Salinity-Dependent Impacts of ProQ, Prc, and Spr Deficiencies on *Escherichia coli* Cell Structure

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ProQ is a cytoplasmic protein with RNA chaperone activities that reside in FinO- and Hfq-like domains. Lesions at *proQ* decrease the level of the osmoregulatory glycine betaine transporter ProP. Lesions at *proQ* eliminated ProQ and Prc, the periplasmic protease encoded by the downstream gene *prc*. They dramatically slowed the growth of *Escherichia coli* populations and altered the morphologies of *E. coli* cells in high-salinity medium. ProQ and Prc deficiencies were associated with different phenotypes. ProQ-deficient bacteria were elongated unless glycine betaine was provided. High-salinity cultures of Prc-deficient bacteria included spherical cells with an enlarged periplasm and an eccentric nucleoid. The nucleoid-containing compartment was bounded by the cytoplasmic membrane and peptidoglycan. This phenotype was not evident in bacteria cultivated at low or moderate salinity, nor was it associated with murein lipoprotein (Lpp) deficiency, and it differed from those elicited by the MreB inhibitor A-22 or the Ftsl inhibitor aztreonam at low or high salinity. It was suppressed by deletion of *spr*, which encodes one of three murein hydrolases that are redundantly essential for enlargement of the murein sacculus. Prc deficiency may alter bacterial morphology by impairing control of Spr activity at high salinity. ProQ and Prc deficiencies lowered the ProP activity of bacteria cultivated at moderate salinity by approximately 70% and 30%, respectively, but did not affect other osmoregulatory functions. The effects of ProQ and Prc deficiencies on ProP activity are indirect, reflecting their roles in the maintenance of cell structure.

Osmotic stress perturbs cell structure, composition, and function (1). Despite retaining their rod-like shape, *Escherichia coli* cells cultivated in high-salinity minimal medium maintain lower hydration, turgor pressure, and growth rate than those cultivated at a lower salinity that is optimal for growth (2). The elastic murein sacculus is believed to buffer effects of osmotically induced water fluxes on cell structure (3). *E. coli* can attenuate osmotically induced dehydration by accumulating small, uncharged, or zwitterionic organic solutes called osmolytes (1, 4, 5). For example, transporter ProP mediates the accumulation of diverse solutes, including proline and glycine betaine, thereby restoring cellular hydration and stimulating bacterial growth in high-salinity media (6, 7).

ProQ is a cytoplasmic protein that binds RNA, facilitating RNA duplexing and strand exchange (8). Previous work showed that *proQ* lesions decreased ProP levels and attenuated ProP activity (the *proQ* transport phenotype). These effects occurred when bacteria expressed ProP from the chromosome or a plasmid-based *pBAD* promoter during growth in low- to moderate-salinity media and were reversed by plasmid-based *proQ* expression (8, 9). Here, we show that *proQ* lesions dramatically slow the growth of *E. coli* populations in high-salinity medium and alter the morphologies of *E. coli* cells (the *proQ* growth and morphological phenotypes). They also impair expression of the downstream locus *prc*. Periplasmic protease Prc (also known as Tsp) has been implicated in cell division and protein quality control (10–14).

This report further defines the impacts of *proQ* and *prc* lesions on *E. coli* cell structure, associates ProQ and Prc deficiencies with distinct mutant phenotypes, and shows that *proQ* lesions do not impair osmoregulatory systems other than ProP. These data suggest that the *proQ* transport phenotype reflects more fundamental roles of ProQ and Prc in the maintenance of cell structure.

MATERIALS AND METHODS

**Bacterial strains and plasmids.** The relevant genotypes and immediate ancestors of the *E. coli* strains and the plasmids used for this study are listed in **Tables 1** and **2**. The kanamycin (Km) resistance cassettes from Keio collection isolates JW5300-1 (*ΔproQ756::kan*) and JW2163 (*Δspr732::kan*) (15) were introduced to strain RM2 by P1 transduction, then deleted as described by Datsenko and Wanner (16) to create deletions in the *proQ* and *spr* loci (*ΔproQ856::FRT* and *Δspr832::FRT*, respectively). Transductions were performed with phage P1 *cml.clr_100* or P1 vir as described by Miller (17).

Routine DNA manipulation, plasmid construction, electrophoresis, and transformation were carried out as described previously (18, 19). Oligonucleotides were purchased from Operon Technologies (Eurofins MWG Operon, Huntsville, AL). PCR was performed as described previously (20). Plasmid pDC77 was created by replacing a fragment of vector pBAD24 (21) flanked by NcoI and HindIII restriction sites with a DNA fragment extending from an NcoI site overlapping the *proQ* initiation codon through a HindIII site adjacent to the *proQ* termination codon (9). Plasmid pCK3 was created by replacing a fragment of vector pBAD33 (21) flanked by SacI and Sall restriction sites with a PCR amplicon obtained using primers *prc*-1, 5′-GGAGAGTCAGAGGAACTGCAATGACAACATGACAGCCGAGCCGGG-3′, and *prc*-2, 5′-GGATCGATTACCTTGAACGCGAGCGGGG-3′, with chromosomal DNA from *E. coli* MG1655 as a

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the template. GFPmut2 was carried by plasmid pMG053 and expressed from a lac promoter under the control of LacI carried in trans by pREP4 (22). A variant of GFPmut3 that localizes to the periplasm of E. coli cells was carried on plasmid pJW1 (23).

**Bacterial cultures.** Bacteria were cultivated in Luria-Bertani (LB) medium (17) or in morpholinopropanesulfonic acid (MOPS) medium (24) with NH₄Cl (9.5 mM) as nitrogen source and glycerol (0.4% [vol/vol]) as carbon source and tryptophan (245 μg/ml) and thiamine (1 mg/ml) to meet auxotrophic requirements. Ampicillin (100 μg/ml), kanamycin (50 μg/ml), and chloramphenicol (30 μg/ml) were added to maintain plasmids. Media osmolalities were adjusted with NaCl and measured as specified by the manufacturer with a vapor pressure osmometer (Wescor, Logan, UT). Cultures were grown at 37°C in a rotary shaker at 200 rpm. Optical densities were monitored with a Bausch and Lomb Spectronic 88 or a Pharmacia Novaspec II spectrometer. A225 (5 μCi/mmol) was incorporated into [1-14C]glycine betaine as the substrate of 0.75 mol/kg, using [1-14C]glycine betaine as the substrate (10 μM; 5 Ci/mole).

**Trehalase assay.** Trehalase accumulation was detected essentially as previously described (26). Bacteria were cultivated in MOPS minimal medium adjusted with NaCl to achieve the desired salinity, and ProP activity was measured using L-[U-14C]proline as the substrate (200 μM; 5 Ci/mole), as described before (23). ProU activity was measured in the same way, at an osmolality of 0.75 mol/kg, using [1-14C]glycine betaine as the substrate (10 μM; 5 Ci/mole).

**Transport assays.** Bacteria were grown in MOPS minimal medium adjusted with NaCl to achieve the desired salinity, and ProP activity was measured using L-[U-14C]proline as the substrate (200 μM; 5 Ci/mole), as described before (23). ProU activity was measured in the same way, at an osmolality of 0.75 mol/kg, using [1-14C]glycine betaine as the substrate (10 μM; 5 Ci/mole).

**Microscopy.** Bacteria were cultivated as for transport assays in MOPS medium supplemented with NaCl (25), and samples were prepared for microscopy as previously described (28). Nucleoids were stained with DAPI (4′,6-diamidino-2-phenylindole; 50 μg/ml), and membranes were stained with FM4-64 [N-[3-triethylammoniumpropyl]-4-(6-(4-(diethylamino)phenyl)hexatrienyl) pyridinium dibromide; 20 μg/ml] as previously described (28). Bacterial viability was tested with the BacLight stain (all stains were from Invitrogen, New London, CN, Canada).

Expression of periplasmic green fluorescent protein (GFP; carried on pJW1) was induced for 45 min by adding anhydrotretracycline (Acros Organics, Geel, Belgium; 15 μg/ml) to the final subculture. After 45 min, expression was stopped by harvesting and resuspending the cells in MOPS medium lacking anhydrotretracycline. The culture was then incubated for a postinduction period of 8 h to attain the desired optical density. Expression of cytoplasmic GFP was not induced.

The peptidoglycan layer was stained by treating cells with HADA (hydroxyly coumarin-carbonyl amino-D-alanine), or with HALA (hydroxyly coumarin-carbonyl amino-L-alanine), or with HALA (hydroxyly coumarin-carbonyl amino-D-alanine) as a negative control, essentially as described by Kuru et al. (29). A stock solution was added to the final 1-ml subculture to bring the culture to 0.5 mM in HADA or HALA and to 1% in dimethyl sulfoxide. The cells were harvested by centrifugation (1 min, 2,000 rpm), 0.9 ml of supernatant was removed, and the cells were resuspended in the remaining medium. To remove the excess HADA, the resulting suspension was applied to a microBioSpin chromatography column packed with 0.1 g of Bio-Gel P-6GD (Bio-Rad Laboratories, Canada, Ltd., Mississauga, ON, Canada) in bacterial growth medium. The column was centrifuged at 2,000 rpm until no more cells were eluted.

**SDS-PAGE and Western blotting.** SDS-PAGE (30) was performed with gels containing 12% (wt/vol) polyacrylamide and 1% (wt/vol) bisacrylamide (for detection of protein ProX) or 10% (wt/vol) polyacrylamide.

**TABLE 2 Plasmids used in this study**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Source</th>
</tr>
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<tbody>
<tr>
<td>pCK3</td>
<td>prc in vector pBAD33 (21)</td>
<td>This study</td>
</tr>
<tr>
<td>pDC77</td>
<td>prQ in vector pBAD24 (21)</td>
<td>9</td>
</tr>
<tr>
<td>pMG053</td>
<td>Carries gene for GFPmut2 (21) inserted into vector pQE12 (Qiagen, Inc.)</td>
<td>22</td>
</tr>
<tr>
<td>pJW1</td>
<td>Carries gene for periplasm-targeted GFPmut2* in vector pASK-IBA3plus (IBA, Göttingen, Germany)</td>
<td>23</td>
</tr>
<tr>
<td>pREP4</td>
<td>Harbors LacI*</td>
<td>Qiagen, Inc.</td>
</tr>
</tbody>
</table>

*All strains were derived from E. coli CSH4 (trp lacZ rpsL thi) (73, 74); RM2 is CSH4 ΔputPAl01 (71).
amide and 0.9% (wt/vol) bis-acrylamide (for detection of proteins ProQ and Prc). Tricine-SDS-PAGE (31) was performed with a separating gel containing 15.5% (wt/vol) polyacrylamide and 1% (wt/vol) bis-acrylamide (for detection of protein Lpp). Gels were stained with Gel-Code Blue (Pierce, Rockford, IL) according to the manufacturer’s instructions. Western blotting was performed to detect ProX, ProQ, Prc, or Lpp, as described previously (32, 33). Western blots were visualized with enhanced chemiluminescence reagents (GE Healthcare, Baie d’Urfé, QC, Canada) according to the manufacturer’s instructions.

RESULTS

High-salinity slows growth and elicits morphological defects in bacteria with proQ mutations. The initial aim of this study was to further characterize the impacts of proQ lesions on the osmoregulatory systems of E. coli. It immediately became apparent that, in contrast to their proQ<sup>+</sup> parent, proQ220::Tn5 bacteria grew very slowly at high salinity (Fig. 1, inverted closed versus inverted open triangles; doubling times of 1.9 h and 3.9 h, respectively). Strain WG1119 (ΔproQ856::FRT) also grew much more slowly at high salinity than its proQ<sup>+</sup> parent (data not shown). DIC microscopy revealed that bacteria with the proQ deletion were elongated after growth at low or high salinity (Fig. 2A). Long cells, swollen cells, enlarged spherical cells, and debris suggestive of cell lysis were evident in micrographs of the high-salinity cultures (Fig. 2, compare panels G and D). Highly refractive, eccentric internal structures were evident within many of the spherical cells (see Fig. S1E in the supplemental material). The spherical cells were viable, as indicated by BacLight staining, although they tended to lyse (data not shown).

Lesions in proQ impair prc expression. The prc locus follows proQ on the E. coli chromosome; the two are separated by only 20 bp, and a putative prc promoter is located within proQ (Fig. 3). Insertion proQ220::Tn5 is upstream from the putative prc promoter, and deletion ΔproQ856::FRT removes it (Fig. 3). Bacteria with prc defects fail to grow at 42°C on solid ½L medium (NaCl-free, half-strength LB), and they form elongated cells when cultured at 42°C in liquid ½L medium (10). We had earlier concluded that the proQ transport phenotype did not result from effects of proQ mutations on prc expression, because those prc phenotypes were not shared by proQ mutant bacteria (9). This question was reconsidered in view of the observations reported above.

Unlike their prc<sup>+</sup> proQ<sup>+</sup> counterparts, bacteria with the mutation Δprc3::kan proQ220::Tn5 or ΔproQ856::FRT lacked the Prc protein (Fig. 4A). (Note that the band immediately below the Prc band, shared in all samples, represents a protein that is not related to Prc [M. Ehrmann, personal communication].) ProQ was absent from the proQ mutant bacteria, and the ProQ level increased with growth medium salinity, as expected (8). In addition, deletion of prc lowered the level of ProQ in bacteria cultivated at low or high salinity (Fig. 4B). Thus, proQ mutant bacteria are ProQ<sup>-</sup> Prc<sup>-</sup>, and prc mutant bacteria are ProQ<sup>+</sup> Prc<sup>-</sup>.

Morphological phenotypes associated with Prc and ProQ deficiencies. Further imaging and complementation analyses associated different morphological phenotypes with ProQ and Prc deficiencies. Fluorescence microscopy of bacteria with mutations in prc or proQ cultivated at high salinity (250 mM NaCl) revealed spherical cells bounded by FM4-64-stained membranes. Eccentric, DAPI-stained nucleoids within these cells were also surrounded by FM4-64-stained membranes (Fig. 5). Many of the spherical cells were much larger than the rod-shaped cells characteristic of wild-type E. coli (Fig. 5, compare with the images of proQ<sup>−</sup> prc<sup>−</sup> bacteria in the top right panel of Fig. 6A; bars designate 2.5 µm in both cases).

To identify the inner and outer membranes evident within the spherical cells, GFP variants targeted to the cytoplasm and periplasm were expressed in proQ<sup>−</sup> and proQ mutant bacteria. Periplasmic targeting via the twin-arginine translocase pathway was achieved by adding a 39-amino-acid TorA signal sequence to Prc [M. Ehrmann, personal communication]. ProQ was ablated from the proQ mutant bacteria, and the ProQ level increased with growth medium salinity, as expected (8). In addition, deletion of prc lowered the level of ProQ in bacteria cultivated at low or high salinity (Fig. 4B). Thus, proQ mutant bacteria are ProQ<sup>-</sup> Prc<sup>-</sup>, and prc mutant bacteria are ProQ<sup>+</sup> Prc<sup>-</sup>.

Complementation analysis to differentiate the proQ and prc mutant phenotypes was based on strain WG1119, in which ΔproQ856::FRT eliminates expression of both proQ and prc, and strain WG703 (proQ<sup>+</sup> Δprc3::kan), which expresses proQ but not prc (Fig. 4). Complementation with a plasmid carrying prc (pCK3) restored Prc expression to both Δprc and ΔproQ bacteria (Fig. 7D). In contrast, complementation with a plasmid carrying proQ (pDC77) restored proQ expression to ΔproQ bacteria (Fig. 7E), but it did not complement the Prc deficiency arising from effects of the chromosomal proQ mutation on prc (Fig. 7D). The levels of ProQ and Prc in bacteria harboring plasmids pDC77 and pCK3 were comparable to those in parent strain RM2 (proQ<sup>+</sup> prc<sup>-</sup>) after cultivation in the same high-salinity medium (Fig. 7D and E).

Growth at high salinity caused bacteria lacking only prc (Δprc3::kan) to form spherical cells (Fig. 2H and 5; see also Fig. S1F in the supplemental material) but not elongated rod-shaped cells (Fig. 2B). Plasmid-based expression of prc during
The growth of *E. coli* WG1119 at high salinity eliminated spherical cells and restored a more normal growth rate (a doubling time of 2.6 h) but not a normal cell length distribution (Fig. 7A). Thus, the formation of spherical cells was due to Prc deficiency, whereas cell elongation was associated with ProQ deficiency. Plasmid-based expression of *proQ* restored a more normal cell length distribution (Fig. 7B) and growth rate to *H9004 proQ856::FRT* bacteria grown at high salinity (the doubling times for strains WG1119 and WG1119 pDC77 were 3.3 h and 2.8 h, respectively), but it did not eliminate spherical cells (Fig. 7B). This was expected, since the bacteria remained Prc deficient (Fig. 7D). With both plasmids, cell morphology became normal (Fig. 7C) but culture growth was still impaired (doubling time, 3.6 h). These results indicate that ProQ-based expression is required for cell elongation in these conditions.

**FIG 2** Impacts of *proQ* and *prc* lesions on bacterial morphology. Bacteria were cultivated in MOPS medium to late exponential phase as described for transport assays (25) and visualized by DIC microscopy (see Materials and Methods). (A and B) Media were unsupplemented (0.25 mol kg⁻¹; closed symbols) or supplemented with 250 mM NaCl (0.75 mol kg⁻¹; open symbols). Length distributions are shown for 100 rod-shaped cells of strains RM2 (circles; *proQ⁺ prc⁺*) and WG1119 (squares; RM2 ΔproQ856::FRT (A) and RM2 (circles) and WG703 (squares; RM2 Δprc::kan) (B). Subsequent experiments revealed that ΔproQ856::FRT renders the bacteria ProQ and Prc deficient (see Fig. 4). For strain WG1119, 3% of measured cells in cultures at low osmolality and 4% of measured cells in cultures at high osmolality were greater than 6 µm in length. No measured cells in cultures of strain RM2 or WG703 at low or high osmolality were greater than 6 µm in length. (C to H) Representative DIC micrographs are shown for strains RM2 (C and F), WG1119 (D and G), and WG703 (E and H) cultivated in unsupplemented medium (low NaCl; C, D, and E) or NaCl-supplemented medium (high NaCl; F, G, and H). Spherical cells with highly refractive, crescent-shaped internal structures are evident in panels G and H. (In Fig. S1 in the supplemental material, the internal structures are more evident in the corresponding panels [E and F].) Bars, 10 µm.

**FIG 3** Organization of *proQ*, *prc*, and flanking loci The *prc* locus follows *proQ* on the *E. coli* chromosome. A putative σ²⁶ promoter for *prc*, with a predicted transcription start site at nucleotide (nt) 1,913,051, falls within the *proQ* open reading frame (at 1,912,860 to 1,913,558) (10, 60). Insertion *proQ220::Tn5* is upstream from the putative *prc* promoter (9), whereas deletion ΔproQ856::FRT removes it (the start codon and the final six codons are retained [16]). A *proQ-prc* transcript may initiate upstream of *proQ* (61). No promoter or transcription start site has been identified for upstream locus msrC (also known as *yebR*), which encodes a methionine-(R)-sulfoxide reductase (75). Independent transcription of the downstream *htpX* locus is mediated by σ⁵⁴ (76).
confirmed that cell elongation results from consequences of proQ defects other than Prc deficiency.

**Origins of spherical cell formation by prc mutant bacteria.** HADA is a fluorescent analogue that is incorporated into peptidoglycan in place of D-alanine (29). To locate the murein layer in Prc-deficient cells, strains WG1119 (ΔproQ856::FRT [phenotypically ProQ−Prc−]) and WG703 (ΔproQ3::kan [phenotypically ProQ−Prc−]) were grown in MOPS medium supplemented with 250 mM NaCl as described in the legend for Fig. 2. Cells from late-exponential-phase cultures were stained with DAPI (the nucleoid; blue fluorescence) and FM4-64 (the membranes; red fluorescence). Micrographs of representative spherical cells are shown. Bar, 2.5 μm.

**FIG 4** Impacts of proQ and prc lesions on Prc, ProQ, and Lpp protein levels. E. coli strains RM2 (proQ+ prc+), WG174 (RM2 proQ220::Tn5 [phenotypically ProQ−Prc−]), WG1119 (RM2 ΔproQ856::FRT [phenotypically ProQ−Prc−]), and WG703 (RM2 ΔproQ3::kan [phenotypically ProQ−Prc−]) were grown in MOPS medium supplemented with 120 mM NaCl (intermediate NaCl) or 250 mM NaCl (high NaCl). Aliquots of cell extracts containing 15 μg of protein were analyzed by Western blotting to detect Prc (A) or ProQ (B), and aliquots containing 1.5 μg of cell protein were analyzed to detect Lpp (C). In panel A, the band immediately below Prc, shared in all samples, represents a protein that is not related to Prc (M. Ehrmann, personal communication). Panel D shows a corresponding GelCode Blue-stained SDS-PAGE gel. M, molecular weight markers; arrows, locations of Prc, ProQ, and Lpp.

**FIG 5** Bacteria with mutations in proQ or prc form spherical cells at high salinity. Derivatives of E. coli strains WG703 (Δprc3::kan), WG1119 (ΔproQ856::FRT), and WG174 (proQ220::Tn5) containing plasmid pMGS053 (carrying the gene for cytoplasmic green fluorescent protein [Cyto-GFP]) or plasmid pJW1 (carrying the gene for periplasmic GFP [Peri-GFP]) were cultivated in MOPS medium supplemented with 250 mM NaCl as described in the legend for Fig. 2. Cells from late-exponential-phase cultures were stained with DAPI (the nucleoid; blue fluorescence) and FM4-64 (the membranes; red fluorescence). Micrographs of representative spherical cells are shown. Bar, 2.5 μm.
rate and suppressed spherical cell formation by
in MOPS medium without or with 250 mM NaCl (low NaCl or high NaCl) and
reovanam on the morphology of
cells (reviewed in references 37 and 38). Prc cleaves the periplas-
matic cross-links, allowing insertion of new material and enlargement of
asases that are redundantly essential for cleavage of peptidoglycan
thesis at low salinity (Fig. 2E). To determine whether cleavage by
of ProQ by Prc is not essential for bacterial growth and morpho-
genesis at low salinity (Fig. 2E). To determine whether cleavage by
Pr is important for PBP3 activity during bacterial growth at high salinity,
bacteria lacking Prc but not ProQ (Fig. 2H and 5) were
pared with ProQ$^{+}$ Prc$^{+}$ bacteria in which aztreonam inhibited
PBP3 during growth at high salinity (Fig. 6A). These cells were
very different (spheres versus filaments), so we concluded that the
phenotype of Prc-deficient bacteria cultivated at high salinity does
not arise because cleavage of PBP3 by Prc is required for PBP3 activity at high salinity.

Spr is a murein D,D-endopeptidase, one of three murein hydro-
lases that are redundantly essential for cleavage of peptidoglycan
links, allowing insertion of new material and enlargement of
the murein sacculus of E. coli (43). Mutations in spr restore the
growth of prc mutant bacteria at high temperature and low osmo-
lity (43, 44). Deletion of spr also restored a more normal growth rate and suppressed spherical cell formation by \(\Delta\text{prc}\) bacteria at high salinity (Fig. 8B and H). Thus, the spherical cell phenotype of Prc-deficient bacteria may result at least in part from un-
controlled Spr activity.

ProQ and Prc deficiencies affect ProP differently but do not
affect other osmoregulatory systems. The proQ locus was originally
identified via effects of proQ lesions on ProP activity (45, 46), so the effects of proQ lesions on transporter ProP are well charac-
terized (8, 9, 47). The initial aim of this study was to define the
effects of ProQ deficiency on other osmoregulatory functions. For
E. coli K-12, cultivation in high-osmotic pressure medium activ-
ates trehalose accumulation mediated by trehalose-6-phosphate
synthase (OtSA) and phosphatase (OtSB). The accumulation of
other osmoles can be mediated by transporters ProP, ProU, and
BetF. ProU is an ABC transporter encoded by the proU operon
and comprised of an ATP-binding cassette (ProV) and mem-
brane-integral (ProW) and periplasmic (ProX) subunits. ProP
and ProU transport proline, glycine betaine, and related compounds, whereas choline is the primary BetF substrate (48).

The impacts of a proQ defect on transporter ProU were as-
essessed, because independent work had revealed suppression of the
proQ transport phenotype by deletions within the proU operon
(49). Insertion proQ220::Tn5 did not affect either glycine betaine
uptake via ProU (Fig. 9A) or the level of the ProX protein (Fig.
9B), despite dramatically attenuating bacterial growth (Fig. 1). This
was consistent with a previous report based on qualitative data (46).

E. coli was cultivated at high salinity without organic osmoprotectants to induce proU expression for measurements of ProU activity (Fig. 9A). Under these conditions, E. coli can also osmo-
regulate by accumulating K$^+$-glutamate and then replacing that
salt with trehalose (50, 51). A compound with the R$_s$ of trehalose
was detected upon chromatographic analysis of cell extracts pre-
pared from both proQ$^{+}$ and proQ mutant bacteria after cultiva-
tion at high salinity (Fig. 9C). As expected, that material was not
 detected in extracts from bacteria cultivated at low salinity (Fig.
9C). Thus, the slow growth (Fig. 1) and morphological phenotypes (Fig. 2 and 5) of proQ mutant strain bacteria did not result from failure to accumulate trehalose.

The impacts of ProQ and Prc deficiencies on ProP activity were also reevaluated. In previous work, radial streak tests revealed no impact of \(\Delta\text{prc}\)kan on ProP activity (9). Transport assays con-
firmed that ProP activity was not eliminated by the prc mutation,
but ProP activity was lowered and its dependence on assay med-
ium osmolarity was altered (Fig. 10A). The bacteria used for these
assays were cultured at an intermediate salinity that is optimal for
ProP expression and function (MOPS medium with 0.12 M NaCl)
but does not elicit spherical cell formation. Complementation with plasmid-harbored Prc fully restored Prc expression (Fig. 7D)
and ProP activity (Fig. 10B). Lesions within proQ, which block
expression of proQ and pcr, impaired ProP activity more severely
than did prc defects (Fig. 10A) (9). Complementation with plas-
mid-encoded ProQ restored expression of ProQ (Fig. 7E) but not
Pc (Fig. 7D), and it restored ProP activity only to levels charac-
teristic of prc mutant bacteria (Fig. 10C). Thus, both ProQ and Prc
are required for full ProP activity under conditions that do not
elicit spherical cell formation. ProQ and Prc are not required by
other osmoregulatory systems (trehalose synthesis or glycine betaine transport via ProU [Fig. 9]).

Glycine betaine accumulation suppresses phenotypes asso-
ciated with ProQ but not Prc deficiency. The data discussed above show that both ProQ and Prc are required for normal cell
growth at high salinity. To further explore the relationship be-
tween the proQ transport and morphological phenotypes, we de-
termined the impact of ProP-mediated glycine betaine accumu-
lation on the morphological phenotypes. Cell length returned to the
normal range (see Fig. S2 in the supplemental material), and the
incidence of spherical cells was dramatically reduced when bacte-
ria lacking ProQ and Prc were cultivated in glycine betaine-sup-

FIG 6 Determinants of bacterial morphology. (A) Impacts of A-22 and az-
treonam on the morphology of E. coli. Strain RM2 (proQ$^{+}$ prc$^{+}$) was cultivated
in MOPS medium without or with 250 mM NaCl (low NaCl or high NaCl) and
without or with A-22 (5 $\mu$g/ml) or aztreonam (1 $\mu$g/ml) as described in Ma-
terials and Methods. Cells were stained with DAPI (the nucleoid; blue fluores-
cence) and FM4-64 (the membranes; red fluorescence), and images were ob-
tained as described in Materials and Methods. Bars, 2.5 $\mu$m. (B) Location of
the peptidoglycan layer. Bacteria were cultivated in MOPS medium with 250
mM NaCl (high NaCl) in the presence HADA (peptidoglycan; blue fluores-
ence) and stained with FM4-64 (membranes; red fluorescence) as described in
Materials and Methods. Bars, 2.5 $\mu$m.
implemented, high-salinity medium (see Fig. S1 in the supplemental material; compare panels H and E). Notably, the accumulation of K\+/-glutamate and trehalose did not have this effect (Fig. 2A and G). In contrast, glycine betaine did not suppress spherical cell formation by bacteria lacking only Prc (see Fig. S1, compare panels F and I). Glycine betaine is much more effective than trehalose in restoring the hydration of E. coli cells under osmotic stress (2, 52–54). These data suggest that cellular dehydration and Prc deficiency have distinct effects on bacterial morphology.

DISCUSSION

ProQ is a soluble, cytoplasmic protein with a trypsin-sensitive linker that connects trypsin-resistant N- and C-terminal domains (residues 1 to 131 and 170 to 232, respectively) (47). These domains are structural homologues of RNA-binding translational regulators FinO (47) and Hfq (8), respectively. FinO regulates F-pilus biogenesis by binding small RNA (sRNA) FinP (55–57), while Hfq is a pleiotrophic regulator with multiple sRNA partners (58). No physiological RNA targets of ProQ have been found (49), but ProQ exerts RNA chaperone activities on FinO substrates. The FinO-like ProQ domain binds RNA with high affinity, and the Hfq-like ProQ domain facilitates RNA strand exchange and duplexing (8).

Prc is an ATP-dependent periplasmic protease with an N-terminal domain of unknown function, a central PDZ domain, and a C-terminal serine protease domain (682 residues in total) (59). Prc was designated a tail-specific protease (Tsp) because it cleaves protein C termini in a sequence-dependent manner (11, 59). Only 20 nucleotides separate prc from proQ (Fig. 3). A putative prc promoter was identified within proQ (10, 60), but Prc was not detected in bacteria with a Tn5 insertion in proQ, 122 bp upstream from the putative prc transcription start site (allele proQ220::Tn5) (Fig. 4A). A proQ-prc transcript was identified (61), mutations in proQ eliminated Prc (Fig. 4A), and replacement of prc lowered ProQ levels (allele prc3::kan) (Fig. 4B). These data suggest that proQ and prc are cotranscribed (Fig. 3), and the reciprocal effects of proQ and prc mutations (Fig. 4) result from alteration of a proQ-prc transcript. ProQ may act as an RNA chaperone to regulate translation of a proQ-prc mRNA.

This study linked ProQ deficiency with cell elongation (Fig. 2 and 7). That elongation was reversed by the osmoprotectant glycine betaine (see Fig. S2 in the supplemental material) but not by accumulation of trehalose (Fig. 2A and 9). Glycine betaine is more effective than trehalose at restoring the hydration of bacteria cultivated at high salinity (53). Diverse perturbations cause cells to elongate. They include the SOS response, a variety of cell division defects, and the inhibition of cell division proteins (e.g., astreinam inhibition of PBP3 [Fig. 6A]). The mechanism by which ProQ deficiency causes cell to elongate is not currently known. No morphological phenotype is evident for bacteria lacking Prc but not ProQ during growth in standard media with optimal

![FIG 7 Complementation of Prc- and ProQ-deficient E. coli strains with plasmids carrying genes for Prc and ProQ. Bacteria were cultivated in MOPS medium supplemented with 250 mM NaCl (0.75 mol kg\(^{-1}\)) to late exponential phase as described for transport assays (25) (A to C) The bacteria were visualized by light microscopy (see Materials and Methods). Cell length distributions and representative light micrographs are shown for WG1119 pCK3 (restoring Prc but not ProQ; open squares) A), WG1119 pDC77 (restoring ProQ but not Prc; open squares) (B), and WG1119 pDC77 pCK3 (restoring ProQ and Prc; open squares) (C) in comparison with WG1119 (lacking ProQ and Prc; closed squares) for all cases. Bars, 10 μm. (D and E) An aliquot of the cell extract from each culture (15 μg of protein) was analyzed by Western blotting to detect Prc (D) or ProQ (E). Strain RM2 (proQ" prc") was included to illustrate the levels of ProQ and Prc attained when each is encoded by its chromosomal gene. To test the levels of expression of these proteins from the plasmid-borne genes, plasmids pCK3 (for Prc) or pDC77 (for ProQ) or both were introduced to strain WG1119 (which otherwise lacks both proteins) (left lanes), or the same plasmids were introduced to strain WG703 (which otherwise lacks Prc but not ProQ) (right lanes). M, molecular weight markers; arrows, locations of ProQ and Prc.]

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salinities (Fig. 2B and E). However, prc mutant bacteria grow slowly and form filaments during cultivation in rich media at very low salinity and high temperature (10,62), and they form spherical cells with an enlarged periplasm and eccentric cytoplasm upon cultivation at the optimal temperature (37°C) and high salinity (Fig. 2H and 5). The spherical cells form despite the accumulation of trehalose (Fig. 9) or the provision of glycine betaine (see Fig. S1I in the supplemental material).

Previous observations have linked Prc to cell division and periplasmic protein quality control. The morphological consequences of Prc deficiency for bacteria cultivated at high salinity reported here (Fig. 2 and 5; see also Fig. S1 in the supplemental material) did not correlate with morphological changes resulting from A-22 inhibition of MreB or aztreonam inhibition of PBP3 (Fig. 6A). However, an spr deletion suppressed the growth and morphological phenotypes associated with cultivation of Prc-deficient bacteria at low (44) or high salinity (Fig. 8). Spr is predicted to associate with the outer membrane of E. coli via an N-terminal signal peptidase recognition sequence, culminating in a membrane-anchoring cysteine. The structure of the C-terminal domain of Spr confirms its membership in the NlpC/P60 protein domain family of peptidases (65), and the murein D,D-endopeptidase activity of that periplasmic domain contributes to enlargement of the murein sacculus (43). Our observations suggest that a Prc deficiency promotes uncontrolled Spr activity, cell rounding, loss of murein layer integrity, and ultimately cell lysis when bacteria are cultivated at high salinity. This hypothesis is consistent with a variety of observations linking the PDZ and serine protease domains of Prc to protein quality control (10–14,33, 66). A tsp (prc) mutation produced synthetic phenotypes with degP, ppiD, surA, fkpA, and ydgD, each with a known or putative role in protein quality control. In addition, bacteria with defects at surA, dsbA, and various combinations of quality control or protease loci grew poorly at high salinity (33).

Like the osmotic stress-dependent morphological phenotypes, the distinct effects of ProQ and Prc deficiencies on the activity of

**FIG 8** Deletion of spr suppresses the prc morphological phenotype. Bacteria were cultivated in MOPS medium to late exponential phase as described for transport assays (25) and visualized by DIC microscopy (see Materials and Methods). Media were unsupplemented (0.25 mol kg⁻¹, low NaCl) (A) or supplemented with 250 mM NaCl (0.75 mol kg⁻¹, high NaCl) (B). Culture optical densities (at 600 nm) were measured with a Pharmacia Novaspec II spectrometer. Representative DIC micrographs are shown for strains WG703 (proQ⁺ prc3::kan spr⁺ [C and F]), WG1457 (proQ⁺ prc⁻ Δspr832::FRT [D and G]), and WG1458 (proQ⁺ Δprc3::kan Δspr832::FRT [E and H]). Bars, 10 μm.
the osmoregulatory transporter ProP link ProQ and Prc to the osmotic stress response. However ProQ and Prc are not general effectors of the osmoregulatory response, since their absence did not impair other osmoregulatory functions (osmoprotectant effectors of the osmoregulatory response, since their absence did not impair other osmoregulatory functions).

For strains RM2 and WG703, nonlinear regression (performed with SigmaPlot) was used to fit the initial rate of proline uptake ($A_0$) at the corresponding assay medium osmolality ($Π/RT$) to the following equation: $A_0 = A_{max} / [1 + e^{-(\frac{Π/RT - Π_{0.5}}{B})}]$, where $A_0$ is the initial rate of substrate (radio-labeled proline) uptake, $A_{max}$ is the rate that would be attained at infinite osmolality, $B$ is a constant, $Π_{0.5}/RT$ is the value of $Π/RT$ at which $A_0 = 0.5A_{max}$, $Π$ is the gas constant (8.314 J·K$^{-1}$·mol$^{-1}$), and $T$ is the temperature (298°K). ProP activity was half-maximal at an osmolality of 284 ± 6 mmol/kg for RM2 and 323 ± 6 mmol/kg for WG703 (means ± standard errors). The amplitude of the osmotic activation response approximately 30% and raises the osmolality required to reach half maximal activity (Fig. 10A, legend). Cardiolipin concentrates at the poles of E. coli cells and ProP concentrates at the cell poles in a cardiolipin-
dependent manner, whereas the membrane-integral PrP component of the PrP system does not (28, 68, 69). The effects of PrP deficiency on PrP activity occur in bacteria cultivated at moderate salinities that do not elicit spherical cell formation. Thus, PrP may indicate subtle effects of PrP deficiency on cell wall and cytoplasmic membrane organization that occur under these moderate conditions.

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