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

J. GOWRISHANKAR,* POHNERKAR JAYASHREE, AND K. RAJKUMARI
Centre for Cellular and Molecular Biology, Hyderabad 500 007, India

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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 TnJ0 insertion, onto a multicopy plasmid directly from the E. coli chromosome. For a given level of osmotic stress, the magnitude of osmorepressive 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, Tn1000 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 proU-lac9 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. 2b), is a derivative of pACYC184 (6) and was obtained after HindIII 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 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 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.

A phage techniques. λ phage lysates were prepared either by UV induction of lysogens or by proagation 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 HindIII 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 selection (30), and temperature induc-
TABLE 1. E. coli K-12 strains used

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype/Source of derivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC4100</td>
<td>F^-Δ(argF-lac)U169 rpsL150 relA1 araD415 blbB5301 deoC1 ptsF25</td>
</tr>
<tr>
<td>JP3301</td>
<td>F^-purE trp hsdR410 argG leu met thi ara xyl mlc Δ(argF-lac)U169 pheR372 recA56 (/pRhaA-lac)</td>
</tr>
<tr>
<td>Xph43</td>
<td>F^-Δ(argF-lac)U169 trp Δ(bnr::phoR24)</td>
</tr>
<tr>
<td>GJ134</td>
<td>MC4100 proU224::Mu d1(lac Ap)</td>
</tr>
<tr>
<td>GJ134</td>
<td>MC4100 ΔproPA101 proP222 Δ(pyr-76::TnlO)</td>
</tr>
<tr>
<td>GJ141</td>
<td>MC4100 proU224::lac stabilized by λ pJ209</td>
</tr>
<tr>
<td>GJ145</td>
<td>GJ134 zfi-900::TnlO</td>
</tr>
<tr>
<td>GJ146</td>
<td>GJ134 ΔproU223</td>
</tr>
<tr>
<td>GJ157</td>
<td>GJ140 ΔproPA101 proP221 proU224::lac Δ(pyr-76::TnlO)</td>
</tr>
<tr>
<td>GJ179</td>
<td>GJ157(pHYD50)</td>
</tr>
<tr>
<td>GJ251</td>
<td>GJ134 ΔproU233</td>
</tr>
<tr>
<td>GJ313</td>
<td>GJ157 recA srl::TnlO</td>
</tr>
<tr>
<td>GJ314</td>
<td>GJ157 Δsrl::TnlO</td>
</tr>
<tr>
<td>GJ315</td>
<td>MC4100 ΔproPA101 proP223 proU224::lac ΔproU225 Δ(pyr-76::TnlO) recA srl::TnlO</td>
</tr>
<tr>
<td>GJ316</td>
<td>GJ134 ΔproU233</td>
</tr>
</tbody>
</table>

* The nomenclature for genetic symbols and for transpositional insertions follows that described by Bachman (1) and by Chamley et al. (9), respectively. Allele numbers are indicated where they are known. The proU224 and ΔproU225 alleles were described as osr2A and ΔosrAoJ in an earlier study (14).

* The zfi-900::TnlO insertion is linked 88% in P1 kc transduction to the proU gene (14).

The nomenclature for genetic symbols and for transpositional insertions follows that described by Bachman (1) and by Chamley et al. (9), respectively. Allele numbers are indicated where they are known. The proU224 and ΔproU225 alleles were described as osr2A and ΔosrAoJ in an earlier study (14).

**Scoring for ProU+ phenotype.** The ProU+ phenotype was easily scored, in strains with mutations in putPA and proP, on the basis of the following 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 pproU-lac9 phage.** Specialized λ transducing phage carrying the proU224::lac fusion was prepared from the original proU::Mu d1(lacAp) strain, GJ2, by a method modified from that of Komeda and Iino (23). The lac fusion in GJ2 was stabilized by λ pL209 lysogenization and subsequent selection for spontaneous temperature-resistant AmpR deletion derivatives as described previously (23, 37). A low-titer λ phage lysate obtained by 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^-3 PFU; a high-frequency transducing phage lysate obtained by the UV induction of one of these strains was designated λ pproU-lac9, and pure stocks of the phage were prepared by propagation from single plaques. λ pproU-lac9 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 pproU-lac9, 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 ΔproU mutant strain, GJ146, as the recipient in plasmid transformation and in complementation experiments. GJ146 was isolated by Tet+ selection from GJ145 (a GJ134 derivative with the zfi-900::TnlO insertion adjacent to the proU+ locus) and was shown on subsequent screening to have become ProU-. This result suggested that a TnlO-promoted deletion had extended into the proU locus in this strain, and the mutation was designated ΔproU233. Infection of GJ146 with λ pproU-lac9 yielded an interesting class of lysogens that were Lac- ProU+·, typified by the strain GJ251. Upon transduction of GJ251 to Tet+ 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 pproU-lac9 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::TnlO insertion (14), and that TnlO-promoted chromosomal deletion occurs in contiguity with the site of original TnlO insertion (22, 36). It postulates that the deletion in GJ146 extends from zfi-900::TnlO to proU+ 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 pproU-lac9 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+ 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.
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::Tn10 allele, which is known to be closely linked to the proU locus, and to screen those among the Tet' colonies that had also acquired the proU' marker. A zfi-900::Tn10 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' and Tet' Cm' colonies. The former were obtained at a frequency of \(10^{-4}/\text{PFU}\), similar to that described previously, and the latter were obtained at \(10^{-7}/\text{PFU}\). One Tet' Cm' colony (GJ179), of 37 that were tested, had also become ProU', suggesting that the contiguous proU' locus had been cloned along with Tn10 on the plasmid vector in this strain. That indeed Tet' and ProU' are plasmid borne in GJ179 was established by the observations that (i) both markers were spontaneously and simultaneously lost with Cm' 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' colonies were also Tet' 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 ISI0R) along with 10.6 kb of contiguous chromosomal DNA beyond ISI0L 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 ΔproU223 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 BglII-HindIII 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 ΔproU 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 EcoRl-SalI 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

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**FIG. 1.** Schematic representation of lysogenization of GJ146 by λ proU-lac9. 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 proU-lac9 (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.
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, Tn10, 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 Tn10 (21). For convenience of representation, the plasmid has been linearized at the HindIII 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, BglII; Ba, BamHI; E, EcoRI; H, HindIII; HpaI; P, PstI; S, SalI; X, XhoI.

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 EcoRI-BglII 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 Tn1000 mutagenesis. We sought to localize, by γ8(Tn1000) 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' transconjugants, and Tet' was used as conraselection 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.
 obrigatory associated with the transposition of \textit{Tn1000} at some site into each of the mobilized plasmid molecules (20).

\textbf{Amp}⁰ exconjugants of GJ313 were obtained at approximately 10⁻⁶ per donor cell. Of 500 such colonies that were initially scored for their \textit{proU} phenotype, around 11% were found to have become \textit{proU}⁺, presumably as a consequence of \textit{Tn1000} insertion into the \textit{proU} locus of the plasmid transferred into each of them. All \textit{proU}⁺ derivatives also showed a strong \textit{Lac}⁺ phenotype on lactose indicator plates supplemented with 0.2 M NaCl, whereas the \textit{proU}⁰ exconjugants exhibited a weak \textit{Lac}⁺ phenotype typical of the multicopy \textit{proU}⁺ strains described above. Additional \textit{proU}⁺-Amp⁰ exconjugants were subsequently directly recovered from the mating mixtures either by selection for DHP resistance (DHP⁺) 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 \textit{Lac} phenotype.

Further analysis of the \textit{proU}⁺ strains obtained above revealed the presence of two phenotypic classes. One class (class I) was typically \textit{proU}⁺ (DHP⁺ 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; Amp⁰ transformants of the \textit{ΔproU}233 strain, GJ316, obtained with a plasmid preparation from a representative strain of this class (pHYD66) were also similarly \textit{proU}⁺ and osmosensitive. The other class (class II) of GJ313 exconjugants was DHP⁺ in the presence of 0.2 M NaCl, but did not show the osmosensitive phenotype. The \textit{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 \textit{proU}⁺ strains (see below); when plasmids prepared from two representative class II strains (pHYD62, pHYD65) were used to transform GJ316 to Amp⁰, all of the transformants remained completely and typically \textit{proU}⁺.

The expression of the chromosomal \textit{proU}:\textit{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 \textit{Tn1000} insertion had not rendered the plasmid \textit{proU}⁺ (pHYD86) was inducible only to the reduced level seen with the other multicopy-\textit{proU}⁺ strains.

\begin{table}
\centering
\begin{tabular}{lcc}
\hline
Plasmid & \textit{β}-Galactosidase sp act (U) after growth in: & \\
& K medium & K medium plus 0.4 M NaCl \\
\hline
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 \\
\hline
\end{tabular}
\caption{Effect of osmolarity on \textit{β}-galactosidase specific activity in \textit{proU224}:\textit{lac} strains carrying various plasmids.}
\label{table:osmolarity}
\end{table}

\textit{GJ313} was the host strain used for the \textit{Tn1000} insertion plasmid derivatives, and \textit{GJ314} was the host strain for the others.

\textit{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.

The sites of \textit{Tn1000} 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 \textit{Tn1000} insertion in the class I strains were clustered (in both orientations) in a 0.4-kb region a little to the right of the \textit{SalI} 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 \textit{SalI} site.

\textbf{Physical characterization of \textit{λ} pproU-lac9.} In an attempt to determine the position of the \textit{proU224}:\textit{lac} fusion in relation to the physical map of the \textit{proU} locus obtained above, DNA was prepared from \textit{λ} pproU-lac9 phage, and its restriction map was constructed (Fig. 5). The \textit{SalI} site derived from this region of the chromosome is situated 0.8 kb upstream from the site of the \textit{proU224}:\textit{lac} fusion. In light of the earlier finding that transcription of \textit{proU}₂:\textit{lac} is directed away from the \textit{zf-900}:\textit{Tn10} insertion (14), we inferred that the \textit{proU224} allele represents an insertion to the right of the \textit{SalI} site in this locus (according to the depiction in Fig. 2 and 4), and therefore that it is homologous to the class I \textit{Tn1000} insertions mapped above. The \textit{BglII} site from the \textit{proU} locus, situated 1.8 kb further upstream from the \textit{SalI} site (Fig. 2), was shown not to be carried on \textit{λ} pproU-lac9, which result indicated that the amount of DNA from this locus carried by the phage is 2.5 kb or less.

\textbf{Complementation in \textit{trans} within the \textit{proU} locus.} The identification of two phenotypic classes of \textit{proU}⁺ strains after \textit{Tn1000} mutagenesis of pHYD58, combined with the finding that the \textit{Tn1000} insertions in these two classes were situated in two discrete clusters, suggested that there perhaps are two cistrons in the \textit{proU} locus which are both required for \textit{proU}⁺ function. The following experiment, in the nature of a formal \textit{trans}-complementation assay, served to substantiate this notion. The plasmid pHYD94 was constructed by subcloning of the \textit{HindIII}-\textit{SalI} fragment shown in Fig. 2 h into the pACYC184-derived vector, pHYD54; when this plasmid was introduced by transformation into the pHYD56 or pHYD66
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 Tn1000 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 Tn1000 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, GJ314 (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 GJ314 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 pHYD66 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**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Maximal concn (M) of NaCl tolerated for growtha</th>
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<tbody>
<tr>
<td>GJ134</td>
<td>0.8</td>
</tr>
<tr>
<td>GJ131</td>
<td>&lt;0.7b</td>
</tr>
<tr>
<td>GJ134(pHYD58)</td>
<td>1.1</td>
</tr>
<tr>
<td>GJ313(pHYD62)</td>
<td>0.8</td>
</tr>
<tr>
<td>GJ313(pHYD66)</td>
<td>&lt;0.7</td>
</tr>
<tr>
<td>GJ313(pHYD86)</td>
<td>1.0</td>
</tr>
<tr>
<td>GJ316(pHYD94)</td>
<td>0.8</td>
</tr>
<tr>
<td>GJ316(pHYD62)</td>
<td>&lt;0.7</td>
</tr>
<tr>
<td>GJ316(pHYD66)</td>
<td>&lt;0.7</td>
</tr>
<tr>
<td>GJ316(pHYD86)</td>
<td>1.0</td>
</tr>
<tr>
<td>GJ316(pHYD94)</td>
<td>&lt;0.7</td>
</tr>
<tr>
<td>GJ316(pHYD66, pHYD94)</td>
<td>0.9</td>
</tr>
</tbody>
</table>

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::Tn10.
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::Tn10 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::Tn10 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 Tn1000 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 pproU-lac9 and the genetic characterization of GJ146 (Fig. 1) it is clear that the proU224::lac insertion is allelic to the Tn1000 insertion mutations of class I strains, whereas the ΔproU233 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 ΔproU233 in GJ316 may therefore be easily explained.

Genetic mapping data have earlier shown that the zfi-900::Tn10 insertion is situated counterclockwise of proU224::lac on the standard E. coli linkage map (14); the cistron identified by Tn1000 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 d1(lac Ap) insertion (which also corresponds to the cistron inactivated by Tn1000 insertions in the class I strains in this study), and that the cistron counterclockwise of proU, identified by the Tn1000 insertions in the class II strains, be designated proV. The results on lysogenization of GJ146 with λ pproU-lac9 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 Tn10 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 Tn1000 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 SalI-HindIII 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.

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 Tn1000-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+ 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 osmoterolization 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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LITERATURE CITED