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Protein ProQ Influences Osmotic Activation of Compatible Solute Transporter ProP in *Escherichia coli* K-12

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ProP is an osmoregulatory compatible solute transporter in *Escherichia coli* K-12. Mutation *proQ220*::Tn*5* decreased the rate constant for and the extent of ProP activation by an osmotic upshift but did not alter *proP* transcription or the ProP protein level. Allele *proQ220*::Tn*5* was isolated, and the *proQ* sequence was determined. Locus *proQ* is upstream from *prc* (*tsp*) at 41.2 centisomes on the genetic map. The *proQ220*::Tn*5* and *prc* phenotypes were different, however. Gene *proQ* is predicted to encode a 232-amino-acid, basic, hydrophilic protein (molecular mass, 25,876 Da; calculated isoelectric point, 9.66; 32% D, E, R, or K; 54.5% polar amino acids). The insertion of PCR-amplified *proQ* into vector pBAD24 produced a plasmid containing the wild-type *proQ* open reading frame, the expression of which yielded a soluble protein with an apparent molecular mass of 30 kDa. Antibodies raised against the overexpressed ProQ protein detected cross-reactive material in *proQ*$^+$ bacteria but not in *proQ220*::Tn*5* bacteria. ProQ may be a structural element that influences the osmotic activation of ProP at a posttranslational level.

Water flows across biological membranes in response to osmotic pressure (osmolality) gradients. Turgor pressure develops if cell walls resist osmotically induced water influx. Osmoregulatory mechanisms adjust cytoplasmic osmolality by modulating the synthesis, catabolism, uptake, or efflux of appropriate solutes in response to osmolality changes. Compatible solutes are organic solutes, accumulated by bacteria exposed to hypertonic environments, which do not impair cellular functions. Physiologists reason that in the absence of osmoregulatory mechanisms, cytoplasmic osmolality would follow environmental osmolality, causing unacceptable fluctuations in cytoplasmic composition, cell volume, and/or turgor pressure (3, 4, 42).

ProP is an osmoregulatory transporter which mediates the active accumulation of diverse compatible solutes, including proline, glycine betaine (*N*-trimethyl glycine), stachydrine (*N*-dimethyl proline) (13, 21), pipecolic acid (8), ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidine carboxylic acid) (16), and taurine (23). Gene *proP* (located at 93 centisomes) is expressed from σ^{70}- and σ^{S}-dependent promoters. Transcription of *proP* is modulated by medium osmolality, carbon source, and culture growth phase (24, 43–45). ProP is activated by an osmotic upshift in whole bacteria (11), cytoplasmic membrane vesicles (26), and proteoliposomes (30). An H^+-compatible solute symporter and member of the major facilitator superfamily, the 500-amino-acid ProP protein differs from sequence homologues not implicated in osmoregulation by possessing a 46-amino-acid carboxyl-terminal extension that is capable of forming a homodimeric α-helical coiled coil of limited stability in vitro (5, 38a).

Mutations *pro-219* and *pro-220*::Tn*5* were selected as increasing the resistance of *Escherichia coli* K-12 derivative RM2 [Δ*(putPA)101*] to toxic proline analogue 3,4-dehydroproline. Mutation *proQ220*::Tn*5* defined a new gene located, by transduction, at 40.4 min on the chromosomal linkage map (27). Whereas no ProP activity could be detected when *proQ220*::Tn*5* bacteria were cultivated in a medium of low osmolality, a partial restoration of ProP activity (41%) was observed when they were cultivated in a hypertonic medium (0.3 M NaCl [27]). The mutation did not alter the transcription of a chromosomal *proP*::*lacZ* operon fusion (9) in response to increased medium osmolality, however (27). This report shows that mutation *proQ220*::Tn*5* impairs the osmotic activation of ProP by acting at a posttranslational level, demonstrates the expression of ProQ by wild-type bacteria, and reveals the predicted sequence of protein ProQ.

MATERIALS AND METHODS

Bacterial strains, plasmids, molecular biological techniques, and growth conditions. The strains and plasmids used for this study are listed in Table 1. Construction of a *prc* deletion strain was carried out through a P1-mediated transduction of *E. coli* RM2 from strain KS1000, yielding strain WG703. Transductants were selected on Luria-Bertani (LB) agar containing kanamycin at a concentration of 50 μg/ml.

Bacteria were grown aerobically in LB medium (25) or MOPS (morpholinepropanesulfonic acid) minimal medium (28) at 37°C. If necessary, antibiotics were added to the medium at the following concentrations: ampicillin, 100 μg ml^{-1}; kanamycin, 50 μg ml^{-1}; chloramphenicol, 40 μg ml^{-1}. The hypotonic medium used to test the *prc* phenotype (1/2L medium, a salt-free, half-strength LB medium [14]) contained Bacto Tryptone (5 g/liter) and Bacto yeast extract (2.5 g/liter). Routine manipulation of DNA, the construction of recombinant plasmids, the isolation of chromosomal DNA, electrophoresis of DNA, and transformation were all carried out by standard techniques described by Sambrook et al. (31). DNA sequencing, based on the method of Sanger et al. (32), was carried out by GenAlyTiC (University of Guelph) or Mobix (Hamilton, Ontario, Canada). Unless otherwise stated, genetic nomenclature and the numbering of DNA sequences are based on release M52 of the *E. coli* MG1655 genome (accession no. U00096).

To characterize mutation *proP219* of *E. coli* WG170, DNA templates were synthesized by PCR amplification with synthetic oligonucleotide primers based on the known sequence of the *E. coli* K-12 *proP* locus (accession no. M83089), and their sequences were determined with the same primers. The overlapping fragments extended from 282 bp upstream through 79 bp downstream of the *proP* open reading frame (ORF), and the full sequence of one DNA strand was

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TABLE 1. *E. coli* K-12 derivatives and plasmids

Strain or plasmid	Genotype or description	Source or reference
E. coli		
DH5α	F⁻ ϕ*80dlacZ*ΔM15 Δ(*lacZYA-argF*) *U169 recA1 endA1 hsdR17* (r_K^- m_K^+) *supE44* λ⁻ *thi-1 gyrA relA1*	BRL (Burlington, Ontario, Canada)
GJ183	F⁻ Δ(*argF-lac*)*U169 rpsL150 relA1 araD139 flbB5301 deoC1 ptsF25* Δ(*putPA*)*101* Δ(*pyr-76*::Tn*10*) *proP227*::Mud1 (*lac* Ap)	9
KS1000	X90 Δ*prc3*::kan *eda-51*::Tn*10*	33
RM2	F⁻ *lacZ trp rpsL thi* Δ(*putPA*)*101*	41
SG13009	F⁻ *his pyrD* Δ(*lon-100*) *rpsL*(pREP4)	7
WG170	RM2 *proP219*	36
WG174	RM2 *proQ220*::Tn*5*	36
WG703	RM2 Δ*prc3*::kan	This work
Plasmids		
pQE60		Qiagen
pJK1	*E. coli* ORF *proQ* inserted into expression vector pQE60 (encodes ProQ S22P)	This work
pBAD24		12
pDC77	*E. coli* ORF *proQ* inserted into expression vector pBAD24 (encodes wild-type ProQ)	This work

determined. PCR and sequencing were repeated to confirm the single observed change, from G to A at nucleotide (nt) 1226 of the *proP* ORF (position 4329305 of the *E. coli* genome), which would truncate the protein at A408 (at the end of putative transmembrane helix 11).

To position the Mud1 (*lac* Ap) insertion of *E. coli* GJ183 (9) in relation to the *proP* promoters, 10 overlapping DNA segments, including *proP* and flanking sequences, were PCR amplified. All reactions yielded DNA products of the expected sizes when *E. coli* K-12 DNA was used as a template, but two of these products were missing when *E. coli* GJ183 DNA was used as a template. Based on the positions of the corresponding primer sequences, insertion *proP227*::Mud1 (*lac* Ap) interrupted the *proP* ORF between nt 4329205 and 4329356, in or after the codon for S375.

Allele *proQ220*::Tn*5* was isolated by selecting bacteriophage Mu *d*II4042-derived recombinant plasmids (10, 40), isolated from *E. coli* WG174 (27), that conferred kanamycin resistance on strain RM2 Mu *cts*. Plasmid transductants were selected on LB medium supplemented with chloramphenicol and kanamycin. Restriction endonuclease analysis of five such plasmids revealed physical maps which aligned with one another and with the 40.4-min region of the *E. coli* genome (17) to which *proQ* had been mapped (27). These plasmids (or their derivatives) served as templates for *proQ* sequencing (both DNA strands) with a primer based on the IS*50* regions of Tn*5* and others predicted by the emerging sequence. The deduced sequence in the region of the Tn*5* insertion was confirmed by PCR amplification and sequencing of the corresponding 609-bp DNA fragment from *E. coli* K-12; it also corresponds to the extended *yebJ* sequence cited in release M52 of the *E. coli* MG1655 genome (accession no. AE000277). In allele *proQ220*::Tn*5*, the transposon had been inserted after nt A314 of the *proQ* ORF (position 1913173 of the *E. coli* genome), interrupting the codon for E105.

To effect *proQ* overexpression, the *proQ* ORF of *E. coli* K-12 was amplified as described previously (2) with primers 5′*proQ* (5′-GGC TCC ATG GAA AAT CAA CCT AAG TTG-3′) and 3′*proQ* (5′-GGA TAA GCT TTC AGA ACA CCA GGT GTT-3′), the former designed to create an *Nco*I site at the *proQ* initiation codon. The amplified fragment and pQE60 (Qiagen, Santa Clarita, Calif.) or pBAD24 (12) vector DNAs were cleaved with restriction endonucleases *Nco*I and *Hin*dIII, and the desired DNA fragments were purified, mixed, and ligated.

Preparation, solubilization, and analysis of cells and subcellular fractions. To analyze ProQ expression in cells on a small scale, a 1-ml overnight culture was centrifuged in a Microfuge for 1 min. Cells were resuspended and boiled in 50 μl of sample buffer (15.625 mM Tris-HCl [pH 6.8], 2% [vol/vol] glycerol, 0.5% [wt/vol] sodium dodecyl sulfate [SDS], 0.05% [wt/vol] bromphenol blue, 1.25% [vol/vol] mercaptoethanol) following a modification of the method of Sambrook et al. (31). ProP expression was analyzed as described above, except that the boiling step was replaced by a 30-min incubation at 37°C. Cells were sheared by repeated passage through a 26-gauge syringe and centrifuged for 5 min in a Microfuge. The supernatant was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE). For larger-scale preparations, cells cultured in LB medium were harvested by centrifugation (Sorvall GS3 rotor; 5,000 rpm for 20 min at 4°C), washed twice with saline (0.85% [wt/vol] NaCl), and resuspended in a 1/300 volume of potassium phosphate buffer (0.1 M; pH 7.1). Washed cells were passed three times through a French pressure cell at a pressure of 1.6×10^8 Pa. The lysate was centrifuged at a low speed (Sorvall SS34 rotor; 10,000 rpm for 20 min at 4°C) to remove cellular debris and inclusion bodies. Soluble and particulate fractions were obtained by ultracentrifugation of the resulting supernatant (Beckman 45 Ti rotor; 36,000 rpm [$145,000 \times g$] for 2 h at 4°C). All fractions were stored at −70°C after resuspension of the pellets in the same buffer. Appropriately diluted samples of these fractions were dissolved in sample buffer as described above. SDS-PAGE analysis of ProQ was performed with gels comprised of 12% (wt/vol) acrylamide and 2.6% bis-acrylamide according to the method of Laemmli (19) with a MiniProtean II cell (Bio-Rad, Mississauga, Ontario, Canada). SDS-PAGE of proteins to resolve ProP was performed with 4 to 15% polyacrylamide gradient Tris-HCl gels (Bio-Rad).

Western blots were carried out according to the method of Towbin et al. (38). Proteins were electrotransferred to a nitrocellulose membrane (Bio-Rad) at 4°C with a constant current of 60 mA in a solution of 15.6 mM Tris, 120 mM glycine, 20% (vol/vol) methanol, and 0.02% (wt/vol) SDS. Membranes were blocked by incubation in phosphate-buffered saline (PBS) (15) containing 5% (wt/vol) skim milk powder for 18 h at 4°C, washed three times with PBS-Tween (PBS supplemented with 0.1% [vol/vol] Tween 20), incubated with either purified anti-ProQ or purified anti-ProP in PBS for 1 h at room temperature, washed three times with PBS-Tween, and incubated with horseradish peroxidase- or alkaline phosphatase-conjugated mouse anti-rabbit immunoglobulin G in PBS. Blots were visualized with the ECL kit (peroxidase; Amersham Life Science) or the BCIP/NBT reagent system (alkaline phosphatase; Sigma, St. Louis, Mo.) according to the manufacturers' instructions. Chemiluminescence was detected by exposing Kodak XAR5 film to the blot for 2 to 5 min.

Affinity purification of anti-ProP antibodies. Anti-ProP antibodies were raised against the partially purified ProP protein, the antibodies were adsorbed with an extract of a *proP* mutant *E. coli* strain, and ProP$(His)_6$ (the ProP protein with six additional, carboxyl-terminal histidine residues) was purified by nickel chelate affinity chromatography as described by Racher et al. (30). Purified ProP$(His)_6$ (2.5 mg) was coupled to CNBr-activated Sepharose 4B (Pharmacia Biotech) according to the manufacturer's instructions. To bind the anti-ProP antibodies to the active resin, 0.5 ml of adsorbed serum was incubated with the resulting resin for 18 h at 4°C on a rotating platform. Bound antibody was eluted from the column by washing it with 0.1 M glycine-HCl buffer, pH 2.5. The eluate was immediately neutralized with 1 M Na_2CO_3. Fractions containing the affinity-purified antibodies were pooled and stored at −40°C.

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HI1670   (1)TDTQLSSQV TDVQTEVQKL TNAKAIITYL AEKFPLCFVL EGEAKPLKIG LFQDLAEALQ DDERVSKTQL RQALRQYTSN (80)
                        +   KL    K +I  L A KFP CF   EGEA+PLKIG +FQDL + +   +  +SKTQL R ALR YTS
ProQ                  (1)MENQPKL NSSKEVIAFL AERFPHCFSA EGEARPLKIG IFQDLVDRVA GEMNLSKTQL RSALRLYTSS (67)
                        +    L  +  + +  L     P  F   +              L D      + LS  +L R AL+  T S
FinO   (51)AELAAKKAQA RQALSIYLNL PTLDDAVNTL KPWWPGLFDG DTPRLLACGI RDVLLEDVAQ RNIPLSHKKL RRALKAITRS(130)


HI1670 (81)WRYLYGCREG AVRVDLQGNP AGVLDAEHVA HAAQQLAEAK ARFAEKRKQK LQLKKHNKKS IRVNLRIKTL KKRVNYH (157)
          WRYLYG + G A RVDL GNP  G LD EHV  HAAQQL EAK AR   +R ++    K+    +              ++
ProQ   (68)WRYLYGVKPG ATRVDLDGNP CGELDEQHVE HARKQLEEAK ARVQAQRAEQ QAKKREAAAT AGEKEDAPRR ERKPRPTTPR(147)
           YL   K G A+R D +G      + ++     A   L+  +      +   Q
FinO   (131)ESYLCAMKAG ACRYDTEGYV TEHISQEEEA YAAERLDKIR RQNRIKAELQ AVLDEK(186)


HI1669                                                          (1)MKVKAG DNAKKATVVE VLKDSARVEL ENGLIMNVAA DRLFA(41)
                                                                   KVKAG  NA  ATV+E + KD  RV+L   G    V A + L
ProQ   (148)RKEGAERKPR AQKPVEKAPK TVKAPREEQH TPVSDISALT VGQALKVKAG QNAMDATVLE ITKDGVRVQL NSGMSLIVRA EHLVF(232)
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FIG. 1. ProQ is a hydrophilic protein with sequence similarities to *E. coli* FinO and other structural elements. The full-length sequence alignment of ProQ, FinO, HI1669, and HI1670 was created by the manual joining of local alignments identified by BlastP (1). +, conservative substitution.

FIG. 2. Position and orientation of *proQ* on the *E. coli* genome. Arrows indicate positions and orientations of ORFs (see the text). Known and suggested functions of some of these loci are listed in Table 2.

Anti-ProQ antibody preparation. The protein overexpressed by *E. coli* SG13009(pJK1) was purified as follows. The fraction recovered by the first low-speed centrifugation of a French press lysate (described above) was washed twice as described by Neugebauer (29). The insoluble residue was dissolved by boiling it for 15 min in Tris-HCl buffer (pH 8.0)–5% (wt/vol) SDS–40 mM dithiothreitol and resolved by SDS-PAGE as described above. The gel was stained with 0.3 M $CuCl_2$, the gel slice containing the overexpressed protein was excised, and the staining was reversed by washing it with 0.25 M EDTA and 0.25 M Tris, pH 9.0 (15). A Bio-Rad electroeluter (model 422) was used to recover the protein from the gel slice by electroelution for 4 h at a constant current of 10 mA in a solution of 25 mM Tris, 192 mM glycine, and 0.1% (wt/vol) SDS. Five milliliters of preimmune serum was taken from each of two New Zealand White female rabbits, before each rabbit was injected intramuscularly with the protein purified from strain SG13009(pJK1). Further immunization and serum collection were conducted as described previously (15), and the antibodies were purified as follows. Cells from a 1-liter overnight culture of *E. coli* SG13009(pQE60) were harvested, resuspended in 15 ml of Na MOPS buffer (0.5 mM; pH 7), and disrupted by four serial passages through a French pressure cell at 15,000 lb/in^2. The cell lysate was coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia Biotech) as specified by the manufacturer, and the resulting affinity matrix was used to remove contaminating antibodies from the anti-ProQ serum. Fractions containing anti-ProQ antibodies were pooled and stored at −40°C.

Transport measurements. The radial streak test (27) was used to estimate the ProP activities of *E. coli* RM2, WG174, and WG703 (as proline analogue sensitivities). Cultures of *E. coli* RM2 and WG174 were prepared and transport measured by filtration assay essentially as described previously (26). MOPS minimal medium (28) was inoculated (0.5% [vol/vol]) with cells from an overnight culture in LB medium. MOPS medium contained NH_4Cl (9.5 mM) as a nitrogen source, glycerol (5 mg ml^{-1}) as a carbon source, L-tryptophan (245 μM), and thiamine hydrochloride (1 μg ml^{-1}). Upon reaching stationary phase, cells were subcultured in the same medium to achieve an optical density at 600 nm (OD_{600}) of 0.5. After growth to an OD of 1, cells were harvested by centrifugation and washed three times in unsupplemented MOPS medium (MOPS medium lacking phosphate, NH_4Cl, and organic nutrients). The optical density of the cells was adjusted to an OD_{600} of 15.0, and amino acid uptake was measured in an assay mixture consisting of unsupplemented MOPS medium containing K_2HPO_4 (2.64 mM), glucose (2 mg ml^{-1}), and NaCl as indicated. Uptake was initiated by the addition of the substrate L-proline (to a concentration of 200 μM; 10 Ci mol^{-1} [370 kBq μmol^{-1}]), following a 3.0-min preincubation of the cells in the assay mixture. The preincubation time was adjusted for analysis of the kinetics of activation, as indicated. The assay mixture was sampled after 20, 40, and 60 s. All assays were done in triplicate, and all experiments were done at least twice. Each set of replicate assays was used to determine the rate of amino acid uptake over the 20- through 60-s interval. The rates are cited as means ± standard deviations.

Protein assays. Protein concentrations were determined by a bicinchoninic acid assay (34) with a kit obtained from Pierce (Rockford, Ill.), with dilutions of bovine serum albumin as the standard.

Nucleotide sequence accession number. The nucleotide sequence of *proQ* was submitted to GenBank and assigned accession no. L48409.

RESULTS

Isolation and sequencing of *proQ*. Allele *proQ220*::Tn*5* was isolated by selecting bacteriophage Mu *d*II4042-derived recombinant plasmids (10, 40) isolated from *E. coli* WG174 (27), and the *proQ* sequence was determined (extending from nt 1913558 through 1912860 of the *E. coli* genome) (see Materials and Methods). The *proQ* ORF was predicted to encode a 232-amino-acid, basic, hydrophilic protein (molecular mass, 25,876 Da; calculated isoelectric point, 9.66; 32% D, E, R, or K; 54.5% polar amino acids) with no obvious N-terminal secretion signal sequence. The Tn*5* insertion interrupted the sequence at codon E105.

Database analysis indicated few protein sequences similar to ProQ. *Haemophilus influenzae* Rd contains two adjacent ORFs (HI1669 and HI1670, one base out of frame) with strong similarities to the N- and C-terminal sequences of ProQ, respectively (Fig. 1). The ProQ sequence is also weakly related to those encoded in *orfR5* (a gene of unknown function within the conjugal transfer region of *Agrobacterium tumefaciens* octopine-type Ti plasmids) and in *finO* of *E. coli*. FinO is believed to reduce the expression of genes required for the conjugative transfer of F and related plasmids by associating with antisense RNA FinP and its target, the *traJ* transcript (39).

ProQ regulates ProP posttranslationally. The recently published *E. coli* genome sequence facilitated the placement of the *proQ* ORF in relation to its neighbors (Fig. 2). Gene *proQ* (identical to the extended ORF *yebJ*; see GenBank accession no. AE000277) occurs at 41.2 centisomes in the segment of the *E. coli* chromosome flanked by loci *cspC* and *holE* (Table 2). ORF b1832, *proQ*, *prc*, and *htpX* constitute a block of genes known (or predicted) to be transcribed counterclockwise (in contrast to the flanking loci). *E. coli* strains defective in *prc* fail to grow at 42°C on solid hypotonic medium (1/2L medium; see Materials and Methods) and have morphologically elongated cells when grown at 42°C in the corresponding liquid medium (14, 33). The previous observation that insertion *proQ220*::Tn*5* impairs ProP activity was attributed to its disruption of locus *proQ* (27, 36). Locus *prc* is downstream from *proQ*, and a putative *prc* promoter exists within the *proQ* ORF (downstream from the *proQ220*::Tn*5* insertion [nt 1913173] at nt 1913074 to 1913048 [14]). It was therefore important to rule out the possibility that the *proQ* insertion exerted its effects on ProP by disrupting *prc* expression, either through polar effects within an operon including both *proQ* and *prc* or by directly disrupting transcription from a *prc* promoter located within *proQ*.

The phenotypes of *proQ* and *prc* mutants were therefore compared. *E. coli* WG703 (RM2 Δ*prc3*::kan) showed the thermosensitivity and morphology characteristic of *prc* mutants

TABLE 2. Proteins encoded by genes adjacent to *proQ*

Gene or ORF	Position (nt)	Function	Reference(s)
cspC (*msmB*)	1905250–1905459	Cold shock protein; suppressor of chromosomal partitioning defects (*mukB*); eukaryotic DNA-binding protein homologue	20, 46
b1827	1907332–1908123	Similar to the IclR family of transcriptional regulators	
htpX	1909719–1910600	Heat shock protein; unknown function	18
prc (*tsp*)	1910792–1912840	Protease responsible for C-terminal cleavage of PBP3 (FtsI)	14, 33
b1835	1918241–1919686	Similar to GTPase-activating, human proliferating-cell nucleolar protein p120 (GAP)	6
holE	1923132–1923362	Theta subunit of DNA polymerase III	37

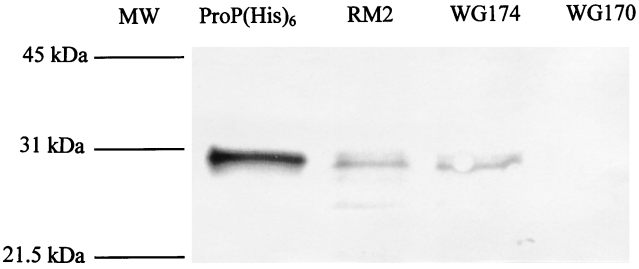

FIG. 3. Mutation *proQ220*::Tn*5* does not alter the level of ProP. Membrane vesicles were prepared from *E. coli* cells grown in NaCl (0.3 M)-supplemented MOPS minimal medium. Membrane proteins (20 μg) were separated by SDS-PAGE, and Western blots were prepared with purified anti-ProP antibodies as described in Materials and Methods. Purified ProP(His)$_6$ (1.3 μg) served as a control. The numbers to the left indicate the positions of molecular size markers (in kilodaltons).

during growth on 1/2L medium at 42°C, but it retained proline analogue sensitivity (indicative of ProP activity) identical to that of *E. coli* RM2 (*proQ*$^+$) and not *E. coli* WG174 (*proQ220*::Tn*5*). In contrast, *E. coli* RM2 and WG174 grew on solid 1/2L medium and did not produce elongated cells during cultivation on the corresponding liquid medium, both at 42°C. Thus the *proQ* and *prc* mutant phenotypes were different, and the effects of the *proQ220*::Tn*5* insertion on ProP were not exerted through *prc*.

By analyzing the impact of *proQ220*::Tn*5* on β-galactosidase activity in bacteria bearing operon fusion *proP227*::Mud1 (*lac* Ap), Milner and Wood (27) showed that the mutation did not alter *proP* transcription. At the time of these experiments the *proP* promoters were not defined, so the position of the fusion in relation to the *proP* promoters was unclear. Subsequent experiments revealed that *proP* is transcribed from two promoters with transcription start sites located 182 bp (P1) and 95 bp (P2) upstream from *proP* (24). In order to ensure that the Mud1 (*lac* Ap) insertion was not between the two promoters, its approximate location was determined as outlined in Materials and Methods. Insertion *proP227*::Mud1 (*lac* Ap) interrupted the *proP* ORF in or after the codon for S375. This observation reinforced the conclusion that the *proQ220*::Tn*5* mutation does not alter the transcription of *proP*.

The fact that ProQ shared some similarity to FinO, a known translational regulator, stressed the importance of examining the effect of the *proQ220*::Tn*5* mutation on the level of ProP. However, mutation *proQ220*::Tn*5* did not influence the level of ProP detected by Western blot analysis in whole cells cultivated in media of elevated osmolarities (data not shown) or in membrane vesicles prepared from cells grown under those conditions (Fig. 3). Thus, neither transcription nor translation of *proP* appears to be altered by mutation *proQ220*::Tn*5*.

ProQ is expressed as a soluble protein in wild-type *E. coli*. The *proQ* ORF was amplified with DNA from *E. coli* K-12 (*proQ*$^+$) as a template and inserted in vector pQE60, yielding plasmid pJK1. This system was designed to amplify the expression of the wild-type *proQ* gene by placing it under the control of an IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible bacteriophage T5 promoter system (Qiagen). An abundant protein with an apparent molecular mass of 30 kDa was present in cells of *E. coli* SG13009(pJK1) which were induced with IPTG but was not in those which were not (data not shown). This protein was contained in a fraction harvested by low-speed centrifugation from a French press lysate of these bacteria, suggesting that it was present as inclusion bodies. It was enriched by washing it with EDTA, deoxycholate, and lysozyme (29), further resolved from contaminants by SDS-PAGE, and eluted from the gel for antibody production. DNA sequence analysis revealed that the *proQ* locus in this plasmid contained mutation C64T, resulting in the predicted protein modification S22P.

To avoid the formation of inclusion bodies and correct the cited mutation, the *proQ* ORF was again amplified, inserted in vector pBAD24 (12) to yield plasmid pDC77, and expressed in strain DH5α(pDC77). Sequence analysis revealed that plasmid pDC77 encoded wild-type ProQ, as expected. Plasmids pJK1 and pDC77 both encoded proteins with apparent molecular masses of 30 kDa, which could be detected by Western blotting with antibodies prepared as described above. The protein expressed from plasmid pDC77 was not concentrated in the pellet obtained by low-speed centrifugation of a French press lysate. It was most abundant in the supernatant obtained after subsequent ultracentrifugation (Fig. 4) and was therefore a soluble protein.

The expression of the putative ProQ protein in *E. coli* RM2 carrying *proQ*$^+$ was analyzed by SDS-PAGE and Western blotting. A protein with an apparent molecular mass of 30 kDa was detected in the soluble fraction from strain RM2 but not in that from strain WG174 carrying *proQ220*::Tn*5* (Fig. 5), suggesting that the 30-kDa protein is the *proQ* gene product.

Mutation *proQ220*::Tn*5* impairs activation of ProP. The rates of proline uptake via ProP in *proQ*$^+$ and *proQ220*::Tn*5*

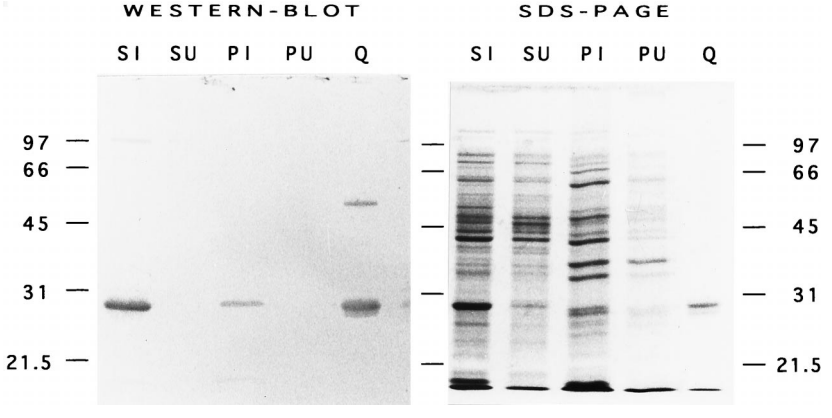

FIG. 4. Expression of *proQ* with vector pBAD24 yields a soluble protein with a molecular mass of 30 kDa. Shown are a Western blot, visualized with the BCIP/NBT reagent system, and an SDS-PAGE analysis of the soluble (S) and particulate (P) fractions derived from *E. coli* DH5α(pDC77) with (I) or without (U) induction of protein expression by arabinose (2 mg/ml). These are compared with the 30-kDa protein overexpressed by *E. coli* SG13009(pJK1) (Q). The numbers indicate the positions of molecular size markers (in kilodaltons).

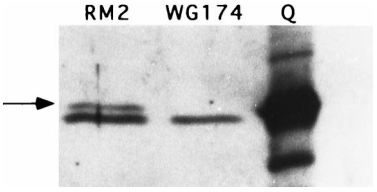

FIG. 5. The ProQ protein is expressed by *E. coli*. A Western blot, visualized with the ECL reagent system, is shown. Soluble fractions derived from *E. coli* RM2 and WG174 and the 30-kDa protein overexpressed by *E. coli* SG13009 (pJK1) (Q) were probed with antibodies raised against the latter protein. The arrow marks the 30-kDa immunoreactive protein that is present in *E. coli* RM2 carrying *proQ*$^+$ but not in *E. coli* WG174 carrying *proQ220*::Tn*5*.

bacteria were measured as a function of the NaCl concentration employed to impose an osmotic upshift (Fig. 6). (The proline uptake observed under these conditions is attributable to transporter ProP, since it is absent from bacteria further defective in locus *proP* [27].) As previously determined, the optimal upshift for activation of ProP in wild-type bacteria was imposed with approximately 0.12 M NaCl. ProP activity was increased sevenfold to a maximum of 22 nmol/min/mg of protein. Activation of ProP in the *proQ220*::Tn*5* strain occurred over a similar range of NaCl concentrations but was very limited (only a threefold increase to a maximum of 2.5 nmol/min/mg of protein). The kinetics of ProP activation after an osmotic upshift imposed with 0.12 M NaCl were determined for *proQ*$^+$ and *proQ220*::Tn*5* bacteria (Fig. 7). The data were fitted to a model describing an exponential increase in ProP activity, postshift (26; Fig. 7). The level of ProP activity approached by the *proQ* mutant bacteria (4.8 ± 0.2 nmol/min/mg of protein) was fivefold lower than that approached by the wild-type strain (22.7 ± 0.3 nmol/min/mg of protein). The rate constant (k) for activation of ProP was reduced 2.6-fold in the *proQ* mutant (from 0.75 ± 0.04 to 0.29 ± 0.04 s^{-1}). Thus, insertion *proQ220*::Tn*5* reduced both the rate and the extent of ProP activation by an osmotic upshift.

The ProQ protein expressed from plasmid pDC77 was capable of complementing the *proQ220*::Tn*5* defect. The proline uptake activities of bacteria cultivated in MOPS minimal medium and subjected to an osmotic upshift (0.12 M NaCl) were 14 ± 4 nmol/min/mg of protein for *E. coli* RM2 carrying *proP*$^+$ *proQ*$^+$, 3 ± 1 nmol/min/mg of protein for *E. coli* WG174 (pBAD24) carrying *proP*$^+$ *proQ220*::Tn*5*, and 12 ± 2 nmol/min/mg of protein for *E. coli* WG174(pDC77). The levels of the ProQ protein expressed by *E. coli* RM2 and WG174(pDC77) were comparable under these conditions, and no ProQ protein was detected in *E. coli* WG174(pBAD24) (Western blotting data not shown). Thus, plasmid-based expression of *proQ* restored ProP activity to wild-type levels in bacteria harboring a chromosomal *proQ* mutation.

DISCUSSION

Cells from diverse organisms can accumulate similar arrays of organic compounds, all known to be compatible with and/or to stabilize protein structure, when challenged by hypertonic

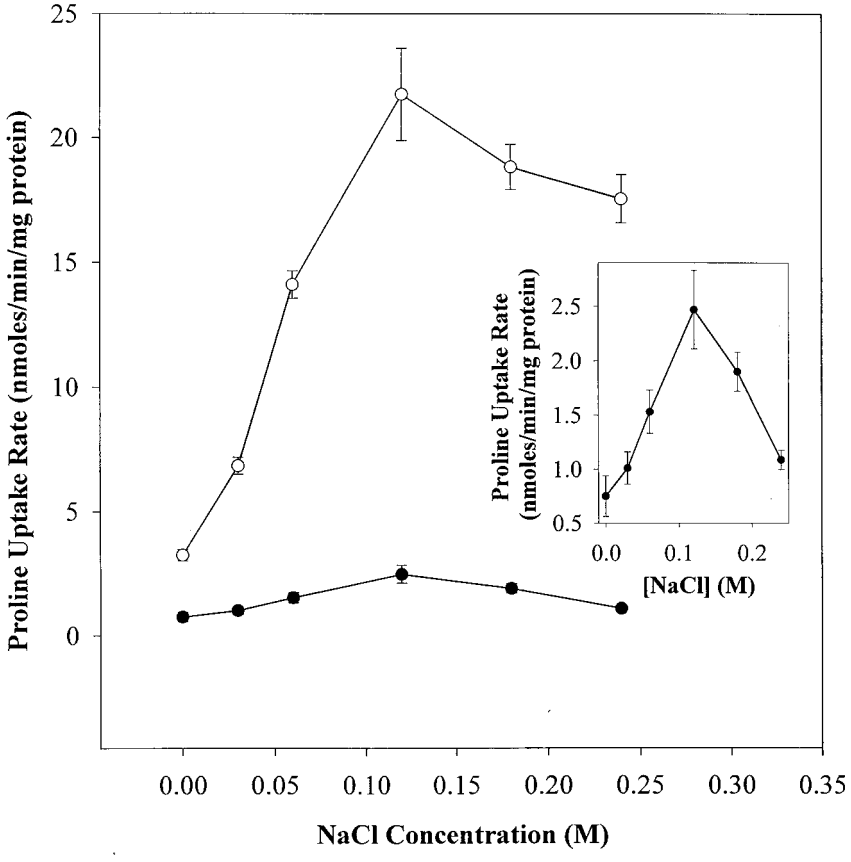

FIG. 6. Mutation *proQ220*::Tn*5* impairs ProP activation. *E. coli* RM2 carrying *proQ*$^+$ (white circles) and WG174 carrying *proQ220*::Tn*5* (black circles) were cultivated in MOPS medium, and proline uptake rates were measured as described in Materials and Methods. Supplementary NaCl was added to the transport assay mixtures at the indicated levels. Error bars indicate standard deviations.

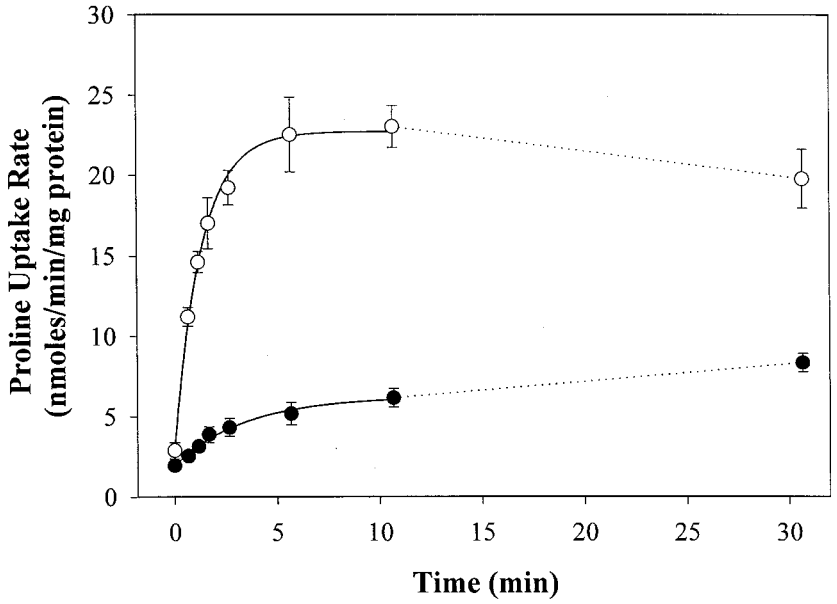

FIG. 7. Mutation *proQ220*::Tn*5* reduces the rate and extent of ProP activation. *E. coli* RM2 carrying *proQ*$^+$ (white circles) and WG174 carrying *proQ220*::Tn*5* (black circles) were cultivated in MOPS medium, and proline uptake rates were measured as described in Materials and Methods. Cells were incubated in NaCl (0.12 M)-supplemented uptake assay mixtures for the indicated periods, before radiolabelled proline was added to initiate the uptake assay. Solid lines represent the outcome of nonlinear regression analyses performed as described by Milner et al. (26). Error bars indicate standard deviations.

environments (35). The machinery of compatible solute accumulation has been described for some organisms (e.g., *E. coli* [3, 4, 42]), but its regulation is not well understood. Although proteins Fis and CAP are involved, no *trans*-acting transcriptional regulatory element specific to locus *proP* has been implicated in the impressive modulation of its transcription by osmotic stress. Transporter ProP is activated, in the absence of protein synthesis, when whole bacteria (11), cytoplasmic membrane vesicles (26), or proteoliposomes incorporating purified ProP (30) are subjected to an osmotic upshift with a membrane-impermeant osmolyte. Our research is designed to elucidate the mechanisms by which ProP senses osmolality changes and mounts its osmoregulatory response. Since ProP activity is impaired by insertion *proQ220*::Tn*5* (27, 36), we are exploring the structure and function of *proQ* as well as its relationship to ProP.

In this study we establish that the effects of the insertion on ProP are due to the altered expression of locus *proQ* and not to polar effects on downstream locus *prc* (see the text and Fig. 2 and 5). The previous conclusion that the Tn*5* insertion in *proQ* does not influence *proP* transcription was confirmed. Database analysis identified two proteins with weak sequence similarities to ProQ. Within this group of homologues, the relationship that appeared most interesting was the weak similarity with translational regulator FinO. This raised the possibility that ProQ could be acting at a translational level to alter the levels of ProP protein. However, this study has shown that the level of ProP protein present in either whole cells (data not shown) or membrane vesicles is not altered by the Tn*5* insertion in locus *proQ* (Fig. 3).

This study has further shown that the rate and extent of ProP activation are significantly reduced in a *proQ220*::Tn*5* strain of *E. coli*. These reports are significant in documenting the only *trans*-acting factor which is known to influence the osmotic activation of ProP. Gene *proQ* is predicted to encode a 232-amino-acid protein that is both basic and hydrophilic in nature. SDS-PAGE and Western blot analysis indicate that the over-expressed ProQ protein is soluble, as predicted (Fig. 4). The subcellular location of the protein in wild-type bacteria remains to be determined, however.

ProP activity is observed in cytoplasmic membrane vesicles (26) and proteoliposomes prepared with purified ProP (30). There are some significant differences between the ProP activities of these vesicle systems and those of whole cells, however. The hyperosmotic shift which gives maximal ProP activity in cells (0.2 osM) is lower than that required in membrane vesicles (0.8 osM) (22). As well, ProP is active in whole cells even without an osmotic shift, whereas ProP activities in proteoliposomes and membrane vesicles absolutely require a hyperosmotic shift (22, 30). Given these contrasting features of ProP activities in cells and vesicle systems, we now believe that ProQ is a structural element which influences the osmotic activation of ProP at a posttranslational level.

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