JOURNAL OF BACTERIOLOGY, Oct. 1985, p. 434–445
0021-9193/85/100434-12$02.00/0

Vol. 164, No. 1

Identification of Osmoresponsive Genes in *Escherichia coli*: Evidence for Participation of Potassium and Proline Transport Systems in Osmoregulation

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Received 11 March 1985/Accepted 17 July 1985

Mu dl(Ap *lac*)-generated operon fusions were used in the identification of genes in *Escherichia coli* whose transcriptional expression is altered by changes in the osmolarity of the growth medium. One such osmoresponsive gene, designated *osrA*, was induced 400-fold when the osmolarity of the medium was increased with the addition of either ionic or neutral impermeable solutes but was not induced with glycerol, which is freely permeable across the cell membrane. *osrA* was mapped to 57.5 min and was shown to be transcribed clockwise on the *E. coli* chromosome. The ability of small concentrations of L-proline to promote the growth of *E. coli* in high-osmolar medium was shown to have been specifically lost in *osrA* mutants; other lines of evidence were also obtained to support the notion that *osrA* codes for an osmoresponsive L-proline transport system and is homologus to *proU* in *Salmonella typhimurium*. A second osmoresponsive operon identified was *kdp*, which codes for an inducible K^+-transport system in *E. coli*. *kdp* expression was elevated 12-fold when the osmolarity of the growth medium was increased with the addition of impermeable ionic solutes but not neutral solutes; furthermore, osmoresponsivity of *kdp* expression was demonstrable only in K^+-limiting media. *kdp* mutants were able to grow normally in high-osmolar media, but strains defective in both *kdp* and *trkA* (a gene for a second major K^+-transport system) displayed an osmosensitive phenotype. The results suggest that transport systems for L-proline and K^+, specified by *osrA* (*proU*) and *kdp*, respectively, play independent and important roles in osmoregulation in *E. coli*. A third osmoresponsive gene that was identified was *lamB*, which codes for an outer membrane protein for maltodextrin transport and λ phage adsorption; its expression was reduced fourfold with increase in the osmolarity of the growth medium.

Escherichia coli, like many other nonhalophilic bacteria, is able to tolerate and grow in media whose osmolarity ranges from nearly 0 to about 1,200 mOsm (corresponding to about 0.7 M NaCl). The central question in our understanding of osmoregulation pertains to the manner in which metabolic activities, particularly those mediated by membrane-associated functions, continue to be maintained in high-osmolar media, in the face of a decrease in turgor pressure that results in plasmolysis of the cell (46, 47). Changes in cell envelope structure and in intracellular concentrations of compatible solutes have been considered as candidate mechanisms of importance in osmoregulation in this organism, but the evidence in support of either mechanism is inconclusive.

Alterations in the relative amounts and activities of several components of the cell envelope have been documented in cells grown on media of different osmolarities; these include changes in membrane phospholipid turnover and composition (37) and changes in the levels of the OmpF and OmpC proteins (29), alkaline phosphatase (51), and membrane-derived oligosaccharides in the periplasm (20) and in transport systems concerned with the uptake of putrescine (38). However, the importance, if any, of these changes in the adaptational response was not established in these studies.

Increases in the intracellular concentrations of several substances have been demonstrated in bacterial cells grown in high-osmolar media, and these have been postulated to play a role in increasing internal osmolarity and restoring cell turgor pressure during adaptation to osmotic stress. With particular reference to the enterobacteria, the solutes include K^+ ions, proline, glutamate, γ-aminobutyrate, polyols, and glycine betaine (12, 27, 32, 40, 45). Substances such as proline, glycine betaine, proline betaine, and γ-butyrobetaine have also been shown to confer enhanced osmotolerance when present in low concentrations in the medium (7, 8, 25–27). However, in most instances, mutant studies have not been reported that could provide causal evidence for the participation of any of these solutes in osmoregulation. In fact, only one class of osmosensitive mutants has been described, whose characterization has implicated the importance of an L-proline transport gene (*proU*) in adaptation to osmotic stress in *Salmonella typhimurium* (10).

In this study, I used the technique of *lac* operon fusions (6) in *E. coli* to identify and examine the regulation of osmoresponsive genes, that is, genes whose expression is specifically either increased or decreased by changes in the osmolarity of the growth medium. This was done in the expectation that genes important in osmoregulation would also be osmoresponsive with regard to their own expression and that they could therefore be characterized by this approach. Mutations in two of the genes so identified, *osrA* and *kdp*, were shown to confer an osmosensitive phenotype to the corresponding strains under defined environmental conditions, providing incontrovertible evidence that the respective functions of L-proline and K^+ transport specified by these genes are both important in osmoregulation in this organism. The *osrA* gene was also shown to be homologous to *proU* in *S. typhimurium* (10).

MATERIALS AND METHODS

Bacterial strains and phage. The bacterial strains used in this study were all derivatives of *E. coli* K-12. They are listed in Table 1.

TABLE 1. *E. coli* K-12 srt

Strain	Genotype[a]	Source or derivation
MC4100	F⁻ Δ(*argF-lac*)*U169 rpsL150 relA1 araD139 flbB5301 deoC1 ptsF25*	4
MAL103	F⁻ *araB*::Mu cts *araD139* Δ(*gpt-lac*)*5 rpsL* [Mu dl(Ap *lac*]	6
CSH57	F⁻ *purE trp his argG met ilv leu thi ara lacY gal malA xyl mtl rpsL*	35
JP3302	F⁺ *purE trp his argG met ilv leu thi xyl mtl pheR372* Δ(*argF-lac*)*U169 zjd-351*::Tn*10 rpsL* (λ *p pheA-lac*)	15
KL209	Hfr(PO18) *thi-1 malB16 supE44*	28
KL252	F⁻ *thr-1 leuB6 proA2 hisG4 trp-35 thyA20 argE3 thi-1 deo-72 ara-14 lacY1 galK2 xyl-5 mtl-1 rpsL31 rpsE2111 tsx-33 supE44*	B. J. Bachmann
KL584	Hfr(PO2a) *metB1 cysG303 relA1? spoT1?* Δ(*gpt-lac*)*5*	B. J. Bachmann
MD3047	*recA srl*::Tn*10*	S. K. Mahajan
TK2205	F⁻ *kdpABC5 trkA405 trkD1 thi nagA lacZ rha*	43
GJ2	MC4100 *osrA2*::Mu dl(Ap *lac*) [*osr-2*::*lac*]	This study
GJ3	MC4100 *kdp-200*::Mu dl(Ap *lac*) [*osr-3*::*lac*]	This study
GJ4	MC4100 *osr-4*::*lac*	This study
GJ5	MC4100 *lamB30*::Mu dl(Ap *lac*) [*osr-5*::*lac*]	This study
GJ11	MC4100 *osrA2*::*lac* stabilized by λ *p*1(209)	This study
GJ18	MC4100 (λ *d kdp-200*::*lac*)	This study
GJ22	GJ18 *kdpD201* or *kdpAo201*	This study
GJ23	GJ18 *kdpD202* or *kdpAo202*	This study
GJ24	GJ18 *kdpD203* or *kdpAo203*	This study
GJ33	GJ18 *kdpD206* or *kdpAo206*	This study
GJ30	GJ18 *trkA200*	This study
GJ39	GJ18 *trkA203*	This study
GJ40	GJ18 *trkA204*	This study
GJ46	MC4100 *zfi-900*::Tn*10*	This study
GJ51	MC4100 *zjb-903*::Tn*10*	This study
GJ54	MC4100 *zbg-901*::Tn*10*	This study
GJ58	GJ18 *zbg-901*::Tn*10*	This study
GJ69	Hfr(PO68)*thi-1 zhc-904*::Tn*10*	From KL14 (28), by P1 *kc* transduction
GJ72	MC4100 *osrA2*::Mu dl(Ap *lac*) nalB92	This study
GJ74	GJ11 *zfi-900*::Tn*10*	From GJ11, by P1 *kc* transduction
GJ95	F *lac*-114(Ts) GJ11 *nalB93 zfi-900*::Tn*10*	From GS74, in two steps
GJ107	CSH57 *rpoB337* (λcI857)	From CSH57, in two steps
GJ113	GJ11 Δ*osrAo12*	This study
GJ121	GJ11 Δ*putPA101 pyr-76*::Tn*10*	From GJ11, by P1 *kc* transduction to Put⁻
GJ125	MC4100 Δ*putPA101 pyr-76*::Tn*10*	From MC4100, by P1 *kc* transduction to Put⁻
GJ126	GJ11 Δ*osrAo12* Δ*putPA101 pyr-76*::Tn*10*	From GJ113, by P1 *kc* transduction to Put⁻
GJ134	MC4100 Δ*putPA101 proP222* Δ(*pyr-76*::Tn*10*)	DHP^r selection from Tet^s derivative of GJ125
GJ135	GJ11 Δ*osrAo12* Δ*putPA101 proP223* Δ(*pyr-76*::Tn*10*)	DHP^r selection from Tet^s derivative of GJ126
GJ157	GJ11 Δ*putPA101 proP221* Δ(*pyr-76*::Tn*10*)	DHP^r selection from Tet^s derivative of GJ121
GJ161	MC4100 Δ*putPA101 proP222* Δ(*pyr-76*::Tn*10*) zjd-351::Tn*10* Δ(*gpt-lac*)*5 araD*⁺	By conjugation, KL584 × GJ134 *zjd-351*::Tn*10*
GJ163	GJ11 Δ*osr Ao12* Δ*putPA101 proP223* Δ(*pyr-76*::Tn*10*) *zjd-351*::Tn*10* Δ(*gpt-lac*)*5 araD*⁺	By conjugation, KL584 × GJ135 *zjd-351*::Tn*10*
GJ166	GJ11 Δ*putPA101 proP221* Δ(*pyr-76*::Tn*10*) Δ(*gpt-lac*)*5 araD*⁺	By conjugation, KL584 × GJ157

[a] The nomenclature for genetic symbols follows that described by Bachmann (1), and the nomenclature for transpositional insertions follows that described by Chumley et al. (9). Allele numbers are indicated if they are known.

The phages λ *p*1(209) (4), λ *c*I857, λ *c*I *h*80 Δ*int*, λ *vir*, and P1 *kc* were obtained from J. Pittard. λNK370 phage, used in the Tn*10* transposition experiments, was provided by N. Kleckner.

Chemicals and media. L-Amino acids, 5-bromo 4-chloro 3-indolyl β-D-galactoside (X-gal), azetidine 2-carboxylic acid (AC), 3,4-dehydro-DL-proline (DHP), *o*-nitrophenyl β-D-galactoside, nalidixic acid, spectinomycin, and rifampin were purchased from Sigma Chemical Co. Ampicillin, streptomycin, and tetracycline were purchased from local pharmaceutical sources. All other chemicals used were of analytical grade.

The growth medium most commonly used in this study was the low-osmolar medium (K medium) described by Kennedy (20). Its composition is (per liter) KH_2PO_4, 1 mM; $(NH_4)_2SO_4$, 1.5 mM; $MgCl_2$, 0.08 mM; $FeSO_4$, 0.5 mg; Casamino Acids (Difco Laboratories), 5 g; and thiamine, 2 mg (pH adjusted to 7.0 with free Tris base). The osmolarity of the medium was around 70 mOsm, and it could sustain growth of *E. coli* to a density of 10^9 cells per ml. When required, the osmolarity of K medium was increased by the addition of 0.4 M NaCl or other solutes as stated. K minimal medium (that is, K medium without Casamino Acids) was supplemented with 0.1% lactose when it was used in Lac⁺ selection. The composition of minimal A medium and L broth (LB) medium has been described earlier (35). Media were solidified by the addition of 1.5% agar (Difco). K medium plates were stored in sealed packets at 4°C until they were used. Growth of cultures in broth was monitored by measurements of optical density either in a Klett colorimeter or at 600 nm in a spectrophotometer.

When used, the final concentrations (μg/ml) of the following chemicals in media were: tetracycline, 5 in minimal and 15 in nutrient medium; ampicillin, 25; streptomycin, 250;

rifampin, 100; spectinomycin, 500; and X-gal, 25. AC and DHP were used at final concentrations of 1 and 0.4 mM, respectively.

The superscripts r and s have been used for phenotypic designations of resistance and sensitivity, respectively, to the proline analogs, AC and DHP, and to: Tet, tetracycline; Amp, ampicillin; Nal, nalidixic acid; Spc, spectinomycin; Str, streptomycin; and UV, UV irradiation.

Preparation of Mu d1(Ap *lac*) lysate and its use in obtaining *lac* operon fusions. The Mu d1(Ap *lac*) lysate was prepared by temperature induction of MAL103 and was used to generate random *lac* operon fusions, by the procedures previously described (6).

Stabilization of Mu d1(Ap *lac*) lysogens. It is known that the Mu d1 (Ap *lac*) prophage is proficient in transposition, a feature that can frustrate attempts to select for regulatory mutants from these *lac* fusion strains (14, 36). To circumvent this problem, the two *lac* fusions that were characterized in some detail in this study were both stabilized so that Mu-mediated transposition could no longer occur. In the case of the *osrA-lac* fusion strain, GJ2, this was achieved by the method described by Komeda and Iino (23), involving lysogenization of the strain with λ *p*1(209), followed by selection for spontaneous temperature-resistant, Amp^s derivatives that have lost the Mu sequences through a RecA-mediated excision event. The resultant strain, GJ11, is λ immune, retains the *osrA-lac* fusion, and continues to be OsrA$^-$ in its phenotype.

In the case of the *kdp-lac* fusion strain, GJ3, stabilization was achieved by a modification of the method of Gowrishankar and Pittard (14). GJ3 was lysogenized by λ *p*1(209), and the λ prophage was then induced by UV irradiation, as described by Miller (35). The lysate so produced was used to infect MC4100, and a defective λ derivative (that had presumably been generated by imprecise excision of the prophage) was isolated which carried the Amp^r determinant and a part, but not including the promoter, of the *kdp-lac* fusion. Lysogens of this defective phage have been obtained by its integration, through homologous recombination, at the *kdp* locus of the recipient strain, thereby reproducing the *kdp-lac* fusion and the Kdp$^-$ phenotype of the original *lac* fusion strain, GJ3. The *lac* fusion, however, has been stabilized in these lysogens, because of the loss of the associated Mu prophage sequences. One such derivative, GJ18, was used in the studies reported below.

Preparation of P1 *kc* lysates. An overnight culture (0.3 ml) of the strain on which a P1 *kc* lysate was to be prepared was infected with approximately 10^7 PFU of P1 *kc* phage in the presence of 2.5 mM Ca^{2+}. After allowing 20 min for phage adsorption, 10 ml of LB medium and 2.5 mM Ca^{2+} were added, and the infected culture was incubated in a flask with gentle shaking at 37°C. The culture usually grew up to a Klett reading of 120 to 160 U before lysis occurred in 2 to 3 h. If bacterial growth continued to densities beyond this range, fresh medium was added, in 3-ml portions, to maintain the cell concentrations within the Klett values specified, until lysis occurred. The titers of P1 lysates prepared routinely by this method varied between 1×10^9 and 5×10^9 PFU/ml. As is already known (B. J. Bachmann, personal communication), the titers of lysates propagated on *recA* strains were 10-fold lower than the above values.

P1 *kc* transduction. The cells of the recipient strain, grown overnight in LB medium plus 2.5 mM $CaCl_2$, were spun down and resuspended in a 1/10 volume of fresh medium; 0.1 ml of the concentrated cell suspension was infected with approximately 10^8 PFU of P1 *kc* lysate, in the presence of 2.5 mM Ca^{2+}, in a total volume of 2 ml of LB medium. After 20 min, the infected cells were spun down, washed once in 0.1 M citrate buffer (pH 5.5), and resuspended in 0.3 ml of the same buffer; 0.1-ml portions of this suspension were then plated for the selection of transductant clones. The plates used for selection contained 2.5 mM sodium citrate, to prevent P1-mediated killing of the transductants. When the selection was for antibiotic resistance, the plates were incubated for 1 h at 30°C for phenotypic expression before the antibiotic was added in a second overlay in an amount that would provide the desired final concentration after diffusion through the entire volume of medium in the plate. With this protocol, 300 to 1,000 transductants were routinely obtained in each selection (per plate), working out to a frequency of around 10^{-5}/PFU.

Conjugation. The methods described by Miller (35) were followed for matings both on solid and in liquid media.

Obtaining random transpositions of Tn*10*. Random transpositions of the tetracycline-resistance transposon, Tn*10*, were obtained in MC4100 with the use of the λNK370 lysate, essentially by the method of Kleckner et al. (21).

Selection for Tet^s clones. Tet^s mutants were selected from Tn*10*-carrying strains by the method of Maloy and Nunn (31), with quinaldic acid as the selective agent. Tet^s derivatives were obtained at a frequency of about 10^{-4} per cell.

Isolation of *nalB* mutants. The method described by Hane and Wood (18) was used for the isolation of *nalB* mutants. Spontaneous Nal^r mutants were obtained on LB agar plates containing 4 μg of nalidixic acid per ml. *nalB* mutants were distinguished from *gyrA* (*nalA*) mutants by the fact that, whereas the latter are resistant to 50 μg of nalidixic acid per ml, the former are resistant to 4 μg and sensitive to 10 μg of this agent per ml. The transfer of the *nalB* mutant allele in transduction and in conjugation was screened in like manner. The phenotypic designation Nal^r and Nal^s have been used in this study to represent the corresponding *nalB* alleles.

Construction of Δ*putPA101* derivatives. The Δ*putPA101* allele was transferred into strains by cotransduction with a linked *pyr*::Tn*10* insertion, as described earlier (49). The inheritance of the Put$^-$ phenotype was scored by the demonstration of the inability of the concerned strains to grow on plates containing 2 mM proline as the sole carbon and nitrogen source (10). In some strains, the *pyr*::Tn*10* insertion was subsequently deleted by Tet^s selection.

Isolation of *proP* mutants. The *proP* locus is known to encode a second proline permease in *E. coli* (49). Spontaneous *proP* mutants were isolated from Δ*putPA* strains by selection for resistance to 0.4 mM DHP, as described previously (49). There has been controversy in the recent literature with reference to the map location of *proP* in *E. coli* and as to whether it is homologous to the *proP* locus in *S. typhimurium* (33, 49). In the present experiments, three independent *proP* mutants (GJ134, GJ135, and GJ157) were transduced to Tet^r with a P1 *kc* lysate prepared on JP3302, a strain that carries the *zjd-351*::Tn*10* insertion very close to *pheR* at 93 min on the linkage map (15); it was observed that 35 to 50% of the Tet^r transductants obtained from each of the three strains had also become *proP*$^+$ (that is, DHP^s). These results placed *proP* at a position on the *E. coli* genetic map that is homologous to the *proP* locus in *S. typhimurium*, contrary to the conclusion of Stalmach et al. (49) based on mapping studies on their *proP* mutants.

Construction of Δ*proBA* strains. The Δ(*gpt-lac*)*5* allele, a deletion which encompasses the *proBA* genes, was introduced into MC4100 derivatives by mating with the Hfr

TABLE 2. β-Galactosidase activities in *osr::lac* fusion strains

Strain	Genotype	β-Galactosidase activity (U) when grown in	
		K medium	K medium + 0.4 M NaCl
GJ2	*osr-2::lac*	2.5	762
GJ3	*osr-3::lac*	34	400
GJ4	*osr-4::lac*	2.7	29.5
GJ5	*osr-5::lac*	290	69

KL584, which transfers this mutation as an early marker in conjugation. MC4100 and its derivatives carry the *araD139* mutation, which renders them arabinose sensitive (4). Selection was made for arabinose-resistant recombinants on plates supplemented with L-proline; streptomycin was used for contraselection against the donor strain. Approximately 40% of the exconjugants, on subsequent screening, were Pro^-, indicating that they had coinherited the Δ(*gpt-lac*)*5* allele from the donor strain.

Starvation for L-proline in batch cultures. Pro^- strains were grown to a density of about 2×10^8 cells per ml in glucose minimal medium supplemented with L-proline. The cells were spun down, washed twice in minimal A buffer, and suspended in the same volume of glucose minimal medium without L-proline. They were then incubated with shaking at 37°C, and samples taken after 1, 2, and 3 h were considered to represent cultures subjected to progressively increasing starvation for L-proline.

Growth of cells and assay of β-galactosidase. Unless otherwise specified, measurements of β-galactosidase activity were made on cultures that had been grown at 30°C for approximately 10 generations (to mid-exponential phase) in media of defined osmolarity. The methods for toluenization of cells, assay of β-galactosidase, and calculation of enzyme specific activity (expressed in units) were described by Miller (35).

RESULTS

Isolation of *osr-lac* fusion strains and preliminary mapping. Mu dl(Ap *lac*) lysogens of Δ*lac* strain MC4100 were obtained by the method of Casadaban and Cohen (6). A total of 3,000 independent Amp^r clones were chosen at random and individually tested for the level of *lac* expression on X-gal indicator plates of K medium and K medium plus 0.4 M NaCl. Several colonies showed qualitative differences in the intensity of the blue developed on the two media and were tentatively identified as carrying *lac* fusions with osmoresponsive (*osr*) genes. The activity of β-galactosidase in the strains so identified as measured after growth in K medium and in K medium plus 0.4 M NaCl, and the osmoresponsivity of *lac* expression in four of these strains was confirmed. The values of enzyme activity in these strains are listed in Table 2. Three of them (GJ2, GJ3, and GJ4) showed an increase in *lac* expression, whereas the fourth (GJ5) showed a decrease in expression, with increase in the osmolarity of the growth medium.

The preliminary mapping of the *lac* fusions in the *osr* strains was done as follows. Random tranpositions of Tn*10* were obtained in MC4100 with the method described by Kleckner et al. (21). A P1 *kc* lysate grown on the population of Tet^r clones was then used to transduce each of the *osr-lac* fusion strains, and the selection was made for Tet^r colonies on plates incubated at 42°C. Because Mu dl(Ap *lac*) lysogens are also temperature sensitive (6), this procedure imposed a selection for strains in which Tet^r had been cotransduced with the wild-type allele for each of the *osr-lac* loci. One transductant was chosen from each of the four *osr* strains in which the Tn*10* insertion had been shown to be closely linked to the corresponding *lac* fusion; the map position of each Tn*10* insertion was then determined by transduction of Tet^r into a panel of Hfr strains followed by conjugation with a suitable polyauxotrophic recipient strain, CSH57. The approximate map position for Tet^r was assumed to indicate the position of the corresponding *osr* locus; the location of the four *osr-lac* fusions obtained in this way is shown on the circular linkage map of *E. coli* in Fig. 1.

Further characterization of the *osr-2*, *osr-3*, and *osr-5* fusions is described below.

***osr-2* represents a new locus in *E. coli*.** Strain GJ2 harbored a *lac* fusion (*osr-2*) which showed a 400-fold induction of β-galactosidase activity upon growth in K medium plus 0.4 M NaCl. Similar increases were obtained with the substitution of NaCl by other ionic and nonionic solutes [0.27 M K_2SO_4 or $(NH_4)_2SO_4$; 0.6 M sucrose, melibiose, mannitol, etc.; data not shown] but did not occur with 0.6 M glycerol, a substance known to be freely diffusible across the cell membrane (3). The results therefore suggested that the expression of the gene to which the fusion had occurred (tentatively designated *osrA*) was specifically stimulated by solutes that tend to decrease the turgor pressure of the bacterial cell.

The plot of β-galactosidase activity in GJ2 against the osmolarity of the growth medium showed a sigmoidal inflection (Fig. 2). The steep portion of the induction curve begins at 0.15 M NaCl; the corresponding medium osmolarity would be 350 mOsm. The β-galactosidase activity in a culture of GJ2 grown in minimal A medium (300 mOsm) was 80 U, which roughly corresponds to the value obtained in K medium with NaCl added to the same osmolarity. The increase in β-galactosidase activity with increasing concentration of NaCl in minimal A medium closely followed the latter part of the sigmoidal curve in Fig. 2 (data not shown). This indicated that the primary determinant of *osrA* expres-

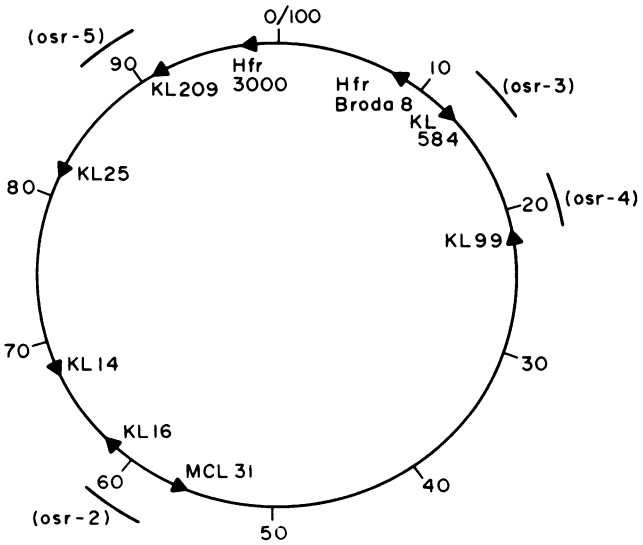

FIG. 1. Circular linkage map of *E. coli* showing the approximate positions of the four *osr::lac* fusions, as determined by conjugation experiments with linked Tn*10* insertions. The points of origin of the various Hfrs used in this study are also marked.

sion was indeed the external osmolarity and that induction was independent of the composition of the medium or of the osmolyte used. Induction of enzyme activity was demonstrable within 20 min of the addition of 0.4 M NaCl to an exponentially growing culture of GJ2 in K medium (data not shown).

A strain (GJ46) with a Tn*10* insertion 89% cotransducible with *osrA* was obtained by the method described above; Hfr mapping experiments with this Tn*10* insertion indicated that the *osrA* locus maps at about 60 min, close to the *srl* genes (Fig. 1). The Tn*10* insertion in GJ46 has been designated *zfi-900*::Tn*10* in the studies described below, in accordance with recommended nomenclature (9).

Further mapping of *osrA* was done by P1 *kc* transduction. A spontaneous *nalB* derivative, GJ72, was obtained from GJ2 by the method previously described (18). GJ72 was used as recipient in each of two transductional crosses, with the P1 *kc* lysates being prepared on MD3047 (*osrA*$^+$ *nalB*$^+$ *recA srl*::Tn*10*) and on GJ46 (*osrA*$^+$ *nalB*$^+$ *zfi-900*::Tn*10*), respectively. In both instances, Tet^r was the marker selected for in transduction, and recombination at the *recA*, *nalB*, and *osrA* loci was scored by checking, respectively, for the acquisition of the UV^s, Nal^s, and Amp^s phenotypes.

The results of the transduction experiments are presented in Table 3. In the first cross with P1(MD3047), *srl*:::Tn*10* was cotransducible 67% both with *recA* and with *nalB* and 23% with *osrA*. In a three-factor analysis of the cross data, the absence of recovery of Tet^r UV^r Nal^r Amp^s transductants suggested that the gene order was *srl*-(*recA*-*nalB*)-*osrA*. The observation made in this experiment, that *recA* and *nalB* are apparently transduced en bloc despite the fact that they are 0.3 min apart on the chromosome, is similar to the one made earlier by Enomoto et al. (11), which involved the P1 transduction of a Tn*10* insertion at another chromosomal location.

In the second cross with P1(GJ46), *zfi-900*::Tn*10* was cotransducible 92% with *osrA* (consistent with the results obtained earlier with GJ2) and 64% with *nalB*. The results of the two transductions, taken together, indicated that *osrA* lies counterclockwise from *nalB* at 57.5 min on the *E. coli* chromosome, but the relative disposition of *zfi-900*::Tn*10* with respect to *osrA* could not be ascertained from these experiments.

Direction of transcription of *osrA*. The F prime F *lac-114*(Ts) was used to mobilize the chromosome of a Δ*lac osrA-lac* (λ) fusion strain, GJ95, in a mating experiment with GJ107, a suitable *his argG rpoB* recipient. It was expected that chromosome transfer from the donor would be initiated within the region of homology shared between the *lac* genes on the F prime and that at the *osrA* locus and that the direction along which the chromosome was mobilized would be determined by the clockwise or counterclockwise disposition of the *lac* genes on the chromosome; the latter in turn would directly be determined by the direction of transcription of *osrA*. *his* and *argG* were chosen as convenient markers for selection in conjugation, because they are situated on either side of, and reasonably close to, the *osrA* locus. Rifampin was used for contraselection against the donor strain. it was also anticipated that the resident λ *p*1(209) prophage in GJ95 would invariably be among the first markers to be transferred in conjugation, irrespective of the direction along which the mobilization of chromosome occurred; the recipient used in conjugation was, therefore, chosen to be a λ *c*I857 lysogen, so that zygotic induction would not occur in the transconjugants. Donor strain GJ95 carries the *zfi-900*::Tn*10* and *nalB93* alleles, and transfer of these alleles was also assessed in the process of chromosome mobilization described above.

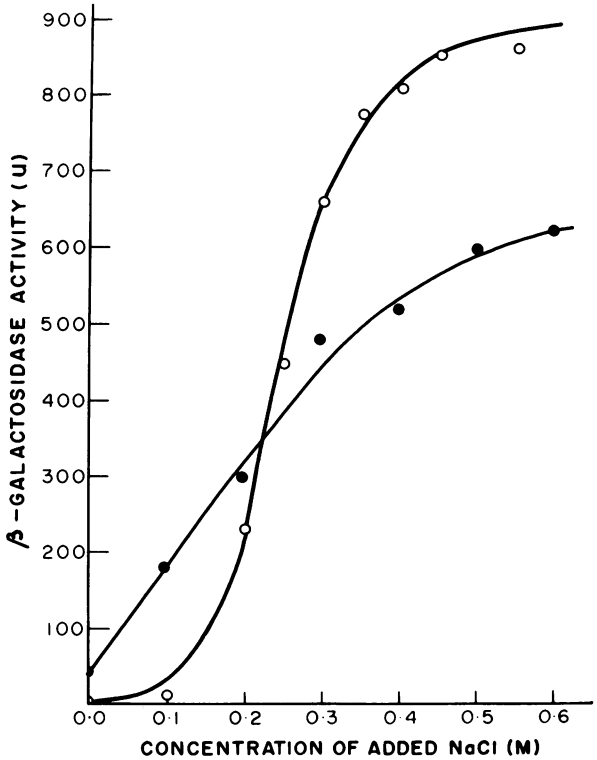

FIG. 2. β-Galactosidase activity in *osr-lac* fusion strains as a function of the concentration of NaCl added to the growth medium. The cultures were grown in K medium plus NaCl (concentrations as specified) for about 10 generations before enzyme activity was measured. Symbols: ○, GJ2 (*osr-2*::*lac*); ●, GJ18 (*osr-3*::*lac*).

The mating experiment yielded Arg$^+$ exconjugants at a frequency of 10^3/ml and His$^+$ exconjugants at <10/ml; of 84 Arg$^+$ exconjugants that were tested, 54 had also become Nal^r. Taken in conjunction with the known relationship between the disposition of *lac* and *oriT* on F *lac*(Ts) (2, 19), these results clearly indicated that *osrA* is transcribed in a clockwise direction on the *E. coli* linkage map and that *nalB* also lies clockwise of *osrA*.

Anomalous results were obtained when the ability of *zfi-900*::Tn*10* to be mobilized in the experiment above was tested. Only 1 of the 84 Arg$^+$ exconjugants tested was Tet^r, but in direct selection, Tet^r exconjugants were obtained at a frequency of 10^2/ml; although approximately 20% of the latter were shown, by a subsequent print mating experiment, to have Tn*10* transposed to F *lac*(Ts), the remainder had indeed recombined for both Tet^r and Nal^r on the chromosome (data not shown). It was initially assumed that the linked transfer of Tet^r and Nal^r in these strains was an indication for the occurrence of *zfi-900*::Tn*10* clockwise of *osrA* (i.e., in the order *nalB*-Tn*10*-*osrA*) and that the lower frequency of recovery of Tet^r conjugants compared with Arg$^+$ exconjugants might have been caused by a decreased frequency of recombination after mobilization, a feature that has been documented earlier for chromosomal markers transferred very early in conjugation (41). Unexpectedly, however, the majority of the Tet^r Nal^r recombinants were found also to have become osmoresponsively Lac$^+$ and temperature resistant, whereas all the Arg$^+$ exconjugants had retained the expected Lac$^-$ temperature-sensitive phe-

TABLE 3. Data on transductional mapping of *osrA*

Cross[a]	Genotype					Transductant classes	No.
	srl	*recA*	*nalB*	*zfi*	*osrA*		
P1(MD3047)	Tn*10*	−	+		+	Tet^r UV^s Nal^s Amp^s	13
×						Tet^r UV^s Nal^s Amp^r	25
GJ72	+	+	*92*		::Mu d1	Tet^r UV^r Nal^r Amp^s	0
						Tet^r UV^r Nal^r Amp^r	19
P1(GJ46)			+	Tn*10*	+	Tet^r Nal^s Amp^s	87
×						Tet^r Nal^r Amp^s	47
GJ72			*92*	+	::Mu d1	Tet^r Nal^s Amp^r	5
						Tet^r Nal^r Amp^r	6

[a] The selected marker for both crosses was Tet^r. The number of transductants scored was 57 for the first cross and 145 for the second.

notype of the parental strain, GJ107. This suggested that the entire *osrA-lac* (λ) fusion of GJ95 had been transferred along with *nalB* into GJ107 in the class of the Tet^r recombinants, a phenomenon which cannot be explained on the basis of mobilization mediated by recombination between the regions of shared *lac* homology on the F prime and on the chromosome. The mechanism by which such anomalous mobilization occurred, albeit at a frequency much lower than the *lac*-mediated event, has not been determined.

That in fact *zfi-900*::Tn*10* lies counterclockwise of *osrA* was established by P1 *kc* transduction. When GJ107 was transduced to Tet^r with a P1 *kc* lysate prepared on GJ95, 28 and 18% of the transductants had become osmoresponsively Lac$^+$ and Nal^r, respectively, and the three-factor analysis of the results indicated a gene order of *nalB-osrA*::*lac*(λ)-Tn*10* (data not shown). The reduction in the frequency of cotransduction of *zfi-900*::Tn*10* with *nalB*, from 64% in GJ46 to 18% in GJ95, was also consistent with the notion that the presence of the integrated λ *p*1(209) prophage at the *osrA* locus had increased the separation between these two markers in the latter strain.

In light of the clockwise direction of *osrA* transcription, the above mapping data placed *zfi-900*::Tn*10* close to the promoter-proximal end of the *osrA* operon. Consonant with this interpretation, 3 of 64 Tet^s mutants selected in GJ74 (a derivative of GJ11 which carries this Tn*10* allele near *osrA-lac*) were found to have lost their osmoresponsive Lac$^+$ phenotype, presumably as a consequence of Tn*10*-induced deletions having removed the *cis* regulatory regions of the *osrA* locus in these strains. One such Δ*osrAo* mutant, GJ113, which had β-galactosidase activity of 0.2 and 0.4 U after growth, in low- and high-osmolar medium, respectively, was used in some of the studies described below on the physiological function of *osrA* in *E. coli*.

Determination of function of the native *osrA* gene. The insertion of Mu d1(Ap *lac*) within *osrA* inactivates the gene, and it was expected that differences in behavior of the *osrA-lac* fusion strain, GJ11, as compared with the parental strain, MC4100, might provide a clue to the function of the native *osrA* gene. The results of preliminary experiments indicated, however, that GJ11 was able to grow just as well as MC4100 itself in high-osmolar media; neither was its ability to adapt to and grow after instantaneous change in medium osmolarity (by the addition of NaCl [to 0.4 M] to cultures growing in K medium) affected significantly (data not shown). Thus, a mutation in *osrA* does not render a strain phenotypically osmosensitive.

After the map position of *osrA* was determined, two possibilities were considered with respect to its identity and function: that it could represent the *gab* gene cluster known to map in the 58-min region in *E. coli* (34) or that it could be the homolog of the gene designated *proU* that had been mapped to the corresponding region of the chromosome in *S. typhimurium*.

The *gab* genes in *E. coli* are concerned with utilization of γ-aminobutyric acid when this compound is provided as the sole nitrogen source in the medium. γ-Aminobutyric acid has also been postulated to a compatible solute in osmoregulation in certain organisms, including *E. coli* (32), and it was considered possible that if *osrA* were to represent this group of genes, they might also be functioning in the synthesis of γ-aminobutyric acid in cells subjected to osmotic stress. However, I could show that MC4100, as well as the *osrA* mutant GJ11 and the Δ*osrAo* mutant GJ113 were equally capable of utilizing γ-aminobutyric acid as the sole nitrogen source in minimal medium. This finding, therefore, served to exclude the possibility that *osrA* represents the *gab* group of genes (34).

Experiments to test whether *osrA* was homologous to *proU* in *S. typhimurium* necessitated the construction, from *osrA*$^+$ and *osrA* strains, of derivatives that carried mutations in *putPA* and *proP*, loci that specify two major transport systems for L-proline in *E. coli* (49, 52). The Δ*putPA101* deletion was introduced into the various strains by P1 *kc* transduction. Spontaneous *proP* derivatives were then isolated from the Δ*putPA* strains by selection for resistance to 0.4 mM DHP, and the *proP* alleles were mapped to 93 min, as described above. Three phenotypic differences were observed between the Δ*putPA proP* derivatives of *osrA*$^+$ and those of *osrA* strains, very similar to those described by Csonka (10) between the *proU*$^+$ and *proU* mutants in *S. typhimurium*.

(i) The ability of small concentrations of exogenous L-proline (0.2 mM) to promote the growth of *osrA*$^+$ and *osrA* strains, in medium of sufficiently high osmolarity to be otherwise inhibitory (minimal A plus 0.65 M NaCl), was tested; it was found that, whereas the *putPA proP osrA*$^+$ strain, GJ134, grew in the proline-supplemented medium with a generation time of about 120 min and was able to form colonies in 60 h on plates, the growth of the isogenic *osrA* strains, GJ135 and GJ157, continued to be completely inhibited, both in liquid and on solid media.

(ii) The *osrA*$^+$ strain (GJ134) was resistant to the toxic analogs of L-proline, AC and DHP, in minimal A medium but was rendered AC^s and DHP^s in minimal A medium supplemented with 0.2 M NaCl (that is, under conditions in which *osrA*$^+$ would be expected to be maximally induced); in contrast, the *osrA* strains (GJ135 and GJ157) were AC^r and DHP^r in both minimal A medium and in minimal A medium plus 0.2 M NaCl.

(iii) Whereas Δ*proBA* derivative (GJ161) of the Δ*putPA proP osrA*$^+$ strain was unable to grow on minimal A medium supplemented with 0.1 mM L-proline and yet was able to do so when the osmolarity of the same medium was increased with the addition of 0.2 M NaCl, isogenic Δ*proBA* derivatives of *osrA* strains (GJ163 and GJ166) were unable to have their auxotrophic requirement satisfied with 0.1 mM L-proline even in the presence of 0.2 M NaCl.

That the osmoprotectant ability of L-proline in high-osmolar media is impaired or lost in *osrA* mutants in comparison with isogenic *osrA*$^+$ strains was demonstrable also in *putP*$^+$ or *proP*$^+$ strains. The relevant experimental results are summarized in Table 4, wherein against each strain within the various pairs of isogenic *osrA*$^+$ and *osrA* mutants is listed the highest concentration of NaCl that permitted growth in the presence and absence of 0.2 mM exogenous L-proline. It can be seen that the loss of the osmoprotectant ability of L-proline in high-osmolar media in *osrA* strains as opposed to *osrA*$^+$ strains was manifest in any genetic background, although the absolute level of osmotolerance achieved in the presence of L-proline progressively decreased with the introduction of *putP* and then the *proP* mutations. In concordance with the observations about *S. typhimurium* (10), the *proP*$^+$ *osrA* mutant (GJ121) itself continued to be protected against increased osmolarity to some extent (albeit less than the *osrA*$^+$ *proP*$^+$ strain) by L-proline; the osmoprotectant role of this imino acid was totally abolished only in the *proP osrA* mutant, GJ135. Thus, both these genes (*proP* as well as *osrA*) appear to be important in mediating the osmoprotectant effect of L-proline in *E. coli*.

Cultures of strain GJ11 grown in K medium plus 0.4 M NaCl and an excess (10 mM) of L-proline continued to show the same magnitude of induction of β-galactosidase activity as in media without L-proline. In another experiment, Pro$^-$ mutants of GJ11 and its *putP* derivative GJ121 were subjected to starvation for L-proline in batch culture as described above. This treatment also did not lead to induction of *osrA-lac* expression in the strains (data not shown). The observations, therefore, indicated that, nothwithstanding the role of *osrA* in L-proline transport, the concentration of L-proline in the medium does not itself affect the expression of the *osrA* operon.

Regulation of *osr-3*::*lac* expression and its identification as *kdp-lac*. Another osmoresponsive Mu d1(Ap *lac*) fusion that was studied in some detail was that designated *osr-3*, originally obtained in GJ3 and then stabilized as described above to yield strain GJ18. β-Galactosidase activity in GJ18 cultures grown in K medium plus 0.4 M NaCl was around 430 U, about 12-fold higher than that in cultures grown in K medium (35 U). A similar magnitude of induction (to 427 U) was observed when 0.27 M $(NH_4)_2SO_4$ was substituted for 0.4 M NaCl in the high-osmolar medium. The pattern of induction with increasing NaCl concentration in the growth medium was roughly linear (Fig. 2), the curve tending to plateau in the higher osmolar range.

The expression of *osr-3*::*lac* in GJ18, unlike that of *osrA-lac* in GJ11, was not induced upon growth in K media whose osmolarity had been increased by the addition (to 0.6 M) of any nonionic solute (seven of which were tested) but instead was maintained at the same level as that obtained during growth in K medium itself (data not shown). Furthermore, when osmoresponsivity of *lac* expression in GJ11 and GJ18 was compared after growth in media in which a defined concentration of one ionic and one nonionic solute together contributed to the external osmolarity, β-galactosidase activity in GJ11 was found to be a function of total medium osmolarity, whereas that in GJ18 was a function only of that fraction of medium osmolarity as determined by its ionic constituents (data not shown).

TABLE 4. Effect of L-proline on osmotolerance of various strains

Strain	Genotype	Highest concn (M) of NaCl that permits growth in[a]:	
		Minimal A medium	Minimal A medium + 0.2 mM L-proline
MC4100	*putP*$^+$ *proP*$^+$ *osrA*$^+$	0.4	0.75
GJ11	*putP*$^+$ *proP*$^+$ *osrA*	0.4	0.65
GJ125	*putP proP*$^+$ *osrA*$^+$	0.4	0.7
GJ121	*putP proP*$^+$ *osrA*	0.4	0.55
GJ134	*putP proP osrA*$^+$	0.35	0.7
GJ135	*putP proP osrA*	0.35	0.35

[a] The concentrations listed are the highest that allowed growth to a colony diameter of 2 mm or more in 60 h at 37°C.

The activity of β-galactosidase in GJ18 was shown also to be regulated by K^+ concentration in the growth medium. The results of an experiment in which β-galactosidase activity in GJ18 was measured as a function of various K^+ concentrations in low-osmolar K medium or in K medium plus 0.4 M NaCl are presented in Fig. 3. For this experiment, the K medium used was modified by the substitution of 1 mM NaH_2PO_4 for 1 mM KH_2PO_4; to this modified medium, with or without added 0.4 M NaCl, KCl was added to give the desired final concentrations of K^+. With increasing K^+ concentrations in the medium, β-galactosidase activity decreased sharply to 1 to 2 U; osmoresponsivity of expression was also not demonstrable in media whose K^+ concentration was >10 mM.

The third factor that affected the expression of *osr-3*::*lac* in GJ18 was the temperature of the growth medium. The β-galactosidase activity of GJ18 grown in K medium at 42°C was 173 U, which was four- to fivefold higher than that obtained upon growth at 30°C. This temperature effect on *osr-3* expression was additive to that of increased osmolarity (β-galactosidase activity in K medium plus 0.4 M NaCl at 42°C, 560 U) but was not seen under the conditions of complete repression obtained with 20 mM K^+ in the medium (approximately 2 to 4 U in low- and high-osmolar medium, both at 30 and 42°C).

A Tn*10* insertion (designated *zbg-901*::Tn*10*) linked 88% to *osr-3*::*lac* was obtained in this study by the strategy outlined earlier and was used in conjugation to map *osr-3* to the 15-min region of the chromosome (Fig. 1). P1 *kc* transduction experiments with a lysate grown on a *nadA*::Tn*10* strain also showed that *osr-3*::*lac* is 15% cotransducible with *nadA* (at 16 min), and the gene order was established as *att*λ-*nadA-osr-3* (data not shown). On the basis of both its map position and its pattern of regulation, it was considered likely that *osr-3* represented a *lac* fusion with *kdp*, a K^+-transport operon which too is situated close to *nadA* and has been shown in an earlier study to be similarly repressed by K^+ and activated by increased osmolarity (24). A P1 *kc* lysate prepared on GJ54 (a *kdp*$^+$ *nagA*$^+$ strain carrying the *zbg-901*::Tn*10* insertion) was used to transduce a *kdpABC5 nagA* strain, TK2205, to Tet^r, and of 82 transductants that were

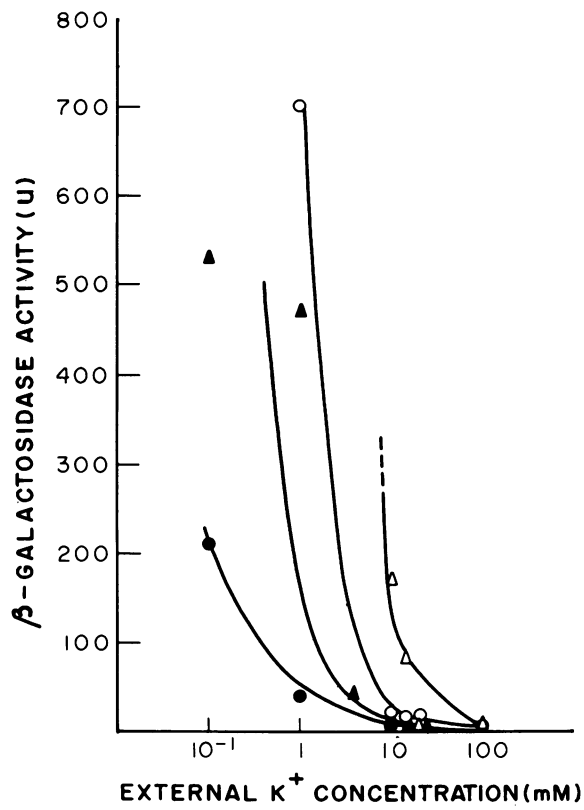

FIG. 3. β-Galactosidase activity in strains GJ18 and GJ39 as a function of external K^+ concentration in the growth medium. Cultures were grown for about 10 generations in (modified) K medium with or without 0.4 M NaCl and with KCl added to the concentrations specified on the abscissa. Symbols: ●, GJ18 in K medium; ▲, GJ18 in K medium plus 0.4 M NaCl; ○, GJ39 in K medium; △, GJ39 in K medium plus 0.4 M NaCl.

tested, 78 (95%) had become Kdp^+ (tested as described in reference 44), and 36 (44%) had become Nag^+. The similarity in transductional linkage values between the *zbg-901*::Tn*10* insertion on the one hand and *osr-3*::*lac* or *kdpABC5* on the other substantiated the notion that the latter two were allelic; it was, therefore, concluded that *osr-3* represents a *lac* fusion within the *kdpABC* operon.

Isolation of mutants altered in *kdp* regulation. The genetics of regulation of *kdp* expression has been well characterized by Epstein and co-workers (44). The operon, consisting of three genes, *kdpA*, *kdpB*, and *kdpC*, is subject to positive control that is mediated by the product of a regulator gene, *kdpD*, situated adjacent to the structural genes. *kdp* regulatory mutants had been selected earlier primarily on the basis of the effects that these mutations had on K^+ transport. I considered that additional regulatory mutants, selected in a *kdp-lac* strain, might prove important in further characterization of the role that the operon plays in osmoregulation.

Essentially the same strategy was used for the isolation of *kdp* regulatory mutants as that used earlier in other *lac* fusion strains (15, 36). GJ18 has sufficiently low β-galactosidase activity in K medium at 30°C so as not to be able to grow on lactose as the sole carbon source under such conditions. Spontaneous mutants were selected from several independent GJ18 cultures on minimal K medium plus lactose at 30°C, and these were tested for *lac* expression on X-gal-containing media of low and high osmolarity, with different K^+ concentrations, and at 30 and 42°C.

The mutants were also transduced to Tet^r with a P1 *kc* lysate grown on GJ58 (a derivative of GJ18 that carries the *zbg-901*::Tn*10* insertion closely linked to the parental *kdp-lac* fusion) to determine whether the mutation in each was linked to the *kdp* gene cluster itself. In all but one class of mutants, the mutations to Lac^+ could be crossed out in a large percentage (90 to 98%) of Tet^r transductants, indicating that they were indeed closely linked to the *kdp* genes. The results obtained on the characterization of the *kdp*-linked Lac^+ mutants are described further here; the results of studies on the derivatives in which the mutations conferring increased *kdp-lac* expression were not linked to *kdp*, are discussed below.

Some of the *kdp*-linked Lac^+ mutants, such as GJ22 and GJ23 (Table 5), had constitutively high levels of β-galactosidase activity which were unaffected by either the osmolarity or the K^+ concentration of the medium. The possibility that some of these strains might actually harbor deletions which have resulted in the fusion of the *lac* genes to a constitutive promoter near the *kdp* locus was not excluded in this study. In a second category of mutants, exemplified by strains GJ24 and GJ33 (Table 5), the expression of *kdp-lac* was rendered partially constitutive in K medium, with full induction being achieved in K medium plus 0.4 M NaCl. The addition of an excess of K^+ (20 mM) to the culture medium did not repress *lac* expression below that seen in K medium itself. The noteworthy feature, however, was that, as in the case of GJ18 itself, the expression of *kdp-lac* in these two mutants was also not affected by the osmolarity of the medium at the high K^+ concentration. It is believed that the mutation in each mutant of this latter category lies at one or the other of the two *kdp* regulatory loci, *kdpD* or *kdpAo*; *cis-trans* tests to distinguish between these two possibilities were not done in this work.

***trkA* mutants as a class with elevated *kdp-lac* expression.** The lone class of Lac^+ mutants of GJ18 which had mutations that were not linked to the *kdp* gene cluster were represented by three independently derived strains, GJ30, GJ39, and GJ40. All three strains could be transduced to Lac^- with the expected frequency of 88% when Tet^r transductants were selected by using a P1 *kc* lysate grown on GJ54, a strain which carries the *zbg-901*::Tn*10* insertion near the wild-type *kdp* operon. This indicated that none of the mutants had suffered transposition of the *lac* genes to a new chromosomal locus.

It was found that all three mutants had also acquired an osmosensitive phenotype: they were able to grow only very slowly in K medium plus 0.4 M NaCl at 30°C and not at all

TABLE 5. β-Galactosidase activity in GJ18 and its Lac^+ mutants grown at 30°C

Strain	β-Galactosidase activity (U)[a] when grown in			
	K medium	K medium + 20 mM KCl	K medium + 0.4 M NaCl	K medium + 0.4 M NaCl + 20 mM KCl
GJ18	48	2.2	462	2.7
GJ22	1,168	1,354	1,282	1,252
GJ23	1,610	899	1,321	1,013
GJ24	769	142	646	265
GJ33	689	63	731	76

[a] Units were as defined by Miller (35).

at 42°C. The growth inhibition of these strains in the high-osmolar medium was partially relieved with the addition of 10 mM K^+ to the medium.

The osmosensitive phenotype itself provided a facile selection strategy for the isolation by P1 *kc* transduction of Tn*10* insertions linked to the mutation conferring osmosensitivity, by a method similar to that described above for obtaining Tn*10* insertions near the sites of the original *osr-lac* fusions. Thus, a P1 *kc* lysate grown on a population of MC4100 Tet^r clones with random Tn*10* insertions was used to select Tet^r transductants in GJ39 on K medium plus 0.4 M NaCl at 42°C; only those colonies could grow that had recombined for both the Tet^r gene and the linked wild-type allele for the osmosensitive locus. Unexpectedly, two classes of Tet^r osmotolerant transductants were obtained in this step, one that had become Lac^- in all media and the other that had become osmoresponsively Lac^+ like the parent, GJ18. This result was interesting because it had two implications.

(i) The recovery of Lac^- Tet^r transductants as one class of osmotolerant recombinants from GJ39 suggested that inactivation of *kdp* function itself by the *lac* fusion contributed to the osmosensitive phenotype in this strain. The Tet^r transductants in GJ30, GJ39, and GJ40, obtained in the earlier crosses with the P1 *kc* lysate grown on GJ54 (*zbg-901*::Tn*10* kdp^+), were reexamined, and it was indeed found that, whereas the Tet^r Lac^- recombinants had also lost their osmosensitive phenotype, those transductants that remained Lac^+ continued to be osmosensitive.

(ii) The fact that the second class of Tet^r osmotolerant recombinants in strain GJ39 had also become osmoresponsively Lac^+ indicated that the second mutation which contributed to osmosensitivity in this strain was also the one responsible for the elevated expression of *kdp-lac* in low-osmolar medium. One Tn*10* insertion from this latter class (designated *zhc-904*::Tn*10* on the basis of the results of mapping experiments described below), which was 90% cotransducible with the osmosensitive allele in GJ39, was also shown to be linked 88 and 80%, respectively, to the corresponding alleles in GJ30 and GJ40 (data not shown). This result served to confirm that the three strains represent a single class of mutants, with mutations at a locus unlinked to *kdp* that acted both to increase *kdp-lac* expression and to contribute to their osmosensitivity.

A strain construction experiment was undertaken in which the *kdp-lac* fusion from GJ18 was first transduced into MC4100, by virtue of its linkage with the *zbg-901*::Tn*10* insertion. The Tn*10* insertion itself was subsequently deleted by Tet^s selection (31), and in the next step, the second osmosensitive allele from GJ39 was introduced by cotransduction with the *zhc-904*::Tn*10* insertion. All the strains so constructed were osmosensitive, thereby confirming the notion that the two mutations described above were both necessary and sufficient for establishing the osmosensitive phenotype.

Hfr mapping experiments suggested that *zhc-904*::Tn*10* was situated around 70 min on the chromosome (data not shown). In a transductional mapping experiment, a P1 *kc* lysate grown on a *zhc-904*::Tn*10* strain, GJ69, was used to transduce an *rpsE* (Spc^r) *rpsL* (Str^r) strain, KL252, to Tet^r. The Tet^r combinants were then scored for cotransduction of the Str^s and Spc^s alleles of the donor strain (Table 6), and *zhc-904*::Tn*10* was shown to be cotransducible 50% with *rpsE* and 34% with *rpsL*. In a three-factor analysis of the cross data (Table 6), the low frequency with which Tet^r Spc^r Str^s recombinants were recovered indicated a gene order of *zhc-904*::Tn*10-rpsE-rpsL*, at 72 min on the *E. coli* genetic map.

TABLE 6. Data on transductional mapping of *zhc-904*::Tn*10*

Cross[a]	Genotype			Transductant classes	No.
	zhc	rpsE	rpsL		
P1(GJ69)	Tn*10*	+	+	Tet^r Spc^s Str^s	25
×				Tet^r Spc^s Str^r	13
KL252	+	*2111*	*31*	Tet^r Spc^r Str^s	1
				Tet^r Spc^r Str^r	37

[a] The selected marker was Tet^r, and 76 transductants were scored.

The expression of *kdp-lac* in strain GJ39, as measured by the activity of β-galactosidase in cultures grown at 30°C, was progressively repressed by increasing concentrations of K^+ (up to 100 mM), both in K medium and in K medium plus 0.4 M NaCl (Fig. 3). The data were qualitatively very similar to those presented for GJ18 itself, except that higher K^+ concentrations were required to obtain the same degree of repression.

The mapping and physiological data, taken together, suggested that the additional locus represented by the mutations in GJ30, GJ39, and GJ40 was the *trkA* gene, which is situated at 72 min and is known to code for a major K^+-transport system that is constitutively expressed in *E. coli* (44). The requirement in these mutants for increased K^+ concentrations to repress *kdp-lac* expression was consistent with the interpretation that a second system of K^+ transport had been lost in these strains. The premise that the strains were indeed *kdp trkA* was confirmed by the observations that (i) *zhc-904*::Tn*10* was cotransducible 85% with the *trkA405* mutation in TK2205 (data not shown) and (ii) TK2205 (which carries *kdpABC5 trkA trkD*) was also unable to grow on K medium plus 0.4 M NaCl at 42°C, whereas isogenic kdp^+ or $trkA^+$ derivatives were able to do so. The derepression of *kdp* expression in *trkA* strains, similar to that seen in GJ30, GJ39, and GJ40, has been described earlier by Laimins et al. (24), although its relationship to osmosensitivity had not been demonstrated by these workers.

Identification of *osr-5* as *lamB-lac*. The third osmoresponsive *lac* fusion that was studied was the one designated *osr-5*, originally isolated in GJ5. β-Galactosidase activity in GJ5 was reduced fourfold in K medium plus 0.4 M NaCl, in comparison with that in K medium (Table 2). A similar reduction in enzyme activity was demonstrable with the substitution of 0.27 M K_2SO_4, 0.27 M $(NH_4)_2SO_4$, or 0.6 M sucrose for 0.4 M NaCl in the high-osmolar growth medium (data not shown). GJ5 was found also to have acquired a λ *vir*-resistant Mal^+ phenotype, whereas MC4100 is λ-sensitive Mal^+. On the basis of this observation (48), I considered it likely that GJ5 had a mutation in *lamB*, a gene which codes for an outer membrane protein that is involved in maltodextrin transport and that also serves as a receptor for λ bacteriophage.

A strain (GJ51) with a Tn*10* insertion 90% cotransducible with the *osr-5* locus was obtained from GJ5 by the strategy outlined above. Transductions to Tet^r with a P1 *kc* lysate grown on GJ51 were done (i) into GJ5, and a complete correlation was established between the loss of Mu dl(Ap *lac*) and the reacquisition of the λ-sensitive phenotype in the transductants and (ii) into a *malB* strain, KL209, and 80% of the transductants were found to have again acquired the Mal^+ λ-sensitive phenotype (data not shown). These results, therefore, localized the site of Mu dl(Ap *lac*) in GJ5 to the *malB* locus on the chromosome. In another experiment,

β-galactosidase activity in GJ5 was shown to be maltose inducible (increasing to 950 U in the presence of 0.2% maltose), consistent with the available evidence on the regulation of the *malK-lamB* operon (48).

Taken together, the observations above provide convincing evidence that GJ5 carries a *lamB-lac* fusion and that the transcription of *lamB* is reduced in media of high osmolarity.

DISCUSSION

Only 3,000 Mu dl(Ap *lac*) lysogens were screened in the work reported here on the identification of osmoresponsive genes in *E. coli*. This might explain why known osmoresponsive genes such as *ompF* and *ompC* (16, 29) or *phoA* (51) were not identified. The possibility that additional unidentified osmoresponsive genes also exist cannot at present be excluded.

Genetics of *osrA* regulation. The experiments described here established the existence of an operon, termed *osrA*, at 57.5 min on the *E. coli* linkage map, whose expression is induced 400-fold by ionic or nonionic impermeable solutes that increase the osmolarity of the culture medium. The slope of the induction curve is maximal in the range of 300 to 800 mOsm; if it is assumed that the optimal osmolarity for growth of *E. coli* is 280 to 300 mOsm, then *osrA* may be expected to function mainly in adaptation to osmolarity that is above the optimal range. The fully induced level of β-galactosidase activity in an *osrA-lac* fusion strain was around 800 U, which approximately corresponds to 8,000 subunit monomers of the enzyme per cell (5); this indicates that *osrA* has a very strong promoter that is tightly regulated. The mechanisms underlying such control of expression, by physical environmental signals such as turgor pressure or medium osmolarity, are not known.

Physiological role of the *osrA* operon. The experiments with different pairs of isogenic *osrA*$^+$ and *osrA* strains permit one to make the inference that the presence of high concentrations of the OsrA gene product(s) within the cell (as obtained during growth of *osrA*$^+$ strains in high-osmolar medium) (i) increases the sensitivity to growth inhibition by the L-proline analogs, AC and DHP, and (ii) decreases the concentration of exogenous L-proline required to satisfy an auxotrophic requirement. This may be regarded as evidence prima facie for a role of the *osrA* operon in the active transport of L-proline into the cell. The observation that the osmoprotectant ability of L-proline is absent or diminished in *osrA* mutants also supports a teleonomic role of this system in osmoregulation in *E. coli*. On the basis of the genetic and physiological data available on the *osrA* system in *E. coli* and *proU* in *S. typhimurium* (10), the identity between the two may be taken as established. Therefore, the *osrA* locus also is being redesignated as *proU* to maintain the similarity in genetic nomenclature in the two organisms.

Regulation of *kdp* expression. My studies have confirmed and extended those of Epstein and co-workers in identifying four factors which affect the expression of the *kdp* operon: (i) medium osmolarity, but only to the extent contributed by ionic solutes, (ii) external K^+ concentration, (iii) temperature of growth, and (iv) the functional activity of the TrkA transport system.

The expression of *kdp* is fully repressed in media with K^+ concentrations >10 mM, and an increase in the osmolarity of the medium does not relieve repression under these conditions. A point that needs emphasis, therefore, is that the effect of medium osmolarity on *kdp* is seen only in low-K^+ media. This is true both in the case of the wild-type *kdp-lac* strain, GJ18, and with the partially constitutive mutant derivatives, GJ24 and GJ33.

Laimins et al. (24) reported that abrupt increases in osmolarity obtained by the addition of ionic or neutral osmolytes to cultures grown in K^+-rich media caused instantaneous increases in *kdp* expression; they postulated, therefore, that it is the turgor pressure across the membrane which acts as the proximate stimulus for the regulation of this operon. It is difficult to reconcile their data with my results, which suggest that *kdp-lac* expression is not elevated in GJ18 cultures grown in the presence of high concentrations of the neutral solutes or when the strain is grown with ionic osmolytes in K^+-rich media. If, as is generally believed (24, 44), the functional role for the stimulation of *kdp* by elevated osmolarity is to increase intracellular solute concentration and maintain cell turgor pressure, then clearly such activation must be sustained throughout the period that the stimulus is maintained, that is, even as the strain continues to grow in that medium. Perhaps the changes in *kdp* expression described by the above workers (24) that follow an abrupt increase in medium osmolarity are only a short-lived consequence of plasmolysis and of the complex changes in cellular metabolism that occur under such circumstances, before the cells adapt themselves and begin to grow in the new conditions. My results also suggest that the mechanism by which *kdp* expression is regulated both by K^+ concentration and by medium osmolarity is probably more complex than that proposed by Laimins et al. (24). Furthermore, the observation that sustained stimulation of *kdp* expression occurs with ionic solutes, but not with nonionic impermeable solutes, also remains unexplained.

Osmoresponsivity of *lamB* expression. The decrease observed in the transcriptional expression of *lamB* with increase in medium osmolarity is similar in magnitude to that described earlier for another outer membrane protein, *ompF* (16, 29). *ompF* and *lamB* have also been shown by other workers to be more or less coordinately regulated in response to growth temperature, procaine, and phenethyl alcohol (30, 39, 42). Osmoresponsivity of *ompF* expression is mediated in a complex way by the products of the *ompR* and *envZ* genes (17); the *envZ* gene product has also been implicated in mediating the procaine effect on *ompF* and *lamB* (13, 50). In light of these observations, it is reasonable to postulate that *envZ* and *ompR* also play a role in the osmoresponsive expression of *lamB*.

Definition of an osmoregulatory gene. Two criteria by which a gene could be considered important is osmoregulation were laid down in this study. (i) The gene must show osmoresponsivity in its expression, or the concerned gene product must show osmoresponsivity in its catalytic activity, and (ii) mutations in the gene that lead to loss of its function must confer an osmosensitive phenotype to the mutant strains. The demonstration of an osmosensitive phenotype may itself necessitate the presence of additional mutations or particular environmental conditions, but so long as this phenotypic difference could be shown between a pair of strains which was otherwise isogenic, this condition could be regarded as having been satisfied.

By these criteria, the *kdp* and *osrA* genes characterized in this study may be considered the first examples of osmoregulatory genes in *E. coli*. That there probably exists no overlap between the osmoregulatory mechanisms mediated by *osrA* (*proU*) and *kdp* was borne out by the observations that (i) the introduction of the *trkA* mutation into the *osrA-lac* fusion strain, GJ11, had no effect on the ability of the strain to grow in high-osmolar media and (ii) the ability of

L-proline to relieve growth inhibition caused by high osmolarity was retained to the expected levels in both GJ18 and GJ39 (data not shown).

ACKNOWLEDGMENTS

I thank P. M. Bhargava, D. Chatterji, R. Nagaraj, J. Pittard, and V. Sitaramam for their comments during the preparation of this manuscript. Saroja Nagaraj and L. Sai Sree provided excellent technical assistance.

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