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Osmoregulation in *Escherichia coli*: Complementation Analysis and Gene-Protein Relationships in the *proU* Locus†

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The *proU* locus in *Escherichia coli* encodes an osmotically inducible transport system for two substrates, glycine betaine and L-proline, whose intracellular accumulation represents an important component in the physiology of osmoregulation. Several osmoresponsive *proU*::*lac* mutants were isolated and tested for complementation with plasmids carrying different functional regions of *proU*. Three classes of mutations were identified which were physically mapped to distinct regions of DNA from this locus. Tn*1000*-insertion mutagenesis of cloned *proU* DNA also yielded three phenotypic classes of mutations whose physical distribution approximately corresponded with those of the chromosomal mutants above. Three proteins, of M_r 44,000, 35,000, and 33,000, were shown to be products of *proU*, and the last of these was localized to the periplasmic space. The data indicate that *proU* is an operon with three genes, designated in order *proV*, *proW*, and *proX*, encoding respectively the gene products above. All three genes were shown to be necessary for exhibition of the *proU*-mediated osmoprotective effects of both glycine betaine and L-proline in *E. coli*.

When exposed to media of sufficiently low water activity, microbial cells suffer an inhibition of growth that is a consequence of intracellular water loss and a reduction in cell turgor. The term osmoregulation refers to the set of physiologic changes that occur which permit the organisms to adapt to and grow under these conditions. In many microorganisms, osmoregulation is associated with the buildup of internal solute content and therefore of intracellular osmolarity, so that cell turgor is restored (31, 34, 42). In the case of *Escherichia coli* and *Salmonella typhimurium*, the solutes which have been shown to accumulate include K^+ ions, glutamate, trehalose, L-proline, and glycine betaine, and several distinct transport systems and biosynthetic mechanisms have been characterized that mediate this broad class of responses towards water stress adaptation (8, 9, 14–22, 26, 30, 34, 37, 39, 41).

One osmoregulatory transport system that has been identified in both *E. coli* and *S. typhimurium* is encoded by the genetic locus *proU*, which maps at 58 min on the chromosome (8, 14, 20, 33). The ProU transporter is involved in the active uptake of glycine betaine and of L-proline from the medium in cells subjected to water stress and in the consequent ability of these compounds, in submillimolar concentrations, to promote growth in media of otherwise inhibitory osmolarity (8, 14, 20, 24, 37). The transcription of *proU* is induced several hundred-fold upon growth in high-osmolarity media, and the activity of the transporter is also increased under these conditions (8, 15, 20, 27, 33).

Recent studies have identified a 31- or 32-kilodalton (kDa) glycine betaine-binding periplasmic protein as a product of the *proU* locus both in *E. coli* and in *S. typhimurium* (4, 5, 18, 28, 33). This finding raised the possibility, by analogy with other well-characterized binding protein-dependent transport systems (1), that ProU is a multicomponent porter with several additional membrane-associated proteins, the genes for all of which are organized in a single operon. In this paper, we describe the isolation and characterization of several *proU*::*lac* mutants and their complementation profiles with plasmids carrying different regions of the cloned *proU* locus. Our results indicate that the locus contains three genes organized in a single unit of transcription; these findings have been corroborated by the nucleotide sequence analysis of *proU*, reported in the accompanying paper (23).

MATERIALS AND METHODS

Chemicals and media. All chemicals and restriction enzymes, [^{32}P]ATP, and [^{35}S]methionine were obtained from commercial sources. The media used in this study included MacConkey, minimal A (36), LB (36), and the low-osmolarity K medium described earlier (20). LBON medium was prepared by omitting NaCl from the composition described for LB.

Antibiotics were used at the following final concentrations (micrograms per milliliter): ampicillin (Amp), 50; kanamycin (Kan), 25; chloramphenicol, 25; and tetracycline (Tet), 15. The indicator dye 5-bromo-4-chloro-3-indolyl-β-D-galactoside was used in plates at a final concentration of 25 μg/ml.

Bacterial strains, plasmids, and phage. All strains in this study were derivatives of *E. coli* K-12 and are listed in Table 1. The plasmids used were those carrying different regions of the chromosomal *proU* locus (many of which have been described earlier; Fig. 1) or derivatives thereof constructed during the course of this work. The Mu dII301(*lac* Ap), Mu dII1734, and λ p*lac*Mu55 phages used in the *lac* fusion studies have been described earlier (11, 12, 33).

P1 *kc* transduction and conjugation. The methods for P1 *kc* transduction and conjugation were as earlier described (20, 36).

Isolation of *proU*::*lac* mutants. In the context of this study, the ProU$^-$ phenotype has been defined as one that manifests, in a *putPA proP* strain background, as (i) resistance to 0.3 mM 3,4-DL-dehydroproline (DHP) in minimal A medium supplemented with 0.2 M NaCl (DHP-NaCl^r) and (ii) an inability of 1 mM glycine betaine or 1 mM L-proline to confer osmoprotection in minimal A medium supplemented with 0.65 M NaCl (20, 24). The ProU$^+$ designation refers to strains exhibiting the converse of the two phenotypes above.

proU::*lac* strains were obtained after mutagenesis with the appropriate vector phage by initial selection for DHP-NaCl^r

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† Dedicated to Pushpa M. Bhargava on his 60th birthday.

TABLE 1. Strains of *E. coli* K-12

Strain	Genotype[a]	Source or reference
MC4100	F⁻ Δ(*argF-lac*)*U169 rpsL150 relA1 araD139 flbB5301 deoC1 ptsF25*	10
POII1734	F⁻ *araD139 ara-3*::Mu cts Δ(*lac*)*X174 galU galK rpsL* (mini-Mu dII1734)	12
MAL315[b]	*malK* or *malT*::Mu cts *araD139 rpsL150* Δ(*ara-leu*)*7697* Δ(*gpt-lac*)*5* [Mu dII301(Ap *lac*)]	11
JP3301	F⁺ *purE trp his argG ilv leu met thi ara xyl mtl* Δ(*argF-lac*)*U169 pheR372 recA56* [λ p(*pheA-lac*)]	25
CSR603	F⁻ *recA1 uvrA6 phr-1 thr-1 leu-6 proA2 argE thi-1 rpsL31*	40
GJ46	MC4100 *zfi-900*::Tn*10*	20
GJ134	MC4100 Δ*putPA101 proP222* Δ(*pyr-76*::Tn*10*)*461*	20
GJ157[c]	MC4100 Δ*putPA101 proP221 proX224*::*lac* Δ(*pyr-76*::Tn*10*)*462*	20
GJ201	GJ134 *proX234*::Mu dII301(Ap *lac*)	This study
GJ202	GJ134 *proX235*::Mu dII301(Ap *lac*)	This study
GJ203	GJ134 *proW236*::Mu dII301(Ap *lac*)	This study
GJ204	GJ134 *proX237*::Mu dII301(Ap *lac*)	This study
GJ205	GJ134 *proW238*::Mu dII301(Ap *lac*)	This study
GJ206	GJ134 *proW239*::Mu dII301(Ap *lac*)	This study
GJ208	GJ134 *proW240*::Mu dII301(Ap *lac*)	This study
GJ210	GJ134 *proW228*::Mu dII301(Ap *lac*)	This study
GJ222	GJ134 *recA srl*::Tn*10 proW230*::λ p*lac*Mu55(Kan)	This study
GJ229	GJ134 *recA srl*::Tn*10 proV229*::λ p*lac*Mu55(Kan)	This study
GJ232	GJ134 *recA srl*::Tn*10 proV232*::λ p*lac*Mu55(Kan)	This study
GJ313	GJ157 *recA srl*::Tn*10*	24
GJ314	GJ157 *recA* Δ(*srl*::Tn*10*)*461*	24
GJ316	GJ134 Δ*proU223 recA srl*::Tn*10*	24

[a] The nomenclature for genetic symbols and for transpositional insertions follows that described by Bachmann (3) and by Chumley et al. (13), respectively. The designations *proV*, *proW*, and *proX* are explained in the text. Allele numbers are indicated where they are known.
[b] MAL315 also has an additional, undefined auxotrophic requirement (11).
[c] The *proX224* allele was described as *proU224* in an earlier study (24).

lysogens; lysates of the two *lac* fusion phages employed for mutagenesis, Mu dII301(*lac* Ap) encoding Amp^r and λ p*lac* Mu55 encoding Kan^r, were each prepared and used as described (7, 11, 33). Each of the lysogens so obtained was then tested for its ProU and Lac phenotypes and also for the ability of the prophage markers to be crossed out when transduced to Tet^r with a P1 *kc* lysate grown on strain GJ46, which has Tn*10* linked 88% to *proU*$^+$ (20). The latter test allowed one to determine whether the strain was a *proU*::Mu*lac* monolysogen.

Transfer of *lac* insertions from chromosome to plasmids. Physical localization of the Mu prophage in each of the *proU*::Mu dII(*lac* Ap) insertions was done after the *lac* fusion was transferred in a two-step procedure from the chromosome onto pHYD58. First, the Mu dII(*lac* Ap) prophage was replaced by the mini-Mu derivative, Mu dII1734 (encoding Kan^r), by transducing the strain to Kan^r Amp^s with a P1 *kc* lysate prepared on a strain lysogenic for the latter transposon. Subsequently, pHYD58 was transformed into one of the transductants, and a fresh plasmid preparation was made from a population of these transformed cells. Derivatives of pHYD58 into which the *proU*::Mu dII1734 allele had been transferred by a RecA-mediated homologous recombination event were expected to be present in the latter plasmid preparation; a *recA* mutant of MC4100 was transformed with this preparation of DNA, and Amp^r colo-

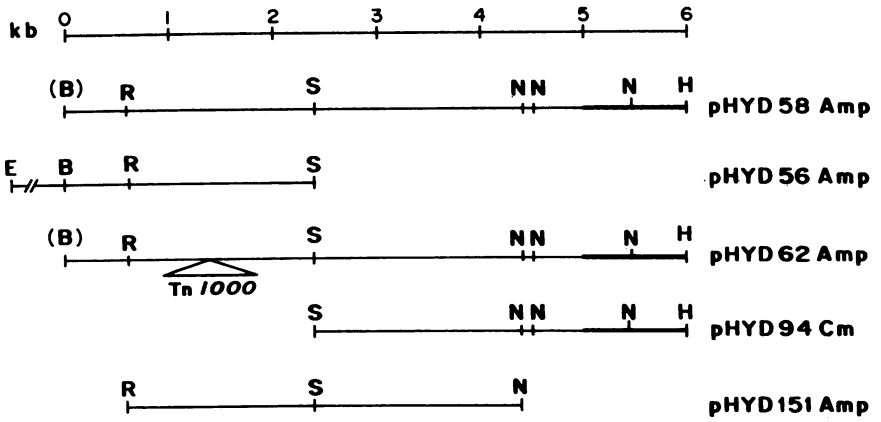

FIG. 1. Alignment and depiction of extent of insert DNA in plasmids carrying different regions of chromosomal *proU* locus. The plasmid number designation and the antibiotic marker carried by the corresponding vector are given at the right end of each line. The thin line represents *proU* DNA, and the thick line represents DNA from the *c* end of Mu phage that had transposed itself to this site in the course of the initial cloning of *proU* (24). Relevant restriction endonuclease cleavage sites are marked: B, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; N, *Nsi*I; R, *Eco*RV; and S, *Sal*I. The *Bgl*II site in *proU* has been lost in the process of construction of pHYD58 (24) and is therefore represented in parentheses on the line corresponding to this plasmid. A kilobase scale is included; the DNA between the *Eco*RI and *Bgl*II sites in pHYD56 is 3.5 kb long.

nies were selected on LBON plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside. Blue colonies identified among the transformants were shown subsequently to have the *proU*::Mu dII(Kan^r) allele transferred onto the *proU* region in pHYD58. It was found that the products of such homologous recombination events could only be recovered if the plasmid preparation used in the second step had been made from cells grown in LBON medium.

Some of the *proU*::Mu dII1734 mutations were also transferred by homologous recombination to plasmids pHYD52 and pHYD53 (24) and to pHYD151 (Fig. 1) by a method similar to that described for pHYD58 above.

Transfer of *recA* mutation into strains. All complementation studies were done in *recA* derivatives of mutant strains. The *recA* allele was introduced into the strains by cotransduction with *srl*::Tn*10* from a suitable donor strain (20).

Tn*1000* mutagenesis of pHYD58. Tn*1000* insertions in pHYD58 were obtained by F-mediated mobilization of the plasmid from a JP3301 derivative, as described (24).

Physical mapping of *proU* mutations. The mapping of *proU*::Mu dII1734 insertions in pHYD58 was done by following the usual procedures for plasmid isolation, restriction enzyme digestion, and agarose gel electrophoresis (32).

In the case of λ p*lac*Mu-generated operon fusions, the site of *proU*::*lac* insertion was determined directly on the chromosome. Chromosomal DNA was prepared, subjected to digestion with *Eco*RV (which cuts once in *proU* [Fig. 1] and then 2.1 kilobase pairs [kb] within the transposon) and gel electrophoresis, and transferred by Southern blotting to a Hybond^N membrane (Amersham, United Kingdom), as described (32). A ^{32}P-labeled probe specific to the 5′ end of the *proU* locus was prepared from the template of RE23.2, a recombinant M13 clone described in the accompanying paper (23), by Klenow-directed extension of hybridization probe primer (35). The probe was then hybridized to DNA immobilized on the membrane (32), and the size of the *proU*::*lac* restriction fragments was calculated from bands visualized after autoradiography.

Maxicell analysis. Plasmid-encoded proteins were labeled with [^{35}S]methionine in strain CSR603 derivatives, as described (40); the labeled proteins were visualized by fluorography after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (29).

Analysis of periplasmic proteins. Periplasmic proteins were isolated from cultures by either osmotic shock (38) or treatment with chloroform (2) and then visualized by Coomassie blue staining after gel electrophoresis as above.

β-Galactosidase assays. The activity of β-galactosidase in cultures was measured, after toluene treatment, by the method of Miller (36).

RESULTS

Isolation and complementation of *proU*::*lac* mutants. Earlier work in this laboratory on the cloned *proU* locus had indicated that it contains at least two cistrons and that the lone *proU224*::*lac* fusion until then characterized was in the one which was the more clockwise of the two on the *E. coli* genetic map (24).

Two approaches were followed in the present study in obtaining additional *lac* operon and gene fusions in the *proU* locus. In the first instance, the gene fusion phage Mu dII301(*lac* Ap) was used to infect GJ134, and selection was made for Amp^r DHP-NaCl^r lysogens. Fourteen of 135 ProU$^-$ colonies tested from this double selection exhibited an osmoresponsive Lac$^+$ phenotype. Ten of these were shown, by the test of transduction to Tet^r with P1(GJ46), to be monolysogens, and the other four were shown to be dilysogens (the second unmapped Mu insertion in each of the latter exhibited a Lac$^-$ phenotype).

The second approach was aimed specifically at obtaining *lac* fusions into the counterclockwise cistron previously designated as *proV* (24). The multicopy plasmid, pHYD62 (Fig. 1), with a Tn*1000* insertion in *proV*, is known to complement the *proU224*::*lac* mutation (24). A pHYD62 transformant of a *recA* derivative of GJ134, therefore, would be expected to give ProU$^-$ (that is, DHP-NaCl^r) Mu lysogens only if the insertion had occurred in *proV*. The *lac* fusion phage used in this experiment was λ p*lac*Mu55, which encodes Kan^r and yields operon fusions, and selection was made for Kan^r DHP-NaCl^r colonies. Thirty-nine ProU$^-$, osmoresponsive Lac$^+$ colonies were obtained in this selection, and the *lac* fusions in them were then transduced afresh into GJ134.

The *lac* fusion strains obtained with the two methods above were tested for their ability to be complemented to ProU$^+$ by each of the plasmids pHYD62 and pHYD94. We had at first thought that the two plasmids were functionally equivalent (see Fig. 1). However, their complementation profiles were dissimilar, and on this basis three classes of *lac* fusions were distinguished: class 1, not complemented by either pHYD62 or pHYD94 (and, therefore, presumptive *proV*::*lac* fusions); class 2, complemented by pHYD94 but not by pHYD62; and class 3, complemented by either plasmid (similar, therefore, to the *proU224*::*lac* fusion originally characterized).

Each of the 14 osmoresponsive Mu dII301-generated *proU*::*lac* mutants was complemented to ProU$^+$ by pHYD94, which result indicated that all of them belonged either to class 2 or to class 3. Further characterization was done only in eight of the monolysogenic strains, in each of which pHYD62 was introduced after the Amp^r marker of Mu dII301 was replaced by Kan^r of Mu dII1734 by homologous recombination; five tested positive for complementation by pHYD62 (class 3), and three tested negative (class 2).

As expected from the method by which they had been selected, none of the 39 λ p*lac*Mu55-generated *proU*::*lac* operon fusion strains was complemented to ProU$^+$ by pHYD62. Thirty-three of them, however, tested positive for pHYD94 complementation (class 2), and only six tested negative (class 1).

The results with pHYD62 and pHYD94 for representative strains of each of the three classes of mutants are summarized in Table 2. Also included in Table 2 are the data on complementation with plasmids pHYD58 and pHYD56 (Fig. 1) in these strains. As expected, pHYD58, which carries the entire *proU* locus, complemented the ProU$^-$ defect in all the strains examined; taken in conjunction with the P1(GJ46) transduction data, this result indicated that all fusions were indeed within the *proU* locus. There was no complementation by pHYD56 in any of the fusion strains, in particular not even in those that were presumptive *proV*::*lac* mutants. These results are discussed and interpreted below in terms of an arrangement of three cistrons within a single *proU* operon, with the three classes of *lac* fusions corresponding to the three complementation groups in this locus.

The magnitude of osmoresponsive induction of *lac* expression in the three classes of fusion strains was similar (approximately 100-fold; Table 2), nor was it different between the operon fusions and the gene fusions. However, the absolute level of induced β-galactosidase activity in gene

TABLE 2. Complementation results of and effect of osmolarity on specific activity of β-galactosidase in *proU*::*lac* fusion strains

Strain	*proU* genotype[a]	Complementation by[b]:				β-Galactosidase sp act[c] (U) after growth in	
		pHYD58	pHYD94	pHYD62	pHYD56	K medium	K medium + 0.4 M NaCl
Class 1							
GJ229	*proV*::*229* (operon)	+	−	−	−	4	325
GJ232	*proV*::*232* (operon)	+	−	−	−	3	200
Class 2							
GJ203	*proW*::*236* (gene)	+	+	−	−	9	1,495
GJ205	*proW*::*238* (gene)	+	+	−	−	7	1,685
GJ207	*proW*::*239* (gene)	+	+	−	−	6	1,500
GJ208	*proW*::*240* (gene)	+	+	−	−	5	1,108
GJ210	*proW*::*228* (gene)	+	+	−	−	9	1,875
GJ222	*proW*::*230* (operon)	+	+	−	−	11	1,090
Class 3							
GJ201	*proX*::*234* (gene)	+	+	+	−	55	5,300
GJ202	*proX*::*235* (gene)	+	+	+	−	50	4,440
GJ204	*proX*::*237* (gene)	+	+	+	−	22	3,740
GJ314	*proX*::*224* (operon)	+	+	+	−	4	560

[a] The nature of *lac* insertion (that is, whether it is an operon fusion or a gene fusion) in each of the strains is indicated within parentheses alongside the corresponding allele number.
[b] The symbols + and − refer respectively to ProU$^+$ and ProU$^-$ phenotypes, described in the text.
[c] Specific activity of β-galactosidase is given in units (U) as defined by Miller (36).

fusion strains of class 3 was significantly higher than it was in gene fusions of the second class (Table 2).

The direction of *lac* transcription in two representative fusion strains from each of the three classes was tested in chromosome-mobilization experiments using F′ (ts)*lac* (as described in reference 13); in all cases transcription was determined to be clockwise on the standard map representation of the *E. coli* chromosome (data not shown).

Definition of the ProU-encoding region on cloned chromosomal DNA in pHYD58. In a Tn*1000* mutagenesis experiment described earlier (24), the clockwise extremity of the *proU* locus defined by transposon insertion was 0.6 kb away from (in Fig. 1, to the right of) the *Sal*I site in the cloned region of chromosomal DNA. In the course of some subcloning experiments, however, we observed that (i) a plasmid, pHYD151 (Fig. 1), carrying the 3.8-kb *Eco*RV-*Nsi*I fragment from this locus did not complement a *proU224*::*lac* or a Δ*proU* mutant strain to ProU$^+$; (ii) furthermore, pHYD151 derivatives of these strains were now rendered extremely osmosensitive (EOS), in that they could grow on LBON medium but not on LB; (iii) these pHYD151 derivatives became ProU$^+$ upon further introduction of the plasmid pHYD94 into them; and (iv) the introduction of pHYD151 into strains that were *proU*$^+$ on the chromosome did not confer an EOS phenotype.

These observations suggested (i) that the region encoding the ProU transporter spans the *Nsi*I site 1.8 kb to the right of the *Sal*I site and (ii) that Tn*1000* insertions in this region had not been identified earlier (24), because the associated EOS phenotype would have precluded growth on LB medium upon which the selection for mutants had been done.

Additional Tn*1000* insertions in pHYD58 were therefore obtained by F-mediated mobilization of the plasmid from a JP3301 derivative into GJ313 (a *proU224*::*lac* strain) as described previously (24), with the modification that exconjugants with mutagenized plasmid derivatives were selected for on LBON medium. Tn*1000* insertion mutants in the cloned *proU* locus were identified by their inability to exhibit (i) a reduced inducibility of *proU*::*lac* expression on MacConkey-NaCl plates and (ii) enhanced osmoprotection by glycine betaine, both of which are characteristic phenotypic features of multicopy-*proU*$^+$ strains (24); such mutants represented approximately 20% of the total number of exconjugants tested. These mutants were further divided into three classes: nonosmosensitive (NOS) that could grow well on LB + 0.2 M NaCl; osmosensitive (OS) that grew well on LB, but poorly on LB + 0.2 M NaCl; and EOS, as defined above. The proportional representation of these three classes was, respectively, 1:1:2. Plasmids from the first two classes, NOS and OS, gave a weak ProU$^+$ phenotype in the *proU224*::*lac* strain (indicating partial complementation) and a ProU$^-$ phenotype in GJ316, a Δ*proU* strain. All three classes of Tn*1000*-generated plasmid mutations failed to show any complementation of the ProU$^-$ phenotype in *proU*::*lac* fusion strains of the first and second categories (that is, other than that of which *proU224*::*lac* is part) described above.

The physical map locations on pHYD58 DNA of 2, 10, and 20 independent Tn*1000* insertions, respectively, from the NOS, OS, and EOS classes were determined (Fig. 2). Each of the three classes of insertions was discretely clustered, and those of the NOS and OS categories were observed to correspond, as expected, respectively to insertions of groups II and I that had been described earlier (24). The EOS class of insertions spanned the region between 0.65 and 2.1 kb to the right (that is, clockwise) of the *Sal*I site in this locus.

Physical mapping of *proU*::*lac* chromosomal fusions. To determine the correlation, if any, between the three complementation groups defined by the chromosomal *lac* fusions in *proU* and the three phenotypic classes of Tn*1000* insertion mutations in pHYD58, we chose to map the sites of Mu insertion in several of our *lac* fusion strains. For this purpose, all eight Mu dII1734 insertions that were constructed from the original gene fusions were transferred by homologous recombination to pHYD58, and their positions on the plasmid were mapped with the aid of restriction enzyme digestions; in the case of the λ p*lac*Mu operon fusions, physical mapping was done by Southern blot hybridization of chromosomal DNA from these strains.

The two fusions from the first group were located to the left of the *Sal*I site, the five from the second group were distributed in the region between 0.15 and 0.7 kb to the right

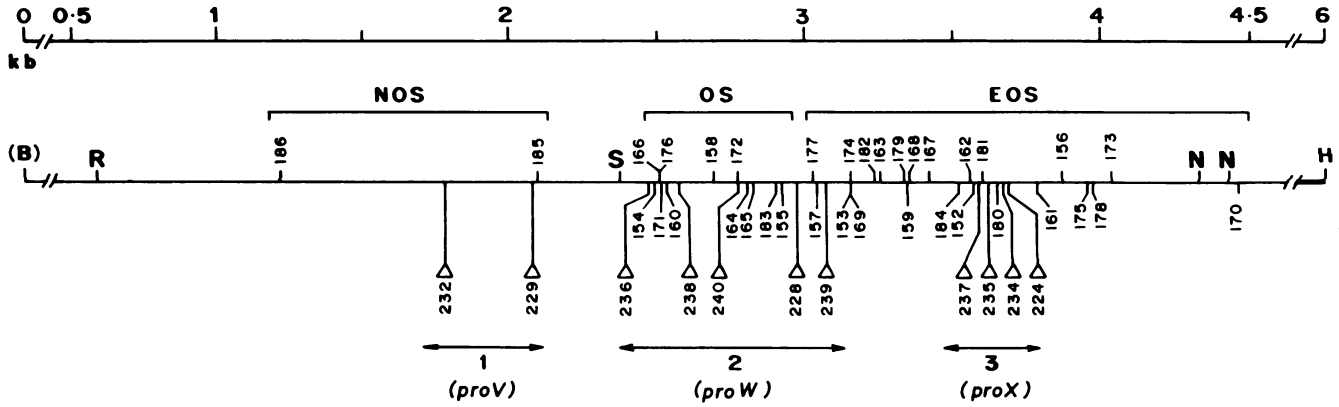

FIG. 2. Physical mapping of *proU*::Tn*1000* and *proU*::*lac* insertions. The insert DNA of pHYD58 is depicted as a horizontal line; line symbols for *proU* DNA and Mu DNA and abbreviations for restriction enzyme recognition sites are the same as those explained in the legend to Fig. 1. A kilobase scale is included for the region of *proU* DNA between the *Eco*RV and *Nsi*I sites, and the coordinates on this scale have been chosen such that they are in register with the scale shown in Fig. 1. Short vertical lines represent the map positions of various independent *proU*::Tn*1000* insertions, and their placement above or below the horizontal line indicates the two orientations of insertion, respectively (as described in reference 24); the corresponding pHYD plasmid number designations are given alongside. The categorization of the Tn*1000* insertions into the NOS, OS, and EOS phenotypic classes is also shown above the line; only two Tn*1000* insertions of the NOS class were chosen for mapping in this study because many others of this class have been mapped to the same region earlier (24). Long vertical lines (ending with Δ) represent the map positions of chromosomal *proU*::*lac* insertions, whose corresponding allele numbers are given alongside. All *lac* insertions mapped were oriented in a single direction (such that *lac* transcription would proceed from left to right in the figure). The classification of the *lac* fusions into groups 1, 2, or 3, corresponding respectively to insertions in *proV*, *proW*, and *proX* (as explained in the text), is also shown.

of the *Sal*I site, and the three fusions from the third group were clustered about 1.2 kb to the right of the same site (Fig. 2). The physical location of the *proU224*::*lac* operon fusion described earlier (24) has been reexamined and mapped more precisely (K. Rajkumari, unpublished data) and shown also to be situated 1.3 kb to the right of the *Sal*I site.

Osmoresponsivity of *proU*::*lac* on plasmids. Two of the *proU*::Mu dII1734 alleles that had been studied on pHYD58 (Fig. 2) were also transferred by homologous recombination from the chromosome onto the plasmid pHYD151 (Fig. 1) and to pHYD52 and pHYD53. The latter two plasmids carry DNA from the *proU* locus inclusive of all that is present on pHYD58 and extending, respectively, 5.5 and 3.5 kb further upstream (24). Each of the mutant plasmid derivatives was introduced into MC4100 *recA*, and the resulting strains were tested for osmotic inducibility of β-galactosidase expression. We noticed that the strains grew very slowly in media of elevated osmolarity and that there was poor reproducibility in steady-state β-galactosidase activity values under these conditions, presumably because of the effects of overexpression of hybrid proteins from the plasmids and the consequent selection for compensatory mutations on them (6). The ability for *lac* expression to be induced upon the instantaneous addition of osmolyte was, therefore, tested in these strains (Table 3), and the observed values were compared with those obtained in derivatives carrying the *lac* fusions on the chromosome. The results indicate that the magnitude of osmoresponsivity of *lac* expression on the various plasmids is approximately the same as that on the chromosome for each of the two fusions examined. In particular, the data also suggest that the *cis* elements responsible for osmotic induction of *proU* expression are contained downstream of the *Eco*RV site in pHYD151.

Identification of proteins encoded by the *proU* locus. The maxicell technique (40) was used in the [^{35}S]methionine labeling and identification of proteins encoded by the cloned *proU* locus; in addition to the vector, pBR322, and the *proU*$^+$ plasmid, pHYD58, one plasmid each from the three classes of Tn*1000* insertion derivatives were chosen for this analysis (Fig. 3). Three proteins, of M_r 44,000, 35,000, and 33,000, were identified as products of pHYD58 that were absent in one or more of the Tn*1000* insertion derivatives. The 33-kDa protein was absent in all three classes of Tn*1000* insertions, the 35-kDa product was absent in the NOS and OS classes and present in the EOS class, and the 44-kDa protein was present in both the OS and EOS classes, but had been replaced by a 39-kDa band in the lane representing the Tn*1000* derivative of the NOS class.

Several workers have identified a 31- or 32-kDa periplasmic glycine betaine-binding protein as product encoded by the *proU* locus in both *E. coli* and *S. typhimurium* (4, 5, 28, 33). We isolated periplasmic proteins by established procedures (2, 38) from the *proU*$^+$ strain GJ134, grown in LB medium and LB + 0.2 M NaCl, and detected a 33-kDa protein that was induced upon growth at high osmolarity (data not shown). The induced level of this protein was

TABLE 3. Osmotic inducibility of *proU* expression on various plasmids

Site of *lac* fusion	β-Galactosidase sp act (U) with[a]:			
	proW238::Mu dII1734		*proW240*::Mu dII1734	
	0 min	15 min	0 min	15 min
Chromosome[b]	7	96	5	64
pHYD52	ND[c]	ND	33	467
pHYD53	22	545	31	553
pHYD58	16	694	28	646
pHYD151	26	647	37	550

[a] Cultures of MC4100 *recA* strains carrying the various plasmid derivatives were grown in K medium at 37°C to early log phase. Specific activity of β-galactosidase in each culture was measured at 0 min and at 15 min after addition of NaCl to 0.4 M.

[b] Osmotic inducibility of the corresponding *lac* fusions on the chromosome was assayed as described above in the Mu dII1734-converted derivatives of strains GJ205 and GJ208.

[c] ND, Not determined.

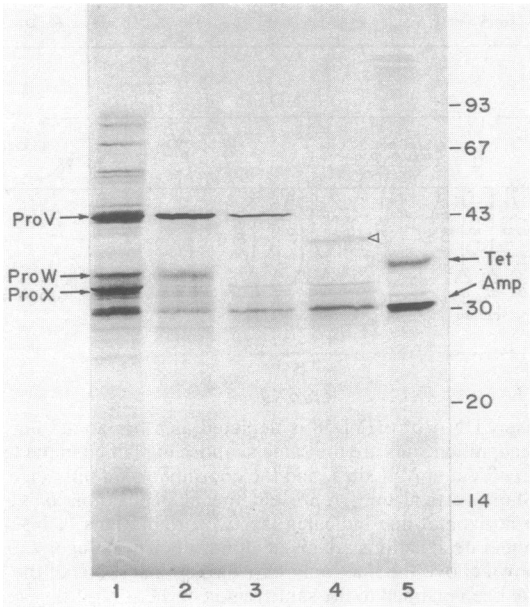

FIG. 3. Analysis of *proU*-encoded proteins after [^{35}S]methionine labeling in maxicells and gel electrophoresis on 10% polyacrylamide-sodium dodecyl sulfate. Different lane numbers correspond to labeled proteins prepared from CSR603 derivatives transformed with the following plasmids (abbreviated plasmid phenotype given in parentheses): 1, pHYD58 (ProU$^+$); 2, pHYD170 (EOS); 3, pHYD172 (OS); 4, pHYD185 (NOS); and 5, pBR322 (vector). The positions and sizes (in kilodaltons) of proteins run as molecular size markers on the same gel are indicated. The Amp and Tet proteins encoded by the vector are marked, and so are the bands corresponding to ProV, ProW, and ProX, whose identification is described in the text. The truncated ProV protein in the lane corresponding to pHYD185 is indicated by the open arrowhead.

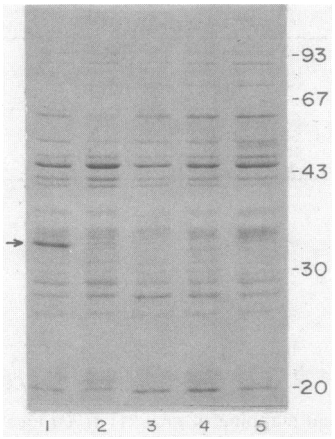

FIG. 4. Analysis of periplasmic proteins from various *proU* strains. Periplasmic proteins were released from cultures grown in minimal A medium + 0.2 M NaCl (unless otherwise stated) by treatment with chloroform (2) and then electrophoresed on a sodium dodecyl sulfate-12% polyacrylamide gel. Lanes: 1, GJ134 (proU$^+$); 2, GJ134 grown in the medium described above supplemented with 1 mM glycine betaine; 3, GJ201 (*proU*::*lac*, class 3); 4, GJ205 (*proU*::*lac*, class 2); and 5, GJ232 (*proU*::*lac*, class 1). The *proU*-encoded periplasmic protein is indicated by an arrow. The positions and sizes (in kilodaltons) of proteins run as molecular size markers on the same gel are indicated. Similar results were obtained upon analysis of periplasmic proteins released by the osmotic shock procedure of Nossal and Heppel (38) (data not shown).

reduced upon glycine betaine supplementation of high-osmolarity medium (Fig. 4), as would be expected of a product encoded by *proU*; furthermore, this protein was absent in the periplasmic protein preparations from all the three classes of *lac* fusion mutant strains grown at high osmolarity (Fig. 4).

DISCUSSION

The results of the maxicell experiments with the plasmids carrying *proU*$^+$ and various Tn*1000* insertions therein are best explained on the assumptions that there are three genes in this locus, organized clockwise in a single operon, and that Tn*1000* insertions in the promoter-proximal genes exert polar effects on the expression of those downstream in the operon. In accordance with the suggestion of Faatz et al. (18) on nomenclature, we propose that the three genes be designated, in order, *proV*, *proW*, and *proX* and that the term *proU* be retained for use in reference either to the entire operon or to mutations in this locus that have not been definitively mapped to one or the other of its constituent genes. The gene now designated as *proV* was identified by the same name in our earlier work as well (24). Three proteins of M_r 44,000, 35,000, and 33,000, detected in the maxicell experiments, are the respective products of *proV*, *proW*, and *proX*. The new 39-kDa band observed with the *proV*::Tn*1000* mutant (Fig. 3) probably represents the synthesis of a truncated ProV protein from the plasmid in this strain, which allows one to calculate that the site of translation initiation of *proV* is situated 0.6 kb downstream from the *Eco*RV site in this locus. The model for a single transcription unit in *proU* is supported also by the observations that all Lac$^+$ *proU*::*lac* operon fusions show the same pattern and magnitude of osmotic induction and that all of them are oriented in the same direction on the chromosome (as examined both by chromosome mobilization and by physical mapping).

We believe that the three classes of *lac* fusion strains identified by complementation analysis correspond to insertions in the three different genes in this locus: the class complemented only by pHYD58 corresponds to *proV* (class 1), that complemented by pHYD94 and pHYD58 but not by pHYD62 corresponds to *proW* (class 2), and that complemented by all three plasmids corresponds to *proX* (class 3). The reasons for the low frequency of recovery of *proV*::*lac* mutations in DHP-NaCl^r selection are not clear. The complementation results are unexpected in terms of the operon model for *proU* organization, but can be explained on the basis of three assumptions. (i) The Mu insertion mutations in *proV* and *proW* on the chromosome have the expected polar effect on expression of the respective downstream genes. The inability of pHYD56 to complement *proV*::*lac* mutants, and of all classes of *proU*::Tn*1000* plasmid derivatives to complement *proV*::*lac* and *proW*::*lac* strains, is therefore explained. (ii) There is a weak internal promoter within *proW* that is capable of expressing *proX* to an extent sufficient for partial complementation (and yet not adequate for detection in maxicell experiments) from a multicopy state. This would account for the earlier interpretation (24) that there are two transcription units in the *proU* locus and would also explain how both pHYD62 and pHYD94 could complement insertions in *proX*. (iii) In the plasmid pHYD94, a functional *proW* gene product is also expressed from a plasmid-borne promoter. Consequently, pHYD94 is able to complement insertions in *proW*, but pHYD62 is unable to do

so because the *proV*::Tn*1000* insertion has abolished expression of the downstream *proW* gene on the latter plasmid.

The fact that the 33-kDa periplasmic protein is absent in all three classes of *proU*::*lac* mutants once again suggests that polarity effects of the Mu insertions are being observed and corroborates the maxicell data indicating that this protein is the product of the third gene, *proX*. The sizes of the three proteins identified in the maxicell experiments are similar to those reported earlier by Faatz et al. (18) from analysis of *proU*-encoded products in minicells. Contrary to their conclusions, however, our data clearly indicate that the periplasmic glycine betaine-binding protein is the product of the third gene in the operon. Furthermore, the organization, extent, and location of the three genes, *proV*, *proW*, and *proX*, deduced from the data reported here, are in accord with the corresponding features of three open reading frames identified from the nucleotide sequence of the *proU* operon, reported in the accompanying paper (23).

We suggest the following explanation for the three phenotypic classes of Tn*1000* insertions in pHYD58. (i) The OS phenotype is associated with maximally induced expression of ProV alone, with little or no ProW and ProX product. (ii) The EOS phenotype is associated with overexpression of the N-terminal region of ProW, perhaps in combination also with overexpression of ProV. (iii) By exclusion, the NOS class represents insertions in *proV*. One important component in the cases of both (i) and (ii) above is the presence of the concerned genes on multicopy plasmids; another important factor in the EOS class is the total absence of expression of ProX, so that there is no possibility of the down-regulation of *proU* expression that is normally observed with the accumulation of glycine betaine or L-proline both in haploid $proU^+$ and in multicopy $proU^+$ strains (24, 33). Consistent with the above suggestions are our additional findings that (i) the OS or EOS phenotype is never observed with any of the plasmids above in strains that are $proU^+$ on the chromosome; (ii) pHYD56 transformants of GJ314 or GJ316 exhibit an OS phenotype (24); and (iii) strains carrying the entire $proU^+$ locus on a multicopy plasmid (such as pHYD58) are unable to grow in minimal A medium supplemented with $\geq$0.2 M NaCl unless glycine betaine or L-proline is also provided (J. Gowrishankar and K. Rajkumari, unpublished data). One corollary of the model above is that Tn*1000* insertions in *proW* that are downstream of the internal promoter in this gene would have an EOS phenotype. This is probably the case with insertions in plasmids pHYD157 and pHYD177 depicted in Fig. 2.

Another interesting observation that emerges from both our work and that of Faatz et al. (18) is that chromosomal *lac* gene fusions (in comparison with the plasmid-borne Tn*1000* insertions) in the third gene, *proX*, are restricted to the promoter-proximal region of the gene. This suggests that fusions further downstream within the gene may not have survived the high-osmolarity conditions used for their selection, perhaps because of toxicity associated with the inability of the corresponding hybrid proteins to be translocated across the inner membrane (6).

Barron et al. (4) have purified the *proU*-encoded glycine betaine-binding protein, but were unable to demonstrate any binding to L-proline by this protein; they therefore speculated that L-proline transport through *proU* is accomplished either with the aid of a distinct, as yet unidentified, periplasmic binding protein, or without the need for any periplasmic component at all. Our results indicate, however, that mutations in *proX* alone abolish the osmoprotectant activity of L-proline and that the activity is restored by complementation with just the three genes of this locus. Indeed, in all our complementation experiments, we have found that the *proU*-encoded phenotypes of osmoprotection by glycine betaine and by L-proline are inseparably linked. It would appear, therefore, that all three protein components of ProU are necessary and sufficient for active transport of both these solutes.

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LITERATURE CITED

1. **Ames, G. F.-L.** 1986. Bacterial periplasmic transport systems: structure, mechanism and evolution. Annu. Rev. Biochem. **55**:397–425.
2. **Ames, G. F.-L., C. Prody, and S. Kustu.** 1984. Simple, rapid, and quantitative release of periplasmic proteins by chloroform. J. Bacteriol. **160**:1181–1183.
3. **Bachmann, B. J.** 1983. Linkage map of *Escherichia coli* K-12, edition 7. Microbiol. Rev. **47**:180–230.
4. **Barron, A., J. U. Jung, and M. Villarejo.** 1987. Purification and characterization of a glycine betaine binding protein from *Escherichia coli*. J. Biol. Chem. **262**:11841–11846.
5. **Barron, A., G. May, E. Bremer, and M. Villarejo.** 1986. Regulation of envelope protein composition during adaptation to osmotic stress in *Escherichia coli*. J. Bacteriol. **167**:433–438.
6. **Bassford, P., and J. Beckwith.** 1979. *Escherichia coli* mutants accumulating the precursor of a secreted protein in cytoplasm. Nature (London) **277**:538–541.
7. **Bremer, E., T. J. Silhavy, and G. M. Weinstock.** 1985. Transposable λ *plac*Mu bacteriophages for creating *lacZ* operon fusions and kanamycin resistance insertions in *Escherichia coli*. J. Bacteriol. **162**:1092–1099.
8. **Cairney, J., I. R. Booth, and C. F. Higgins.** 1985. Osmoregulation of gene expression in *Salmonella typhimurium*: *proU* encodes an osmotically induced betaine transport system. J. Bacteriol. **164**:1224–1232.
9. **Cairney, J., I. R. Booth, and C. F. Higgins.** 1985. *Salmonella typhimurium proP* gene encodes a transport system for the osmoprotectant betaine. J. Bacteriol. **164**:1218–1223.
10. **Casadaban, M. J.** 1976. Transposition and fusion of *lac* genes to selected promoters in *Escherichia coli* using bacteriophage lambda and Mu. J. Mol. Biol. **104**:541–555.
11. **Casadaban, M. J., and J. Chou.** 1984. *In vivo* formation of gene fusions encoding hybrid β-galactosidase in one step with a transposable Mu-*lac* transducing phage. Proc. Natl. Acad. Sci. USA **81**:535–539.
12. **Castilho, B. A., P. Olfson, and M. J. Casadaban.** 1984. Plasmid insertion mutagenesis and *lac* gene fusion with mini-Mu bacteriophage transposon. J. Bacteriol. **158**:488–495.
13. **Chumley, F. G., R. Menzel, and J. R. Roth.** 1979. Hfr formation directed by Tn*10*. Genetics **91**:639–655.
14. **Csonka, L. N.** 1982. A third L-proline permease in *Salmonella typhimurium* which functions in media of elevated osmotic strength. J. Bacteriol. **151**:1433–1443.
15. **Dunlap, V. J., and L. N. Csonka.** 1985. Osmotic regulation of L-proline transport in *Salmonella typhimurium*. J. Bacteriol. **163**:296–304.
16. **Epstein, W., and L. Laimins.** 1980. Potassium transport in *Escherichia coli*: diverse systems with common control by osmotic forces. Trends Biochem. Sci. **5**:21–23.
17. **Epstein, W., and S. G. Schultz.** 1965. Cation transport in *Escherichia coli*. V. Regulation of cation content. J. Gen. Physiol. **49**:221–234.
18. **Faatz, E., A. Middendorf, and E. Bremer.** 1988. Cloned struc-

tural genes for the osmotically regulated binding-protein-dependent glycine betaine transport system (ProU) of *Escherichia coli* K-12. Mol. Microbiol. **2**:265–279.

19. **Giaever, H. M., O. B. Styrvold, I. Kaasen, and A. R. Strøm.** 1988. Biochemical and genetic characterization of osmoregulatory trehalose synthesis in *Escherichia coli*. J. Bacteriol. **170**: 2841–2849.
20. **Gowrishankar, J.** 1985. Identification of osmoresponsive genes in *Escherichia coli*: evidence for participation of potassium and proline transport systems in osmoregulation. J. Bacteriol. **164**: 434–445.
21. **Gowrishankar, J.** 1986. *proP*-mediated proline transport also plays a role in *Escherichia coli* osmoregulation. J. Bacteriol. **166**:331–333.
22. **Gowrishankar, J.** 1988. Osmoregulation in *Enterobacteriaceae*: role of proline/betaine transport systems. Curr. Sci. **57**:225–234.
23. **Gowrishankar, J.** 1989. Nucleotide sequence of the osmoregulatory *proU* operon of *Escherichia coli*. J. Bacteriol. **171**: 1923–1931.
24. **Gowrishankar, J., P. Jayashree, and K. Rajkumari.** 1986. Molecular cloning of an osmoregulatory locus in *Escherichia coli*: increased *proU* gene dosage results in enhanced osmotolerance. J. Bacteriol. **168**:1197–1204.
25. **Gowrishankar, J., and J. Pittard.** 1982. Molecular cloning of *pheR* in *Escherichia coli* K-12. J. Bacteriol. **152**:1–6.
26. **Grothe, S., R. L. Krogsrud, D. J. McClellan, J. L. Milner, and J. M. Wood.** 1986. Proline transport and osmotic stress response in *Escherichia coli* K-12. J. Bacteriol. **166**:253–259.
27. **Gutierrez, C., J. Barondess, C. Manoil, and J. Beckwith.** 1987. The use of transposon Tn*phoA* to detect genes for cell envelope proteins subject to a common regulatory stimulus: analysis of osmotically regulated genes in *Escherichia coli*. J. Mol. Biol. **195**:289–297.
28. **Higgins, C. F., L. Sutherland, J. Cairney, and I. R. Booth.** 1987. The osmotically regulated *proU* locus of *Salmonella typhimurium* encodes a periplasmic betaine-binding protein. J. Gen. Microbiol. **133**:305–310.
29. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
30. **Landfald, B., and A. R. Strøm.** 1986. Choline-glycine betaine pathway confers a high level of osmotic tolerance in *Escherichia coli*. J. Bacteriol. **165**:849–855.
31. **Le Rudulier, D., A. R. Strøm, A. M. Dandekar, L. T. Smith, and R. C. Valentine.** 1984. Molecular biology of osmoregulation. Science **224**:1064–1068.
32. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
33. **May, G., E. Faatz, M. Villarejo, and E. Bremer.** 1986. Binding protein dependent transport of glycine betaine and its osmotic regulation in *Escherichia coli* K-12. Mol. Gen. Genet. **205**: 225–233.
34. **Measures, J. C.** 1975. Role of amino acids in osmoregulation of non-halophilic bacteria. Nature (London) **257**:398–400.
35. **Messing, J.** 1983. New M13 vectors for cloning. Methods Enzymol. **101**:20–78.
36. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
37. **Milner, J. L., D. J. McClellan, and J. M. Wood.** 1987. Factors reducing and promoting the effectiveness of proline as an osmoprotectant in *Escherichia coli*. J. Gen. Microbiol. **133**: 1851–1860.
38. **Nossal, N. G., and L. H. Heppel.** 1966. The release of enzymes by osmotic shock from *Escherichia coli* in exponential phase. J. Biol. Chem. **241**:3055–3062.
39. **Perroud, B., and D. Le Rudulier.** 1985. Glycine betaine transport in *Escherichia coli*: osmotic modulation. J. Bacteriol. **161**:393–401.
40. **Sancar, A., A. M. Hack, and W. D. Rupp.** 1979. Simple method for identification of plasmid-coded proteins. J. Bacteriol. **137**: 692–693.
41. **Strøm, A. R., P. Falkenberg, and B. Landfald.** 1986. Genetics of osmoregulation in *Escherichia coli*: uptake and biosynthesis of organic osmolytes. FEMS Microbiol. Rev. **39**:79–86.
42. **Yancey, P. H., M. E. Clark, S. C. Hand, R. D. Bowlus, and G. N. Somero.** 1982. Living with water stress: evolution of osmolyte systems. Science **217**:1214–1222.