Isolation and Preliminary Characterization of Amino Acid Substitution Mutations That Increase the Activity of the Osmoregulated ProP Protein of *Salmonella Enterica* Serovar Typhimurium

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In *Enterobacteriaceae*, the ProP protein, which takes up proline and glycine betaine, is subject to a post-translational control mechanism that increases its activity at high osmolarity. In order to investigate the osmoregulatory mechanism of the *Salmonella enterica* ProP, we devised a positive selection for mutations that conferred increased activity on this protein at low osmolarity. The selection involved the isolation of mutations in a proline auxotroph that resulted in increased accumulation of proline via the ProP system in the presence of glycine betaine, which is a competitive inhibitor of proline uptake by this permease. This selection was performed by first-year undergraduates in two semesters of a research-based laboratory course. The students generated sixteen mutations resulting in six different single amino acids substitutions. They determined the effects of the mutations on the growth rates of the cells in media of high and low osmolarity in the presence of low concentrations of proline or glycine betaine. Furthermore, they identified the mutations by DNA sequencing and displayed the mutated amino acids on a putative three-dimensional structure of the protein. This analysis suggested that all six amino acid substitutions are residues in trans-membrane helices that have been proposed to contribute to the formation of the transport pore, and, thus, may affect the substrate binding site of the protein.

Introduction

CELLS OF ALL ORGANISMS adapt to changes in the osmotic strength of their surroundings by raising or lowering the concentrations of cytoplasmic solutes in parallel with changes that occur in the external osmolarity. Solutes that are used to regulate the osmolarity of the cytoplasm are accumulated by *de novo* synthesis or transport from the medium when cells are challenged with high osmolarity, and they are excreted or degraded in response to a decrease in the external osmolarity. There are a limited number of special solutes, called osmoprotectants, that can have a profound stimulatory effect on the growth rate of bacteria when they are available in media of high osmolarity. The most common osmoprotectants for *Enterobacteriaceae* are proline, glycine betaine (N,N,N-trimethyl glycine), and about a dozen other structurally related compounds (Csonka and Epstein, 1996).

Salmonella enterica serovar Typhimurium (*Salmonella typhimurium*, hereafter) and *Escherichia coli* K-12 accumulate proline, glycine betaine, and other osmoprotectants via the ProP and ProU transport systems (Anderson *et al.*, 1980; Csonka, 1983; Cairney *et al.*, 1985a; Cairney *et al.*, 1985b; Gowrishankar, 1985; Gowrishankar, 1986; MacMillan *et al.*, 1999). These organisms have a third transporter, PutP, that is needed to take up proline when it is used as a carbon or nitrogen source, but PutP does not recognize glycine betaine and has no role in osmoregulation. Both the ProU and ProP systems are regulated by the osmolarity of the medium. The *proU* operon, which encodes the components of the former system, is induced by osmotic stress (Balaji *et al.*, 2005), whereas the latter is regulated by a combination of transcriptional induction (Balaji *et al.*, 2005) and *in situ* activation (Kunte *et al.*, 1999), resulting in up to 20-fold increase in activity by osmotic stress (Grothe *et al.*, 1986). In addition

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to ProU and ProP, *S. typhimurium* LT2 has an additional transport system, OsmU, which can take up glycine betaine in media of high osmolarity (Gutierrez and Csonka, 1989), encoded by the STM1491 to STM1494 genes. This transporter, which does not take up proline, is absent from *E. coli* K-12.

Kaback and Deuel (1969) observed that proline transport into cell free membrane vesicles of *E. coli* was stimulated by high osmolarity. Since at that time, PutP was the only known proline transporter, they attributed this effect to the stimulation of that permease, but after the discovery of ProP, it was demonstrated that osmotic stress enhances the activity of the latter transporter in whole cells by a post-translational mechanism (Dunlap and Csonka, 1985; Grothe *et al.*, 1986). The laboratory of Dr. Janet Wood reproduced the osmotic stress-dependent stimulation of ProP in membrane vesicles obtained from whole cells (Milner *et al.*, 1988) and in a reconstituted system consisting of purified ProP protein and phospholipid vesicles (Racher *et al.*, 1999). These studies demonstrated that high osmolarity can act on membrane-bound ProP protein to stimulate its activity and that this protein itself is an osmosensor.

ProP, which is a member of the major facilitator superfamily (MFS) of proteins (www.tcd.org/superfamily.php), is energized by the cotransport of H⁺ ions. The structure of the *E. coli* ProP protein has been modeled on the crystallographic structure of one of the members of the MFS family, the GlpT (glycerol 3-phosphate:phosphatase antiport) protein of *E. coli*, which has 17% amino acid sequence identity with ProP (Wood *et al.*, 2005). This model predicted that the ProP protein has 12 transmembrane domains, TMI to TMXII, that are connected by alternating periplasmic and cytoplasmic loops, P1 to P6 and C1 to C5, respectively, (A version of this model has been reproduced in Fig. 2, in a slightly modified form to show the *S. typhimurium* ProP sequence). TMI, TMIV, TMVII, and TMX, in addition to portions of TMII, TMV, TMVIII, and TMXI, were proposed to form the substrate-conducting pore. Extensive site-directed mutagenesis, involving the replacement of various amino acids with cysteines followed by chemical labeling of the cysteines, led to the identification of residues in TMI, P1, TMII, TMIV, P6, and TMXII that are important for the activity and/or osmotic regulation of the protein (Wood *et al.*, 2005; Liu *et al.*, 2007; Culham *et al.*, 2008; Keates *et al.*, 2010). The ProP protein of *E. coli* and related bacteria contains a 45-amino acid C-terminal extension that has been predicted to form a cytoplasmic α -helical coiled-coil (Culham *et al.*, 2000). Based, in part, on the observation that such a C-terminal extension was lacking from members of the MFS that were not involved in osmoregulation, it was proposed that this unique feature of ProP might be responsible for its function as an osmosensor (Culham *et al.*, 1993). However, subsequently, it was reported that while mutations in the C-terminal extension can attenuate the regulation of ProP, this domain is not essential for osmotic activation of the protein (Tsatskis *et al.*, 2005).

Although the targeted replacement of amino acids with cysteines has been informative as to the functions of various residues in ProP, the understanding of the mechanism of osmotic control of ProP activity and the amino acids that mediate this response is incomplete. Therefore, as an alternative approach to site-directed replacement of amino acids, we devised a positive selection procedure for mutations that

increased the activity of the *S. typhimurium* ProP protein at low osmolarity, with the expectation that these mutations are likely to include amino acid changes that alter the conformation of the ProP protein such that it mimics the state it adopts at high osmolarity. This project has been carried out by first-year undergraduate students in a research-based laboratory class during the Spring 2010 and 2011 semesters as part of a Center for Authentic Science Practice in Education (CASPiE) in the Department of Biological Sciences at Purdue University. The students used three different mutagenesis protocols, localized the mutations by classical genetic mapping, identified mutations by polymerase chain reaction (PCR) amplification and DNA sequencing, and superimposed the mutated amino acids on a predicted secondary structure of the ProP protein. Comparisons of the growth rates of the mutants to the wild-type strain were also performed to provide a finer functional characterization of ProP.

In this article, we present the results of this project. We have isolated six independent missense mutations that confer the ability of growth of proline auxotroph bacteria with only ProP to take up proline in the presence of glycine betaine antagonism. Each of these mutations are in different locations in the ProP protein and confer different growth phenotypes.

Materials and Methods

Media and growth conditions

The rich medium Luria Broth (LB) was made according to the recipe of Davis *et al.* (1980), and minimal medium 63 (M63) was made as described by Dunlap and Csonka (1985). Unless otherwise stated, the carbon source in M63 was 10 mM glucose. When used, melibiose was at 5 mM. Solid media contained an additional 20 g/l Bacto agar (Difco). Cultures were grown with aeration at 37°C in a standing incubator (on plates) or in a shaking water bath (in tubes and flasks). When used, sterile antibiotic solutions were added to the media after autoclaving at the following concentrations (mg/L): spectinomycin hydrochloride (Sp), 300; kanamycin sulfate (Km), 25; sodium ampicillin, 100; chloramphenicol (Cm), 25; tetracycline (Tc), 20. Resistance and sensitivity to antibiotics is indicated by superscripts ^R and ^S, respectively.

Bacterial strains

All bacterial strains were derived from *Salmonella enterica* serovar Typhimurium LT2. The genotypes and derivation of the strains used in this work are shown in Supplementary Table S1 (Supplementary Data are available online at www.liebertonline.com/dna). Generalized transductions for mapping or strain construction were carried out using bacteriophage P22 HT105/1 *int-201* (called P22 hereafter) according to the procedures of Davis *et al.*, (1980). The *proP* mutations were transduced from the original isolates into the clean genetic background of strain TL4513 (*proP4::Tn10dCm ΔproBA47 ΔputPA573 proU1884::MudJ*), using the linked *melA::Tn10* as the selected marker. Tc^R recombinants that were likely to have inherited the *proP* point mutations were recognized by the fact that they lost the Cm^R, and the presence of the *proP* mutations was confirmed by tests for growth on M63 + 0.1 mM proline + 2 mM glycine betaine. Strains TL4554–TL4559 and the isogenic *proP*⁺ strain TL4553 were constructed in this manner. Proline

prototrophic (*proB*⁺*A*⁺) derivatives of these strains were constructed by transducing them to growth on M63 without proline, generating strains TL4533-TL4535, TL4544-TL4546, and TL4560. Finally, the third glycine betaine transport system, OsmU, was inactivated in the latter strains by two sequential transductions, the first involving the replacement of the *proU1884::MudJ* (Km^R) insertion with the *proU1872::MudA* insertion, followed by the introduction of the *osmU::kan* marker, yielding strains TL4627-TL4633.

Isolation of *proP* mutations

As described next and more fully in the Results section, the procedure for the isolation of the *proP* mutations involved the selection of strains in which the ProP protein could take up proline rapidly in the presence of excess concentrations of glycine betaine, which is a competitive inhibitor of proline uptake by this permease. The starting strains for the mutant isolation were the proline auxotrophs TL1673 (*proP*⁺ Δ *proBA* Δ *putPA* *proU::MudJ*) and TL4500, which, in addition to the *proP*⁺ Δ *proBA* Δ *putPA* *proU::MudJ* alleles, carries the *mutT174::spc* mutation that inactivates 8-oxo-dGTPase and thereby increases the frequency of incorporation of the mutagenic 8-oxoG into DNA. These strains in which ProP is the only functional proline transporter can grow in media containing 0.1 mM proline but not in media containing 0.1 mM proline plus 2 mM glycine betaine. Derivatives that could grow in the latter medium were obtained from TL1673 as spontaneous mutants or after mutagenesis by ethyl methane sulfonate (EMS) and from TL4500 by *mutT*-dependent mutagenesis. For the spontaneous and *mutT* mutagenesis, TL1673 and TL4500, respectively, were grown from single colonies overnight (O/N) in liquid LB, and 100 μ L samples containing $\sim 4 \times 10^8$ cells were spread on M63 glucose+0.1 mM proline+2 mM glycine betaine plates and incubated at 37°C until colonies began to appear (36 h to 48 h). Mutants that could grow on the selective medium arose spontaneously at the approximate frequency of 10^{-7} per cell in TL1673 and at about four to five times higher frequency in strain TL4500. For the EMS mutagenesis, 100 μ L samples of O/N cultures of TL1673 in LB were spread on M63 glucose+0.1 mM proline+2 mM glycine betaine plates. After the plates were dry, ~ 1 cm diameter sterile Whatman filter disks were placed at the center of the plates, and 10 μ L of EMS was introduced on the disks. For each selection, cells from 10 to 20 colonies were restreaked on M63 glucose+0.1 mM proline+2 mM glycine betaine plates to confirm the phenotype and to purify the mutants from the parental strain.

Genetic and genomic characterization of the mutations

In the selection that we used, any mutation that would increase the acquisition of proline would enable the *proP*⁺ Δ *proBA* Δ *putPA* *proU::MudJ* mutants to grow on M63 glucose+0.1 mM proline+2 mM glycine betaine plates. As discussed in the Results section, in addition to mutations in *proP*, mutations in other genes might conceivably result in the selected phenotype. Since we were primarily interested in identifying alterations in *proP*, we used two alternative P22 mapping strategies to recognize strains in which the mutations were in or closely linked to this gene. Flow charts summarizing these mapping strategies are shown in Figure 1.

Mapping procedure I

The first procedure consisted of two sequential transductions in which the initial step involved the transduction of the *proP::Tn10dCm* insertion from strain TL1901 into the mutants on LB+Cm plates. Although the derivatives obtained in this step were now deficient for all three proline transport systems ProP, ProU, and PutP, they were able to grow on LB, because this rich medium probably contains prolyl peptides that are taken up by oligopeptide permeases (Csonka, 1983). These resultant transductants were screened for growth on M63 glucose+0.1 mM proline+2 mM glycine betaine plates with the expectation that if the ability of the original mutants to grow on this medium resulted from a change in the activity of the ProP protein, then inactivation of the *proP* gene should abolish growth on M63 glucose+0.1 mM proline+2 mM glycine betaine, whereas if the growth were due to mutations that are unrelated to ProP, then the inactivation of this transporter would not affect growth on the selective medium. Although the loss of ability to grow on M63 glucose+0.1 mM proline+2 mM glycine betaine upon inheritance of the *proP::Tn10dCm* would indicate that the growth on the original selective medium in the mutants was dependent on ProP, this criterion is not sufficient to show that the mutations are in *proP*, because it is possible that the growth on the selective medium could be due to a mutation in some unknown protein that interacts with ProP or in an unlinked regulatory gene that results in elevated expression of *proP*. To distinguish between these possibilities, the mutants were characterized by a second transduction.

The prospective *proP* mutations had been isolated in the genetic background of a strain harboring a *melA::Tn10* insertion, which is 13 kbp from the *proP* gene and blocks the utilization of melibiose as carbon source. In the second mapping step, the *proP::Tn10dCm* *melA::Tn10* derivatives were transduced with P22 grown on TL1 (*mel*⁺ *proP*⁺) to growth on M63 melibiose plates containing 1 mM proline. Due to the close linkage of *proP* and *melA*, approximately 40% of the *Mel*⁺ transductants inherited the *proP*⁺ allele, as indicated by loss of Cm^R. *Mel*⁺ *proP*⁺ (Cm^S) transductants were tested for growth on M63 glucose+0.1 mM proline+2 mM glycine betaine. Approximately 95% of the mutants that successfully passed the first mapping tests were found to be unable to grow in M63 in M63 glucose+0.1 mM proline+2 mM glycine betaine on the inheritance of the *proP*⁺ allele in the second transduction. The ancestors of these strains were judged to carry mutations that were in or closely linked to the *proP* gene, because if the growth were due to an unlinked regulatory mutation, then the *proP*⁺ transductants obtained in the last set of crosses should have retained the ability to grow on the selective medium.

Mapping procedure II

The second mapping procedure utilized only one transduction. For this transduction, P22 was grown on the presumed *proP* *melA::Tn10* mutants, and the lysates were used to transduce TL4513 (*mel*⁺ *proP::Tn10dCm* Δ *proBA* Δ *putPA* *proU::MudJ*) to Tc^R on LB+Tc plates. The transductants were first screened for growth on LB+Cm plates, and the recombinants that showed Cm^S phenotype, which were likely to have inherited the *proP* allele of the respective donor

FIG. 1. Flow chart showing the transductional mapping procedures used to determine whether the mutations that allowed growth in M63+0.1 mM proline+2 mM glycine betaine are linked to *proP*. Details of the procedures are described in Materials and Methods. Panel A: mapping procedure I; Panel B: mapping procedure II. Pro is proline, GB is glycine betaine.

FIG. 1. (Continued)

strains, were tested for growth on M63 glucose+0.1 mM proline+2 mM glycine betaine plates. Growth on the latter medium indicated the mutation was in or closely linked to *proP*.

PCR amplification and sequencing of the mutated *proP* genes

A 1937 bp DNA fragment containing the *proP* gene together with 321 bp upstream sequences that include the promoters and 113 base pairs downstream of the gene was amplified by PCR from the original isolates of the strains that were shown by the P22 mapping to carry mutations in *proP*, using primers ProPFM217 (5'-GGTTACACTCGAATAACCGCTTT-3') and ProPR1720 (5'-ACACTACACAGGGTCGTCAAA-3'). The amplicons were sequenced by the Purdue university Core Genomics Center with six primers: the two primers that were used for the amplification as well as ProPM56 (5'-GGTAT

GCCAGTGCCCGCCGTA-3'), ProPF904 (5'-CTGGAGCAGGGCGACCGC-3'), ProPR530 (5'-GCCAACCGAGAAGCCCTG-3'), and ProPR903 (5'-GACCGATACATGACAACAGGCTAC-3'), which hybridize to various places in the *proP* gene on the two strands and generate overlapping sequences in both directions.

The sequences of the amplicons were compared with the sequence of *S. typhimurium* LT2 (Genbank ID AE006468.1), using the blastn algorithm (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). A consistent difference from the wild type sequence that was found in three or more overlapping sequences obtained with different primers was taken as evidence for the presence of a mutation.

Determination of growth rates

For the determination of the growth rates of strains in liquid cultures, strains were grown to saturation in LB,

inoculated at a 100-fold dilution into M63-glucose medium, which was supplemented with 1 mM proline for proline auxotrophs. After O/N growth, the strains were subcultured at a density of $\sim 5 \times 10^7$ cells/mL ($OD_{600} = \sim 0.05$) into 15 mL of M63 glucose without or with 0.3 M NaCl and 0.1 mM proline or 0.1 mM glycine betaine, as indicated in the figure legends. The cultures were incubated with aeration at 37°C, and the cell density was monitored by measurement of light scattering at OD_{600} with a Shimadzu UV-Vis spectrophotometer every hour until the cultures reached stationary phase. To correct for multiple scattering at high cell densities, when the OD_{600} of the cultures exceeded > 1 , the OD_{600} was determined from samples that were diluted 10-fold into the respective growth media. The specific exponential growth rate constants of the cultures were calculated from least-squares fit of the data points to an exponential function of time over the exponential growth phase of the cells. The growth rate results were calculated as the averages $\pm$ standard deviation obtained in three independent experiments carried out in each case with duplicate cultures. Significant differences between the growth rates of the *proP* mutants and of the respective control *proP*⁺ strains were calculated using the two-tailed *t*-test.

In silico analysis of the predicted structure of the ProP protein

Tertiary structure analysis of the ProP protein was carried out using the Phyre² (protein homology/analogy recognition engine) sequence-to-structure alignment server v. 2.0 (Kelley and Sternberg, 2009). Three-dimensional structures were visualized using PyMol molecular visualization software (<http://pymol.sourceforge.net/faq.html>; PyMol Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC).

Results

Isolation of *proP* mutations

Our mutant selection procedure was based on the fact that glycine betaine is a competitive inhibitor of proline uptake via ProP (MacMillan *et al.*, 1999), and as a result, proline

auxotrophic strains in which ProP is the only functional proline transport system are unable to grow in media containing low concentration of proline and excess concentration of glycine betaine. Therefore, the selection of derivatives that are able to grow in this medium provides a positive selection for mutants that are able to acquire sufficient proline in the presence of otherwise inhibitory concentrations of glycine betaine. Genetic alterations that could confer growth under these conditions would be expected to include mutations in the osmosensing part of ProP that render it more active in the absence of osmotic stress or mutations in the substrate-binding site of ProP that increase its affinity for proline or decrease it for glycine betaine. Although we were mainly interested in isolating amino acid substitutions that change the regulation or activity of ProP, it is possible that the selected phenotype could be the result of other types of alterations, for example, mutations in the *proP* promoter or in regulatory genes that elevate the expression of *proP*, mutations that change the expression or activity of a transport protein for some other metabolite so that it can now use proline as a substrate (Liao *et al.*, 1997), or mutations that bypass the Δ *proBA* biosynthetic defect (Berg and Rossi, 1974). As initial screening, we carried out transductional mapping described in Materials and Methods to identify the mutations that were in or closely linked to the *proP* gene. DNA fragments containing the *proP* gene and flanking sequences were amplified from the mutants, their sequences were determined with three forward and three reverse primers that hybridize to different sites in the amplicons, and mutations in *proP* were identified by blastn comparison to the database sequence of the wild type *S. typhimurium* LT2.

This procedure yielded sixteen mutations resulting in predicted substitutions at six different amino acids in ProP, some of which were isolated independently a number of times. The nucleotide and amino acid changes that were obtained are summarized in Table 1. Each of these mutations generated a single amino acid substitution in the predicted ProP sequence. One of the strains that was recovered after EMS mutagenesis carried two *proP* mutations, one of which was c $\rightarrow$ t transition at nucleotide 218 of the gene and resulted in a predicted change of serine to a phenylalanine at residue

TABLE 1. *PROP* MUTATIONS ISOLATED

Number of isolates ¹	Nucleotide change ²	Amino acid change ³	Isolated in strain	Mutagens
2	t194g	M65R	TL4500	<i>mutT</i>
3	c218t	S73F	TL1673	EMS ⁵
1 ⁴	c218t, g1242a	S73F	TL1673	EMS ⁵
1	t266g	L89R	TL1673	Spontaneous
7	t548g	V183G	TL4500	<i>mutT</i>
1	a850c	S284R	TL4500	<i>mutT</i>
1	t900g	I300M	TL4500	<i>mutT</i>

¹Number of times this mutation has been isolated independently.

²Nucleotide positions of mutations in the *proP* gene. The number between the lowercase letters is the nucleotide position where the mutation occurred, counted from translation start site as 1; the letter before the number is the wild-type base in the coding strand, and the letter after the number is the base in the mutant.

³Amino acid substitution predicted from nucleotide sequence. The number between the uppercase letters is the amino acid position in the predicted protein where we obtained a substitution, counted from the translation start site; the letter before the number is the wild-type amino acid, and the letter after the number amino acid is the mutant.

⁴Double mutant: in addition to the c218t mutation, it has a silent mutation, g1242a, which changed the S414 codon tcg to a synonymous tca serine codon.

⁵EMS, ethyl methane sulfonate.

73 of the protein and the other one was $g \rightarrow a$ transition at nucleotide 1242 that changed codon 414 from tgc to tca . Since both these latter codons specify serine, this unselected mutation would not be expected to have an effect on the structure of the protein, and it may have been generated in close proximity to the $c218t$ amino acid substitution mutation by an error-prone repair mechanism induced by the EMS. We did not recover any mutations that were in the promoter or other transcriptional regulatory site of the gene. The base pair changes we obtained are in accord with the known specificities of EMS for promoting $c:g \rightarrow t:a$ transitions and of *mutT* generating $a:t \rightarrow c:g$ transversions (Sekiguchi, 1996). The fact that we recovered multiple isolates of the same mutations in independent experiments (two $t \rightarrow g$ at nucleotide position 194, four $c \rightarrow t$ at position 218, and seven $t \rightarrow g$ at position 548) in mutagenized cultures may be due to the fact that these sites may be especially susceptible to the respective mutagens that result in amino acid changes, that would permit growth on the selective medium or to the fact that we may have saturated the target of mutations. However, before we accept the latter conclusion, it will be necessary to carry out additional selections using spontaneous mutations or other mutagens besides EMS and *mutT*.

Locations of the amino acid substitutions in the predicted ProP structure

Figure 2 shows the locations of the six amino acids where we obtained mutations overlaid on the proposed secondary structure of ProP (Culham *et al.*, 2008), which has been modified slightly to show the sequence of the *Salmonella* protein, instead of the *E. coli* ortholog. Three mutated residues, M65R, S73F, and L89R, are in the putative TMII, and V183G, S284R, and I300M are mutated sites in TMV, TMVII, and TMVIII, respectively. The M65R, L89R, and S284R mutations are major changes that replaced a neutral amino acid

with a positively charged one in these membrane-spanning domains. The S73F mutation replaced a small hydrophilic amino acid with a bulky hydrophobic one in the middle of a transmembrane region, and the V183G and I300M were conservative alterations. None of our mutations were in the C-terminal tail of the protein, which had been suggested to be involved in the stimulation of transport activity by high osmolarity (Culham *et al.*, 2008). However, as a negative result, the absence of mutations here is not compelling evidence against a role of this domain in the osmotic control of ProP activity.

Physiological characterization of the *proP* mutants

We carried out a number of growth studies to analyze the effect of the *proP* mutations on the uptake of proline and glycine betaine. First, we determined how well the mutated ProP proteins could support the growth of proline auxotrophs in M63 glucose containing 0.1 mM proline. The results of this experiment are shown in Figure 3A. Each of the six *proP* mutants grew significantly more rapidly than the isogenic *proP*⁺ control strain, as demonstrated by the fact that the specific exponential growth rate constants (k 's) of the *proP* mutants ranged from 0.50 to 0.74 h⁻¹, whereas the growth rate of the *proP*⁺ control strain was 0.34 h⁻¹. When cells are grown in the normal M63, which has moderately low osmolarity, proline is accumulated primarily for protein synthesis, rather than as an osmoprotectant. Since at low osmolarity, the wild-type ProP protein is not maximally active, it cannot take up 0.1 mM proline to support optimal growth rate of proline auxotrophs. This conclusion can be seen from the result that the *putP*⁺ *proP*⁺ strain, which had two functioning proline transporters, grew more rapidly ($k=0.67$ h⁻¹) than the *proP*⁺ *putP* mutant, in which ProP⁺ is the only functioning proline permease ($k=0.34$ h⁻¹). The activities of ProP proteins carrying four of the mutations,

FIG. 2. Illustration of the *Salmonella typhimurium* ProP protein structure showing the amino acid sequence organized into the twelve transmembrane regions (in rectangles, labeled in roman numerals) connected to each other by periplasmic and cytoplasmic loops. Rectangles of identical shading have been suggested to be located near each other in the three-dimensional protein structure. Adapted with Permission from Biochemistry 2005, 44, 5634-5446. Copyright American Chemical Society.

FIG. 3. The growth rates of the *proP* mutants supported by 0.1 mM proline at low and high osmolarity. The growth rates of the strains in M63 + 0.1 mM proline (**Panel A**) and M63 + 0.3 M NaCl + 0.1 mM proline (**Panel B**) were determined as described in Materials and Methods. In addition to the $\Delta proBA47 \Delta putPA573 proU1884::MudJ melA361::Tn10$ mutations, the strains used carried the indicated *ProP* amino acid replacements, where the name of the strain is indicated in parentheses: WT (*proP*⁺, TL4553); M65R (TL4558); S73F (TL4554); L89R (TL4559); V183G (TL4556); S284 (TL4555); I300M (TL4557); *PutP*⁺ *ProP*⁺ (TL191).

M65R, S73F, S284R, and I300M, were apparently enhanced sufficiently so that they could support growth with 0.1 mM proline at rates that were comparable to or greater than that seen with the *putP*⁺ *proP*⁺ strain.

Since in addition to being used for protein synthesis, proline also functions as an osmoprotectant, we investigated how the transport of proline was affected by the *proP* mutations in M63 containing 0.3 M NaCl and 0.1 mM proline (Fig. 3B). Although high osmolarity is generally inhibitory, the control *ProP*⁺ strain nevertheless grew more rapidly in M63 with 0.3 M NaCl + 0.1 M proline than in M63 + 0.1 mM proline without NaCl ($k = 0.47 \text{ h}^{-1}$ in the presence of 0.3 M NaCl vs. 0.34 h^{-1} in normal M63). This result can be explained on the basis that at low osmolarity, 0.1 mM proline is not taken up by *ProP* rapidly enough to support maximal growth rate, but high osmolarity stimulates the activity of *ProP* so that the increased accumulation of proline can sustain faster growth. The growth rates of the M65R, S73F, L89R, S284R, and I300M mutants in the presence of 0.3 M NaCl + 0.1 mM proline were statistically similar to that of the *proP*⁺ strain ($p > 0.05$, two-tailed *t*-test). However, the k of 0.34 h^{-1} of the V183G mutant was significantly slower than that of the *proP*⁺ strain in M63 + 0.3 M NaCl + 0.1 mM proline ($p < 0.05$, two-tailed *t*-test).

We also assessed the effect of the *proP* mutations on the utilization of glycine betaine as an osmoprotectant, using proline prototrophic (*proB*⁺ *A*⁺) versions of the mutants, which in addition carried an *osmU::kan* insertion to eliminate interference from the third glycine betaine transport system. For this experiment, the strains were grown in M63 + 0.3 M NaCl without and with 0.1 mM glycine betaine (Fig. 4). These results reproduce the well-documented osmoprotective effect of glycine betaine, as demonstrated by the fact that the k of the *proP*⁺ strain increased from 0.43 h^{-1} in the absence of glycine betaine to 0.58 h^{-1} in the presence of this supplement. There were no statistically significant differences between the growth rates of the six *proP* mutants and the *proP*⁺ strain in the absence of glycine betaine, and the M65R, S73F, L89R, and S284R mutants also grew with the same rates as the *proP*⁺ in the presence of glycine betaine ($p > 0.05$, two-tailed *t*-test). However, the V183G mutant showed a statistically significant 1.4-fold reduction in its growth rate in M63 + 0.3 M NaCl + 0.1 mM glycine betaine compared with the *proP*⁺ control strain ($p < 0.05$, two-tailed *t*-test). This result is consistent with the impaired growth seen with the V183G mutant in medium containing 0.3 M NaCl and 0.1 mM proline (Fig. 3B). Although not significant at the 95% confidence level, the I300M mutant exhibited a faster growth

FIG. 4. The growth rates of the *proP* mutants in medium of high osmolarity in the absence and presence of 0.1 mM glycine betaine. The growth rates of strains were determined in M63+0.3 M NaCl without (light gray histograms) and with 0.1 mM glycine betaine (dark gray histograms), as described in Materials and Methods. In addition to the $\Delta putA573 proU1872::MudA melA361::Tn10 osmU::kan$ mutations, the strains used carried the indicated ProP amino acid replacements, where the name of the strain is indicated in parentheses: WT (*proP*⁺, TL4553); M65R (TL4558); S73F (TL4554); L89R (TL4559); V183G (TL4556); S284R (TL4555); I300M (TL4557).

Mutation	M65R	S73F	L89R	V183G
	↓	↓	↓	↓
<i>S. typhimurium</i>	62 SVQMIAALATFSVPFLIRPLGGLFFGMLGDKY			93 . . . 158 DRKRGFMSGWLDFGSIAGFVLGAGVVVLI 186
<i>E. coli</i>	62 SVQMVAALATFSVPFLIRPLGGLFFGMLGDKY			93 . . . 158 DRKRGFMSGWLDFGSIAGFVLGAGVVVLI 186
<i>P. putida</i>	63 SVQMIAALATFSVPFLIRPLGGLFFGALGDRF			94 . . . 159 DRKRGFMSGWLDFGSIAGFVLGAGVVVLI 187
<i>A. tumefacies</i>	53 AVQTVAAALATFSVPFLIRPLGGIIFFGAMGDKF			84 . . . 149 DRKRGFMSGWLDFGSIAGFVLGAGLVVLL 177
<i>B. petrii</i>	66 TVQIIAALGTFSPVFLVRPLGGVFFFGVMGDRF			97 . . . 162 DRRRGFLGSLWLDFGSIAGFVLGAGLVVLL 190
<i>A. xylosoxidans</i>	68 AVQTIAALGTFSPVFLVRPLGGIIFFGVMGDKF			99 . . . 164 DRQRGFLGSLWLDFGSIAGFVLGAGMVLLL 192
<i>Streptomyces</i>	65 GAQVVQTFATFAAAFLVRPLGGLVFGPLGDRI			96 . . . 161 DKRRGFFGSLWLDFGTFVGYSLGSLVTVL 189
<i>B. dolosa</i>	98 SAQLLATFGTFAAAFFVVRPLGGMVFGPLGDRI			129 . . . 194 DKRRGFMSGFLEFGTLIGYVMGAGVVALL 222
<i>X. gardneri</i>	77 TAQLIATFATFTVAFLVRPIGGMVFGPLGDRI			108 . . . 173 DRNRGLMSGWLEFGTLGGYIAGAATVTAL 201
<i>A. pasteurianus</i>	72 ATRLIATFATFTVAFLVRPLGGMVFGPLGDRI			103 . . . 168 DKNRGLMSGWLEFGTLAGYVAGAGIVTAL 196
<i>Geobacillus</i>	68 SLQIIYSFATFAVAFIVRPIGGMFFGMLGDRI			99 . . . 164 DKKRSFMASGLEVTGLVGSAGAGLVTTLL 192
Consensus	: : :	: : :	: : :	: : :

Mutation	S284R	I330M	
	↓	↓	
<i>S. typhimurium</i>	. . . 259 RSLLSICGLVIATNVTYMLLTYP	SYLSHNLHYSEDHGVLI	IIAIMIGMLFVQPMGLLSDR 321
<i>E. coli</i>	. . . 259 RSLLTICGLVIATNVTYMLLTYP	SYLSHNLHYSEDHGVLI	IIAIMIGMLFVQPMGLLSDR 321
<i>P. putida</i>	. . . 260 RSLVTCIGVVIATNVTYMLLTYP	SYLSHNLHYSEDHGVLI	IIAIMVGMFLVQPIIGLLSDK 322
<i>A. tumefacies</i>	. . . 250 RSLAICIGMVLVTNVTYMLLTYP	TYLSKTLNHYSEDHGVLI	IIAVMGMFLVQPAIGLLSDR 311
<i>B. petrii</i>	. . . 263 KPLLVCGMVLVTNITYMLLTYP	TYLSGSLGYSEEHGVLI	IIVVMIGMLFVQPVVGFSSDK 325
<i>A. xylosoxidans</i>	. . . 265 KALLVCIGMVLTTNITYMLLTYP	TYLSGSLHYEQHGVLI	IIVVMGMFLVQPGVGFVSDK 327
<i>Streptomyces</i>	. . . 267 EAILICMGLVLLYNVTNMTSYLPT	YMSSETLDESETTAQLL	VLGTMVLVLTITTVGRSSDR 329
<i>B. dolosa</i>	. . . 291 RALLCVGLVLIFNVTDYMLVSLY	SFMSSTLHFDHSLVL	VLIVMVLMMPLTLAAGRSLSDK 353
<i>X. gardneri</i>	. . . 270 PQLLKCVGLVLVFNVTYMLLTYP	SYLSVTMGYAESKGLLI	IIVMLVMMPLNIVGGFLSDK 332
<i>A. pasteurianus</i>	. . . 263 RQLLKCMGLVLVFNVTYMLLTYP	SYLTVALGYAESKGLLI	IIVMLMMPLNVMGGYLSDK 325
<i>Geobacillus</i>	. . . 258 KPLLVCIIVVFYNVDYMLVTYP	SYMQLGYGEMRGLVL	TLVSMFMMPFILMGYLSDR 320
Consensus	: : :	: : :	: : :

FIG. 5. Multiple alignment of ProP orthologs from diverse bacteria for transmembrane (TM) domains containing the sites of mutated amino acids in the *S. typhimurium* protein. Multiple alignments were carried out with ClustalW2 (www.ebi.ac.uk/Tools/msa/clustalw2). The mutations isolated in the *S. typhimurium* ProP protein are shown above the blocks of multiple alignments. The consensus symbols are, as defined in the ClustalW2 website: "*" (asterisk), positions that have a completely conserved residue; ":" (colon), positions containing strongly similar residues; "." (period) containing weakly similar residues. The numbers at the beginning and end of each block of alignments indicate the amino acid positions in the ProP orthologs in the indicated bacteria. The accession numbers of the ProP orthologs are: *S. typhimurium* LT2, gi|16767540; *Escherichia coli* K-12, gi|324114647; *Pseudomonas putida* F1, gi|148547991; *Agrobacterium tumefaciens* str. C58, gi|159186117; *Bordetella petrii* DSM 12804, gi|163856137; *Achromobacter xylosoxidans* AXX-A, gi|338783639; *Streptomyces* spSA3_actG, gi|318062180; *Burkholderia dolosa* AUO158, gi|254252716; *Xanthomonas gardneri* ATCC 19865, gi|325924461; *Acetobacter pasteurianus* IFO 3283-01, gi|258543207; *Geobacillus* sp. G11MC16, gi|196248809.

rate than the *proP*⁺ in 0.3 M NaCl plus 0.1 mM glycine betaine ($k=0.65\text{ h}^{-1}$ for the mutant vs. 0.58 h^{-1} for the wild type), suggesting the possibility that this mutation might have increased the transport of glycine betaine.

Discussion

Using a procedure that involved the selection of mutations that increased the ability of the ProP transporter to take up proline more efficiently in the presence of high concentration of glycine betaine in a medium of low osmolarity, we isolated and characterized six amino acid substitution muta-

tions in this protein in *S. typhimurium*. The majority of these mutations were isolated and characterized by first-year, undergraduate students in a one-semester, research-based, introductory biology laboratory course.

The result that all six *proP* mutations conferred faster growth rate in M63+0.1 mM proline to the $\Delta\text{proBA } \Delta\text{putPA } \text{proU}::\text{MudJ}$ mutant compared with the *proP*⁺ control strain (Fig. 3A) is consistent with the possibility that these mutations increased the accumulation of proline by ProP at low osmolarity. However, none of these mutations resulted in improved growth rate in M63+0.3 M NaCl+0.1 mM proline, compared with the *proP*⁺ control strain (Fig. 3B). Unlike the *proP*⁺ control strain, whose growth rate was stimulated by 0.3 M NaCl in medium containing 0.1 mM proline, the *proP* mutants were inhibited by the increased osmolarity. Except for the V183G mutant, the growth rates of the other five mutants were not significantly different from that of the *proP*⁺ strain in medium containing 0.3 M NaCl and 0.1 mM proline, suggesting that the growth rates of these strains is limited by the high osmolarity rather than by the acquisition of proline. The V183G mutation caused a statistically significant 1.4-fold decrease in the growth rate compared with the *proP*⁺ control in 0.3 M NaCl+0.1 mM proline. This mutation resulted in a similar reduction in the growth rate in M63+0.1 mM glycine betaine (Fig. 4), suggesting that while it increased the activity of the ProP protein at low osmolarity, it may have compromised it at high osmolarity.

There are several ways in which mutations could increase the activity of the ProP protein: by altering the osmotic control of the protein so that it is more active in the absence of osmotic stress, by decreasing its K_m for proline, or by enhancing the translation or stability of the protein or its insertion into the membrane. It will be necessary to carry out extensive kinetic characterization by transport assays using radioactive substrates and by western blot analyses to determine whether these mutations affect the functioning, translation, or stability of the protein. However, these experiments were not feasible in the scope of an undergraduate class.

FIG. 6. The model of the *S. typhimurium* ProP protein was constructed and visualized as described in Materials and Methods. Amino acids are colored by “chainbows,” where the N-terminus starts blue and progressively changes colors to red at the C-terminus. **Panel A** shows the perspective with the N-terminus on the left, and **Panel B** shows the perspective with the N-terminus on the right. The transmembrane domains are represented as alpha helices and are numbered with roman numerals. The approximate locations of the membrane-spanning regions are based on structural predictions of Culham *et al.* (2008). **Panel C** shows the predicted structure of ProP, as visualized from the periplasmic side of the protein looking down through the substrate pore of the protein toward the cytoplasmic side. The predicted location of each mutated amino acid is color coded as follows: M65, white; S73, magenta; L89, red; K134, yellow; V183, orange; S284, green; I300, purple. Transmembrane domains are colored as follows: TM I—dark blue, TM II—blue, TM III—light blue, TM IV—turquoise, TM V—green, TM VI—lime green, TM VII—yellow, TM VIII—yellow-orange, TM IX—light orange, TM X—orange, TM XI—red-orange, and TM XII—red.

The superimposition of the mutations on the proposed secondary structure of ProP (Wood *et al.*, 2005) suggested that M65R, S73F, and L89R changed amino acids located in the proposed TMII, V183G affected an amino acid in TMV, S284R targeted a residue in TMVII, and I300M altered an amino acid in TMVIII (Fig. 2). The fact that three mutations (M65R, S73F, and L89R) affect residues in TMII is consistent with the suggestion that TMII is a part of the active site of the protein, and, therefore, these mutations may have changed the K_m of the protein for proline. The conclusion that residues in TMII are a part of the active site is supported by the observation that the arginine at position 79, which is conserved in all ProP orthologs, is essential for activity (Keates *et al.*, 2010). The locations of the V83G, S284R, and I300M mutations are also consistent with the hypothesis that TMV, TMVII, and TMVIII also contribute to the formation of the transport pore (Culham *et al.*, 2008).

Since the conservation of amino acids in the sequences of orthologous proteins from a wide range of organisms can give powerful clues about which residues are important for the function of the protein (Dayhoff *et al.*, 1978), we carried out a multiple sequence alignment of ProP proteins from 11 diverse Gram-negative and Gram-positive bacteria that exhibited a range of 95% to 50% blastp sequence identity/99% to 70% sequence similarity to the *Salmonella* protein. Figure 5 shows an abbreviated version of the multiple sequence alignment for the TM regions that contain the sites of mutations in the *Salmonella* protein. In all 11 species, the amino acid at the site corresponding to residue 183 of *Salmonella* is uniformly a valine. Although this high extent of conservation could indicate that this hydrophobic amino acid has an important role, nevertheless, it can be replaced by the smaller G without destroying the activity of the protein. Positions 284 and 300 are very nearly conserved, as they are occupied by very similar residues (S or T at the former position, I or L at the latter). The other three positions where we obtained mutations are less conserved: the amino acid corresponding to the *Salmonella* 89 can be an L or M, position 73 can contain an S, A, or T, and position 65 can be filled by M, T, I, V.

In order to visualize the sites of our mutations in the native protein, we repeated the modeling of the *Salmonella* ProP sequence on the GlpT structure, using the Phyre² algorithm (Kelley and Sternberg, 2009). The two views of the predicted structure are presented in Figure 6, which highlight the sites of our mutations. Figure 6 shows the structure as seen from a cross-section of the membrane. As can be seen in panel A of this figure, M65 and L89 are in an alpha helix in TMII. These residues are near the periplasmic and cytoplasmic edges of TMII, respectively; by replacing the non-polar residues M and L with a positively charged R, these mutations might weaken the docking of TMII into the phospholipid bilayer. Interestingly, M65 is predicted to come in close contact with K134 located in domain IV, and, therefore, mutation of M65 to R might also alter the conformation in this region by juxtaposing two positively charged residues in close proximity. S73 is also in TMII, although according to our *in silico* analysis, it is predicted to be in a random loop instead of the alpha helix. However, since previous ProP structural modeling has not suggested that this is the case (Culham *et al.*, 2008; Keates *et al.*, 2010; Liu *et al.*, 2007; Wood *et al.*, 2005), this prediction needs to be confirmed experimentally.

Figure 6C depicts the protein from the periplasmic side, perpendicular to the plane of the membrane. This view shows that the TMII, TMIV, TMV, TMVII, TMVIII, and TMXI domains form a pore, suggesting that residues in these domains might contact the substrates during transport. Of the three residues in TMII where we obtained mutations, only S73 is predicted to be located so that it faces the inner pore, whereas M65 and L89 are on the outer face of the helix away, from the pore. The replacement of the hydrophilic S73 with a bulky, hydrophobic F may alter the interaction of the pore with the transported substrates.

V183 is predicted to be located on the inner helix face of TMV, in the vicinity of the transport pore. The V183G alteration, which was isolated independently seven times, is the most intriguing among our mutations, because the replacement of valine with glycine does not entail a large physicochemical change and, therefore, would not be predicted to have a substantial effect on protein structure. However, V183 is conserved in all 11 organisms examined (Fig. 5), suggesting it is important for some aspect of ProP function. The importance of a V at this position is underscored by the result that the V183G mutation appears to impair the activity of the ProP at high osmolarity (Figs. 3B and 4).

Amino acid 300, which is an I or an L in all ProP orthologs, is predicted to be on the outer face of TMVIII, away from the pore. The near conservation of this residue suggests that it might have an important role in the activity or the structure of the protein, but evidently, the protein can tolerate the replacement of the I at this position with an M. However, since this mutation resulted in improved growth rate in M63+0.3 M NaCl in the presence of glycine betaine, it may have increased the activity ProP at high osmolarity with this substrate.

S284 is in TMVII, which has been proposed to line the inner pore of the protein (Culham *et al.*, 2008). Our model (Fig. 6C) predicts that S284 is at the inner face of the TMVII helix, facing the interior substrate pore, and that a part of this transmembrane helix near the periplasmic side is tilted inward towards the pore. In this position, S284 could come into close contact with the transported substrate, and mutation from a serine to an arginine could significantly alter this interaction.

The selection that we employed might also be applied to isolate mutations in potential *cis*-acting transcriptional sites or *trans*-acting proteins that regulate the expression or activity of the ProP protein, or in genes which encode other proteins that could take up proline. Although we may very well have obtained some of these mutations in this project, their identification has not been feasible in our one-semester laboratory courses. However, the isolation of these other types of mutations could be a productive future direction for this project.

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