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ProQ Is an RNA Chaperone that Controls ProP Levels in *Escherichia coli*

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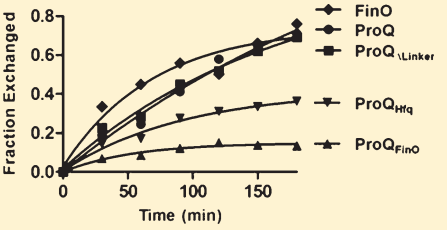
ABSTRACT: Transporter ProP mediates osmolyte accumulation in *Escherichia coli* cells exposed to high osmolality media. The cytoplasmic ProQ protein amplifies ProP activity by an unknown mechanism. The N- and C-terminal domains of ProQ are predicted to be structurally similar to known RNA chaperone proteins FinO and Hfq from *E. coli*. Here we demonstrate that ProQ is an RNA chaperone, binding RNA and facilitating both RNA strand exchange and RNA duplexing. Experiments performed with the isolated ProQ domains showed that the FinO-like domain serves as a high-affinity RNA-binding domain, whereas the Hfq-like domain is largely responsible for RNA strand exchange and duplexing. These data suggest that ProQ may regulate ProP production. Transcription of *proP* proceeds from RpoD- and RpoS-dependent promoters. Lesions at *proQ* affected ProP levels in an osmolality- and growth phase-dependent manner, decreasing ProP levels when *proP* was expressed from its own chromosomal promoters or from a heterologous plasmid-based promoter. Small RNA molecules are known to regulate cellular levels of sigma factor RpoS. ProQ did not act by changing RpoS levels since *proQ* lesions did not influence RpoS-dependent stationary phase thermotolerance and they affected ProP production and activity similarly in bacteria without and with an *rpoS* defect. Taken together, these results suggest that ProQ does not regulate *proP* transcription. It may act as an RNA-binding protein to regulate *proP* translation.



Osmoregulatory processes control cellular hydration. Uptake of small organic solutes (osmoprotectants) via transporters ProP, ProU, BetT, and BetU stimulates the growth of *Escherichia coli* in high osmolality media.[1−3] Secondary transporter ProP mediates the uptake of proline, glycine betaine, and related osmoprotectants.[4] ProP activity and *proP* transcription are osmoregulated.[5,6] RpoD-dependent transcription from *proP* promoter P1 increases with increasing growth medium osmolality.[7] Transcription from promoter P2 is activated by Fis, a nucleoid protein that attains high levels upon subculture of cells into a nutrient-rich medium,[8] and RpoS, an alternative sigma factor (σ^{38}) that attains high levels in the stationary phase.[9] This dependence on Fis and RpoS causes a pulse of *proP* expression in late exponential to early stationary phase.[10]

In *E. coli*, lesions at the *proQ* locus attenuate ProP activity without altering its osmolality-dependence.[11,12] Among bacterial genomes, *proQ* and *proP* are not genetically linked; each can be found without the other,[13] and a *proQ* lesion altered the rates of uptake of multiple amino acids, the impact on ProP being the largest detected.[11,12] Thus, the role of ProQ may extend beyond the regulation of ProP. ProQ is a soluble protein in which a trypsin-sensitive linker connects trypsin-resistant N- and C-terminal domains (residues 1−131 and 170−232 of *E. coli* ProQ, respectively).[14] Plasmid-based production of full-length ProQ, of a ProQ variant without the linker, or of the N-terminal domain raised ProP activity in bacteria with a *proQ* deletion.[12] The C-terminal domain did not complement and neither domain interfered with the activity of full-length ProQ.[14]

No protein of known function is homologous in sequence to full-length ProQ. However, *E. coli* FinO is 35% identical to the N-terminal half of ProQ, and structure prediction servers GenThreader and 3D-PSSM give strong matches of ProQ to the known FinO crystal structure 1DVO (Figure 1A).[13,15] On this basis, the ProQ domain can be modeled convincingly on the FinO crystal structure (1DVO), and analysis of the purified N-terminal domain by circular dichroism (CD) spectroscopy confirmed that it is predominantly α-helical.[13−15] The C-terminal domain of ProQ was initially modeled on the SH3-like myosin motor domain from *Dictyostelium disodium* (1LVK).[13] However, the CD spectrum of this ProQ domain was characteristic of β structure (with a weak minimum near 220 nm) and did not show the positive ellipticity with a maximum at 220 nm characteristic of some SH3 (SRC Homology 3) proteins.[14] The secondary structure of the ProQ C-terminal domain (residues 177−232) can be aligned with that of Hfq from *Methanococcus jannaschii* (residues 15−71), an Sm-like protein. The Sm-like domain is the nearest neighbor to the SH3 domain in structure classifications such as CATH.[16] Despite their limited sequence similarity (Figure 1B), a homology model for the C-terminal domain of ProQ can be derived from the Hfq crystal structure (2QTX)[17] (Figure 1C). This model is more consistent with the secondary structure of this ProQ domain indicated by CD

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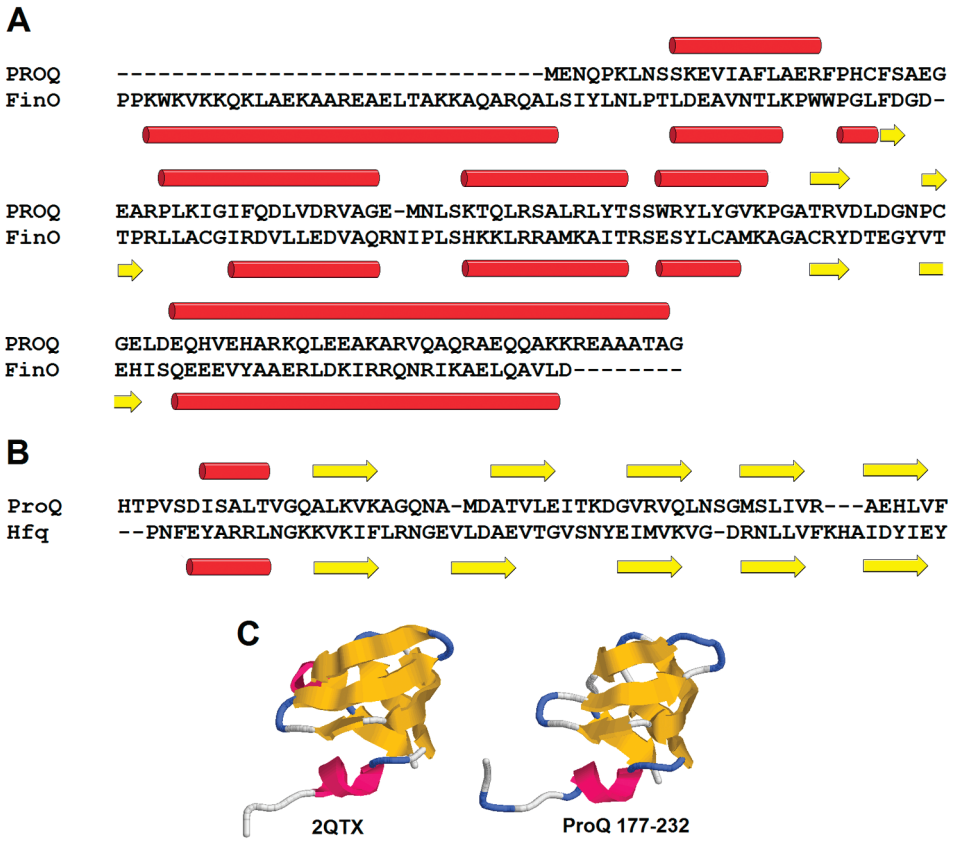


Figure 1. Homology model of the C-terminal domain of ProQ based on the crystal structure of Hfq A. Alignment of the secondary structure of ProQ (residues 1−129) with FinO from *E. coli* (residues 33−184). Helices are indicated by red cylinders and beta strands by yellow arrows. Secondary structure of FinO was taken from the crystal structure 1DVO, and secondary structure of ProQ was as predicted by PsiPred.[73] (B) Alignment of the secondary structure of ProQ (residues 177−232) with Hfq from *Methanococcus jannaschii* (residues 15−71). Secondary structure of ProQ was predicted by PsiPred, and secondary structure of Hfq was taken from the crystal structure 2QTX. (C) Structure of one chain of Hfq from *M. jannaschii* (Protein Databank Identifier 2QTX) and the derived ProQ model for residues 177−232, colored by secondary structure where red is α helix and yellow is β sheet. In 2QTX, Hfq is found as a hexameric ring. The initial alignment with *S. aureus* Hfq (1KQ2) was detected using GenThreader.[74] The sequence of *M. jannaschii* Hfq (2QTX) was selected as the best match to the ProQ C-terminal from an alignment with Hfq homologues of known structure. The final alignment of ProQ with *M. jannaschii* Hfq was adjusted manually to optimize the match of secondary structure and location of polar and nonpolar residues. This alignment was used to model the ProQC on 2QTX using MODELER.[75,76]

spectroscopy. Sm-like proteins have the same β-sheet topology as SH3 proteins but lack a small 3_{10} helix which may account for the differences in CD spectra between SH3 and Sm proteins. Thus, on a structural basis, the N- and C-terminal domains of ProQ are designated as FinO-like and Hfq-like, respectively.

FinO is encoded by F-like plasmid family members.[18] It represses F plasmid gene expression through an antisense RNA-based mechanism that targets the mRNA of a key plasmid transcription factor, TraJ. FinO binds the plasmid-encoded antisense RNA FinP,[19,20] protecting it from degradation by RNaseE[21] and facilitating its interactions with *traJ* mRNA.[15,20,22−24] This may sequester the Shine-Dalgarno sequence of the *traJ* message and results in a 3−5-fold reduction in the transcription of *traJ*.[25]

Biochemical and structural studies have provided detailed insights into the mechanism of FinO RNA chaperone activity.[15,22,23,26,27] FinO adopts an extended and largely helical structure[15] and binds with little sequence specificity to hairpin RNAs with 5′ and 3′ single-stranded tails.[21] RNA chaperone activity is essential to FinO function. The protein facilitates sense−antisense RNA pairing between FinP and *traJ* mRNA that is otherwise inhibited due to internal secondary structures of these RNAs.[20,22−25] FinO also can facilitate strand exchange between a two-stranded mimic of a hairpin RNA and a complementary single strand, suggesting that FinO chaperone function may rely on FinO-induced destabilization of internal RNA hairpin structures.[22]

Hfq is a member of the Sm family of proteins which form hexameric ring structures when they interact with RNA molecules.[28] Unlike FinO, which has a specific sRNA target, Hfq mediates a wide range of sRNA−mRNA interactions involved in regulation of diverse functions.[28,29] The crystal structures of seven Hfq orthologues reveal similar folds (e.g., ref 17). Hfq binds poly(A) regions of RNA and AU-rich regions preceding or following stem-loop structures.[30−33] Many sRNAs are known to interact with Hfq.[34] Hfq may stabilize important stem-loop structures of sRNAs, allowing them to act on their target mRNA molecules through base-pairing interactions.[35,36]

Early experiments revealed no significant effects of insertion *proQ220*::Tn5 on the β-galactosidase activity of bacteria harboring insertion *proP227*::Mud1(*lac* Ap)[11] or on ProP protein levels.[12] The Mud1(*lac* Ap) insertion interrupted the *proP* open reading frame at the codon for S375, leaving the *proP* promoters and 5′-untranslated regions intact.[12] These experiments led to

Table 1. *E. coli* Strains and Plasmids

strain or plasmid[a]	genotype or description	source or reference
BL21 Gold	*E. coli* B F$^-$ *ompT hsdS* (r_B- m_B-) *dcm*$^+$ *Tet*^{R} *gal endA* Hte	Stratagene
JM520	MC4100 *trpDC700::putPA1303*::[*kan*^{R}-520 bp *proP-lacZ*]	67
MC4100	F$^-$ *araD139* Δ*(argF-lac)U169 rpsL150 relA1 deoC1 rbsR flhD5301 fruA25 λ*$^-$	68
RH90	MC4100 *rpoS*::Tn*10*	69
RM2	F$^-$ *trp lacZ* Δ*putPA101 rpsL thi*	70
WG170	RM2 *proP219*	71
WG174	RM2 *proQ220*::Tn*5*	11
WG210	RM2 *proU205*	72
WG584	RM2 *trpDC700::putPA1303*::[*kan*^{R}-520 bp *proP-lacZ*]	this study
WG914	WG210 Δ*proQ676*	13
WG1065	WG914 Δ*proP771*::*kan*	this study
WG1072	RM2 Δ*proQ756*::kan	this study
WG1119	WG584 Δ*proQ870*::*FRT*	this study
WG1120	WG584 *rpoS359*::Tn*10*	this study
WG1121	WG1119 *rpoS359*::Tn*10*	this study
pBAD24	expression vector	38
pDC79	pBAD24 derivative encoding ProP	39
pMS1	pQE60 derivative encoding ProQ-His_6	13
pMS10	pQE82L derivative encoding His_6-$ProQ_{FinO}$	14
PMS13	pQE82L derivative encoding His_6-$ProQ_{Hfq}$	14
pMS14	pQE82L derivative encoding His_6-$ProQ_{DLinker}$	14
pQE-60	expression vector, used with plasmid pREP4	Qiagen
pQE-82 L	expression vector	Qiagen

[a] Strains are derived from *E. coli* K-12 unless otherwise stated.

the proposal that ProQ may act directly to amplify ProP activity through protein−protein interactions.[37] We reassessed ProQ function based on the structural homology of ProQ to FinO and Hfq.

Here we show that ProQ acts as an effective RNA chaperone, with significant RNA binding, RNA strand exchange, and RNA duplexing activities. In addition, we show that *proQ* lesions lower *proP* levels in a manner that is independent of the *proP* promoters and of sigma factor RpoS. These results suggest that ProQ may regulate ProP activity through an RNA-based, post-transcriptional mechanism.

EXPERIMENTAL PROCEDURES

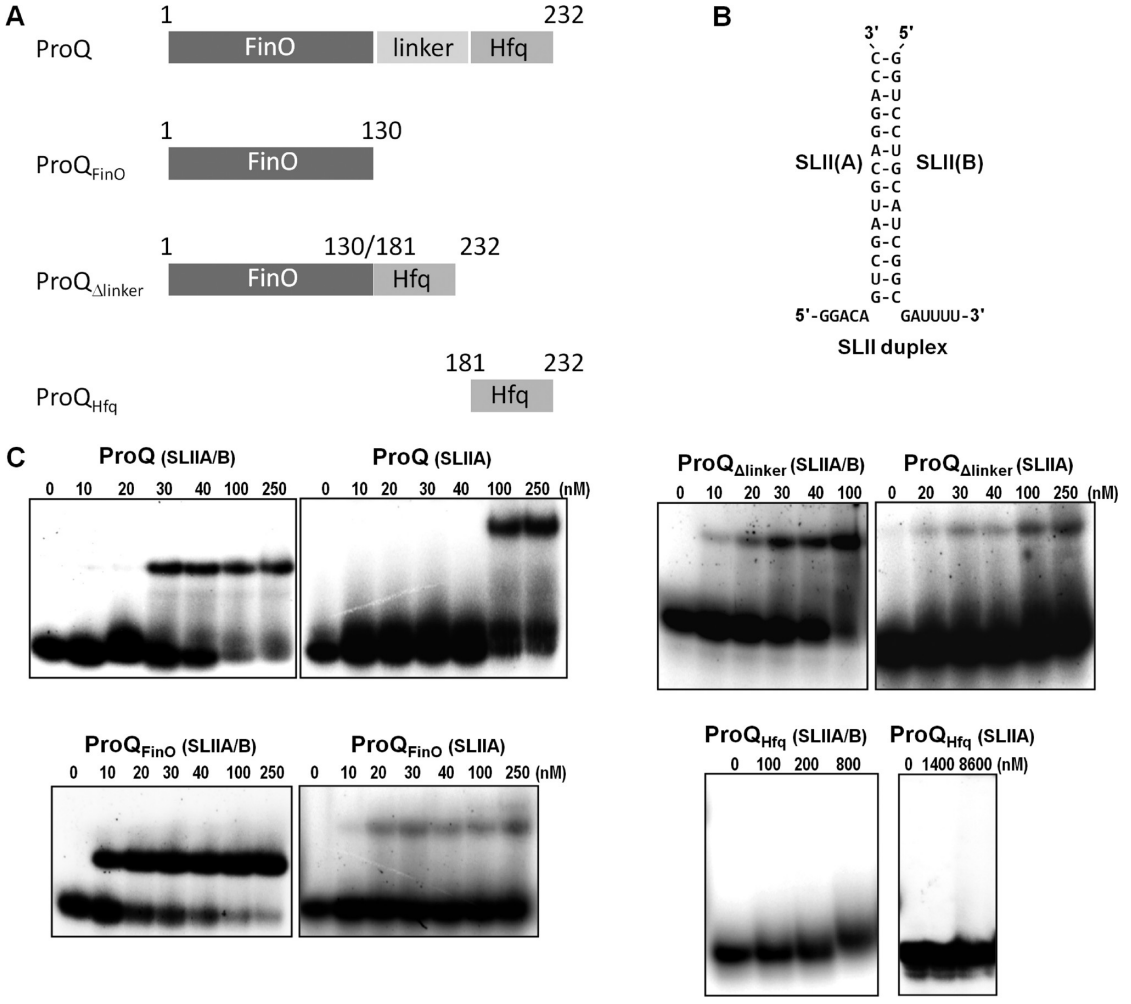
Bacterial Strains and Plasmids. The genotypes of the *E. coli* strains used for this study are listed in Table 1. Plasmid pDC79 was created by replacing a fragment of vector pBAD24[38] flanked by NcoI and *Hin*dIII restriction sites with a DNA fragment extending from an NcoI site overlapping the *proP* initiation codon through a *Hin*dIII site 105 bp downstream of the *proP* termination codon.[39] Plasmid isolation was performed using QIAprep Spin Miniprep Kits (Qiagen, Mississauga, ON). Routine DNA manipulation, plasmid construction, electrophoresis, and transformation were carried out as described previously.[40,41] Transductions were performed with phage P1 (*cml clr_100*) as described by ref 42. The polymerase chain reaction (PCR) was performed as described previously[43] using *Pfu* turbo polymerase (Invitrogen, Burlington, ON), and oligonucleotides were purchased from Cortec DNA Services (Kingston, ON). The Molecular Biology Supercenter (University of Guelph, Guleph, ON) performed DNA sequencing to verify all plasmid constructs.

Strain WG1065 was constructed by introducing the Keio collection[44] *proP::Km* replacement into strain WG914 by P1 transduction, then deleting the Km resistance cassette as described by Datsenko and Wanner.[45] Strain WG584 was created by P1 transduction of *trpDC700::putPA1303*::[*kan*^{R}-520 bp *proP-lacZ*] from *E. coli* JM520 into *E. coli* RM2. The *proQ* locus was deleted from strain WG584 as described above to yield strain WG1119. Mutation *rpoS359::*Tn*10* was introduced to strains WG584 and WG1119 by P1 transduction from strain RH90 to create strains WG1120 and WG1121, respectively.

Bacterial Cultures. Bacteria were cultivated in Luria−Bertani (LB) medium[42] or in NaCl-free MOPS medium[46] with NH_4Cl (9.5 mM) as the nitrogen source, glycerol (0.4% v/v) as the carbon source, and tryptophan (245 μM) and thiamine (1 mg/mL) to meet auxotrophic requirements. Ampicillin (100 μg/mL) was added to maintain plasmids. Medium osmolalities were adjusted with NaCl and measured with a Wescor vapor pressure osmometer (Wescor, Logan, UT, USA). Cultures were grown at 37 °C in a rotary shaker at 200 RPM. Optical densities were monitored with a Bausch and Lomb Spectronic 88 spectrometer.

SDS−Polyacrylamide Gel Electrophoresis and Western Blotting. SDS−PAGE was performed as described by Laemmli[47] with gels containing 12% (w/v) polyacrylamide and 0.9% (w/v) bisacrylamide. Gels were stained with Gel-Code Blue (Pierce, Rockford, IL) according to the manufacturer's instructions. Western blotting was performed to detect ProQ[12,13] and ProP[48] as previously described. Western blots were visualized with ECL reagents (GE Healthcare, Baie d'Urfe, QC) according to the manufacturer's instructions.

Protein Assays. Protein concentrations were determined using the BCA (bicinchoninic acid) assay[49] with reagents from



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RNA	ProQ	$ProQ_{\Delta linker}$	$ProQ_{FinO}$	$ProQ_{Hfq}$
dsRNA	31 ± 1.5 nM	35 ± 5 nM	8 ± 0.9 nM	≥800 nM
ssRNA	~200 nM	≥250	≥250	N/A

Figure 2. RNA binding activity of ProQ. (A) ProQ protein fragments used in these experiments. Shaded boxes indicate the FinO-like, linker, and Hfq-like domains. Amino acid numbers corresponding to the domain boundaries are shown. (B) The FinP-derived RNA substrates used for RNA binding studies were SLIIA/B duplex for dsRNA binding and SLIIA for single-stranded binding. SLIIA was ^{32}P 5′ end labeled for measurement of protein binding to single- or double-stranded RNA. (C) Electrophoretic gel mobility shift assays were used to assess interactions between ProQ and its deletion mutants and a double-stranded RNA (SLIIA/B, left panels) or a single-stranded RNA (SLIIA, right panel). The small size of $ProQ_{Hfq}$ resulted in a relatively small electrophoretic mobility shift preventing accurate binding affinity determination. (D) Table listing the affinities of ProQ and its fragments for single- or double-stranded RNA.

Pierce, (Rockford, IL) according to the manufacturers' instructions and bovine serum albumin as the standard.

Transport Assays. To determine the initial rates of proline uptake by cells, bacteria were grown in MOPS-based minimal medium adjusted with NaCl to achieve the desired osmolality. Transport assays were performed as described previously.[50]

Protein Overproduction and Purification for RNA Assays. ProQ and its fragments were overexpressed and purified essentially as described.[14] BL21 Gold cells harboring the relevant expression vector were grown to OD_{600} of ~1, then brought to 22 °C and induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) overnight. Cell pellets were resuspended in lysis buffer (50 mM Hepes (pH 7.3), 600 mM NaCl, 1 mM DTT, 5% (v/v) glycerol, 1 mM EDTA) containing protease inhibitor tablets (Roche, as per manufacturer's instructions) and lysed by sonification. Cleared lysates were subjected to precipitation with 0.3% (w/v) polyethylenimine (PEI), and the resulting supernatants were precipitated with ammonium sulfate (70% (w/v) ammonium sulfate). Precipitated protein was resuspended in lysis buffer containing 20 mM imidazole and purified by Ni(NTA) affinity chromatography using lysis buffer containing 500 mM NaCl for elution followed by size exclusion chromatography in lysis buffer.

RNA Preparation. RNAs were prepared by in vitro runoff transcription reactions using bacteriophage T7 RNA polymerase with the appropriate DNA template and complementary primer

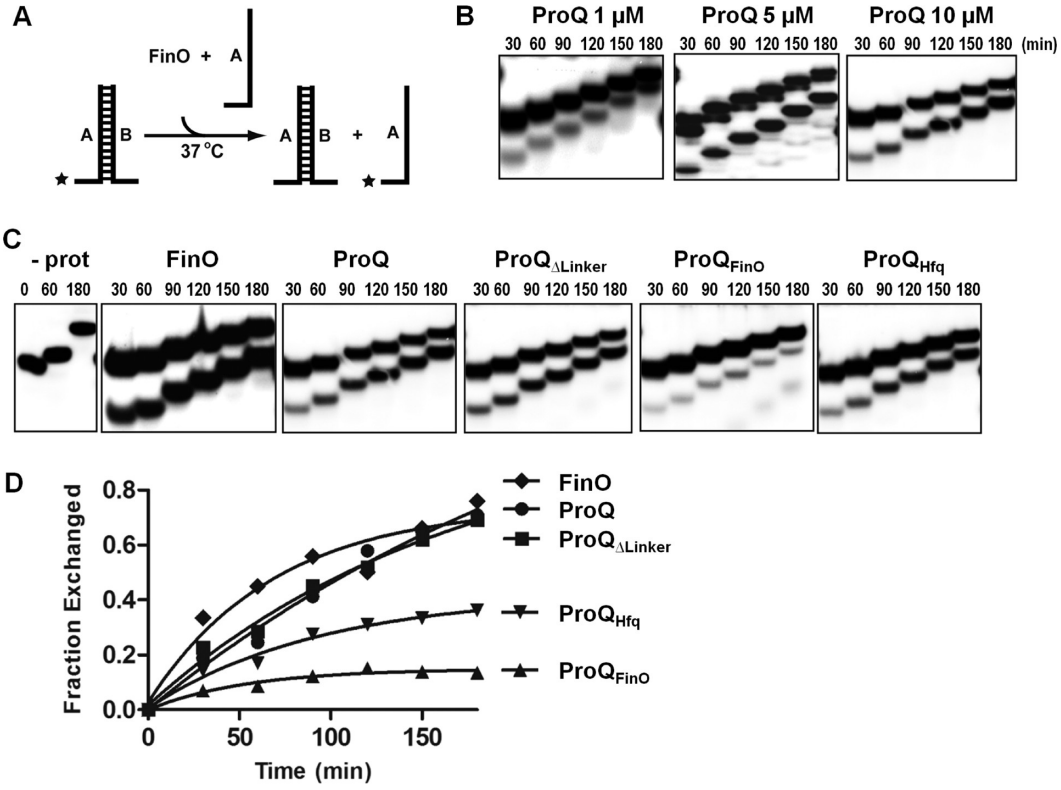

Figure 3. RNA strand exchange activity of ProQ. (A) Schematic diagram of the strand exchange assay. A two-stranded mimic of FinP SLII (SLIIA/B) in which the A strand is labeled with ^{32}P (*) is challenged with an excess of unlabeled SLIIA. Exchange of the labeled SLIIA from duplex to single strand is monitored by gel electrophoresis. (B) Strand exchange activity increases with increasing ProQ concentration. Reactions were carried out at the indicated ProQ concentrations for the times indicated and reaction products were monitored by electrophoresis on a continuously running native polyacrylamide gel. (C) Strand exchange of ProQ or the indicated deletion variants was compared to FinO or a no protein (no prot) control. Protein concentrations were 10 μM. (D) Quantitation of the data in C was used to compare the rates of strand exchange for the various ProQ derivatives with the corresponding reactions mediated by FinO and in the no protein control.

as described.[8] The primer binding sites are underlined in the following sequences.

SLIIx: 5′-GGA CTC GCC GAT GCA GGG AGA CGT GAA CTC CCT GCA TCG ACT GTC CTA TAG TGA GTC GTA TTA-3′

SLIIc$_x$: 5′-GGA CAG TCG ATG CAG GGA GTT CAC GTC TCC CTG CAT CGG CGA GTC CTA TAG TGA GTC GTA TTA-3′

SII(A): 5′-GGT CCT GCA TCG ACT GTC CTA TAG TGA GTC GTA TTA-3′

SII(B): 5′-AAA ATC GCC GAT GCA GGA CCT ATA GTG AGT CGT ATT A-3′

RNA Binding Electrophoretic Mobility Shift Assay (EMSA). Binding experiments were performed on ice in 100 μL reaction mixtures based on binding reaction buffer (25 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM EDTA), containing ~50000 cpm of ^{32}P 5′ end-labeled SLIIA or ^{32}P 5′ end-labeled SLIIA duplexed with an equimolar amount of unlabeled SLIIB. Prior to their addition, SLIIA or SLIIA/B were heat denatured at 90 °C in binding reaction buffer and cooled slowly to room temperature (2 °C/min). Reaction mixtures were incubated for 20 min, on ice, before 10 μL of 20% glycerol was added and subjected to 6% (19:1) nondenaturing PAGE (190 mM glycine, 25 mM Tris, pH 8, 2−3 h at 180 V). Gels were exposed to Molecular Dynamics storage phosphor screen and exposed screens were scanned using a Storm 840 phosphorimager (Molecular Dynamics). Bands were quantified with ImageQuanNT software (Molecular Dynamics) and binding affinities were obtained using GraphPad PRISM 5.02.

Strand Exchange Assay. Strand exchange assays were performed as previously described[22] on ice in 12 μL reaction mixtures containing 25 mM Tris (pH 8.1), 50 μg/mL bovine serum albumin (BSA), 5% (v/v) glycerol, 0.05% (v/v) β-mercaptoethanol, and 10 mM NaCl, 2 units (1 μL) of RNAguard (Amersham), 1, 5, or 10 μM protein, 5 nmol of the labeled SIIA/B duplex (described above) to give a final duplex concentration of ~400 pM) and 2.5 μmol of the unlabeled SIIA strand (to give a final concentration of ~200 nM). Strand exchange assays were initiated by placing reaction tubes at 37 °C and stopped at the indicated times by adding an equal volume of stop solution (5% (v/v) glycerol, 0.4% (w/v) SDS, and 20 mM EDTA). Samples were subjected to 15% (19:1) nondenaturing PAGE (190 mM glycine, 25 mM Tris, pH 8, 180 V for 2 h) at room temperature to separate the free and duplexed ^{32}P-labeled strands. Gels were exposed and analyzed as described above.

Duplexing Assays. Duplexing between SLII$_x$ and SLIIc$_x$ was measured as previously described[22] using 96 μL reaction mixtures containing 25 mM Tris (pH 8.1), 50 μg/mL BSA, 5% (v/v) glycerol, 0.05% (v/v) β-mercaptoethanol, 20 mM NaCl, 10 μM protein, 1 μM SLIIc$_x$, and 50 nM ^{32}P-labeled SLII$_x$. To initiate duplexing, labeled SLII$_x$, preincubated at 37 °C, was added to reaction mixtures, also preincubated at 37 °C and lacking SLII$_x$.

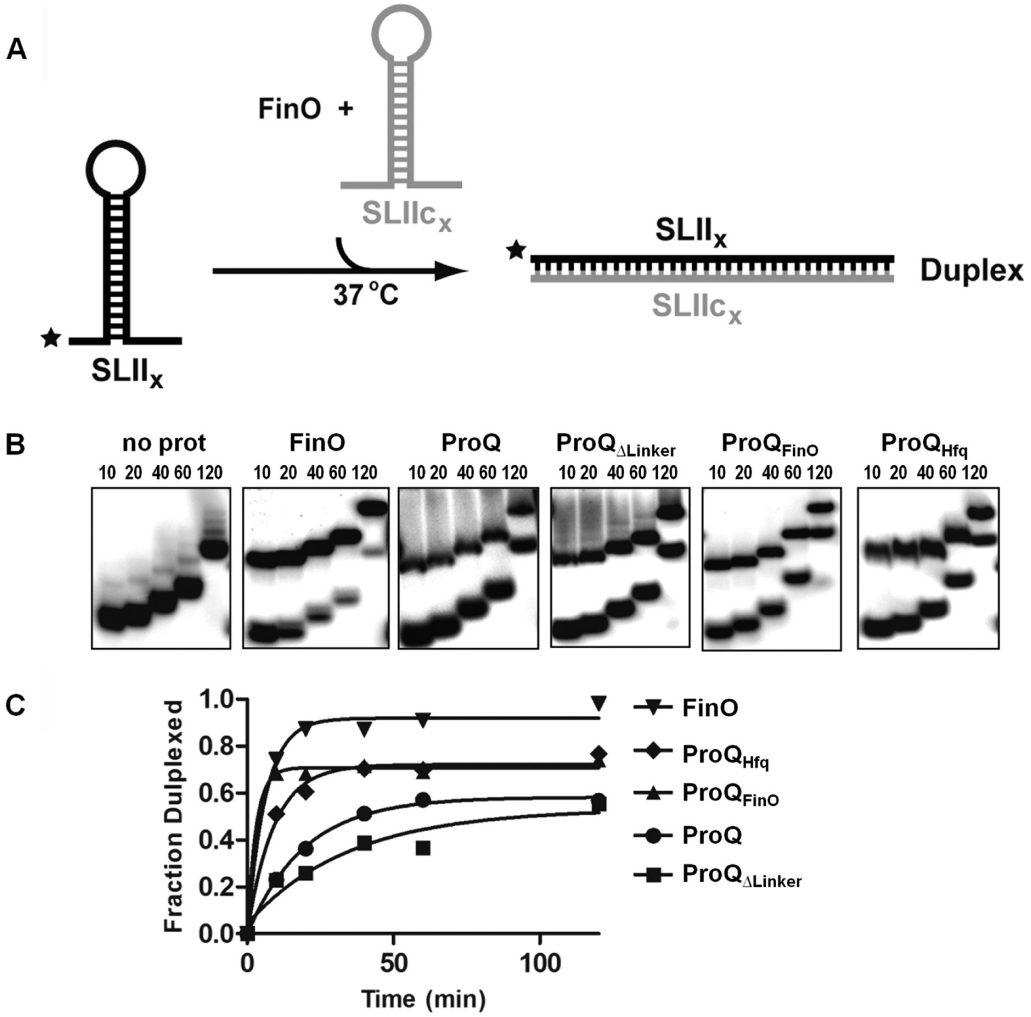

Figure 4. RNA duplexing activity of ProQ. (A) Schematic diagram of the RNA duplexing assay. Stem-loop II from FinP (SLII$_x$) is labeled with ^{32}P (*) and an excess of unlabeled, complementary SLIIc$_x$ from *traJ* mRNA 5′ UTR. Duplex formation between these RNAs is monitored as a function of time by native gel electrophoresis. (B) Duplex formation between SLII$_x$ and SLIIc$_x$ RNAs monitored by electrophoresis on a continuously running native polyacrylamide gel over a 120 min time course catalyzed by the indicated ProQ derivatives, FinO, or no protein control, as indicated. (C) Quantitation of the data in B was used to compare the duplexing rates for the various ProQ derivatives in comparison to FinO and the no protein control.

Twelve microliter aliquots were taken at various time points and added to 12 μL of stop solution (as above). Samples were subjected to 10% (19:1) nondenaturing PAGE (190 mM glycine, 25 mM Tris, pH 8, 180 V 2 h) prior to visualization and quantification of the labeled SLII$_x$ as described above.

RESULTS

The FinO-like Domain of ProQ acts as a High Affinity RNA Binding Module. Limited proteolysis and sequence analysis indicate that ProQ contains an N-terminal FinO-like domain (residues 1−130), and a C-terminal Hfq-like domain (residues 181−232) separated by a flexible, protease-sensitive linker.[13,14] To evaluate the roles of these regions, the FinO-like domain (ProQ$_{FinO}$), the Hfq-like domain (ProQ$_{Hfq}$), and a version of ProQ lacking the flexible linker (ProQ$_{\Delta linker}$) were purified and analyzed in RNA chaperone assays in comparison to full-length ProQ and FinO (Figure 2A). Substrates based on the FinO target, FinP RNA, were used because no ProQ-specific RNA target has yet been identified.

Electrophoretic mobility shift assays (EMSAs) were used to characterize the binding of ProQ and its derivatives to a two-stranded version of stem-loop II (SLII) from FinP. The two-stranded molecule lacks the terminal hairpin loop but contains the 5′ and 3′ single-stranded tails that are critical for high affinity binding by FinO (Figure 2B).[22] Full-length ProQ bound this RNA to form a well-defined species with an apparent K_d of 31 ± 1.5 nM, similar to the affinity of FinO for this RNA under the same conditions (Figure 2C). Deletion of the internal linker (ProQ$_{\Delta linker}$) did not have a noticeable effect on binding affinity, suggesting that the linker plays little if any role in contacting this RNA species. The isolated FinO domain (ProQ$_{FinO}$) bound this RNA with slightly higher affinity (K_d = 8 ± 0.9 nM), whereas the isolated Hfq-like domain exhibited greatly reduced affinity for SLII ($K_d \geq$ 800 nM). Binding to a single-stranded RNA (SLIIA) was also tested. All of the ProQ constructs showed significantly reduced affinity for ssRNA compared to dsRNA. This data indicate that ProQ$_{FinO}$ functions as a high-affinity RNA binding domain, recognizing a validated FinO target RNA with affinity similar to FinO (Figure 2D).

ProQ Exhibits Efficient RNA Strand Exchange Activity. RNA strand exchange activity, a critical determinant of FinO RNA chaperone function, was measured using the two-stranded mimic of SLII (Figure 3). In this assay, one strand of the duplex (SLII(A)) is labeled with ^{32}P and is challenged with an excess of an unlabeled version of SLII(A), and conversion of SLII(A) from duplex to single strand is monitored by native gel electrophoresis (Experimental Procedures, Figure 3A). In the absence of RNA chaperone, strand exchange is extremely slow with little detectable exchange. FinO facilitates exchange in a concentration-dependent manner, with maximal exchange rates observed at 1 μM FinO.[22] ProQ also catalyzed strand exchange at 1 μM, albeit less efficiently than FinO. However, as ProQ concentration increased to 10 μM, the rate of strand exchange increased significantly to levels similar to the maximum rates achieved with FinO (Figure 3B).

Next, strand exchange was assayed for ProQ$_{\Delta linker}$, ProQ$_{FinO}$, and ProQ$_{Hfq}$ in comparison to the full-length protein, to test the importance of the different regions of ProQ for RNA strand exchange activity (Figure 3C,D). ProQ$_{\Delta linker}$ gave nearly identical strand exchange activity to full-length ProQ, indicating that the linker region plays little role in strand exchange. ProQ$_{Hfq}$ also showed significant strand exchange activity, albeit at a reduced level compared to ProQ and ProQ$_{\Delta linker}$. ProQ$_{FinO}$ displayed the lowest level of strand exchange activity, albeit at a significantly higher level than the no protein control. Taken together, these results indicate that ProQ has significant RNA strand exchange activity and that this activity critically relies on the ProQ Hfq-like domain.

Both the FinO- and the Hfq-like Domains Contribute to ProQ RNA Duplexing. FinO dramatically facilitates the pairing of complementary hairpin RNAs that otherwise do not associate due to the stability of the intramolecular hairpin structures. RNA duplexing assays were carried out for ProQ and its derivatives using the complementary hairpins, FinP SLII$_x$ and *traJ* SLIIc$_x$, in which SLII$_x$ was ^{32}P-labeled and SLIIc$_x$ was not (Figure 4A). Duplexing of these RNAs is essentially undetectable in the absence of FinO but is enhanced by more than 2 orders of magnitude in the presence of FinO, consistent with previous results (ref 22; Figure 4B). All four ProQ constructs facilitated pairing of SLII$_x$ and SLIIc$_x$. The isolated N- and C-terminal domains showed rapid initial duplexing kinetics, similar to FinO, however, the extent of duplexing reached a plateau at only ~70% completion, compared to FinO, which goes to ~90−100% completion (Figure 4C).

Mutations at the *proQ* Locus Affect ProP Levels. We previously reported that ProQ deficiency did not alter ProP levels in bacteria grown in MOPS medium containing 0.35 M NaCl (osmolality approximately 0.8 mol/kg).[12] Here we reproduce that result but find that a *proQ* defect does reduce ProP levels in bacteria grown at lower osmolalities (0.4 mol/kg to 0.7 mol/kg) (Figure 5A, Western Blot Image). In Figure 5, the equivalent loading of the set of lanes representing each growth osmolality (ProP$^+$ ProQ$^+$, ProP$^+$ ProQ$^-$ and ProP$^-$ ProQ$^+$) is illustrated with a stained replica of the gel used for the Western Blot (Figure 5A, Gel Image). Note that ProP is not detected by Western Blotting in ProP$^-$ bacteria or by Gel Code Blue staining in ProP$^+$ or ProP$^-$ bacteria.

The dependence of *proP* transcription on Fis and RpoS results in growth phase-dependent *proP* expression when bacteria are grown in rich medium (LB, osmolality 0.4 mol/kg).[10] We therefore asked whether the growth phase would affect the impact of *proQ* on ProP levels. A *proQ* defect had no effect on

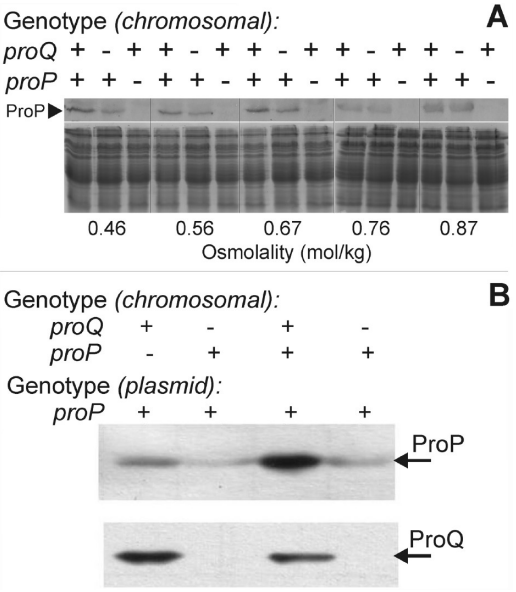

Figure 5. Effects of ProQ on ProP expression. (A) ProP levels in cells grown at various osmolalities. Strains RM2 (*proQ*$^+$ *proP*$^+$), WG174 (*proQ220*::Tn*5* *proP*$^+$), and WG170 (*proQ*$^+$ *proP219*) were grown in MOPS minimal medium supplemented with NaCl to reach the noted osmolalities. The levels of ProP were determined by Western blotting as described in Experimental Procedures. The top panel shows representative Western Blots and the bottom panel shows a Gel Code Blue-stained replica of the gel employed for this Western Blot. (B) ProQ modulates *proP* expression from a heterologous promoter. Plasmid pDC79, encoding ProP, was introduced to strains (left to right) WG170 (*proP219* *proQ*$^+$), WG174 (*proP*$^+$ *proQ220*::Tn*5*), RM2 (*proP*$^+$ *proQ*$^+$), and WG1072 (*proP*$^+$ Δ*proQ756*::*kan*). Cells were grown as described for transport assays and the levels of ProP and ProQ were analyzed by Western blotting as described in Experimental Procedures. Panel A shows that no protein with the electrophoretic mobility of ProP is detected by anti-ProP antibodies in *proP219* bacteria.

the growth of *E. coli* in LB medium (Figure 6A). ProP levels increased immediately after subculturing, decreased during exponential phase, and increased again during the transition from exponential to stationary phase (Figure 6B). These changes are consistent with documented variations in Fis, RpoS, and ProP levels.[10,51] Fis levels increase transiently upon subculture of *E. coli* in LB medium and the levels of Fis and RpoS decrease during the exponential phase (2−3 h following subculture). Fis levels drop as cells approach the stationary phase (4 h following subculture), but induction of *rpoS* results in a pulse of *proP* expression. The ProQ deficiency reduced the ProP level during the pulse of *proP* expression in late exponential and stationary phase (after 3 h of subculturing in LB medium). ProP levels declined in ProQ$^+$ and ProQ$^-$ bacteria during the stationary phase (Figure 6B). The equivalent loading of the pair of lanes representing each time point (ProQ$^+$ and ProQ$^-$ bacteria) is illustrated in Figure 6C with a stained replica of the gel used for the Western Blot. These results suggested that ProQ affects the synthesis of ProP in response to both osmotic induction and the onset of stationary phase.

The Impact of ProQ on ProP Activity Reflects ProP Levels and Is Promoter-Independent. RpoD-mediated transcription of *proP* from promoter P1 yields a transcript with a 182 nucleotide 5′-untranslated region (5′-UTR), whereas RpoS-mediated transcription from promoter P2 yields a transcript

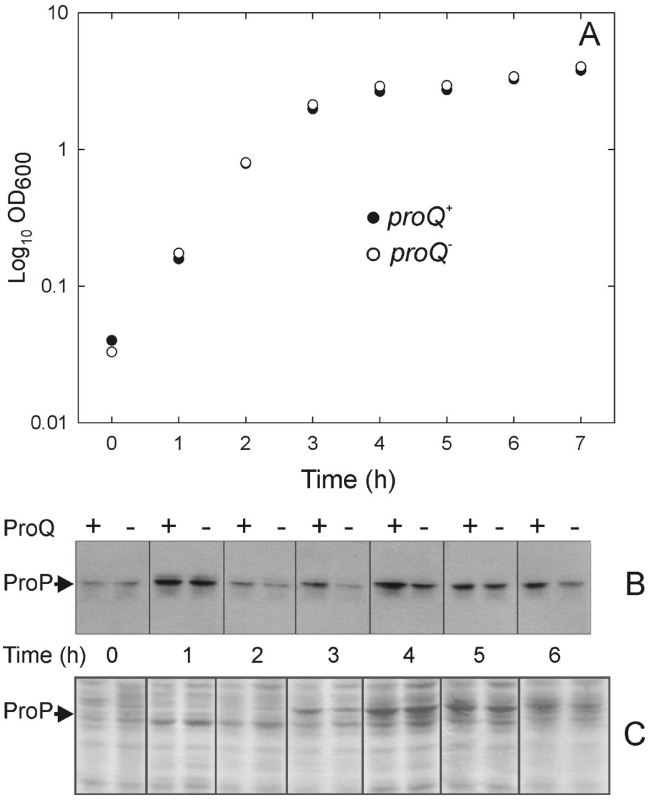

Figure 6. Effects of ProQ on ProP levels as a function of culture growth phase. (A) Growth of *E. coli* strains RM2 (*proQ*$^+$ *proP*$^+$, closed symbols) and WG174 (*proQ*$^-$ *proP*$^+$, open circles) was monitored after inoculation of LB medium to an initial OD_{600} of 0.04. (B) Cells were harvested from aliquots of these cultures taken immediately after subculturing (time 0 h) and at each of the indicated times after inoculation, to 6 h. They were resuspended in saline (0.85% (w/v) NaCl) and the ProP contents of samples containing 10 μg of cell protein were determined by Western blotting as described in Experimental Procedures. No protein remained in the gel post-transfer as indicated by staining with Gelcode Blue (Thermo Scientific). (C) Replica of the polyacrylamide gel used to produce the Western Blot shown in B. Proteins were stained with Gelcode Blue. The ProP expression level is not sufficient for detection in this way but the position of ProP in the gel is marked for comparison to B.

with a 95 nucleotide 5′-UTR. The experiments described above reflected expression of *proP* from both promoters. The ability of ProQ to modulate ProP production was further explored using *E. coli* strain WG584 in which there are 2 *proP* loci, each under the control of the P1 and P2 promoters. One locus is at the native chromosomal location and encodes wild type ProP; the other was inserted in the *trp* locus, includes 383 base pairs upstream from *proP* and encodes a fusion protein in which LacZ follows the first 190 residues of ProP.[7] Mellies et al. found that bacteria harboring this gene fusion had similar β-galactosidase activities to others in which *lacZ* was inserted after the stop codon for *proP* (1614 bp after the transcriptional start site).[7] Thus, strain WG584 permits parallel monitoring of ProP production, through measurements of β-galactosidase activity, and ProP activity, through transport assays. A derivative of this strain lacking *proQ* (Δ*proQ870::FRT*) was prepared and its β-galactosidase and proline uptake activities were measured after growth in LB medium to an optical density of 0.8 (exponential phase) or for 24 h (stationary phase). The *proQ* defect had similar impacts on ProP production (β-galactosidase in Table 2B) and ProP activity (proline uptake activity in Table 2B). Further, the ratio of β-galactosidase activity to proline uptake activity was similar for *proQ*$^+$ and *proQ*$^-$ bacteria cultivated to exponential or stationary phase (Table 2B). Thus, the impact of ProQ on ProP activity mirrored the impact of ProQ on ProP production and was growth phase-independent.

The impact of a *proQ* defect on ProP production based on *proP* expression from a heterologous promoter was also examined (Figure 5B). In plasmid pDC79, *proP* is controlled by the arabinose-inducible *araBAD* promoter of vector pBAD24.[38,39] Neither *proP* promoter nor any cognate regulatory or untranslated sequences are present since the initiation codon of *proP* coincides with the NcoI site within the multiple cloning site of the vector. The level of ProP attained when *proP* is expressed from plasmid pDC79 in the absence of arabinose is similar to that attained when *proP* is expressed from the chromosome of osmotically stressed bacteria.[52] The levels of ProP expressed from pDC79 were significantly lower in *proQ*$^-$ than in *proQ*$^+$ bacteria. ProQ is unlikely to modulate *proP* transcription since *proQ* mutations influence ProP production based on *proP* expression from its own chromosomal promoters and from a heterologous promoter.

ProQ Does Not Act through RpoS. Expression of *proP* is RpoS-dependent and RpoS is subject to complex controls, including modulation of *rpoS* message stability by small noncoding RNAs and the RNA-binding protein Hfq.[9] Thus, ProQ might influence *proP* expression indirectly, via RpoS. To test this possibility, derivatives of *E. coli* WG584 lacking *rpoS* (*rpoS359::*Tn*10*) were prepared and their β-galactosidase and proline uptake activities were measured after growth in LB medium to an optical density of 0.8 (exponential phase) or for 24 h (stationary phase).

The *rpoS* defect reduced the production of ProP during stationary phase, as expected, and the ratios of the β-galactosidase activities of *proQ*$^+$ to *proQ*$^-$ strains were similar for exponential and stationary phase bacteria (Table 2B). Further, the ratios of ProP production (β-galactosidase activity) to ProP activity (proline uptake activity) in the presence and absence of *rpoS* were similar (Table 2B). Thus, ProQ affected ProP production in a manner that was independent of the effects of RpoS and bacterial growth phase.

Given the complex regulation of RpoS, an independent assay was used to rule out any impact of the *proQ* lesion on RpoS function. Stationary phase thermotolerance requires RpoS-dependent production of proteins OtsA and OtsB, which catalyze trehalose synthesis. Wild type *E. coli* survives exposure to high temperature (55 °C) after growth into the stationary phase, but *rpoS*-deficient bacteria do not.[53] The stationary phase thermotolerance of bacteria with defects at *rpoS* and/or *proQ* was examined (Figure 7). The *rpoS* defect impaired thermotolerance, as expected, but the *proQ* defect did not. Thus, ProQ is not relevant for RpoS-mediated thermotolerance and it does not act on *proP* via RpoS.

Taken together these results, as well as the structural (Figure 1)[13] and functional analyses reported above (Figure 2−4) suggest that ProQ may act at the level of translation.

DISCUSSION

RNA chaperones constitute a large family of proteins that control RNA-based processes by modulating RNA folding, RNA−protein interactions, and RNA−RNA annealing.[54−57]

Table 2. Dependence of *proP* Expression and ProP Activity on Culture Growth Phase, ProQ and RpoS

	exponential[a]		stationary[b]	
A: relevant genotype	β-galactosidase[c]	proline uptake[d]	β-galactosidase[c]	proline uptake[d]
proQ$^+$ *rpoS*$^+$	0.58 ± 0.03	12.23 ± 0.52	0.60 ± 0.08	4.12 ± 0.16
proQ$^-$ *rpoS*$^+$	0.41 ± 0.07	7.89 ± 0.20	0.38 ± 0.05	2.43 ± 0.06
proQ$^+$ *rpoS*$^-$	0.58 ± 0.03	12.04 ± 0.63	0.31 ± 0.03	0.46 ± 0.02
proQ$^-$ *rpoS*$^-$	0.39 ± 0.09	7.24 ± 0.07	0.21 ± 0.02	0.24 ± 0.01
	relative activity (*proQ*$^+$/*proQ*$^-$)			
B: relevant genotype	β-galactosidase[c]	proline uptake[d]	β-galactosidase[c]	proline uptake[d]
rpoS$^+$	1.4 ± 0.3	1.55 ± 0.08	1.6 ± 0.3	1.70 ± 0.08
rpoS$^-$	1.5 ± 0.3	1.66 ± 0.09	1.5 ± 0.2	1.92 ± 0.11

[a] Bacteria were harvested from an LB culture at an optical density of 0.8. [b] Bacteria were harvested from an LB culture after 24 h of growth. [c] β-Galactosidase activity in units of μmol min^{-1} mg cell protein^{-1} ± the standard error. [d] Proline uptake activity in nmol min^{-1} mg cell protein^{-1} ± the standard error.

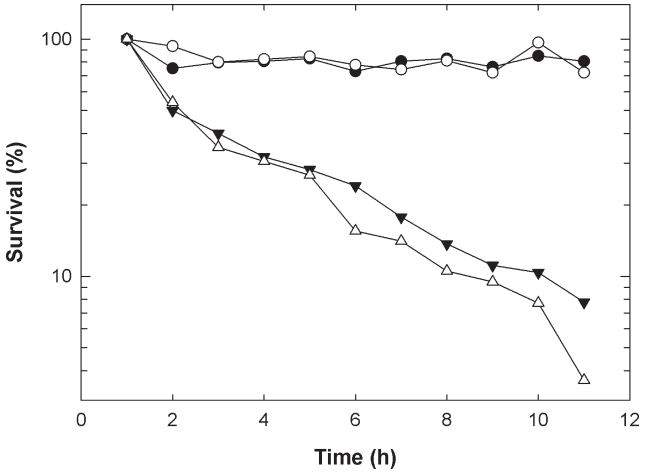

Figure 7. ProQ does not alter the stationary phase thermotolerance of *E. coli*. Strains MC4100 (closed circles), MC4100 *proQ220::*Tn*5* (open circles), RH90 (MC4100 *rpoS359::*Tn*10* closed inverted triangles), and RH90 *proQ220::*Tn*5* (open inverted triangles) were grown overnight in MOPS based minimal medium supplemented with 50 mM NaCl. Cells were harvested by centrifugation, resuspended in 0.85% (w/v) NaCl to an OD_{600} of 0.7 and incubated at 55 °C. Samples of each culture were removed at 1 min intervals and dilutions plated on LB medium to determine viable cell count.

Small RNAs (sRNAs) provide a critical mechanism for the regulation of bacterial gene expression, often working with RNA chaperone proteins to modulate the translational efficiency of mRNAs by binding the Shine-Dalgarno sequences within the 5′ UTR of the targeted mRNA.[58,59] FinO is a specific RNA chaperone, acting on antisense RNA FinP and the complementary region of the *traJ* mRNA 5′-UTR. In contrast, Hfq is a multipurpose chaperone involved in the regulation of numerous genes.[28,34] Data presented in this paper suggest that the FinO-like and Hfq-like domains of ProQ provide RNA chaperone activities that may regulate ProP production through an RNA-based mechanism.

ProQ Amplifies ProP Production. Defects at *proQ* clearly impair ProP production by *E. coli*. Lesions at *proQ* reduced ProP levels in cells cultivated to late exponential phase in MOPS media with moderate osmolalities (0.4−0.7 mol/kg) (Figure 5A) or to the transition from late exponential to stationary phase in LB medium (Figure 6). Merodiploid cells in which ProP and a ProP::LacZ fusion protein are each expressed under *proP* promoter control were used to further explore the impact of a *proQ* defect on ProP (Table 2). For cells from exponential and stationary phase LB cultures, a *proQ* defect reduced both ProP activity and β-galactosidase activity approximately 1.5-fold (Table 2). These effects on ProP activity were smaller than those reported previously for bacteria cultivated in minimal medium (3−5-fold) (e.g., ref 12). This may reflect physiological differences between bacteria cultivated in rich and minimal media, including the presence of osmoprotectants in LB medium that are known to attenuate the increase in ProP activity otherwise observed in response to high medium osmolality.[60,61]

How might ProQ control ProP levels? A number of observations suggest that ProQ may be an RNA-binding translational regulator. ProQ was found during an analysis of ribosome-associated proteins in *E. coli*.[62] A protease-sensitive linker connects N- and C-terminal ProQ domains that can be modeled on the crystal structures of known RNA chaperones FinO[15] and Hfq,[63] respectively[13,14] (Figure 1). ProQ binds RNA and possesses RNA chaperone activities (Figures 2−4, discussed further below). These observations suggest that ProQ may work with a small regulatory RNA to control *proP* translation. Efforts to identify such an sRNA have been unsuccessful. RNA copurifies with ProQ but only rRNA sequences were recovered upon synthesis, cloning, and sequencing of the corresponding cDNA.[64] Thus, ProQ serves as an RNA chaperone in vitro but its native RNA targets remain unknown.

ProQ could regulate ProP production directly or indirectly. In other work, DNA microarrays were used to assess the impact of lesion *proQ220*::Tn*5* on the transcriptome of bacteria cultivated under conditions that reveal the impact of ProQ on ProP activity.[65] Differential expression was observed for only one genetic locus. A modest elevation of the *ytfA* message level was observed in *proQ*$^+$ versus *proQ*$^-$ bacteria. The *ytfA* locus is believed to be a pseudogene, and the putative 108 residue protein encoded by the *E. coli* K-12 *ytfA* locus is identical to the C-termini of larger proteins encoded by the corresponding loci in wild type *E. coli* genomes. In sum, the available data suggest that, at least under the physiological conditions that affect ProP levels, ProQ

is not a global regulator and it does not control a global regulator of transcription.

The fact that an *rpoS* defect did not significantly alter the impact of a *proQ* defect on ProP production (Table 2) suggests that effects of ProQ on ProP levels do not depend on the promoter used for *proP* transcription or the length of the 5′-UTR (182 and 95 base pairs, respectively, for RpoD dependent transcription from the P1 promoter and RpoS-dependent transcription from the P2 promoter, respectively). Indeed, *proQ* lesions also reduced the level of ProP encoded by a pBAD24-based plasmid and expressed under the control of the *araBAD* promoter to yield a transcript that retains none of the *proP* 5′-UTR (Figure 5B). Thus, either the putative RNA targets of ProQ do not include the *proP* 5′UTR, or ProQ targets *proP* indirectly.

The FinO-like Domain May Provide an RNA-Binding Anchor for the Hfq-like RNA Chaperone Domain of ProQ. Significant insight into ProQ function is provided by structural modeling of ProQ on FinO and Hfq (Figure 1) plus analysis of the actions of ProQ and its domains on validated FinO substrates (Figures 2−4). FinO acts on FinP and the complementary region of the *traJ* mRNA 5′-UTR. These molecules contain highly stable stem-loop structures which provide a significant barrier to sense-antisense annealing. The individual hairpins from these RNAs, together with the adjacent 5′ and 3′ single-stranded tails, constitute minimal, high-affinity FinO binding targets.[19] The X-ray crystal structure of FinO revealed a novel, extended structure consisting of a folded domain and flexible N- and C-terminal extensions.[15] Biochemical analysis shows that the FinO region most critical for RNA binding, the folded core domain, is distinct from the region essential for chaperone function, the flexible N-terminal tail.[22] The N-terminal region corresponding to residues 1−61, shows little detectable RNA binding in electrophoretic mobility shift assays but nevertheless shows significant strand exchange and duplexing activities. A similar separation of function may occur in ProQ. The FinO-like domain may be primarily responsible for specific, high-affinity binding of a double-stranded FinO substrate and possesses little chaperone activity. ProQ lacks an extended flexible region N-terminal to the folded FinO-like domain, and the domain used in our studies ($ProQ_{FinO}$: residues 1−130) corresponds closely to the N-terminal FinO truncation mutant, FinO-(45−186), which has no RNA chaperone activity but binds RNA with high affinity.[22] However, the ProQ N-terminal domain partially complemented a *proQ* defect in vivo, restoring ProP activity.[14] This domain may have stronger chaperone activities with its cognate substrates than with the FinO substrates used for this study.

Intriguingly, another member of the FinO family of RNA chaperones has recently been discovered.[66] The crystal structure of this protein, NMB1681 from *Neisseria meningitides*, shows strong similarity to FinO, although NMB1681, like ProQ, lacks the extended, flexible N-terminus found in FinO. NMB1681 displays RNA strand exchange and duplexing activities on FinO substrates and moreover can rescue a *finO* deficiency to effect repression of plasmid conjugation in *E. coli*. Thus, while native sRNA substrates for NMB1681 have not been discovered, it is highly likely that this protein functions as an RNA chaperone in *Neisseria* and suggests that a FinO conjugation family could be a common RNA chaperone module throughout bacterial species.

The C-terminal domain of ProQ provides both RNA strand exchange and duplexing activities (Figures 3 and 4). This domain is clearly not related to the flexible, N-terminal region of FinO. It is rich in β-sheet structure and may adopt an Hfq-like fold.[14] This suggests that ProQ may contain two chaperone domains. The region linking the ProQ N- and C-terminal domains is dispensable for RNA binding, strand exchange, and duplexing. This indicates that the linker does not play a direct role in RNA chaperone function, and the way in which the N- and C-terminal domains are joined is not critical for chaperone activity.

In summary, these data suggest that ProQ acts on unknown RNA substrates, regulating translation in a manner that directly or indirectly amplifies ProP production. This phenomenon influences ProP synthesis based on *proP* transcription from RpoD- and RpoS promoters in response to both the osmotic pressure and bacterial growth phase. Defects at *proQ* reduce ProP activity 2−13-fold, depending on bacterial growth and assay conditions, and those effects are not confined to high osmolality conditions.[11,12] Thus, ProQ may also regulate ProP production in response to signals that have not yet been explored. The N-terminal and C-terminal domains of ProQ appear to be members of RNA chaperone families, typified by FinO and Hfq, which play highly specific roles in the regulation of bacterial gene expression.

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ABBREVIATIONS USED

BCA, bicinchoninic acid; bp, base pairs; BSA, bovine serum albumin; CD, circular dichroism; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid; EMSA, electrophoretic mobility shift assay; Hfq, host factor required for the replication of Qβ RNA bacteriophage; IPTG, isopropyl-β-D-thiogalactopyranoside; LB, Luria−Bertani medium; MOPS, 4-morpholinopropanesulfonic acid; Ni(NTA), nickel nitrilotriacetate; OD, optical density; PCR, polymerase chain reaction; PEI, polyethylenimine; RPM, revolutions per minute; SDS, sodium dodecyl sufate; PAGE, polyacrylamide gel electrophoresis; $ProQ_{FinO}$, the FinO-like

N-terminal domain of ProQ; $ProQ_{Hfq}$, the Hfq-like C-terminal domain of ProQ; $ProQ_{\Delta linker}$, a ProQ variant in which the FinO-like N-terminal domain is coupled directly to the Hfq-like C-terminal domain and the linker is absent; SH3 Domain, SRC Homology 3 Domain; Sm proteins, RNA binding heteroheptameric proteins; Tris, tris(hydroxymethyl)aminomethane

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