was analyzed at various growth stages (Fig. 2A). Proline dehydrogenase activity appeared at the beginning of growth and increased as the growth rate increased in exponential phase. Proline dehydrogenase activity then decreased when growth slowed down at stationary phase. Growth phase regulation of proline dehydrogenase production could be manifest at either the transcriptional or posttranscriptional level. To distinguish between these two possibilities, the levels of *putA* mRNA were monitored during growth. The same amount of total RNA was isolated from ATCC 29307 cells at different stages of growth. The results indicated that the *putA* mRNA levels decreased as the culture entered stationary phase and suggested that growth phase-dependent production of proline dehydrogenase is regulated at the level of transcription (Fig. 2B).

Northern analysis of the *put* transcripts. To further characterize the expression of *putAP*, Northern analysis was per-

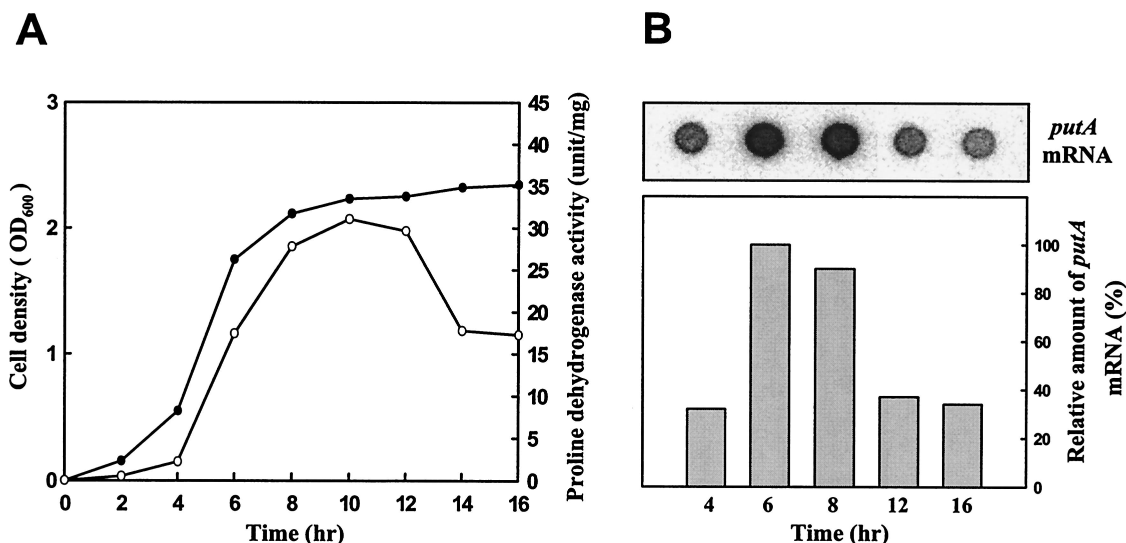

FIG. 2. Expression of *putA* as a function of growth. Samples of the culture of *V. vulnificus* ATCC 29307 in LBS were removed at the time points indicated. Cell density (●) and proline dehydrogenase activity (○) (A) and amount of *putA* mRNA (B) in samples are shown. The relative amounts of *putA* transcript of each dot are presented, with the amount of *putA* transcript at 6 h set at 100%.

formed. When total RNA was isolated from *V. vulnificus* ATCC 29307 cells grown to log phase in LBS and hybridized with the PUTPP DNA probe, only a single, approximately 5.4-kb transcript, was detected (Fig. 3). On the basis of the DNA sequence of *putP*, it was anticipated that the *putP* mRNA would be approximately 1.5 kb long. Cotranscription of *putA* and *putP* was predicted to produce a 5.4-kb transcript. Therefore, it appeared that the single mRNA coded for both PutA and PutP. As a further test of this possibility, Northern blot analysis was performed again using PUTAP as a DNA probe. PUTAP also hybridized to a 5.4-kb RNA. In contrast to the result with PUTPP, however, another band of 3.5 kb hybridized with the PUTAP probe (Fig. 3). Since the *putA* mRNA that is approximately 3.5 kb long was expected on the basis of the DNA sequence of *putA*, the 3.5-kb band may represent the *putA* gene transcript. It is noteworthy, however, that the level of transcript AP was lower than that of transcript A in the total RNA we used.

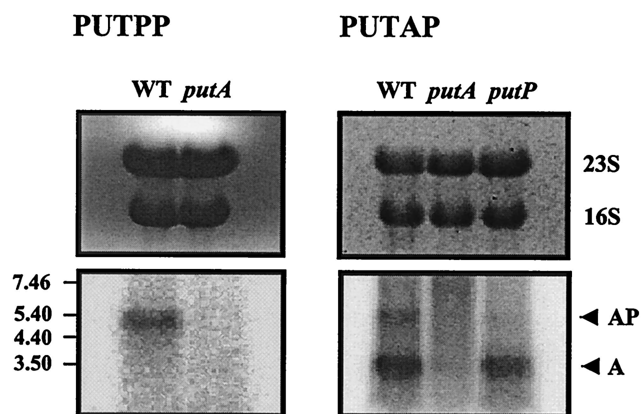

FIG. 3. Northern blot analysis of the *put* transcripts. Total RNAs were isolated from cultures of the wild-type (WT) or *putA* or *putP* mutants grown to log phase in LBS. The RNAs were separated (top) and hybridized to a ^{32}P DNA probe corresponding to the internal coding regions of *putA* (PUTAP) or *putP* (PUTPP) (bottom). The positions of molecular size markers and of hybridization (in kilobases) are shown to the left of the gel. The positions of *put* transcript A and transcript AP are shown to the right of the gel.

These results supported the possibility that transcription of the *putAP* genes results in two different *put* transcripts. However, it was possible, though unlikely, that the two bands resulted from nonspecific hybridization with PUT DNA probes. To examine this possibility, total RNAs isolated from the *V. vulnificus* isogenic mutants, in which either *putA* or *putP* was disrupted by the insertion of *nptI* (19, 28), were analyzed by Northern blotting. When total RNA was isolated from the *putA* mutant (HJK001 [Table 1]), the bands corresponding to the two *put* transcripts were not detected in the Northern blot, irrespective of the DNA probe (Fig. 3). This result demonstrated that the two RNA bands resulted from specific hybridization of PUT DNA probes to the *put* mRNA rather than from nonspecific hybridization. These data indicate that transcription of the *putAP* genes results in two different species of *put* transcripts, transcript AP (a polycistronic *putAP* transcript) and transcript A (a shorter *putA* transcript).

Transcript A could be the result of a 3′-end decay of the longer transcript, transcript AP. To further examine this possibility, Northern analysis of total RNA isolated from the isogenic *putP* mutant (HJK002) (Table 1) was performed. When hybridized with the PUTAP DNA probe, transcript AP was not detected from the total RNA, indicating that transcript AP is not produced in HJK002 (Fig. 3). However, the shorter transcript, transcript A, was still detected, and there was no significant difference in band intensity of the transcript A between the wild-type strain and HJK002. Because transcript A was detected in RNA that lacked transcript AP, it was apparent that transcript A is not the result of a degradation of the longer transcript, transcript AP.

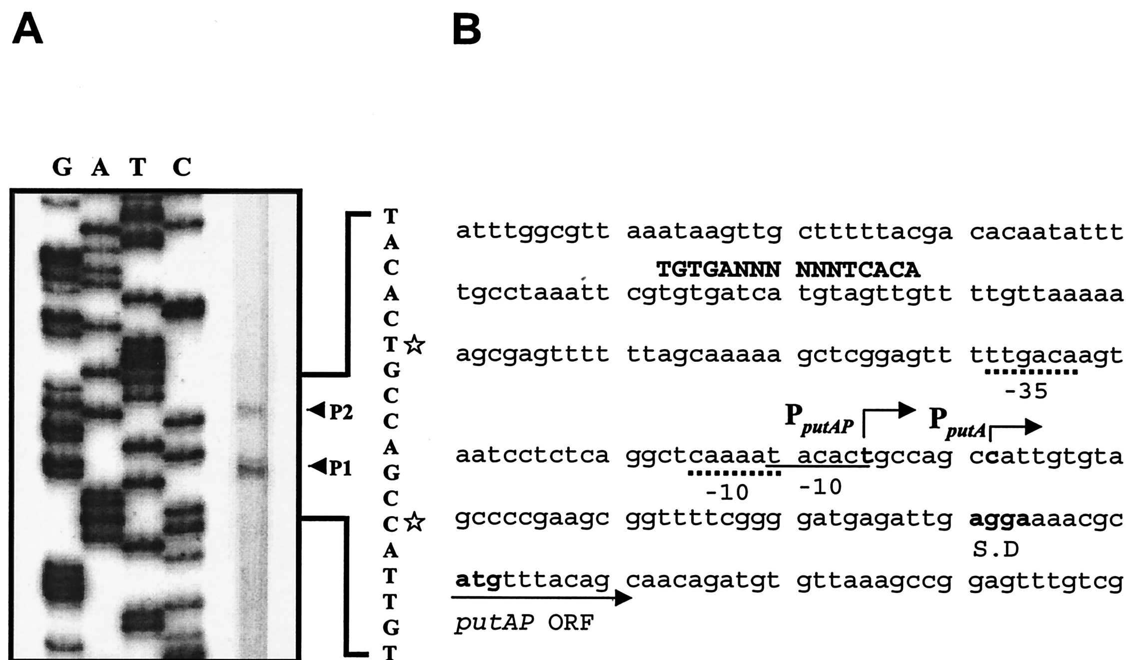

FIG. 4. Primer extension analysis of *put* transcripts and sequence of *putAP* upstream region. (A) The transcription start sites were determined by primer extension of the RNA derived from *V. vulnificus* ATCC 29307 grown to log phase in LBS. Lanes G, A, T, and C represent the nucleotide sequencing ladders of pHJK002. The transcription start sites for P1 (C) and P2 (T) are indicated by white stars. (B) Transcription start sites are indicated by bent arrows. Possible promoters (−10 and −35) are shown underlined with continuous and broken lines for the promoters P_{putA} and P_{putAP}, respectively. Conserved nucleotide sequences for CRP binding (4) are indicated by capital letters. The ATG translation initiation codon and putative ribosome binding site (AGGA) are indicated in boldface type. ORF, open reading frame.

Primer extension analysis of the *put* transcripts. Several different explanations were still possible for production of these different types of *put* transcripts. One is that the destiny of the transcripts is determined after initiation of the transcription through a promoter. That is, transcription of *put* is regulated by attenuation to result in the shorter transcript A and/or by antitermination (read-through) to result in transcript AP. The other possibility is that the two types of transcripts are determined at the transcription initiation step by two different promoters.

To examine these two possibilities, the transcription start sites of *putAP* were mapped by primer extension analysis. Total RNA was prepared from *V. vulnificus* ATCC 29307 grown to log phase in LBS. The primer extension analysis revealed two different transcription start sites, P1 and P2 (Fig. 4A). P1 was located 48 bp upstream of the translational initiation codon of PutA, and P2 was 6 bp from P1 (Fig. 4B). Although other explanations are possible, these results supported the possibility that two different promoters were used for transcription of the two *put* transcripts. We were unable in several attempts to identify any other transcription start sites by primer extension analysis using different sets of primers (data not shown).

The putative promoters upstream of the transcription start sites were named P_{putA} (consisting of P1) and P_{putAP} (consisting of P2). Based on the intensity of the bands of the reverse transcripts, the activity of P_{putAP} was lower than that of P_{putA}. The level of transcript A was higher than that of transcript AP in the total RNAs as determined by Northern blotting (Fig. 3). This observation suggests that transcription from the stronger promoter, P_{putA}, results in transcript A and that transcription from the weaker promoter, P_{putAP}, results in transcript AP.

Effect of *crp* mutation on proline dehydrogenase activity. Proline dehydrogenase activity of an isogenic *crp* mutant of *V. vulnificus*, which was constructed by allelic exchange (15), was compared with that of the parental wild type. Cultures of ATCC 29307 and the *crp* mutant KC74 were grown in LBS to log phase (OD_{600} of 0.6), and the proline dehydrogenase activity of each culture was determined (Fig. 5A). While proline dehydrogenase activity was present at about 10 U per mg of cellular protein in the wild-type strain, KC74 appeared to produce much less proline dehydrogenase. The residual level of proline dehydrogenase activity in KC74 corresponded to approximately 1/10 that in the wild-type strain (Fig. 5A). The reduced production of proline dehydrogenase due to the disruption of *crp* suggested that cyclic AMP receptor protein (CRP) acts as a positive regulator in the production of proline dehydrogenase, at least in log-phase cultures.

To rule out the possibility that the decreased proline dehydrogenase activity was due to polar effects of the *crp* insertion on downstream genes, we examined whether reintroduction of *crp* on a plasmid could complement the mutation. To do this, plasmid pKC0004 that had been constructed by subcloning *crp* into pRK415 (15, 17), was transferred into *V. vulnificus* KC74 by conjugation. The proline dehydrogenase activity expressed by KC74(pKC0004) was about twofold higher than that of ATCC 29307 (Fig. 5A). Therefore, the decreased proline dehydrogenase activity of KC74 resulted from the inactivation of *crp* rather than any polar effects on any genes downstream of *crp*.

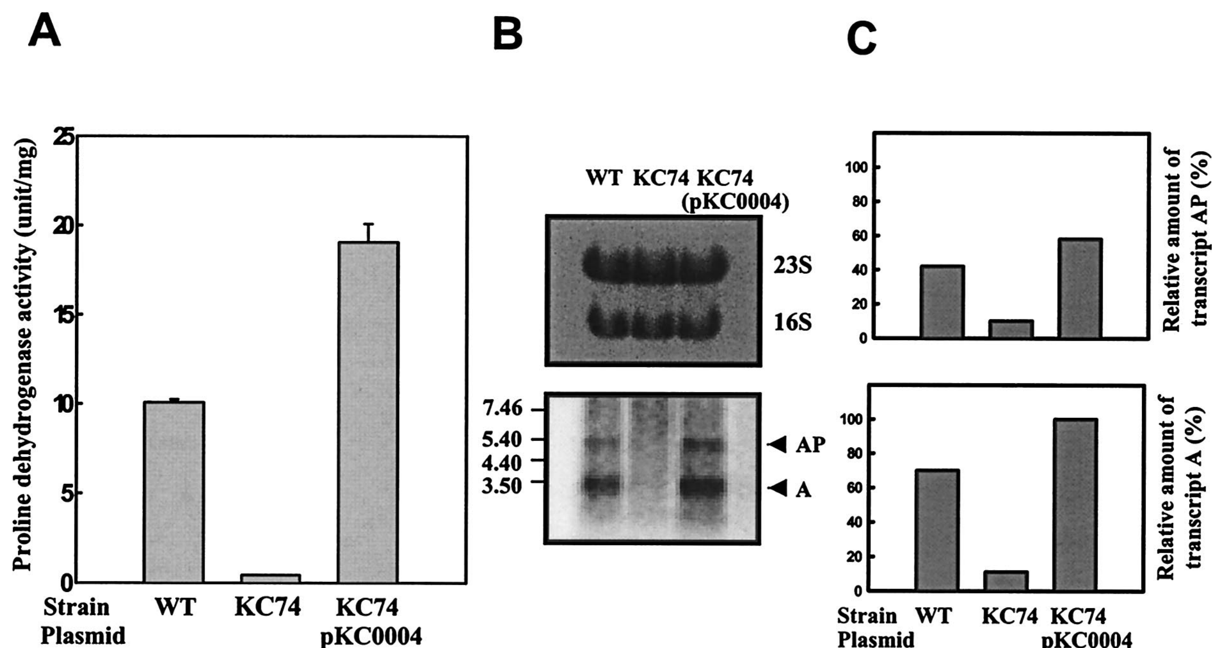

FIG. 5. Dependence of proline dehydrogenase production of *V. vulnificus* on *crp*. Proline dehydrogenase activities (A) and relative amounts of the *put* transcripts (B and C) were determined for the wild-type (WT) strain and the isogenic *crp* mutant, KC74, as indicated. Complementation of the *crp* mutation by functional *crp* (pKC0004) is also presented. Samples removed from each culture grown to log phase in LBS were analyzed for proline dehydrogenase activity and *put* transcripts. Means ± SEM (error bars) are shown. PUTAP was used as a DNA probe, and the Northern blot is presented as described in the legend to Fig. 3. The relative amounts of the *put* transcripts in each band are expressed relative to the amount of the transcript A of KC74(pKC0004).

In order to characterize the role of CRP in more detail, the levels of *put* transcript in the wild-type strain and KC74 were compared by Northern blot analysis (Fig. 5B and C). When PUTAP was used as the DNA probe, the *put* transcripts were readily detected in the wild-type strain and in KC74(pKC0004). Transcript A and transcript AP were barely detected in KC74, indicating that CRP exerts its effects on the production of proline dehydrogenase at the level of transcription from both *put* promoters. Furthermore, the levels of both *put* transcripts in KC74(pKC0004) recovered, and the pattern of the recovery was similar to that observed when the proline dehydrogenase activities were compared directly. Taking these results together led us to conclude that expression of *putAP* in *V. vulnificus* is under the positive control of CRP and that CRP affects the level of proline dehydrogenase production by activating both *put* promoters.

Effects of proline and glutamate on the expression of *putAP*. To examine whether expression of *putAP* is modulated by proline, proline dehydrogenase activities of cultures in M9 broth (32) supplemented with different levels of proline were analyzed. When the amount of proline was increased to 200 mM, the level of proline dehydrogenase activity reached higher levels than that observed in the absence of proline (Fig. 6A). A Northern blot analysis using a PUTAP DNA probe revealed that the addition of proline resulted in increased levels of transcript A and transcript AP (Fig. 6B and C). It was apparent from these results that the expression of *putAP* is subject to induction by proline in *V. vulnificus*.

To further investigate regulation of *putAP* expression, the effect of adding glutamate on the production of proline dehydrogenase was examined. To do this, cultures of *V. vulnificus* were grown in M9 broth supplemented with 200 mM proline alone or with 200 mM proline and glutamate together. As shown in Fig. 7A, the level of proline dehydrogenase activity induced in the presence of proline alone was repressed by the addition of glutamate. It was possible that the decrease of proline dehydrogenase activity was due to inhibition of the enzyme activity itself by increasing glutamate. However, the cellular level of *put* transcripts determined by a Northern blot analysis using a PUTAP DNA probe was lower in response to the presence of glutamate (Fig. 7B and C). This reduction of *put* transcripts brought about by glutamate indicates that the expression of the *V. vulnificus putAP* genes is repressed by glutamate.

Effects of hyperosmotic stress on proline dehydrogenase activity and cellular level of glutamate. It has been proposed that since inactivation of *putA* renders *V. vulnificus* more sensitive to hyperosmotic stress, proline dehydrogenase contributes to the osmotic tolerance of the bacterium (19). As a further test of this hypothesis, we examined whether expression of the *put* genes is modulated in response to osmotic stress. When cultures in M9-N medium with proline were grown with increasing levels of NaCl, the levels of proline dehydrogenase activity increased (Fig. 8A). Northern blot analysis using a PUTAP DNA probe demonstrated that the level of transcript A also increased in response to excess NaCl. However, no significant increase in the level of transcript AP was observed in the culture grown with NaCl, indicating that

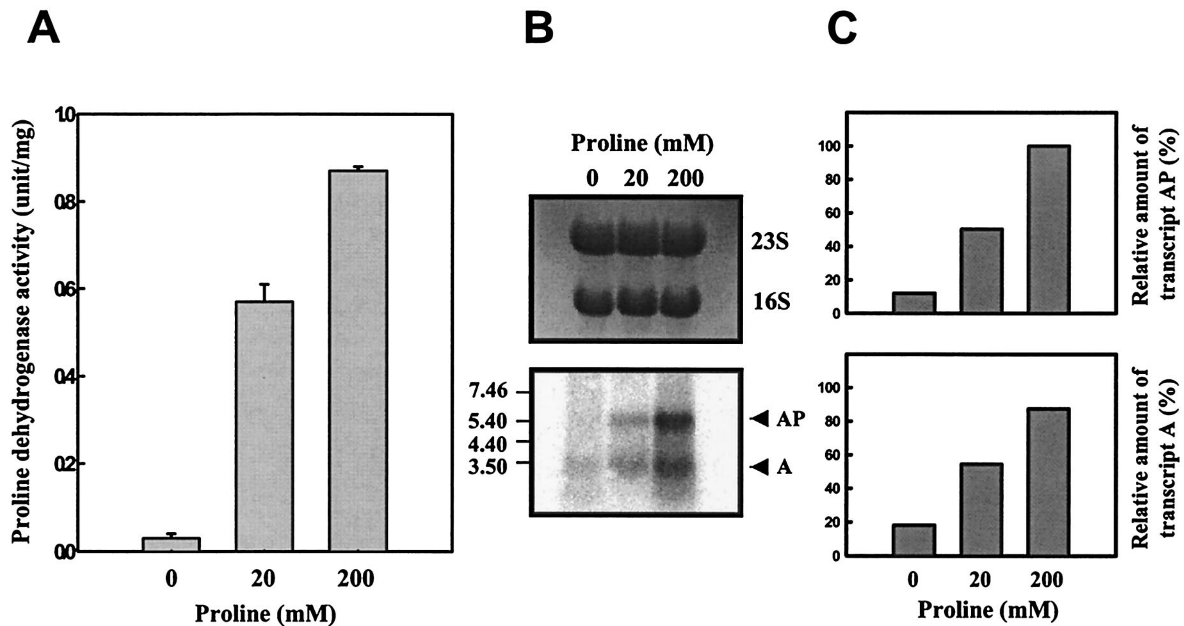

FIG. 6. Induction of expression of *V. vulnificus putAP* genes by proline. Samples were removed from the culture of ATCC 29307 grown to log phase in M9 medium supplemented with various levels of proline as indicated. Samples were analyzed for determination of proline dehydrogenase activity (A) and *put* transcripts (B and C). Means ± SEM (error bars) are shown. PUTAP was used as a DNA probe, and the Northern blot is presented as described in the legend to Fig. 3. The relative amounts of the *put* transcripts in each band are expressed relative to the amount of transcript AP as expressed in the presence of 200 mM proline.

the elevated level of transcript A is responsible for the increased proline dehydrogenase activity (Fig. 8B and C).

The activities of the *put* promoters were compared by primer extension of the total RNAs isolated from cultures of ATCC 29307 grown with or without NaCl (Fig. 9). The activity of promoter P_{putA} increased in response to NaCl, but the activity of P_{putAP} was not significantly affected. This indicates that the higher level of proline dehydrogenase activity observed in the

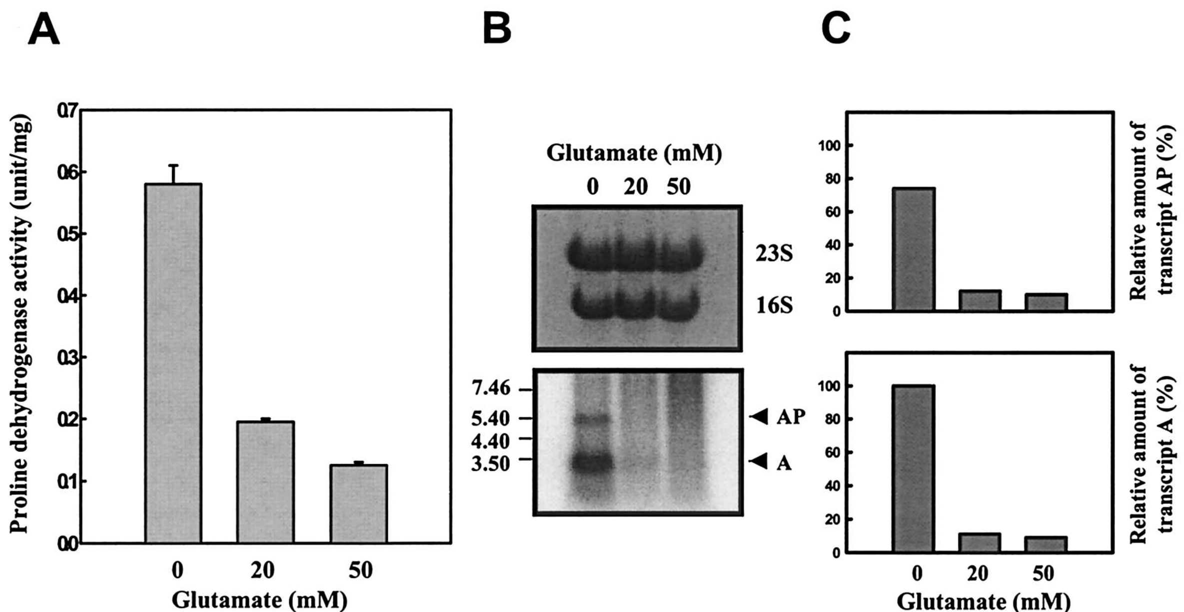

FIG. 7. Repression of expression of the *V. vulnificus putAP* operon by glutamate. Samples were removed from the culture of ATCC 29307 grown to log phase in M9 medium supplemented with 200 mM proline and various levels of glutamate as indicated. Samples were analyzed for determination of proline dehydrogenase activity (A) and *put* transcripts (B and C). Means ± SEM (error bars) are shown. PUTAP was used as a DNA probe, and the Northern blot is presented as described in the legend to Fig. 3. The relative amounts of the *put* transcripts in each band are expressed relative to the amount of transcript A as expressed in the absence of glutamate.

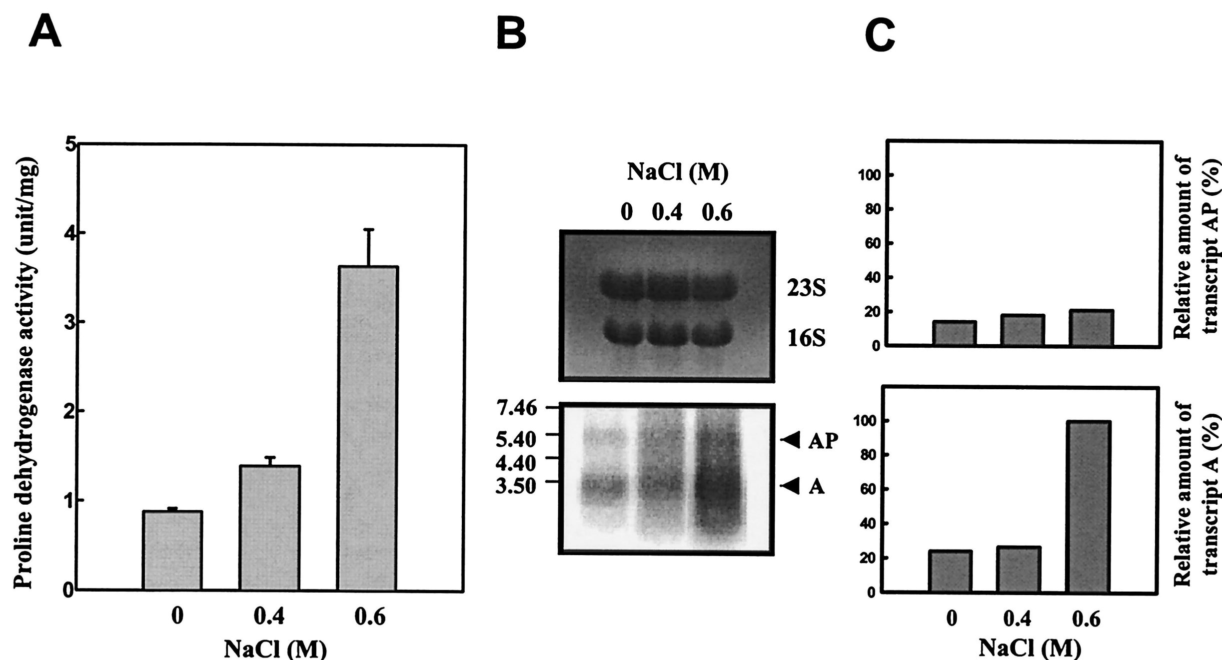

FIG. 8. Induction of expression of the *V. vulnificus putAP* operon by NaCl. Samples were removed from the culture of ATCC 29307 grown to log phase in M9-N medium supplemented with 200 mM proline and various levels of NaCl as indicated. Samples were analyzed for determination of proline dehydrogenase activity (A) and *put* transcripts (B and C). Means ± SEM (error bars) are shown. PUTAP was used as a DNA probe, and the Northern blot is presented as described in the legend to Fig. 3. The relative amounts of the *put* transcripts in each band are expressed relative to the amount of transcript A as expressed in the presence of 0.6 M NaCl.

presence of NaCl is mainly due to the increased activity of the P_{putA} promoter. These results combined together agreed with our previous hypothesis that transcription of transcript A is directed by promoter P_{putA} and that transcription of transcript AP is directed by promoter P_{putAP}. In contrast, the activities of both P_{putA} and P_{putAP} were reduced when the RNA isolated from the cells grown in the presence of glutamate was analyzed by primer extension (Fig. 9). This observation indicates that the activities of both P_{put} promoters were repressed by glutamate. This result was not unexpected, since both transcript A and transcript AP were lower in the cells grown with glutamate as determined by Northern blot analysis (Fig. 7B and C).

Since proline dehydrogenase converts proline into glutamate, it is reasonable to assume that glutamate, rather than proline, plays a role in counteracting osmotic stress. To test this possibility, the accumulation of glutamate was measured in cells grown with 0.6 M NaCl. Compared with cells grown in M9-N medium alone, cells of *V. vulnificus* in M9-N medium supplemented with NaCl accumulated greater amounts of glutamate (Table 3). To examine whether this increased level of proline dehydrogenase activity and glutamate accumulation occurred as a result of increasing osmotic stress or NaCl itself, the effects of other osmolytes on proline dehydrogenase activity and on the accumulation of glutamate were analyzed. As shown in Table 3, the cells accumulated excess glutamate and produced higher levels of proline dehydrogenase when they were osmotically stressed, regardless of the type of osmolytes. Since the number and impermeability of the dissolved particles are different, osmolarity of the osmolytes in culture broth would be not same. However, it was obvious that glutamate accumulated in excess and proline dehydrogenase activity increased when cells were exposed to increased osmolarity. These results indicate that the increased level of proline dehydrogenase activity and glutamate accumulation results from increased osmotic strengths in culture media and that glutamate, rather than proline, is a possible compatible solute counteracting hyperosmotic stress in *V. vulnificus*.

In summary, we have found that transcription of the *V. vulnificus putAP* genes is initiated by two different promoters, P_{putA} and P_{putAP}. The level of *putAP* expression is growth phase dependent and is under the control of CRP. Transcription of the *putAP* genes is induced by proline and hyperosmotic stress and repressed by glutamate. Accumulation of glutamate in response to hyperosmolarity indicates that glutamate plays a physiologically significant role as a compatible solute for osmoprotective purposes in *V. vulnificus*.

DISCUSSION

Proline is catabolized via the gene products of *putA* and *putP* (23, 25). The *putP* gene encodes a proline permease with a high affinity for proline (22, 31). Proline is then converted into glutamate by the action of two enzymes, proline dehydrogenase and $\triangle^1$-pyrroline-5-carboxylate dehydrogenase. In enteric bacteria, *putA* encodes the PutA protein, which catalyzes both steps of proline utilization (23, 25). Although the functions of *putAP* gene products are quite conserved among the different microorganisms, the genetic organization and regulatory mechanisms that control the expression of the genes are highly divergent (8, 18). In *Agrobacterium tumefaciens* and *Rhodobacter*

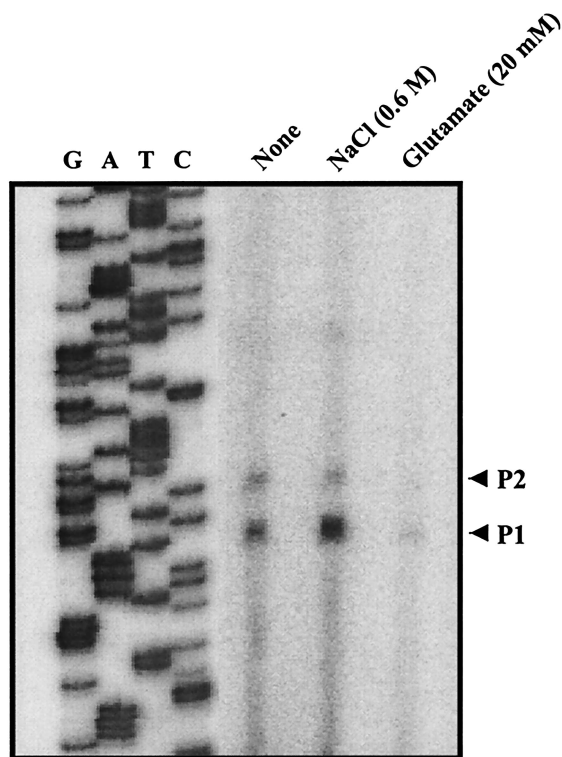

FIG. 9. Effects of NaCl and glutamate on the activities of *put* promoters. Promoter activities were determined by primer extension of the RNA derived from the culture of ATCC 29307 grown to log phase in M9-N (None) and in M9-N supplemented with NaCl (0.6 M) or glutamate (20 mM) as indicated. Lanes G, A, T, and C contain the nucleotide sequencing ladders of pHJK002. The transcription start sites for P_{putA} (P1) and P_{putAP} (P2) are indicated by arrows.

capsulatus, *putA* is organized as a monocistronic transcriptional unit, and *putP* is not located adjacent to *putA* (8, 18). In *E. coli*, *S. enterica* serovar Typhimurium, and *Pseudomonas putida*, the *putA* and *putP* genes are transcribed divergently (23, 34). In these bacteria, the PutA protein is a bifunctional enzyme; in addition to its enzymatic activities, PutA functions as a proline-responsive repressor of the *putA* and *putP* genes (1, 22, 24, 26, 30). Besides this autoregulation by PutA, the *put* genes are catabolite repressed, requiring cyclic AMP and CRP (7). In *Klebsiella aerogenes*, the expression of proline utilization genes is positively regulated by the Nac (nitrogen assimilation control) protein that is expressed under conditions of general nitrogen limitation (21).

As observed in *E. coli* and *S. enterica* serovar Typhimurium, the *V. vulnificus putA* gene belongs to the *put* operon together with the *putP* gene, which encodes the major proline permease (19). The *putA* gene encodes a proline dehydrogenase, revealing that the function of the gene product of *putA* is well conserved as observed in other enteric bacteria (19). However, the amino acid sequence homology of *V. vulnificus* PutA with those of *E. coli* and *S. enterica* serovar Typhimurium is quite lower than that observed between PutA proteins from *E. coli* and *S. enterica* serovar Typhimurium (Fig. 1). Furthermore, the organization of the *putAP* genes of *V. vulnificus* is different from that of other enteric bacteria, such that the transcription orientations of both genes are the same direction rather than divergent. This information that its sequence and genetic organization differ from those of *putAP* of other enteric bacteria suggests that the *putAP* genes are probably not expressed and modulated in the same way as observed in other enteric bacteria. To determine whether *V. vulnificus putAP* genes are autoregulated by PutA, we assessed the expression of *putA* by constructing a *put-lux* fusion reporter. When introduced with the reporter, the levels of luminescence in the wild-type and *putA* mutant cells were comparable (data not shown). This result suggests that the *V. vulnificus putAP* operon is not autoregulated by the PutA protein.

TABLE 3. Glutamate accumulation and proline dehydrogenase activity in response to osmotic stress

Osmolyte[a]	Amount of glutamate (nmol mg of protein^{-1})	Proline dehydrogenase activity (U mg of protein^{-1})
None (control)	253.85	1.37
NaCl	575.35	5.85
KCl	559.45	5.74
Sucrose	485.45	4.14

[a] NaCl, KCl, and sucrose were present at a final concentration of 0.6 M.

Recently, the *putAP* operon from *P. aeruginosa* was identified (27). The features of the *P. aeruginosa putAP* operon are remarkably similar to those of the *putAP* operon of *V. vulnificus*. Transcription of the *P. aeruginosa putAP* genes is in the same direction and results in two transcripts, one for *putA* and the other for *putAP*. Moreover, it is apparent that the *P. aeruginosa putAP* genes are not autoregulated by the PutA protein. Expression of the *P. aeruginosa putAP* genes are activated by a transcriptional activator, PruR, a product of the *pruR* gene located upstream of *putA*. Transcription of the *putAP* genes from a promoter appeared to be attenuated by a stem-loop terminator structure located 10 bp downstream of *putA*, and only a small portion of the transcription resulted in the *putAP* transcript. However, an extensive sequence analysis was not able to identify a *pruR* gene (or homologs of *pruR*) in the upstream region of *V. vulnificus putAP* operon (data not shown). Searching for any secondary structures of RNA or sequences conserved for attenuation or antitermination in the *putA-putP* intergenic region of *V. vulnificus* was not successful (DNASIS version 2.6, Hitachi Software Engineering Co., Ltd., Tokyo, Japan).

Northern blot and primer extension analyses were performed to determine whether expression of the *putAP* operon results in two different types of *put* transcripts. In the meanwhile, it was obvious that transcription of *V. vulnificus putAP* is directed by two different promoters. The two *put* promoters appear to be independent of one another, as P_{putA} is induced but P_{putAP} is not by the presence of increasing osmotic stress (NaCl). Furthermore, the observation that the relative amount of each transcript is changed in distinct ways in different growth conditions suggests that the shorter transcript, transcript A, is probably not produced simply by the 3′-end degradation of the longer transcript, transcript AP. Taken together, these results suggested that transcription initiating from P_{putA} results in transcript A and that transcription initiating from P_{putAP} results in transcript AP. However, additional

work, such as construction of mutants which lack either P_{putA} or P_{putAP}, is needed to establish the specific mechanisms which result in the two different types of *put* transcripts. Nevertheless, the differential utilization of the two promoters, requiring distinct regulation, may permit precise adjustment of *put* expression in response to environmental signals.

We have previously shown that the *V. vulnificus* strain with a null mutation in the *putA* gene exhibits impaired growth with proline as the sole carbon and nitrogen source and that its survival is impaired under hyperosmotic stress (19). These pleiotropic effects of *putA* mutation can be explained if proline dehydrogenase converts proline into glutamate for a dual purpose, for dissimilation and/or assimilation of glutamate and for accumulation of glutamate as an osmoprotectant. Many bacteria accumulate glutamate in response to osmotic stress (6, 11). The availability of compatible solutes in the environment determines whether synthesis or uptake will predominate in the cell (6). The endogenous glutamate that is accumulated in response to osmotic stress is synthesized by glutamate dehydrogenase in enteric bacteria (11). Our data revealed that expression of proline dehydrogenase and accumulation of glutamate in cells increased in response to hyperosmotic stress. These results suggest that the increased level of proline dehydrogenase plays a significant, if not the sole, role for the increased accumulation of glutamate in response to increasing osmotic stress in *V. vulnificus*. However, when exogenous glutamate is abundant, it has also been observed that the *putA* mutant HJK001 can take up and utilize glutamate as the sole carbon and nitrogen source and accumulate excess glutamate in response to hyperosmotic stress (data not shown).

In the present study, it was found that expression of the *V. vulnificus putAP* operon is induced in response to hyperosmotic stress. It has been reported previously that many of the stress-induced genes in enteric bacteria are induced at the onset of stationary phase and are regulated by RpoS (σ^{38}), an alternative sigma factor (13, 33). However, the proline dehydrogenase activity appeared at the beginning of growth and reached a maximum in late exponential phase. Consistent with this, neither the cellular level of the *put* transcripts nor the proline dehydrogenase activity was affected by the mutation of *rpoS* (data not shown). On the basis of homology to consensus sequences of the *E. coli* σ^{70} promoter, the putative *put* promoter sequences consisting of -10 and -35 segments were identified (Fig. 4B). The assigned sequences for -10 regions (CAAAAT or TACACT) scored a 67% homology to the -10 consensus sequences (TATAAT) of the *E. coli* σ^{70} promoter. Although no sequence of the -35 region assigned with respect to P_{putA} revealed a reasonable homology to -35 consensus sequences, the P_{putAP} -35 region (TTGACA) is apparently identical to the consensus sequence. The homology of *put* promoters with the consensus sequences of the *E. coli* σ^{70} promoter suggests that *put* promoters are most probably recognized by the *V. vulnificus* homolog of the σ^{70} RNA polymerase. In addition to these sequences, a location for a possible CRP binding site was identified by consensus sequences (TG TGA$N_{6\text{-}8}$TCACA [4]). However, it has not yet been established whether CRP activates *put* promoters directly by binding to the upstream sequences of *putAP*.

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REFERENCES

1. **Allen, S. W., A. Senti-Willis, and S. R. Maloy.** 1993. DNA sequence of the *putA* gene from *Salmonella typhimurium*: a bifunctional membrane-associated dehydrogenase that binds DNA. Nucleic Acids Res. **21:**1676.
2. **Blake, P. A., R. E. Weaver, and D. G. Hollis.** 1980. Diseases of humans (other than cholera) caused by vibrios. Annu. Rev. Microbiol. **34:**341–367.
3. **Botsford, J. L., M. Alvarez, R. Hernandez, and R. Nichols.** 1994. Accumulation of glutamate by *Salmonella typhimurium* in response to osmotic stress. Appl. Environ. Microbiol. **60:**2568–2574.
4. **Botsford, J. L., and J. G. Harman.** 1992. Cyclic AMP in prokaryotes. Microbiol. Rev. **56:**100–122.
5. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principles of protein-dye binding. Anal. Biochem. **72:**248–254.
6. **Bremer, E., and R. Kramer.** 2000. Coping with osmotic challenges: osmoregulation through accumulation and release of compatible solutes in bacteria, p. 79–98. *In* G. Storz and R. Hengge-Aronis (ed.), Bacterial stress responses. American Society for Microbiology, Washington, D.C.
7. **Chen, L. M., and S. Maloy.** 1991. Regulation of proline utilization in enteric bacteria: cloning and characterization of the *Klebsiella put* control region. J. Bacteriol. **173:**783–790.
8. **Cho, K., and S. C. Winans.** 1996. The *putA* gene of *Agrobacterium tumefaciens* is transcriptionally activated in response to proline by an Lrp-like protein and is not autoregulated. Mol. Microbiol. **22:**1025–1033.
9. **Choi, H. K., N. Y. Park, D. Kim, H. J. Chung, S. Ryu, and S. H. Choi.** 2002. Promoter analysis and regulatory characteristics of *vvhBA* encoding cytolytic hemolysin of *Vibrio vulnificus*. J. Biol. Chem. **277:**47292–47299.
10. **Csonka, L. N.** 1989. Physiological and genetic responses of bacteria to osmotic stress. Microbiol. Rev. **53:**121–147.
11. **Csonka, L. N., and A. D. Hanson.** 1991. Prokaryotic osmoregulation: genetics and physiology. Annu. Rev. Microbiol. **45:**569–581.
12. **Galinski, E. A.** 1995. Osmoadaptation in bacteria. Adv. Microb. Physiol. **37:**273–328.
13. **Hengge-Aronis, R.** 1996. Regulation of gene expression during entry into stationary phase, p. 1497–1512. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. American Society for Microbiology, Washington, D.C.
14. **Imhoff, J. F.** 1986. Osmoregulation and compatible solutes in eubacteria. FEMS Microbiol. Rev. **39:**57–66.
15. **Jeong, H. S., K. C. Jeong, H. K. Choi, K. J. Park, K. H. Lee, J. H. Rhee, and S. H. Choi.** 2001. Differential expression of *Vibrio vulnificus* elastase gene in a growth phase-dependent manner by two different types of promoters. J. Biol. Chem. **276:**13875–13880.
16. **Jeong, K. C., H. S. Jeong, S. E. Lee, S. S. Chung, J. H. Rhee, A. M. Starks, G. M. Escudero, P. A. Gulig, and S. H. Choi.** 2000. Construction and phenotypic evaluation of a *Vibrio vulnificus vvpE* mutant for elastolytic protease. Infect. Immun. **68:**5096–5106.
17. **Keen, N. T., S. Tamaki, D. Kobayashi, and D. Trollinger.** 1988. Improved broad-host-range plasmids for DNA cloning in gram-negative bacteria. Gene **70:**191–197.
18. **Keuntje, B., B. Masepohl, and W. Klipp.** 1995. Expression of the *putA* gene encoding proline dehydrogenase from *Rhodobacter capsulatus* is independent of NtrC regulation but requires an Lrp-like activator protein. J. Bacteriol. **177:**6432–6439.
19. **Kim, H. J., J. H. Lee, J. E. Rhee, H. S. Jeong, H. K. Choi, H. J. Chung, S. Ryu, and S. H. Choi.** 2002. Identification and functional analysis of the *putAP* genes encoding *Vibrio vulnificus* proline dehydrogenase and proline permease. J. Microbiol. Biotechnol. **12:**318–326.
20. **Klontz, K. C., S. Lieb, M. Schreiber, H. T. Janowski, L. M. Baldy, and R. A. Gunn.** 1988. Syndromes of *Vibrio vulnificus* infections. Clinical and epidemiologic features in Florida cases, 1981–1987. Ann. Intern. Med. **109:**318–323.
21. **Macaluso, A., E. A. Best, and R. A. Bender.** 1990. Role of the *nac* gene product in nitrogen regulation of some NTR-regulated operons of *Klebsiella aerogenes*. J. Bacteriol. **172:**7249–7255.
22. **Maloy, S., and J. Roth.** 1983. Regulation of proline utilization in *Salmonella typhimurium*: characterization of *put*::Mud(Ap *lac*) operon fusion. J. Bacteriol. **154:**561–568.
23. **Maloy, S. R.** 1987. The proline utilization operon, p. 1513–1519. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.

24. **Maloy, S. R., and V. Stewart.** 1993. Autogenous regulation of gene expression. J. Bacteriol. **175:**307–316.
25. **Menzel, R., and J. Roth.** 1992. Purification of the *putA* gene product: a bifunctional membrane-bound protein from *Salmonella typhimurium* responsible for the two-step oxidation of proline to glutamate. J. Biol. Chem. **256:**9755–9761.
26. **Muro-Pastor, A. M., P. Ostrovsky, and S. Maloy.** 1997. Regulation of gene expression by repressor localization: biochemical evidence that membrane and DNA binding by the PutA protein are mutually exclusive. J. Bacteriol. **179:**2788–2791.
27. **Nakada, Y., T. Nishijyo, and Y. Itoh.** 2002. Divergent structure and regulatory mechanism of proline catabolic system: characterization of the *putAP* proline catabolic operon of *Pseudomonas aeruginosa* PAO1 and its regulation by PruR, an AraC/XylS family protein. J. Bacteriol. **184:**5633–5640.
28. **Oka, A., H. Sugisaki, and M. Takanami.** 1981. Nucleotide sequence of the kanamycin resistance transposon Tn903. J. Mol. Biol. **147:**217–226.
29. **Ostrovsky, P., K. O'Brien, and S. Maloy.** 1991. Regulation of proline utilization in *Salmonella typhimurium*: a membrane-associated dehydrogenase binds DNA in vitro. J. Bacteriol. **173:**211–219.
30. **Ostrovsky, P., and S. Maloy.** 1993. PutA protein, a membrane-associated flavin dehydrogenase, acts as a redox-dependent transcriptional regulator. Proc. Natl. Acad. Sci. **90:**4295–4298.
31. **Ratzkin, B., and J. Roth.** 1978. Cluster of genes controlling proline degradation in *Salmonella typhimurium*. J. Bacteriol. **133:**744–754.
32. **Sambrook, J., and D. W. Russell.** 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
33. **Tanaka, K., Y. Takayanagi, N. Fujita, A. Ishihama, and H. Takahashi.** 1993. Heterogeneity of the principal σ factor in *Escherichia coli*: the *rpoS* gene product, σ^{38}, is a second principal σ factor of RNA polymerase in stationary-phase *Escherichia coli*. Proc. Natl. Acad. Sci. **90:**3511–3515.
34. **Vilchez, S., L. Molina, C. Ramos, and J. L. Ramos.** 2000. Proline catabolism by *Pseudomonas putida*: cloning, characterization, and expression of the *put* genes in the presence of root exudates. J. Bacteriol. **182:** 91–99.
35. **Witt, I.** 1974. Determination with glutamate dehydrogenase and 3-acetyl pyridine analogue of NAD (APAD), p. 1713. *In* U. Berger (ed.), Methods of enzymatic analysis, vol. 4. Academic Press, New York, N.Y.