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Identification of a PutP Proline Permease Gene Homolog from *Staphylococcus aureus* by Expression Cloning of the High-Affinity Proline Transport System in *Escherichia coli*

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The important food-borne pathogen *Staphylococcus aureus* is distinguished by its ability to grow at low water activity values. Previous work in our laboratory and by others has revealed that proline accumulation via transport is an important osmoregulatory strategy employed by this bacterium. Furthermore, proline uptake by this bacterium has been shown to be mediated by two distinct transport systems: a high-affinity system and a low-affinity system (J.-H. Bae, and K. J. Miller, Appl. Environ. Microbiol. 58:471–475, 1992; D. E. Townsend and B. J. Wilkinson, J. Bacteriol. 174:2702–2710, 1992). In the present study, we report the cloning of the high-affinity proline transport system of *S. aureus* by functional expression in an *Escherichia coli* host. The sequence of the staphylococcal proline permease gene was predicted to encode a protein of 497 amino acids which shares 49% identity with the PutP high-affinity proline permease of *E. coli*. Analysis of hydropathy also indicated a common overall structure for these proteins.

The important food-borne pathogen *Staphylococcus aureus* is believed to be responsible for as much as one-third of all food-borne illness in the United States (5, 6). Because of its high osmotic tolerance (e.g., some strains may grow at water activity values as low as 0.83), this pathogen is a particular problem in foods of lower moisture content, where it may grow without competition from other microbial flora (see reference 38 for a review).

Recently, the osmoregulatory strategies employed by *S. aureus* have been the focus of study in several laboratories. These studies have revealed that proline and glycine betaine are principal compatible solutes for this bacterium (17, 22, 26). That is, both of these compounds are accumulated to extremely high intracellular concentrations when *S. aureus* is grown in complex media of high osmotic strength. The accumulation of these compounds as compatible solutes has been shown to be mediated by transport processes (3, 4, 31, 37). Kinetic analyses of proline uptake have revealed the presence of two transport systems: a high-affinity (K_m of approximately 2 to 7 μM) system and a low-affinity (K_m of approximately 100 to 400 μM) system (4, 37). Similar analyses of glycine betaine uptake have also revealed the presence of a high- and a low-affinity system (3, 31). Recently, Pourkomailian and Booth have provided evidence that the low-affinity glycine betaine transport system and the low-affinity proline transport system may, in fact, be the same transporter (31).

Interestingly, the gram-negative enteric bacteria *Escherichia coli* and *Salmonella typhimurium* have also been shown to utilize proline and glycine betaine as compatible solutes (see reference 9 for a review). However, the growth of these bacteria is restricted to a relatively narrow osmotic strength range (e.g., water activity values greater than 0.95). Proline transport in these bacteria has been shown to be mediated by three transporters: PutP, ProP, and ProU (see references 9 and 39 for reviews). While PutP is a high-affinity proline permease (K_m of approximately 1 μM), ProP and ProU are low-affinity proline transporters (K_m values of approximately 300 and 200 μM, respectively). Furthermore, both ProP and ProU have been shown to transport glycine betaine, and glycine betaine has been shown to be the preferred substrate for both of these transport systems (K_m values of approximately 1 and 40 μM for ProU and ProP, respectively).

On the basis of the above-described similarities between the osmoregulatory strategies of *S. aureus* and the gram-negative enteric bacteria, it is intriguing that *S. aureus* is able to grow over a much wider range of osmotic strength. This ability may reflect a greater tolerance of the proline and glycine betaine transport systems of *S. aureus* to high-osmolarity conditions. Consistent with this possibility, previous studies have revealed that the low-affinity proline transport system of *S. aureus* is optimally activated at NaCl concentrations (e.g., 0.75 to 1 M) that are inhibitory to the ProP and ProU transport systems of the gram-negative enteric bacteria (4, 15, 18).

Since the remarkable salt tolerance of *S. aureus* may depend upon the unique properties of its proline and glycine betaine transport systems, we have initiated studies aimed at characterizing these transport systems at the molecular level. In the present study, we report the identification of the structural gene encoding the high-affinity proline permease of *S. aureus*. The gene was cloned by functional expression in an *E. coli* host that is otherwise completely defective for proline transport.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are described in Table 1. *E. coli* WG389 was grown at 37°C either in LB medium (24) containing 200 mM proline, 0.001% tryptophan, and 0.0002% thiamine or in M9 medium (24) containing 200 mM proline, 0.001% tryptophan, 0.0002% thiamine, and 0.4% glucose. *S. aureus* ATCC 12600 was grown in Trypticase soy broth medium (Becton Dickinson, Cockeysville, Md.) at 37°C.

Genomic DNA and plasmid isolation. Genomic DNA was isolated from *S. aureus* ATCC 12600 by the CsCl gradient centrifugation method described by

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Description	Source
S. aureus ATCC 12600	Type strain	American Type Culture Collection, Rockville, Md.
E. coli WG389	Δ(*brnQ phoA proC*) Δ(*putPA*) *101* Δ(*proU*)*600* Δ(*proP mel*) *212 trp thi*	Janet Wood, University of Guelph, Ontario, Canada
pUC19	Ap^r	GIBCO BRL
pPW1	pUC19 containing a 3.8-kbp *S. aureus* genomic DNA fragment	This study
pPWC1	pUC19 containing the 2.46-kbp *Kpn*I fragment from pPW1	This study

Dyer and Iandolo (14). Small-scale plasmid DNA isolation from *E. coli* was performed by methods described by Sambrook et al. (33). Large-scale plasmid DNA isolation from *E. coli* was performed by using a Maxi-prep Plasmid Isolation Kit (Qiagen, Inc., Chatworth, Calif.) according to procedures described by the manufacturer. These plasmid preparations were further purified with Magic DNA Clean-up columns (Promega Corp., Madison, Wis.) or Magic DNA Prep columns (Promega Corp.).

Construction of a *S. aureus* genomic library. The genomic DNA preparation from *S. aureus* ATCC 12600 was partially digested with *Sau*3A, and 3- to 10-kbp fragments were retrieved from electrophoresed agarose gels (0.7%) by elution onto DEAE paper (NA-45; Schleicher & Schuell, Keene, N.H.) by procedures recommended by the manufacturer. The genomic fragments were then ligated with pUC19 plasmid DNA that had been previously digested with *Bam*HI and dephosphorylated with calf intestine alkaline phosphatase. Ligation products were used to transform *E. coli* WG389. Transformation was performed by electroporation using an *E. coli* Gene Pulser (Bio-Rad, Inc., Richmond, Calif.) following the protocol provided by the manufacturer. Transformants were plated onto selective M9 medium containing 50 μg of ampicillin per ml and 25 μM proline.

Southern analysis. DNA preparations (genomic and plasmid) were digested with *Eco*RI, electrophoresed within agarose gels, and subsequently blotted onto nitrocellulose membranes (GIBCO BRL, Gaithersburg, Md.) as described by Sambrook et al. (33). A 1.65-kbp biotinylated DNA probe was used for these experiments. This probe was generated by PCR using a GeneAmp kit (Perkin-Elmer/Cetus, Norwalk, Conn.) and biotin-dUTP. The oligonucleotide primers used were 5′-TGGTATGCAAGTATTTCAAAA-3′ and 5′-TTTTTCTCTAAC GATGTCACG-3′, and the amplified probe corresponded to an internal fragment within plasmid pPWC1 beginning at nucleotide 747 and ending at nucleotide 2398 (see Fig. 3). Detection of probe-target DNA complexes was performed with the BluGENE nonradioactive detection system (GIBCO BRL). Hybridization, washing, and development procedures were those recommended by the manufacturer.

DNA sequencing and sequence analysis. DNA sequencing was performed by the dideoxy nucleotide chain termination method (34) with Sequenase (version 2; U.S. Biochemical Corp., Cleveland, Ohio) and [α-^{35}S]dATP as the radioactive label. The sequencing reactions were performed in both directions on denatured, double-stranded plasmid DNA with synthetic oligonucleotide primers. Computer analysis of DNA and protein sequences was performed at The Pennsylvania State University Center for Computational Biology with programs from Intelligenetics (Mountain View, Calif.) and the University of Wisconsin Genetics Computer Group (12). DNA and peptide homology searches were performed at the National Center for Biotechnology Information with E-mail server. Peptide alignments were performed with the FastDB and GenAlign programs of the Intelligenetics software package.

Assay for proline transport. *E. coli* WG389 containing plasmid pUC19 or pPWC1 was grown overnight in 5 ml of LB medium containing 200 mM proline, 0.001% tryptophan, 0.0002% thiamine, and 50 μg of ampicillin per ml. The entire preculture was used to inoculate 250 ml of the same medium containing no added proline. Cultures were incubated for 4 to 5 h at 37°C with shaking at 150 rpm. Cells were then harvested by centrifugation and washed twice with 50 ml of 50 mM potassium phosphate buffer at pH 7.5 at 5°C. Cells were resuspended in 50 mM potassium phosphate buffer containing 40 mM glucose and 25 mM NaCl at a concentration of approximately 100 μg of total cellular protein per ml. Transport experiments were performed at room temperature using the filtration method previously described by Bae and Miller (4). Proline uptake was measured at 10, 20, and 30 s after the addition of L-[2,3-^{3}H]proline (specific activity, 80,000 cpm/nmol) at a final concentration of 2.5 μM.

Chemicals and enzymes. Restriction enzymes were purchased from U.S. Biochemicals Corp., GIBCO BRL, or Boehringer Mannheim (Indianapolis, Ind.). Biotin-labeled dUTP was purchased from Boehringer Mannheim. [α-^{35}S]dATP and L-[2,3-^{3}H]proline were purchased from New England Nuclear (Boston, Mass.). Synthetic oligonucleotide primers were obtained from either the Midland Certified Reagent Co. (Midland, Tex.) or the Protein and DNA Analysis Laboratory of the Biotechnology Institute at The Pennsylvania State University (University Park).

Nucleotide sequence accession number. Nucleotide sequence data have been submitted to GenBank under accession number U06451.

RESULTS

Complementation of *E. coli* WG389. *E. coli* WG389, constructed by Culham and coworkers, is deleted for all three proline transport systems (PutP, ProP, and ProU), lacks detectable proline transport activity, is unable to synthesize proline, and requires extremely high concentrations of proline (e.g., 200 mM) for optimal growth (10). On the basis of the growth properties of strain WG389, Culham and coworkers suggested that complementation of this strain could provide an approach to identify proline transporter genes from other bacteria. We therefore decided to use this strategy to identify a proline permease gene from *S. aureus*. For these experiments, strain WG389 was transformed with a *S. aureus* ATCC 12600 genomic DNA library within pUC19 as described in Materials and Methods. Transformants were then plated onto minimal M9 medium containing 50 μg of ampicillin per ml and 25 μM proline (a concentration too low to support the growth of strain WG389).

Among 24,000 recombinant transformants of *E. coli* WG389 containing *S. aureus* ATCC 12600 genomic DNA inserts within pUC19, one colony which was capable of growth on selective medium containing 25 μM proline was identified. The plasmid isolated from this clone was designated pPW1 and was found to contain a 3.8-kbp insert of *S. aureus* genomic DNA. When this plasmid was purified and used to transform *E. coli* WG389, all transformants were found to grow on media containing 25 μM proline. Southern hybridization experiments confirmed that the insert within plasmid pPW1 was derived from *S. aureus* ATCC 12600 genomic DNA (data not shown). A partial restriction map of the 3.8-kbp genomic DNA insert within plasmid pPW1 is shown in Fig. 1.

In order to further localize the region within pPW1 necessary for complementation of *E. coli* WG389, subcloning experiments were performed. These studies revealed that the 2.46-kbp *Kpn*I fragment isolated from pPW1 was able to fully complement strain WG389. The plasmid containing this 2.46-kbp *Kpn*I fragment (ligated within the *Kpn*I site of pUC19), was designated pPWC1. Further attempts to isolate a smaller genomic DNA fragment from plasmid pPWC1 which still permitted complementation of *E. coli* WG389 were not successful.

Functional expression of a staphylococcal proline permease is the basis for complementation of *E. coli* WG389. Although *S. aureus* ATCC 12600 is auxotrophic for proline (26), the possibility that complementation of *E. coli* WG389 with staphylococcal genomic DNA resulted from proline biosynthesis existed. In order to further examine this possibility, additional growth experiments were performed. As shown in Fig. 2, *E. coli* WG389(pUC19) was capable of growth only in minimal media containing very high concentrations of proline (200 mM), as expected. Also as expected, *E. coli* WG389(pPWC1) was capable of growth in minimal media containing 25 μM proline. However, *E. coli* WG389(pPWC1) was not able to grow in minimal media which lacked proline.

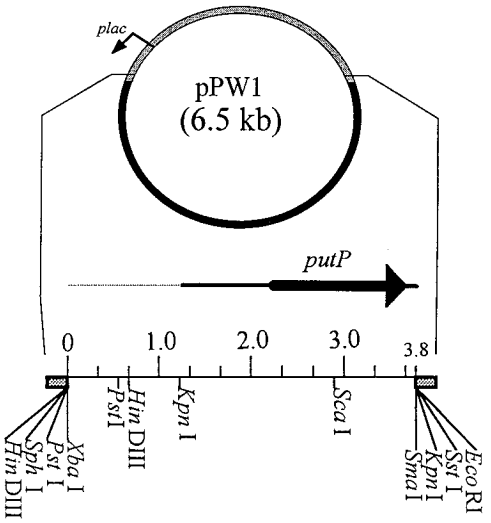

FIG. 1. Partial restriction map of the 3.8-kbp *S. aureus* ATCC 12600 *Sau*3A genomic fragment within plasmid pPW1. The genomic fragment was inserted at the *Bam*HI site within the polylinker region of pUC19 as described in the text. Subcloning studies revealed that the *S. aureus putP* homolog was present on the 2.46-kbp *Kpn*I fragment from this plasmid. Sequence analysis of the 2.46-kbp *Kpn*I fragment revealed the location of the *putP* gene (also see Fig. 3).

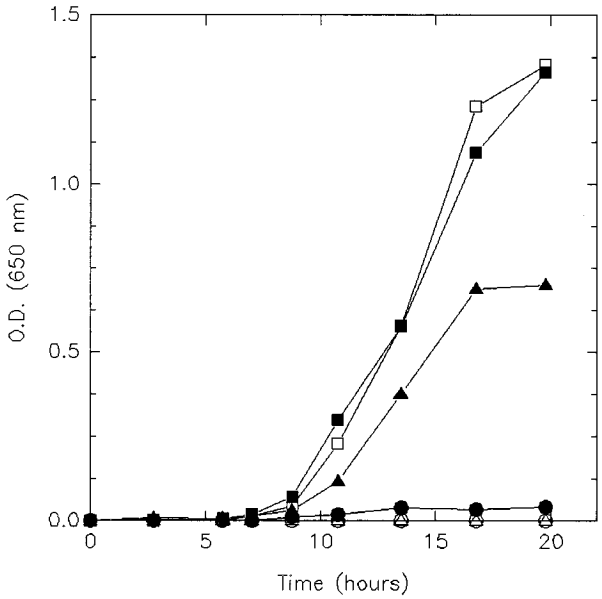

FIG. 2. Growth of *E. coli* WG389 transformants in a defined liquid medium containing different concentrations of proline. Precultures of *E. coli* WG389 containing either pUC19 (open symbols) or pPWC1 (closed symbols) were grown overnight on solid M9 medium containing 200 mM proline, ampicillin (50 μg/ml), 0.001% tryptophan, and 0.0002% thiamine at 37°C. Colonies (5 to 10) were removed and suspended by vortexing in 2 ml of M9 medium containing ampicillin (50 μg/ml), 0.001% tryptophan, and 0.0002% thiamine but no added proline. Aliquots (250 to 500 μl) were then used to inoculate 250-ml-capacity sidearm flasks containing 50 ml of M9 medium supplemented with ampicillin (50 μg/ml), 0.001% tryptophan, 0.0002% thiamine, and three different concentrations of proline (0, 25 μM, and 200 mM). Cultures were incubated at 37°C with shaking at 150 rpm. Growth was measured turbidometrically at 650 nm (O.D., optical density). Squares, 200 mM proline; triangles, 25 μM proline; circles, no added proline.

The results of the growth experiments described above strongly suggested that the basis for complementation of strain WG389 was through the functional expression of a staphylococcal proline transport system. In order to examine this possibility directly, proline transport experiments were performed. Consistent with results of an earlier study (10), we were unable to measure proline uptake by strain WG389(pUC19) (i.e., values were less than 0.1 nmol/min/mg of total cellular protein). However, proline uptake (approximately 1.5 nmol/min/mg of total cellular protein) was detected when strain WG389 was transformed with plasmid pPWC1. Thus, these results confirm that the basis of complementation of strain WG389 is through the functional expression of a *S. aureus* proline transport system.

Sequence analysis of the 2.46-kbp *S. aureus* genomic DNA fragment within plasmid pPWC1. The nucleotide sequence of the entire 2.46-kbp insert within pPWC1 was determined, and the sequence is presented in Fig. 3. Inspection of the sequence in all possible coding frames revealed only one major open reading frame of 1,494 bp mapping from positions 865 to 2358. Although two nearby translational start codons are possible (positions 817 and 856), the translational start codon at position 865 is the only one preceded by the possible ribosome-binding site (Shine-Dalgarno site) GGAG (36).

The nucleotide sequence of the open reading frame has an overall AT content of 64%, consistent with the overall AT content of the *S. aureus* genome, which ranges between 64 and 68% (7, 29). Analysis of the codon usage of this open reading frame revealed a marked bias for incorporation of A or U in the third position (Table 2) and was similar to the codon usage bias previously reported for the staphylococcal *lac* operon structural genes (7, 29).

The open reading frame is predicted to encode a protein of 497 amino acids with a molecular mass of 54,428 Da. When the program BLAST (1) was used to search the PIR (release 38; 61,248 entries), SWISS-PROT (release 27; 33,329 entries), and GenBank (release 80; 86,895 entries) databases for sequences similar to the open reading frame, the sequences with highest homology were shown to correspond to the PutP proline transporters of *E. coli* and *S. typhimurium* (the probability that the degree of similarity arose by chance is $\leq 10^{-79}$). Significant homology was also found with the sodium-pantothenate symporter of *E. coli* (the probability that the degree of similarity arose by chance is 10^{-26}).

When homology between the putative PutP protein of *S. aureus* and the PutP transporters of *E. coli* and *S. typhimurium* was further examined, a very high degree of similarity became evident. An optimal alignment between the predicted *S. aureus* protein and the *E. coli* PutP protein is shown in Fig.4. Not only are the proteins of similar size (497 and 502 amino acid residues for the *S. aureus* and *E. coli* proteins, respectively), but also the two proteins share 245 identical amino acids (49% identity). When conservative substitutions are permitted, the apparent homology increases to 72%.

Hydropathy analysis. Previous hydropathy analyses of the PutP proteins of *E. coli* (28) and *S. typhimurium* (25) have suggested that these proteins have 12 membrane-spanning regions. When the putative *S. aureus* PutP permease was examined by the method of Kyte and Doolittle (23), the hydropathy profile was found to be extremely similar to that generated for the *E. coli* and *S. typhimurium* PutP proteins (Fig. 5). Interestingly, the level of nonpolar amino acids (69.4%) in the staphylococcal protein was essentially identical to that (70%) reported for the *E. coli* and *S. typhimurium* PutP permeases (25, 28), and these values are typical of those found in integral membrane proteins (8).

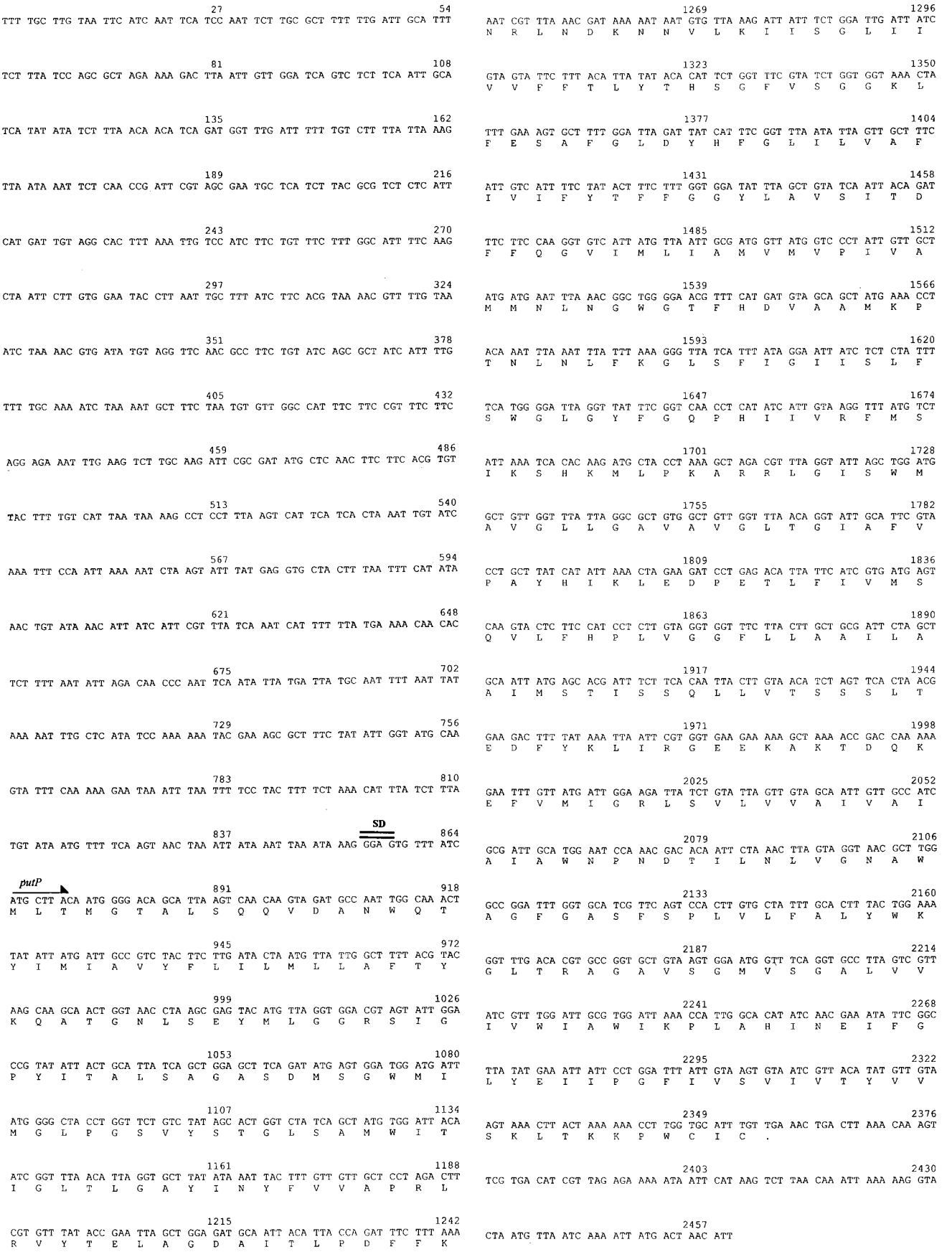

FIG. 3. Nucleotide sequence of the 2.46-kbp *S. aureus* genomic DNA fragment within plasmid pPWC1. The predicted amino acid sequence of the *S. aureus* PutP permease is given below the nucleotide sequence in single-letter code. "SD" indicates the putative Shine-Dalgarno sequence.

TABLE 2. Codon usage of the PutP proline permease gene of *S. aureus*

Amino acid	Codon	Preference (%)	Amino acid	Codon	Preference (%)
Phe	UUU	51.4	Tyr	UAU	73.7
	UUC	48.6		UAC	26.3
Leu	UUA	55.5	His	CAU	90.0
	UUG	7.9		CAC	10.0
	CUU	14.3	Gln	CAA	100.0
	CUC	1.6		CAG	0.0
	CUA	20.6	Asn	AAU	52.6
	CUG	0.0		AAC	47.4
Ile	AUU	68.3	Lys	AAA	77.8
	AUC	18.3		AAG	22.2
	AUA	13.3	Asp	GAU	75.0
Met	AUG	100.0		GAC	25.0
Val	GUU	34.8	Glu	GAA	81.2
	GUC	13.0		GAG	18.8
	GUA	41.3	Cys	UGU	50.0
	GUG	10.9		UGC	50.0
Ser	UCU	27.0		UCC	0.0
	UCA	27.0	Arg	CGU	58.3
	UCG	5.4		CGC	0.0
	AGU	29.7		CGA	0.0
	AGC	10.8		CGG	0.0
Pro	CCU	68.8		AGA	33.3
	CCC	0.0		AGG	8.3
	CCA	25.0	Gly	GGU	54.2
	CCG	6.3		GGC	6.3
Thr	ACU	25.0		GGA	33.3
	ACC	7.1		GGG	6.3
	ACA	53.6	Trp	UGG	100.0
	ACG	14.3			
Ala	GCU	50.0			
	GCC	13.6			
	GCA	27.3			
	GCG	9.1			

DISCUSSION

It is likely that the *S. aureus* PutP homolog identified in the present study corresponds to the high-affinity proline transport system previously detected within whole cells and membrane vesicles (4, 35, 37). In fact, these earlier studies revealed that the high-affinity proline transport system of *S. aureus* shares several properties with the PutP permeases of *E. coli* and *S. typhimurium*. First, the high-affinity proline permease of *S. aureus* and the PutP permeases of *E. coli* and *S. typhimurium* have similar affinities for proline (the K_m value for the high-affinity transport system of *S. aureus* has been reported to range between 1.7 and 7 μM [4, 35, 37], and the K_m values for the PutP permeases of *E. coli* and *S. typhimurium* have been reported to range between 0.4 and 5 μM [39]). Second, the activity of the high-affinity proline transport system of *S. aureus* is stimulated by millimolar concentrations of NaCl (4, 37), suggesting that this system, like PutP, is also a sodium-proline symport system. Third, the high-affinity proline permease of *S. aureus*, like the PutP permeases of *E. coli* and *S. typhimurium*, is not osmotically stimulated (4). Thus, it is unlikely that this system contributes to the accumulation of proline as a compatible solute. Instead, it has been proposed that the primary function for the high-affinity proline permease of *S. aureus* may be to provide proline as a carbon or nitrogen source (4).

The identification of a PutP homolog in *S. aureus* should provide new and important insight concerning the amino acid residues and/or domains of this permease that are critical for function. Recently, Reizer et al. (32) have shown that the PutP permeases of *E. coli* and *S. typhimurium* fall within a family of symporters referred to as the sodium-solute symporter family (SSF). These researchers identified 14 members of this family which include six gram-negative bacterial proteins and eight mammalian proteins. Although members of this family of proteins transport a wide variety of substrates (e.g., sugars, amino acids, vitamins, nucleosides, and cyclic polyols), all are obligatory Na^+-solute symporters. The striking homology between the *S. aureus* PutP homolog and the PutP permeases of *E. coli* and *S. typhimurium* indicates that this protein represents the first gram-positive bacterial member of this family.

In their analysis of the SSF, Reizer et al. (32) reported that the N-terminal hydrophobic domains of members of this family exhibited the greatest degree of similarity. On the basis of this finding, it was suggested that the N-terminal hydrophobic domains of these proteins are therefore of the greatest structural or functional significance. In fact, when these corresponding portions of the *E. coli* and *S. aureus* PutP proteins are compared, the amino acid identity is increased to 60% (versus 49% when the entire amino acid sequences of these proteins are compared).

A multiple alignment of a portion of the N-terminal hydrophobic domains (approximately 160 amino acid residues) of 13 members of the SSF was presented by Reizer et al. (32), and 10 amino acid residues were found to be conserved in all of these proteins. Interestingly, these 10 amino acid residues are also conserved in the *S. aureus* PutP homolog (these residues correspond to arginine 51, glycine 74, glycine 77, glycine 83, threonine 120, arginine 128, leucine 142, glycine 168, glycine 189, and glycine 190 in the *S. aureus* protein), providing further evidence that these residues may have important structural and/or functional roles.

An additional feature of proteins within the SSF is a proposed sodium ion binding (SOB) motif (11). This motif is approximately 50 amino acid residues in length, begins within the putative eighth membrane-spanning domain of PutP, and extends into the ninth membrane-spanning domain. Five amino acid residues which correspond to glycine 328, alanine 366, leucine 371, glycine 375, and arginine 376 of the *E. coli* PutP permease have been shown to be conserved in the proposed SOB motif. In Fig. 6, an alignment of the SOB motifs of the *E. coli* PutP permease, the *S. typhimurium* PutP permease, and the *S. aureus* PutP homolog is presented. This alignment reveals that glycine 328, alanine 366 (although it is shifted 2 residues towards the amino terminus), glycine 375, and arginine 376 are conserved in the *S. aureus* PutP homlog (note that the residue number used here corresponds to that present in the *E. coli* protein). Leucine 371 is not present in the *S. aureus* protein.

Prior to the identification of the SSF by Reizer and coworkers (32), studies from several laboratories provided evidence that additional amino acid residues within PutP were critical for function. These studies were based on limited alignment comparisons with one or two members of the now recognized SSF. For example, Hediger and coworkers directly compared the amino acid sequence of the PutP permease from *E. coli* with the human intestinal Na^+-glucose cotransporter (20). These researchers were particularly interested in conserved tyrosine residues because previous biochemical studies with the intestinal Na^+-glucose cotransporter suggested that a tyrosyl residue is at or near the Na^+ binding site (30). Of the 25 tyrosine residues present within the Na^+-glucose cotransporter, 4 were found to also be present within the *E. coli* PutP

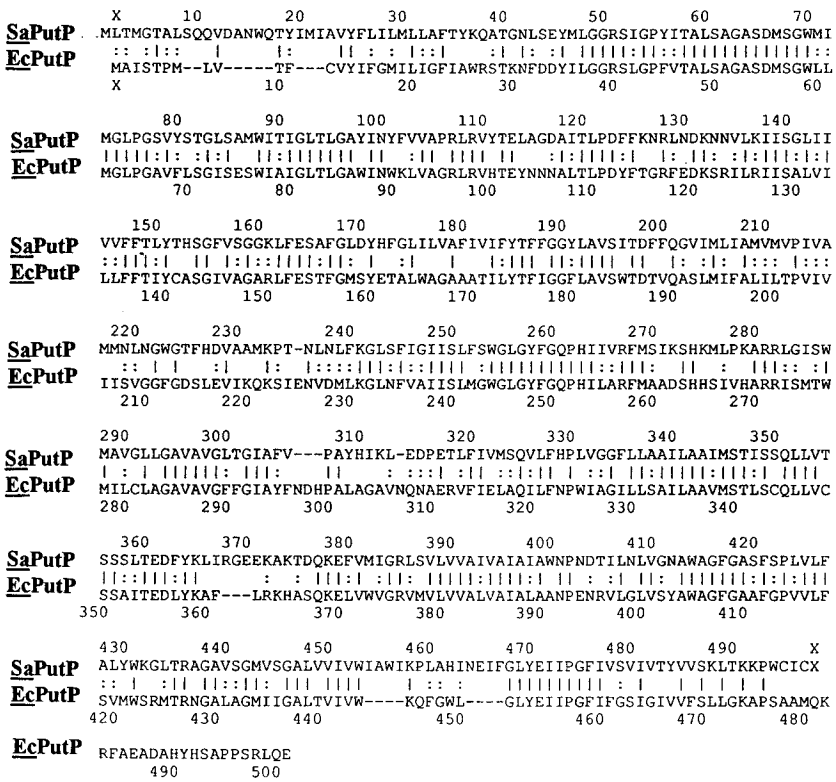

FIG. 4. Amino acid alignment of the putative *S. aureus* PutP permease with the PutP permease of *E. coli*. The FastDB program was used with a gap penalty of 1, a similarity threshold level of 10%, a window size of 25, and a mutation data scoring matrix value of 150 PAMS (16). Vertical lines indicate identity; colons indicate conservative substitutions; dashes indicate gaps. Sa, *S. aureus*; Ec, *E. coli*.

permease (these correspond to tyrosine residues at positions 113, 174, 358, and 453 of PutP). Examination of the predicted amino acid sequence of the *S. aureus* PutP homolog reveals that tyrosine residues at positions 174, 358, and 453 are also conserved in this protein, providing further evidence that they may have important roles in Na^+ binding. Interestingly, it is noted that tyrosine 358 is located within the proposed SOB motif (Fig. 6).

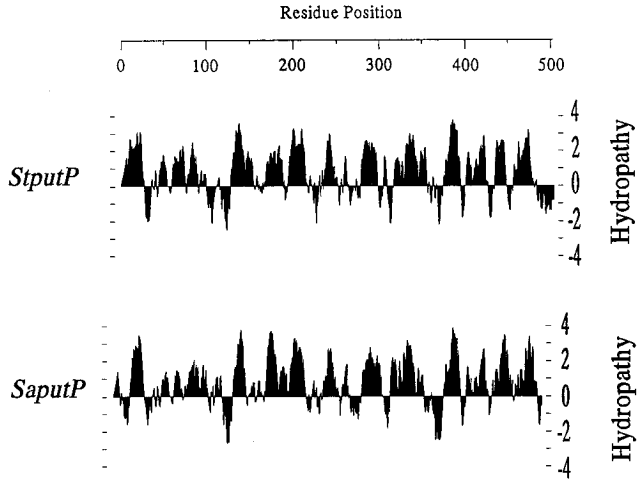

FIG. 5. Comparative hydropathy plots for the *S. aureus* PutP homolog and the *S. typhimurium* PutP permease. The plots were constructed by using the method of Kyte and Doolittle (23) and the PEP Peptide Analysis Program from Intelligenetics. Values were averaged over a window of 6 amino acid residues.

While the alignment analyses described above provide powerful approaches to identify amino acid residues and/or domains within PutP that may be important for function, it should be noted that a variety of mutagenesis studies with the PutP permeases of *E. coli* and *S. typhimurium* have also provided important insight. For example, using this approach, Maloy and coworkers (13, 27) have concluded that multiple regions of PutP are involved in the binding of proline. Their studies have also revealed that mutations at the 5′ and 3′ termini of the *putP* gene alter the cation binding properties of the protein.

Yamato and coworkers (40, 42) have also utilized *putP* mutants in order to gain insight concerning the cation and proline binding domains of this protein. The results of their studies suggest that arginine 257, glycine 22, and cysteine 141 are involved in sodium binding. Interestingly, arginine 257 is also conserved in the *S. aureus* PutP homolog. It is noted, however, that neither glycine 22 nor cysteine 141 is conserved in the *S. aureus* protein.

The PutP permease has been shown to be inactivated by *N*-ethylmaleimide (2, 21). Furthermore, proline and sodium have both been shown to protect the permease against inactivation by *N*-ethylmaleimide (19), suggesting that reactive cysteine residues are located near the binding sites for both proline and Na^+. On the basis of studies with mutant carriers (in which cysteine residues have been altered to serine residues), Hanada and coworkers have proposed that cysteine 344 is involved in the binding of Na^+ and proline (19). It should be

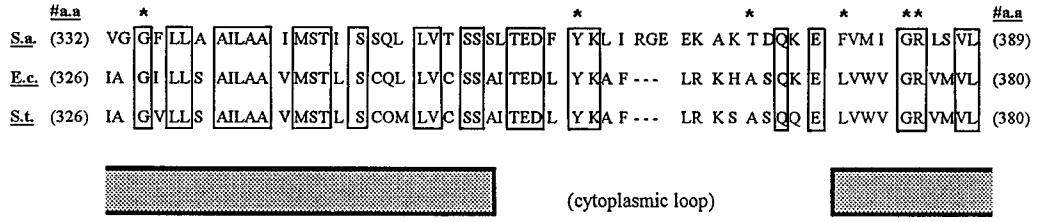

FIG. 6. Alignment of the predicted SOB domains of the *E. coli* (E.c.) PutP permease, the *S. typhimurium* (S.t.) PutP permease, and the *S. aureus* (S.a.) PutP homolog. Amino acid residues identical in all three permeases are boxed. The residues corresponding to glycine 328, alanine 366, leucine 371, glycine 375, and arginine 376 of the *E. coli* PutP permease are marked by asterisks. The conserved tyrosine residue (at position 358 in the *E. coli* PutP permease) is also marked by an asterisk. The shaded boxes represent the positions of putative membrane-spanning domains 8 and 9.

noted, however, that this cysteine residue is not conserved in the *S. aureus* PutP homolog. In fact, we further note that none of the five cysteine residues present within the *E. coli* PutP permease are conserved within the *S. aureus* PutP homolog, indicating that cysteine residues are not essential for PutP function.

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ADDENDUM

Yamato et al. (41) have recently shown that arginine 376 (which is within the SOB motif and conserved in the *S. aureus* PutP homolog) of the *E. coli* PutP permease is not essential for proline-Na^+ symport.

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