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Molecular Cloning of an Osmoregulatory Locus in *Escherichia coli*: Increased *proU* Gene Dosage Results in Enhanced Osmotolerance

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The *proU* locus in *Escherichia coli* encodes an important osmoregulatory function which mediates the growth-promoting effect of L-proline and glycine betaine in high-osmolarity media. This locus was cloned, in contiguity with a closely linked Tn*10* insertion, onto a multicopy plasmid directly from the *E. coli* chromosome. For a given level of osmotic stress, the magnitude of osmoresponsive induction of a single-copy *proU*::*lac* fusion was reduced in strains with multiple copies of the *proU*$^+$ genes; in comparison with haploid *proU*$^+$ strains, strains with the multicopy *proU*$^+$ plasmids also exhibited enhanced osmotolerance in media supplemented with 1 mM L-proline or glycine betaine. Experiments involving subcloning, Tn*1000* mutagenesis, and interplasmid complementation in a deletion mutant provided evidence for the presence at this locus of two cistrons, both of which are necessary for the expression of ProU function. We propose the designations *proU* for the gene originally identified by the *proU224*::Mu d1(*lac* Ap) insertion and *proV* for the gene upstream (that is, counterclockwise) of *proU*.

In a large variety of microorganisms, adaptation to growth in water-stressed environments is associated with, and dependent upon, the intracellular accumulation of certain solutes such as K^+, L-proline, and glycine betaine (28, 29, 32, 39). Genetic studies have led to the identification in enterobacteria of transport systems for each of these solutes (3, 4, 10, 13–15, 19, 24, 35) and of a pathway for synthesis of glycine betaine from choline (25, 38), all of which are activated under conditions of osmotic stress. One important osmoregulatory locus so identified in both *Escherichia coli* and *Salmonella typhimurium* is *proU*, which had earlier been shown to encode an active transport system for L-proline (10, 13, 14) and more recently has also been implicated in the active transport of glycine betaine in *S. typhimurium* (4). Both L-proline and glycine betaine are able, at submillimolar exogenous concentrations, to promote the growth of organisms of the family *Enterobacteriaceae* in high-osmolarity media (7, 8, 10, 14, 27, 29). The *proU* locus has been mapped to 58 min, and its transcription shown to be stimulated 400-fold in media of elevated osmolarity (4, 10, 13, 14, 19).

In an approach toward a molecular characterization of the *proU* locus, we describe below its cloning on multicopy plasmids directly from the *E. coli* chromosome. Our studies have shown that this locus is comprised of at least two cistrons that are together required for ProU function and, furthermore, that the presence of multiple copies of *proU*$^+$ increases osmotolerance in *E. coli*.

MATERIALS AND METHODS

Bacterial strains and phage. All bacterial strains were derivatives of *E. coli* K-12 and are listed in Table 1. The phages λ p1(209) (5), and P1 *kc* were from our laboratory collection (14). The preparation of λ p*proU-lac*9 phage is described below.

Plasmids. The plasmids pBR322 (2) and pBC4042 (18) have been described earlier. The pHYD plasmids constructed in this study are described in the legends to Fig. 2 and 4. pHYD54, the vector used in the construction of pHYD94 (see Fig. 2h), is a derivative of pACYC184 (6) and was obtained after *Hin*dIII digestion and ligation of pHYD50; it represents the circularized version of the DNA fragment between the kilobase-pair (kb) coordinates 4.6 and 8.6 in Fig. 2a.

Chemicals and media. All antibiotics and chemicals, including restriction endonucleases and T4 DNA ligase, were purchased from commercial sources. The growth media that were used included minimal A and LB media (33) and the low-osmolar K medium with or without 0.5% Casamino Acids (14). Indicator media, used in the screening of *proU-lac* expression, included MacConkey agar and eosin methylene blue-lactose agar and were supplemented with 0.2 M NaCl as appropriate.

Antibiotics were routinely used in the following concentrations: tetracycline, 15 μg/ml in nutrient medium and 5 μg/ml in minimal medium; ampicillin, 50 μg/ml; and chloramphenicol, 25 μg/ml. Amp^r selection was done at 100 μg of ampicillin per ml in the pHYD58 mobilization experiment. Strains with the plasmids pHYD52, pHYD53, or pHYD55 expressed Tet^r only to 5 μg of tetracycline per ml even in nutrient media.

The L-proline analogs azetidine 2-carboxylic acid and 3,4-dehydro-DL-proline (DHP) were used at final concentrations of 1 and 0.3 mM, respectively, in minimal A medium containing 0.2% glucose as the carbon source.

λ phage techniques. λ phage lysates were prepared either by UV induction of lysogens or by propagation from single plaques (33). The methods for obtaining lysogens and of testing for lysogeny have been described earlier (14).

Recombinant DNA techniques. The protocols described by Maniatis et al. (31) were followed for the preparation of DNA from λ phage and plasmids, restriction endonuclease digestion, gel electrophoresis, ligation, and transformation. Restriction fragment sizes were calculated from their mobility on agarose gels with the aid of the computer program of Duggleby et al. (12), with λ *Hin*dIII fragments as standards.

Other methods. Growth of strains in broth was monitored by measurements of optical density in a Klett-Summerson colorimeter. The methods for conjugation (33), P1 *kc* transduction (14), Tet^s selection (30), and temperature induc-

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TABLE 1. *E. coli* K-12 strains used

Strain	Genotype[a]	Source or derivation
MC4100	F⁻ Δ(*argF-lac*)*U169 rpsL150 relA1 araD139 flbB5301 deoC1 ptsF25*	5
JP3301	F⁺ *purE trp his argG ilv leu met thi ara xyl mtl* Δ(*argF-lac*)*U169 pheR372 recA56* [λ p(*pheA-lac*)]	17
Xph43	F⁻ Δ(*argF-lac*)*U169 trp* Δ(*brnQ-phoR*)*24*	18
GJ2	MC4100 *proU224*::Mu d1(*lac* Ap)	14
GJ134	MC4100 Δ*putPA101 proP222* Δ(*pyr-76*::Tn*10*)	14
GJ141	MC4100 *proU224*::*lac* stabilized by λ p1(209)	This study
GJ145	GJ134 *zfi-900*::Tn*10*[b]	By P1 *kc* transduction
GJ146	GJ134 Δ*proU233*	This study
GJ157	MC4100 Δ*putPA101 proP221 proU224*::*lac* Δ(*pyr-76*::Tn*10*)	14
GJ179	GJ157(pHYD50)	This study
GJ251	GJ134 Δ*proU233* [λ p*proU-lac*9]	This study
GJ313	GJ157 *recA srl*::Tn*10*	By P1 *kc* transduction
GJ314	GJ157 *recA* Δ(*srl*::Tn*10*)	From GJ313, by Tet[s] selection
GJ315	MC4100 Δ*putPA101 proP223 proU224*::*lac* Δ*proU225* Δ(*pyr-76*::Tn*10*) *recA srl*::Tn*10*	From GJ135 (14), by P1 *kc* transduction
GJ316	GJ134 Δ*proU233 recA srl*::Tn*10*	From GJ146, by P1 *kc* transduction

[a] The nomenclature for genetic symbols and for transpositional insertions follows that described by Bachmann (1) and by Chumley et al. (9), respectively. Allele numbers are indicated where they are known. The *proU224* and Δ*proU225* alleles were described as *osrA2* and Δ*osrAo12* in an earlier study (14).

[b] The *zfi-900*::Tn*10* insertion is linked 88% in P1 *kc* transduction to the *proU* gene (14).

tion of Xph43(pBC4042) (18) have been described earlier. β-Galactosidase specific activity was measured by the method of Miller (33), and the values are expressed in the units defined therein.

Scoring for ProU⁺ phenotype. The ProU⁺ phenotype was easily scored, in strains with mutations in *putPA* and *proP*, on the basis of the follwing characteristics (14): (i) resistance to DHP or azetidine 2-carboxylic acid in minimal A medium and sensitivity to both analogs in minimal A medium supplemented with 0.2 M NaCl and (ii) osmoprotection by 1 mM L-proline in minimal A medium supplemented with 0.65 M NaCl. Cairney et al. (4) have recently reported that, in *S. typhimurium*, *proU* mediates osmoprotection by glycine betaine as well as by L-proline. Their finding was confirmed in this study to be the case also in *E. coli*, and the ability of 1 mM glycine betaine to permit growth of *putPA proP* strains in minimal A medium containing 0.7 M NaCl was employed as an additional test in screening for the ProU⁺ phenotype.

Preparation of λ p*proU-lac*9 phage. Specialized λ transducing phage carrying the *proU224*::*lac* fusion was prepared from the original *proU*::Mu d1(*lac* Ap) strain, GJ2, by a method modified from that of Komeda and Iino (23). The *lac* fusion in GJ2 was stabilized by λ p1(209) lysogenization and subsequent selection for spontaneous temperature-resistant Amp[s] deletion derivatives as described previously (23, 37). A low-titer λ phage lysate obtained by the UV induction of one such strain, GJ141, was used to infect GJ134, and Lac⁺ lysogens were selected on lactose-K minimal medium plates supplemented with 0.4 M NaCl (that is, under conditions which would permit growth of the desired *proU*::*lac* transductants). Lac⁺ lysogens were obtained at 10^{-1}/PFU; a high-frequency transducing phage lysate obtained by the UV induction of one of these strains was designated λ p*proU-lac*9, and pure stocks of the phage were prepared by propagation from single plaques. λ p*proU-lac*9 lysogens of GJ134, in which integration of the prophage had been shown by P1 *kc* transduction to have occurred at the *proU* locus, were osmoresponsive Lac⁺ (β-galactosidase activity, 9 and 660 U, respectively, after growth in K medium and K medium with 0.4 M NaCl) and continued to be ProU⁺. The latter result suggested that the phage did indeed carry the entire *proU224*::*lac* fusion, so that the *lac* and *proU*⁺ genes in the lysogens were being independently expressed from two separate *proU* promoters (16). The structure of λ p*proU-lac*9, as obtained by a genetic characterization of the phage, is shown in Fig. 1c.

RESULTS

Isolation and characterization of GJ146. The studies described below on the cloned *proU* locus were aided in large part by the use of a *recA* derivative of the Δ*proU* mutant strain, GJ146, as the recipient in plasmid transformation and in complementation experiments. GJ146 was isolated by Tet[s] selection from GJ145 (a GJ134 derivative with the *zfi-900*::Tn*10* insertion adjacent to the *proU*⁺ locus) and was shown on subsequent screening to have become ProU⁻. This result suggested that a Tn*10*-promoted deletion had extended into the *proU* locus in this strain, and the mutation was designated Δ*proU233*.

Infection of GJ146 with λ p*proU-lac*9 yielded an interesting class of lysogens that were Lac⁻ ProU⁺, typified by the strain GJ251. Upon transduction of GJ251 to Tet[r] with P1 *kc* (GJ145), the λ prophage was crossed out in 98% of transductants, whereas the 2% that remained λ immune had now become osmoresponsive Lac⁺. A scheme of λ p*proU-lac*9 integration into the chromosome of GJ146 that would account for the observations above is shown in Fig. 1. The model is based also on the known facts that transcription of the *proU*⁺ gene is directed away from the *zfi-900*::Tn*10* insertion (14), and that Tn*10*-promoted chromosomal deletion occurs in contiguity with the site of original Tn*10* insertion (22, 36). It postulates that the deletion in GJ146 extends from *zfi-900*::Tn*10* up to and beyond the *proU* promoter, but that it has stopped short of that site in the wild-type structural gene which is allelic to the site of the original *proU224*::*lac* insertion. Recombination between the homologous *proU* regions carried on λ p*proU-lac*9 and on GJ146 would, therefore, lead to reconstitution of the *proU*⁺ gene and placement of *lac* adjacent to the chromosomal deletion (Fig. 1d). Transduction of such a lysogen to Tet[r] with P1 *kc* (GJ145) would be expected to yield only two classes of recombinants, λ-sensitive Lac⁻ and λ-immune osmoresponsive Lac⁺, as observed.

Primary cloning of *proU*. The in vivo method of Groisman et al. (18) was used in the primary cloning of *proU* directly from the *E. coli* chromosome. The method as described makes use of a strain [Xph43(pBC4042)] that carries a Mu *c*(Ts) helper prophage and a Cm[r] plasmid replicon as part of a mini-Mu phage genome. Temperature induction of this strain yields a lysate in which some of the packaged Mu particles are expected to carry regions of chromosomal DNA

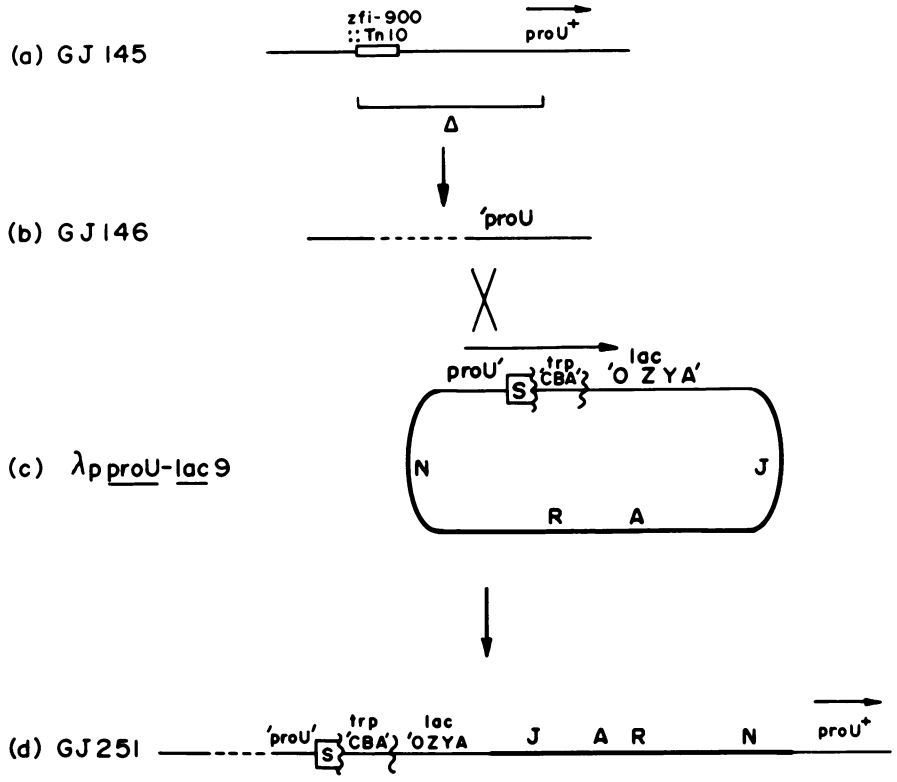

FIG. 1. Schematic representation of lysogenization of GJ146 by λ p*proU-lac*9. The postulated extent of the deletion event (Δ) in GJ145 (a) to yield GJ146 (b) is shown, and the deletion itself is represented by the interrupted line segment. The site of recombination between λ p*proU-lac*9 (c) and GJ146 and the genetic organization in the resulting lysogen, GJ251 (d), are also indicated. The following symbols have been used: overhead arrows indicate the direction of transcription; a prime next to a genetic symbol shows that it is interrupted or deleted on the corresponding side; λ phage DNA is represented by the thick line; and the Mu *S* end DNA has been identified by an open box. The figure is not to scale.

flanked by the mini-Mu phage sequences. Recombination between the flanking sequences after introduction of each of these hybrid molecules by infection into a suitable recipient cell permits the establishment of the chromosomal genes as part of a multicopy plasmid molecule. This method thus enables the shotgun cloning of contiguous chromosomal DNA sequences up to 18 kb long.

The strategy adopted in this study was to select in the cloning experiment for inheritance of the *zfi-900*::Tn*10* allele, which is known to be closely linked to the *proU* locus, and to screen those among the Tet^r colonies that had also acquired the *proU*$^+$ marker. A *zfi-900*::Tn*10* derivative of Xph43(pBC4042) was constructed by P1 *kc* transduction. The Mu lysate obtained by temperature induction of this strain was used to infect the *proU224* strain, GJ157, and selection was made for Cm^r and Tet^r Cm^r colonies. The former were obtained at a frequency of 10^{-4}/PFU, similar to that described previously, and the latter were obtained at 10^{-7}/PFU. One Tet^r Cm^r colony (GJ179), of 37 that were tested, had also become ProU$^+$, suggesting that the contiguous *proU*$^+$ locus had been cloned along with Tn*10* on the plasmid vector in this strain. That indeed Tet^r and ProU$^+$ are plasmid borne in GJ179 was established by the observations that (i) both markers were spontaneously and simultaneously lost with Cm^r at a frequency of 60 to 100% in the absence of selection, and (ii) in transformation into GJ157 with a plasmid preparation from GJ179, all Cm^r colonies were also Tet^r and ProU$^+$.

A limited restriction map of the plasmid from GJ179, pHYD50, was constructed (Fig. 2a), which indicated that it is 35.8 kb long and that it carried 8.5 kb of *zfi-900*::Tn10 (all but 0.8 kb of IS*10*R) along with 10.6 kb of contiguous chromosomal DNA beyond IS*10*L that presumably spanned the *proU*$^+$ locus. On the basis of this map, the subcloning experiments described below were done.

Subcloning of *proU*$^+$ from pHYD50. Various fragments derived from the chromosomal region of the insert in pHYD50 were subcloned in the plasmid vector pBR322 and introduced by transformation into GJ314 or GJ316, *recA* derivatives of strains carrying, respectively, the *proU224* or Δ*proU233* mutation. The extent of chromosomal DNA in each of the subcloned plasmid derivatives is indicated in Fig. 2b through g. The plasmids pHYD52, pHYD53, pHYD55, and pHYD58 were all able to complement the ProU$^-$ phenotype in these strains; pHYD58 carries the *Bgl*II-*Hin*dIII fragment from the right end of the insert (with 5 kb of chromosomal DNA) and was the smallest obtained in these experiments that was able to convert both GJ314 and GJ316 to ProU$^+$. This plasmid could also complement the ProU$^+$ phenotype in another Δ*proU* deletion mutant, GJ315, suggesting that this plasmid does indeed carry the entire *proU*$^+$ locus as part of its insert.

In the course of the subcloning experiments, we observed that the plasmid pHYD56, carrying the *Eco*RI-*Sal*I chromosomal fragment, produced an unexpected phenotype upon introduction into GJ314. Not only was the resultant strain ProU$^-$, it had also become osmosensitive in the sense that its growth rate in LB medium supplemented with 0.2 M NaCl

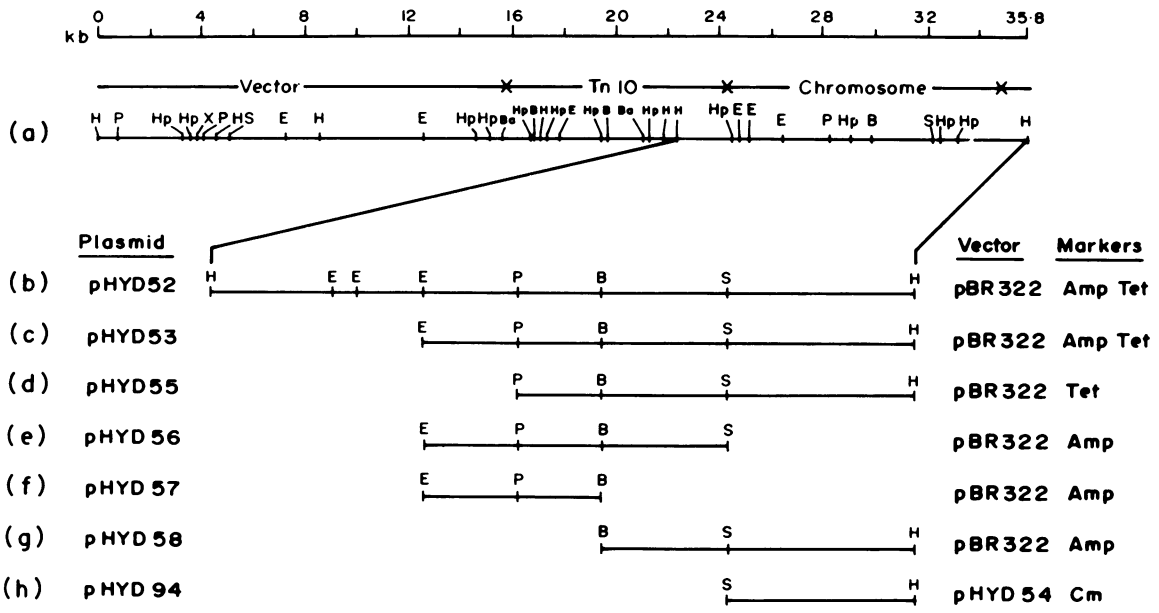

FIG. 2. Physical map of pHYD50 and of its subcloned derivatives. (a) The physical map of the entire pHYD50 plasmid is shown; the regions of DNA corresponding to vector, Tn*10*, and chromosome are marked, and a 'kb scale is included. The construction of this physical map was facilitated by its comparison with the published restriction maps of pBC4042 (18) and Tn*10* (21). For convenience of representation, the plasmid has been linearized at the *Hin*dIII site 1 kb to the right of the Muc end in the vector. (b to h) Representation of the extent of insert DNA in the subcloned plasmid derivatives; the scale has been doubled from the corresponding depiction in (a). The designated plasmid number, the vector used in subcloning and the antibiotic-resistance markers on each plasmid are indicated. Abbreviations for restriction enzyme sites: B, *Bgl*II; Ba, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; Hp, *Hpa*I; P, *Pst*I; S, *Sal*I; X, *Xho*I.

was half that for GJ314 itself in the same medium (Fig. 3); on MacConkey or LB agar plates supplemented with 0.2 M NaCl, colonies of GJ314(pHYD56) after overnight incubation were pinpoint sized, whereas those of GJ314 were normal in size. There was no difference in growth rates of the two strains in unsupplemented LB medium alone or in glucose-minimal A medium containing 0.2 M NaCl (data not shown). An identical osmosensitive phenotype in rich medium was also observed for pHYD56 transformants of GJ316.

A plasmid carrying the *Eco*RI-*Bgl*II chromosomal fragment, pHYD57, did not also complement the *proU* mutation in GJ314 or GJ316; unlike pHYD56, however, it did not retard the growth rates of the corresponding strains in nutrient medium supplemented with 0.2 M NaCl (data not shown).

Effect of multicopy *proU*$^+$ on *proU-lac* expression. Derivatives of GJ314 that had been obtained after transformation with the plasmids constructed in the subcloning experiments above were assayed for β-galactosidase activity in low- and high-osmolarity media to examine the effects which each of these plasmids exerted on expression of the chromosomal *proU*::*lac* fusion in this strain (Table 2). All of the derivatives retained the same low level of β-galactosidase activity (2 to 3 U) in the low-osmolarity medium; in the high-osmolarity medium, the magnitude of induction of *proU*::*lac* was not altered in the presence of plasmids which did not complement the ProU$^-$ phenotype (pBR322, pHYD56), whereas strains with multicopy-*proU*$^+$ plasmids (pHYD53, pHYD55, pHYD58) exhibited only 1/12 as much induction of expression under the same conditions. These two classes of strains could also easily be differentiated on lactose indicator plates supplemented with 0.2 M NaCl. The significance of this finding is further discussed below.

Localization of *proU*$^+$ on pHYD58 by Tn*1000* mutagenesis. We sought to localize, by γδ(Tn*1000*) mutagenesis, the region of DNA on pHYD58 that encodes the ProU$^+$ function. To this end, a pHYD58 transformant of an F$^+$ *recA* strain, JP3301, was used as donor in conjugation with a *proU*::*lac recA* strain, GJ313; selection was made for Amp^r transconjugants, and Tet^r was used as contraselection against the donor strain. It is known that F-mediated mobilization between *recA* strains of pBR322-derived plasmids is

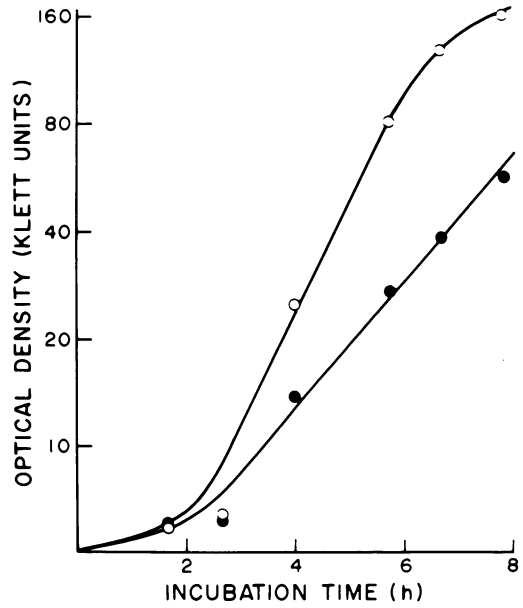

FIG. 3. Growth curves of GJ314 (○) and GJ314(pHYD56) (●) in LB medium supplemented with 0.2 M NaCl.

obligatorily associated with the transposition of Tn*1000* at some site into each of the mobilized plasmid molecules (20).

Amp^r exconjugants of GJ313 were obtained at approximately 10^{-6} per donor cell. Of 500 such colonies that were initially scored for their ProU phenotype, around 11% were found to have become ProU$^-$, presumably as a consequence of Tn*1000* insertion into the *proU* locus of the plasmid transferred into each of them. All ProU$^-$ derivatives also showed a strong Lac$^+$ phenotype on lactose indicator plates supplemented with 0.2 M NaCl, whereas the ProU$^+$ exconjugants exhibited a weak Lac$^+$ phenotype typical of the multicopy *proU*$^+$ strains described above. Additional ProU$^-$ Amp^r exconjugants were subsequently directly recovered from the mating mixtures either by selection for DHP resistance (DHP^r) in the presence of 0.2 M NaCl or by selection on NaCl-containing lactose indicator plates on which the colonies of interest could easily be identified on the basis of their distinctive Lac phenotype.

Further analysis of the ProU$^-$ strains obtained above revealed the presence of two phenotypic classes. One class (class I) was typically ProU$^-$ (DHP^r in the presence of 0.2 M NaCl and unable to grow in minimal A medium containing 0.7 M NaCl and 1 mM glycine betaine) and also exhibited an osmosensitive phenotype very similar to that described for the pHYD56 transformants above; Amp^r transformants of the Δ*proU233* strain, GJ316, obtained with a plasmid preparation from a representative strain of this class (pHYD66) were also similarly ProU$^-$ and osmosensitive. The other class (class II) of GJ313 exconjugants was DHP^r in the presence of 0.2 M NaCl, but did not show the osmosensitive phenotype. The ProU phenotype in these strains was only partially defective in that the osmoprotective effect of L-proline or glycine betaine was not completely lost in them, although it was considerably less than that seen for the multicopy *proU*$^+$ strains (see below); when plasmids prepared from two representative class II strains (pHYD62, pHYD65) were used to transform GJ316 to Amp^r, all of the transformants remained completely and typically ProU$^-$.

The expression of the chromosomal *proU::lac* fusion in both class I and class II strains was inducible to approximately the same extent as that in GJ314 itself (Table 2), whereas that in a strain in which Tn*1000* insertion had not rendered the plasmid ProU$^-$ (pHYD86) was inducible only to the reduced level seen with the other multicopy-*proU*$^+$ strains.

TABLE 2. Effect of osmolarity on β-galactosidase specific activity in *proU224::lac* strains carrying various plasmids[a]

Plasmid	β-Galactosidase sp act (U) after growth in[b]:	
	K medium	K medium plus 0.4 M NaCl
pBR322	2.1	696
pHYD53	2.4	69
pHYD55	2.7	43
pHYD56	2.4	950
pHYD58	2.1	50
pHYD86	2.1	75
pHYD62	2.5	915
pHYD66	2.5	810
pHYD71	2.8	905

[a] GJ313 was the host strain used for the Tn*1000* insertion plasmid derivatives, and GJ314 was the host strain for the others.

[b] β-Galactosidase specific activity was measured after strains had been grown in the medium specified, along with appropriate antibiotic selection, for at least 10 generations.

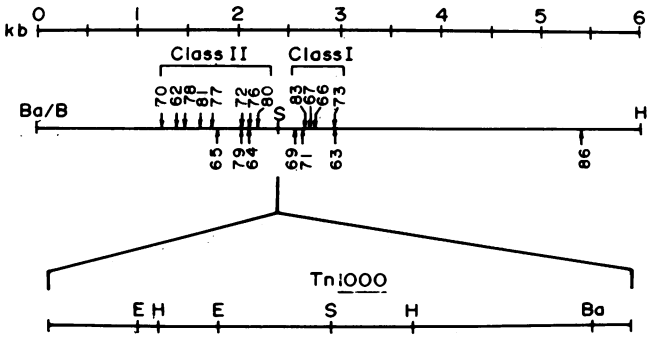

FIG. 4. Representation of sites of Tn*1000* transposition into the chromosomal insert in pHYD58. The sites of several independent Tn*1000* insertions are shown by the arrows, and the corresponding pHYD plasmid number designations are marked. Arrows below the line represent insertions of Tn*1000* in the orientation shown, and those above the line represent insertions in the reverse orientation. The discrete clustering of insertions in plasmids from the class I and from the class II strains is also depicted. The Tn*1000* insertion in pHYD86 does not affect the ProU$^+$ phenotype. A kb scale is included; the map of Tn*1000* (taken from reference 20) is also to scale. Abbreviations for restriction enzyme sites: Ba, *Bam*HI; Ba/B, ligation joint between *Bam*HI and *Bgl*II ends; E, *Eco*RI; H, *Hin*dIII; S, *Sal*I.

The sites of Tn*1000* insertion in plasmids obtained from seven independent class I and 11 class II strains were mapped after restriction endonuclease digestion and agarose gel electrophoresis; the position and orientation of transpositional insertion in each of them are depicted in Fig. 4. The sites of Tn*1000* insertion in the class I strains were clustered (in both orientations) in a 0.4-kb region a little to the right of the *Sal*I site in the insert DNA, whereas those of the class II strains were distributed over a 1.1-kb region to the left of the *Sal*I site.

Physical characterization of λ p*proU-lac*9. In an attempt to determine the position of the *proU224::lac* fusion in relation to the physical map of the *proU* locus obtained above, DNA was prepared from λ p*proU-lac*9 phage, and its restriction map was constructed (Fig. 5). The *Sal*I site derived from this region of the chromosome is situated 0.8 kb upstream from the site of the *proU224::lac* fusion. In light of the earlier finding that transcription of *proU::lac* is directed away from the *zfi-900*::Tn*10* insertion (14), we inferred that the *proU224* allele represents an insertion to the right of the *Sal*I site in this locus (according to the depiction in Fig. 2 and 4), and therefore that it is homologous to the class I Tn*1000* insertions mapped above. The *Bgl*II site from the *proU* locus, situated 1.8 kb further upstream from the *Sal*I site (Fig. 2), was shown not to be carried on λ p*proU-lac*9, which result indicated that the amount of DNA from this locus carried by the phage is 2.5 kb or less.

Complementation in *trans* within the *proU* locus. The identification of two phenotypic classes of ProU$^-$ strains after Tn*1000* mutagenesis of pHYD58, combined with the finding that the Tn*1000* insertions in these two classes were situated in two discrete clusters, suggested that there perhaps are two cistrons in the *proU* locus which are both required for ProU$^+$ function. The following experiment, in the nature of a formal *trans*-complementation assay, served to substantiate this notion. The plasmid pHYD94 was constructed by subcloning of the *Hin*dIII-*Sal*I fragment shown in Fig. 2 h into the pACYC184-derived vector, pHYD54; when this plasmid was introduced by transformation into the pHYD56 or pHYD66

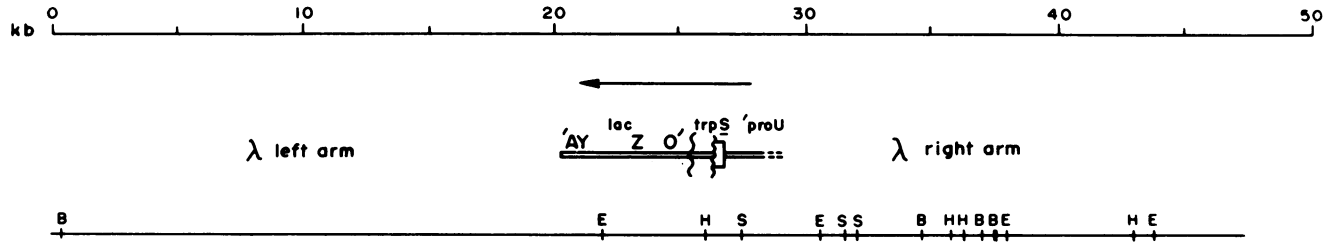

FIG. 5. Physical map of λ p*proU-lac*9 with respect to recognition sites for the restriction enzymes *Hin*dIII (H), *Eco*RI (E), *Bgl*II (B), and *Sal*I (S). A kb scale is included. The physical map, represented on the lower line, has been correlated with the genetic organization in the central substituted region of the phage (between the flanking left and right arms of λ). Symbols for the latter are as in Fig. 1. The broken-line segment at the junction between *'proU* and the λ right arm indicates the extent of uncertainty in determining the length of substitution in this region. The construction of this map was facilitated by its comparison with the published restriction maps of wild-type λ (11), λ p1(209) (26), and Mu d1(*lac* Ap) phage (34).

derivative of GJ316, the resultant double-plasmid strains were all shown to have become ProU$^+$. In contrast, derivatives of GJ316 carrying pHYD56, pHYD66, or pHYD94 alone or pHYD94 with pHYD62 (a Tn*1000* plasmid from a class II strain) were all ProU$^-$. The results clearly indicated that the plasmid pHYD94 is able to complement the function inactivated by Tn*1000* insertion in the plasmid pHYD66, but not that in pHYD62, and supported the hypothesis that there are at least two cistrons in the *proU* locus.

pHYD94 transformants of GJ314 also exhibited osmoprotection by L-proline and glycine betaine, to an extent similar to that described above for the class II derivatives of GJ313 but less than that obtained for the pHYD58 transformants (Table 3). These results are interpreted and further discussed below in the context of complementation of the *proU224* allele by pHYD94 and plasmids of the class II strains.

Multicopy-*proU*$^+$ enhances osmotolerance in *E. coli*. The observation that the multicopy *proU*$^+$ plasmids served to reduce the expression of chromosomal *proU*::*lac* in high-osmolarity medium, whereas insertional inactivation of ProU function in plasmids of the class I and class II strains resulted in restoration of the normal magnitude of osmoresponsive *lac* expression, had several interesting implications. For one, the latter result argued against possible models for direct autoregulation of *proU* expression. Second, it suggested that the presence of multiple copies of the *proU* promoter does not by itself influence the regulation of *proU* expression, as could be envisaged for example by titration of a putative limiting regulator gene product by the plasmids in these cells. The data instead lent support to the notion that it was increased expression of the ProU function in these strains that had an indirect feedback-negative effect on *proU*::*lac* expression, perhaps as a result of an attenuation of the inducing signal.

β-Galactosidase activity in multicopy *proU*$^+$ strains grown in the high-osmolarity medium (K medium with 0.4 M NaCl) is equivalent to that observed in the parental *proU*::*lac* strain after growth in medium supplemented with 0.25 M lower concentration of NaCl (14). This suggested that the concerted functioning of multiple *proU*$^+$ copies in a single cell serves to attenuate the osmolarity signal to an extent roughly equivalent to that exerted by 0.25 M NaCl, and that the osmotolerance of multicopy *proU*$^+$ strains should therefore be increased over that of the haploid *proU*$^+$ strains to a corresponding degree. This prediction was tested in experiments aimed at measurements of growth rates of various strains in glycine betaine-supplemented high-osmolarity media and of their maximal osmotolerance. We could show that the maximal osmotolerance (in the presence of 1 mM glycine betaine) of the multicopy *proU*$^+$ derivatives of GJ314 was increased to around 1.1 M NaCl from that of 0.8 M NaCl obtained for the isogenic haploid *proU*$^+$ strains, GJ134 (Table 3). At any single NaCl concentration tested (again in the presence of 1 mM glycine betaine), the growth rate of the multicopy *proU*$^+$ strain was substantially greater than that of GJ134 in the same medium (Fig. 6).

The maximal osmotolerance of derivatives of GJ314 and GJ316 carrying other plasmids was also measured (Table 3). In GJ314, pHYD66 (class I) provided no osmotolerance at all, whereas the plasmids pHYD62 (class II) and pHYD94 provided a haploid level of osmotolerance. The growth rates of the latter strains in the high-osmolarity media also paralleled those of GJ134 depicted in Fig. 6 (data not shown). On the other hand, in GJ316, neither the class I nor the class II plasmid tested nor pHYD94 conferred osmotolerance; the pHYD86 derivative exhibited the enhanced level of osmotolerance described above for GJ314(pHYD86), whereas that carrying the complementing pair of plasmids, pHYD66 and pHYD94, had an intermediate level of osmotolerance.

The ability of pHYD58 to increase osmotolerance was demonstrable also in a *put*$^+$ *proP*$^+$ background in strain MC4100 (data not shown).

TABLE 3. Maximal osmotolerance of various strains in the presence of glycine betaine

Strain	Maximal concn (M) of NaCl tolerated for growth[a]
GJ134	0.8
GJ314	<0.7[b]
GJ314(pHYD58)	1.1
GJ313(pHYD62)	0.8
GJ313(pHYD66)	<0.7
GJ313(pHYD86)	1.0
GJ314(pHYD94)	0.8
GJ316(pHYD62)	<0.7
GJ316(pHYD66)	<0.7
GJ316(pHYD86)	1.0
GJ316(pHYD94)	<0.7
GJ316(pHYD66, pHYD94)	0.9

[a] The value listed for each strain is the maximal concentration of NaCl in minimal A medium supplemented with 1 mM glycine betaine that permitted at least a 20-fold increase in cell density in 60 h at 37°C.

[b] No growth in 0.7 M NaCl, the lowest concentration tested in this experiment.

DISCUSSION

Cloning of *proU*$^+$ in physical contiguity with *zfi-900*::Tn*10*. In the strategy for in vivo cloning adopted in this study, plasmids complementing the *proU224* mutation in the recipient strain were sought not by direct selection for ProU$^+$, but instead by the screening of those colonies obtained after selection for inheritance of the adjacent *zfi-900*::Tn*10* allele. An unambiguous interpretation was therefore rendered possible with regard to the nature of the cloned gene in pHYD50. The size of pHYD50 is toward the upper limit of that which can be obtained by the mini-Mu cloning method above (18), and the distance of 8 kb between the site of *zfi-900*::Tn*10* insertion and the *proU*$^+$ locus is consistent with the 88% linkage in P1 transduction between these two loci (14). The method used may, therefore, be generally applicable for the cloning of any gene in contiguity with a transposon insertion close to it, provided that the cotransduction frequency between the two is around 85% or higher.

Presence of two cistrons at the *proU* locus. The data presented above on the two classes of Tn*1000* insertion mutants, taken together with the complementation results in GJ316, provide strong evidence for the presence of at least two cistrons whose expression is necessary for ProU function. Furthermore, from the physical mapping data on λ p*proU-lac*9 and the genetic characterization of GJ146 (Fig. 1) it is clear that the *proU224*::*lac* insertion is allelic to the Tn*1000* insertion mutations of class I strains, whereas the Δ*proU233* mutation would have abolished the expression of both cistrons in this locus. The observations that all of the plasmids from the class II strains, and also the plasmid pHYD94, can complement the *proU224* mutation in GJ313 or GJ314 but that none of them is able to complement Δ*proU233* in GJ316 may therefore be easily explained.

Genetic mapping data have earlier shown that the *zfi-900*::Tn*10* insertion is situated counterclockwise of *proU224*::*lac* on the standard *E. coli* linkage map (14); the cistron identified by Tn*1000* insertions in the class II strains would, therefore, also be counterclockwise to that similarly identified in the class I strains. We propose that the designation *proU* be retained for the gene originally identified by the *proU224*::Mu dl(*lac* Ap) insertion (which also corresponds to the cistron inactivated by Tn*1000* insertions in the class I strains in this study), and that the cistron counterclockwise of *proU*, identified by the Tn*1000* insertions in the class II strains, be designated *proV*. The results on lysogenization of GJ146 with λ p*proU-lac*9 would also indicate that the *proV*$^+$ gene is carried intact on the genome of this transducing phage.

It is known that transcription of the *proU224*::*lac* fusion is in the direction away from the *zfi-900*::Tn10 insertion (14). Two alternative possibilities which may be considered are that the two cistrons identified above constitute a single operon whose transcription is in the direction away from the Tn*10* insertion, or that they constitute independent units of transcription. Our results indicate that the former possibility is less likely, because in that case one would have expected at least some of the Tn*1000* insertions in the class II strains to exhibit a polar effect on expression of the downstream gene. The observation that pHYD94 (which carries only the *Sal*I-*Hin*dIII insert) is able to complement the *proU224*::*lac* mutation also supports the interpretation that there are two independent transcription units in this locus. The alternative explanation for our observations would be that *proV* and *proU* do constitute a single operon, but that sufficient residual expression of the *proU* gene occurs from a secondary internal promoter in the multicopy plasmids above for the complementation results to be positive.

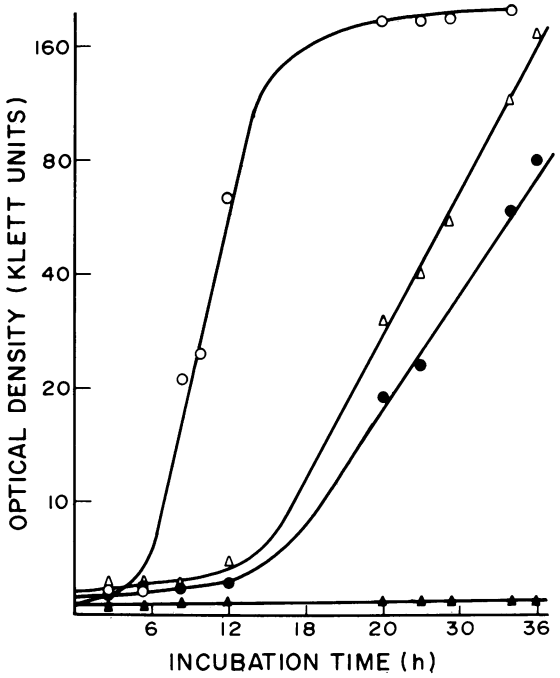

FIG. 6. Growth curves of GJ134 (●, ▲) and GJ314(pHYD58) (○, △) in minimal A medium with 1 mM glycine betaine and 0.7 M NaCl (●, ○) or 0.9 M NaCl (▲, △).

Phenotype of strains with multiple copies of *proU*$^+$ or *proV*$^+$. The phenotype of osmosensitivity, described above for derivatives of GJ314 and GJ316 carrying either pHYD56 or the class I plasmids, is associated with the presence in these cells of multiple functional copies of *proV* along with complete absence of expression of *proU*. When the same plasmids were introduced by transformation into the haploid *proU*$^+$ strain GJ134 the phenotype of osmosensitivity was not seen (data not shown). Multiple copies of *proU*$^+$ in a haploid *proV*$^+$ strain (as obtained with the Tn*1000*-insertion plasmids in the class II strains) do result in osmoprotection by glycine betaine and L-proline, but the strain also continues to be DHP^r in the presence of 0.2 M NaCl unlike the haploid *proU*$^+$ *proV*$^+$ strain GJ134. These results provide some clues to the nature of the structural or functional interactions between the products of the two operons at the *osrA* locus, but the molecular details of such interaction are not known.

Enhancement of osmotolerance (in the presence of 1 mM glycine betaine) is seen only in those strains that have multiple copies of both the *proU*$^+$ and *proV*$^+$ genes. This observation is consistent with the finding in *S. typhimurium* that this locus encodes the structural component(s) of a transport system for glycine betaine (4) and with the hypothesis that osmotolerance is limited by the ability of the cell to restore turgor pressure under conditions of water stress.

It has been suggested that the signal regulating *proU* expression in enterobacteria is the turgor pressure across the membrane (14). The results described above on the effect of multicopy *proU*$^+$ *proV*$^+$ plasmids on chromosomal *proU*::*lac* expression lend support to this notion. The mo-

lecular mechanisms underlying the regulation of *proU* expression have, however, yet to be characterized.

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