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Biochemical and Genetic Characterization of Osmoregulatory Trehalose Synthesis in *Escherichia coli*

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It has been shown previously that *Escherichia coli* accumulates endogenously synthesized trehalose under osmotic stress. We report here that *E. coli* contained an osmotically regulated trehalose-phosphate synthase which utilized UDP-glucose and glucose 6-phosphate as substrates. In the wild type, the synthase was induced by growth in glucose-mineral medium of elevated osmotic strength and the synthase itself was strongly stimulated by K^+ and other monovalent cations. A laboratory strain which expressed the synthase at a high constitutive level was found. GalU mutants, defective in synthesis of UDP-glucose, did not accumulate trehalose. Two genes governing the synthase were identified and named *otsA* and *otsB* (osmoregulatory trehalose synthesis). They mapped near 42 min in the *flbB-uvrC* region. Mutants with an *otsA-lacZ* or *otsB-lacZ* operon fusion displayed osmotically inducible β-galactosidase activity; i.e., the activity was increased fivefold by growth in medium of elevated osmotic strength. Mutants unable to synthesize trehalose (*galU*, *otsA*, and *otsB*) were osmotically sensitive in glucose-mineral medium. But an osmotically tolerant phenotype was restored in the presence of glycine betaine, which also partially repressed the synthesis of synthase in the wild type and of β-galactosidase in *ots-lacZ* fusion mutants.

In order to prevent osmotic dehydration and sustain a proper turgor pressure, bacteria have to maintain an osmotic strength of the cytoplasm which exceeds that of the environment. It is now well documented that in an environment of elevated osmotic strength, *Escherichia coli* and other eubacteria osmoregulate by accumulating K^+ together with a few types of organic molecules, e.g., betaines, amino acids, and sugars (13, 19, 20, 24–26, 38). However, the mechanisms for uptake and synthesis of these osmolytes, and particularly the regulatory mechanisms involved, remain an intriguing field for biochemical and genetic research.

The osmotic tolerance of *E. coli* depends on the composition of the growth medium, particularly the availability of osmoprotective compounds (26). The highest level of osmotic tolerance in *E. coli* is achieved by uptake of glycine betaine or proline betaine (24–26) and by synthesis of glycine betaine from choline (22, 39); a somewhat lesser tolerance is achieved by uptake of γ-butyrobetaine or proline (6, 24, 25). ProP and ProU have been identified as the transport systems for proline and glycine betaine (6, 7), and the Bet system is responsible for high-affinity choline uptake and oxidation of choline to glycine betaine (22, 39). ProP is synthesized constitutively (6), whereas the syntheses of the ProU (7, 15, 29) and the Bet (22, 39) systems depend on osmotic stress. The only difference between *E. coli* and the closely related *Salmonella typhimurium* with respect to the utilization of the osmoprotective compounds appears to be that the latter organism lacks the Bet system and is therefore not protected against osmotic stress by choline (19).

Osmoregulation of *E. coli* in glucose-mineral medium without any osmoprotective compounds seems to involve endogenous synthesis of trehalose (10, 24, 38), together with a lesser synthesis of glutamic acid (24). This contention was originally based on the finding that the intracellular accumulations of these compounds increase with the osmotic strength of the growth medium. Trehalose has also been shown to be an osmoregulatory solute in phototrophic bacteria (36), and it accumulates in many organisms which can withstand dehydration (8). The enzyme trehalose-phosphate synthase, which utilizes UDP-glucose and glucose 6-phosphate as substrates, has been characterized in a number of organisms (11, 12, 23, 34) but not in relation to osmoregulation. In the present study, we have partially characterized trehalose-phosphate synthase of *E. coli*. We report that the enzyme is osmotically inducible and K^+ activated. Mutants of *E. coli* which are defective in the synthesis of the synthase have an impaired osmotic tolerance in glucose-mineral medium. Some comparative biochemical studies were carried out with *S. typhimurium*.

MATERIALS AND METHODS

Bacterial strains, bacteriophages, and plasmids. The bacterial strains, phages, and plasmids used are listed in Table 1.

Growth media and growth conditions. The minimal growth medium used was medium 63 (31) with 22 mM glucose, 22 mM galactose, 22 mM trehalose, or 11 mM lactose and 1.7 mM sodium citrate as carbon sources. (When not stated otherwise, glucose was used). The osmotic strength of the medium was increased by the addition of 0.2 to 0.75 M NaCl, and in some cases glycine betaine (1 mM) was added as an osmoprotectant, as stated below. The pH was adjusted to 7.2 with NaOH when necessary. Amino acids and other growth factors were added as required by the strains. Half-strength medium 63 contained half the amount of the inorganic salts and the full amount of glucose. For measurements of β-galactosidase activity of *lacZ* fusion mutants and for some studies on the effect of osmotic shock, the cells were grown in the low-osmolarity minimal medium (LOM-glucose) with the appropriate amount of NaCl (6). The rich medium used was LB (31). The bacteria were grown aerobically at 37°C. Cells used for enzyme assays or determination of osmolyte accumulation were harvested in exponential growth phase by centrifugation at 8,000 × *g* for 10 min (4°C).

Antibiotics. The following concentrations of antibiotics were used in the growth media where appropriate: ampicil-

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TABLE 1. Bacterial strains, bacteriophages, and plasmids

Strain, bacteriophage, or plasmid	Characteristic(s)[a]	Construction or source[b]
Escherichia coli		
L60	Hfr PO2A Δ(*bet-lac*) *rel-1 tonA22* T2^r Rif^r	39
CSH7	F$^-$ *lacY rpsL thi*	31
MC4100	F$^-$ *araD139* Δ(*argF-lac*) *U169 flbB5301 relA1 rpsL150 deoC1 ptsF25 rbsR*	CGSC6152
FF1000	F$^-$ MC4100 *recA56 srl-300*::Tn*10*	MC4100 × JC10240
FF69	Hfr PO2A L60 *otsA1*::Tn*10*	This study
FF70	Hfr PO2A L60 *otsA2*::Tn*10*	This study
FF103	Hfr PO2A L60 *otsA3*::Tn*10*	This study
FF108	Hfr PO2A L60 *otsA4*::Tn*10*	This study
FF110	Hfr PO2A L60 *otsA5*::Tn*10*	This study
FF770	F$^-$ CSH7 *otsA2*::Tn*10*	CSH7 × P1 (FF70)
FF4169	F$^-$ MC4100 *otsA1*::Tn*10*	MC4100 × P1 (FF69)
FF5169	F$^-$ FF4169 Δ(*otsA1*::Tn*10*)	This study
FF6169	F$^-$ FF5169 *recA56 srl-300*::Tn*10*	FF5169 × JC10240
FF741	F$^-$ MC4100 Φ(*otsA-lacZ*)*6* (λ p*lac*Mu55)	This study
FF2032	F$^-$ MC4100 Φ(*otsA-lacZ*)*7* (λ p*lac*Mu55)	This study
FF1112	F$^-$ MC4100 Φ(*otsB-lacZ*)*8* (λ p*lac*Mu55)	This study
FF1608	F$^-$ MC4100 Φ(*otsB-lacZ*)*9* (λ p*lac*Mu55)	This study
FF4011	F$^-$ FF4169 Φ(*otsB-lacZ*)*8* (λ p*lac*Mu55)	FF4169 × P1 (FF1112)
FF4001	F$^-$ MC4100 *galU95*	This study
FF4002	F$^-$ MC4100 *galU106*	This study
ED1039	Hfr Δ(*gpt-lac*)*5 supE44 TP4relA1*? *rpsE2123 thi-1* λ$^-$	CGSC 5396
KL96	Hfr *thi-1 relA1 spoT1* λ$^-$	CGSC 4243
Hfr44	Hfr *ilv-282 argA42 argR40 argS41 galT23* λ$^-$	CGSC 4230
FF4020	Hfr KL96 *otsA1*::Tn*10*	KL96 × P1 (FF4169)
FF4021	Hfr ED1039 *otsA1*::Tn*10*	ED1039 × P1 (FF4169)
FF4022	Hfr Hfr44 *otsA1*::Tn*10*	Hfr44 × P1 (FF4169)
LCB107	F$^-$ *tre-1 trpA43 metB1 lacY1 malA1* (λ^r) *rpsL134 supE44*?	CGSC 6407
FF4023	F$^-$ LCB107 *otsA1*::Tn*10*	LCB107 × P1 (FF4169)
FF4024	F$^-$ LCB107 Φ(*otsB-lacZ*)*8* (λ p*lac*Mu55)	LCB107 × P1 (FF1112)
FF4018	F$^-$ MC4100 *tre-1 zcf-229*::Tn*10*	This study
FF4019	F$^-$ FF4018 Δ(*zcf-229*::Tn*10*)	This study
FF4025	F$^-$ FF4019 Φ(*otsB-lacZ*)*9* (λ p*lac*Mu55)	FF4019 × P1 (FF1608)
FF4029	F$^-$ FF4019 *otsA1*::Tn*10*	FF4019 × P1 (FF4169)
CA10	Hfr *galU95 relA1 spoT1* λ$^-$	CGSC 4973
CA198	Hfr *galU106 relA1 spoT1* λ$^-$	CGSC 4983
DFF1/JC1553	F′ 150/*leuB6 tonA2 lacY1 supE44 gal-6* λ$^-$ *hisG1 recA1 argG6 rpsL104 malA1* (λ^r) *xyl-7 mtl-2 metB1*	CGSC 4326
F500/GMS724	F′ 500/*aroD6 recA1 metB1 lacY1 galK2 man-4 rpsL70 tsx-29*? *supE44*?	CGSC 5505
KL717	F′ 148/*proA2 aroD5 his-4 gyrA12 recA1* λ$^-$ *thi-1 xyl-5* (or *7*) *tsx-1*? (or *29*?) *supE44*?	CGSC 4302
TK2204 *trpB*::Tn*10*	F$^-$ *thi rha lacZ*(Am) *trkA405 trkD1 kdpA4 trpB*::Tn*10*	W. Epstein
JC10240	Hfr *thr-300 recA56 srl*-300::Tn*10 relA1 ilv-318 spoT1 thi-1 rpsE2300* λ$^-$	CGSC 6074
AB1884	F$^-$ *thr-1 ara-14 leuB6* Δ(*gpt-proA*)*62 lacY1 tsx-33 supE44 galK2* λ$^-$ rac$^-$ *uvrC34 hisG4 ribD1 rpsL31 kdgK51 xyl-5 mtl-1 argE3 thi-1*	CGSC 1884
NK5526	F$^-$ *hisG213*::Tn*10* λ$^-$ IN(*rrnD-rrnE1*)*1*	CGSC 6416
FF4016	F$^-$ FF1112 *hisG213*::Tn*10*	FF1112 × P1 (NK5526)
BL203	F$^-$ *thr-1(Am) leuB6 his-4 metF159*(Am) *thi-1 eda rpsL136 lacY1 ara-14 xyl-5 mtl-1 tonA31 zec-748*::Tn*10*	B. Lowsky
BL230[c]	F$^-$ *thr-1*(Am) *leuB6 his-4 metF159*(Am) *thi-1 eda rpsL136 lacY1 ara-14 xyl-5 mtl-1 tonA31 (flbB)*::Tn*10*	B. Lowsky
CY313	F$^-$ *tonA2 lacY1 tsx-70 supE44 gal-6* λ$^-$ *zcf-229*::Tn*10 trp-45 his-68 tyrA2 rpsL125 malT1*(λ^r) *xyl-7 mtl-2 thi-1*	CGSC 6429
BK2106	F$^-$ *tag ada his*	E. Seeberg
FF4031	F$^-$ FF4011 Δ[*otsA1*::Tn*10* Φ(*otsB-lacZ*)*8*]*1*	This study
Salmonella typhimurium LT2	Wild type	C. F. Higgins
Phages		
P1	*cml clr-100*	K. Carlson; 31
λNK55	*b221 c*I857 *c*IIII67::Tn*10 ind*$^+$ *O*am29	21
λ p*lac*Mu55	MucIts62 *ner*$^+$ *A*′am 1039 *′uvrD′* Mu *S′* ′trp′ *lacZ*$^+$ *lacY*$^+$ *lacA′ kan imm* λ$^-$	E. Bremer; 29
λ pMu507	MucIts62 *A*$^+$ *B*$^+$ *S*am7 *c*Its857	E. Bremer; 29
Plasmids		
cos4	Ap^r	37
pFF47	Ap^r	This study

[a] The symbol φ indicates the presence of a *lacZ* fusion.
[b] CGSC, *E. coli* Genetic Stock Center, Yale University, New Haven, Conn. All CGSC strains were obtained from B. J. Bachmann.
[c] The Tn*10* of BL230 is 100% cotransducible with *flbB*; see text.

lin, 100 μg/ml; kanamycin, 60 μg/ml; streptomycin, 100 μg/ml; and tetracycline, 5 to 15 μg/ml.

In vitro trehalose synthesis. The harvested cells were washed once in 10 mM sodium phosphate (pH 7.5) by centrifugation and for practical reasons were stored at −80°C. To permeabilize the cells, 10% (vol/vol) toluene was added to 3 to 5 ml of ice-cold cell suspension containing 100 to 200 mg of cell protein. The mixture was vortexed for 1 min; 6 ml of 10 mM Tris hydrochloride (pH 7.5) was then added, and the mixture was centrifuged at 6,000 × *g* for 5 min (4°C). The cells were washed twice by centrifugation in 10 ml of Tris buffer and then suspended at a protein concentration of 20 mg/ml in Tris buffer containing 2 mM dithiothreitol.

The standard reaction mixture for determination of trehalose-phosphate synthase activity contained in a 0.3-ml volume 0.75 μmol of UDP-glucose (Sigma Chemical Co., St. Louis, Mo.), 1.5 μmol of glucose 6-phosphate (Sigma), 0.75 μmol of $MgCl_2$, 10 μmol of Tris hydrochloride (pH 7.5), 75 μmol of KCl, and 1 mg of cell protein. In experiments in which the effects of other salts (i.e., NaCl, LiCl, $CaCl_2$, or potassium glutamate) were tested, KCl was omitted. The reaction mixture was incubated at 37°C for various times up to 6 min, and the reaction was terminated by heating for 5 min in a boiling water bath. Sucrose (0.3 μmol in a 30-μl volume) was then added as an internal standard, and denatured protein was removed by centrifugation. A sample of 280 μl was withdrawn, the pH was adjusted to 8.0 with 2.5 μmol of Tris base, and 1 U of alkaline phosphatase (Sigma) was added. After incubation at 37°C for 2 h, the reaction was stopped by heating. For desalting, a sample of 250 μl was applied to a 0.5 by 2 to 6 cm column packed with equal amounts of Dowex 50X4-200 in H^+ form and Dowex 1X8-400 in formate form. Free sugars were washed through the column with 1 to 3 ml of water, and the eluate was freeze-dried and analyzed by gas chromatography as described below. The enzyme unit was nanomoles of trehalose formed per minute at 37°C. The present method was a modification of one published previously (11).

Trehalase activity. To release periplasmic proteins, freshly harvested cells were subjected to cold osmotic shock as described previously (33) and the shock fluid was dialyzed against distilled water overnight (4°C). The standard reaction mixture for determination of trehalase activity contained in a 1-ml volume 5 μmol of sodium phosphate (pH 6.0 or 7.5), 1 μmol of trehalose, and the appropriate amount of shock-released cell protein or toluene-treated cells. The mixture was incubated at 37°C for up to 30 min. The reaction was stopped by boiling for 5 min, the sample was freeze-dried, and the remaining trehalose was determined by gas chromatography as described below, using sucrose as an internal standard.

Gas chromatographic determination of trehalose. An HP5890A gas chromatograph, equipped with an HP3393A integrator, and an HP1 capillary column (25 m by 0.31 mm [inner diameter]; Hewlett-Packard Co., Avondale, Pa.) was used. Helium was used as the carrier gas. The injector and detector temperatures were 250 and 300°C, respectively. The column temperature was kept at 190°C for 2 min, followed by a temperature increase of 30°C per min to 250°C, after which the temperature was kept at 250°C for 10 min. The freeze-dried trehalose samples were dissolved in 20 μl of dimethylformamide and then trimethylsilylated by the addition of 20 μl of bis(trimethylsilyl)-trifluoroacetamide containing 1% trimethylchlorosilane.

Determination of trehalose accumulation in cells. The cells were washed by centrifugation in medium 63 without sugar and containing an appropriate amount of NaCl. Trehalose accumulation was routinely determined by the anthrone method after the reducing sugars were destroyed by boiling with alkali as described previously (23, 24). In some cases, the results were verified by gas chromatographic analysis (see above).

Genetic procedures and strain constructions. P1 phage transduction, F′ complementation, and conjugation were performed as described by Miller (31). *galU* mutations were introduced into strain MC4100 by cotransduction with *trpB*. First, MC4100 was transduced with P1 lysate prepared from strain TK2204 *trpB*::Tn*10*, and then a resulting Trp^- Tc^r mutant was transduced with P1 lysate prepared from the *galU* mutant CA10 or CA198. In the latter crosses, we selected for Trp^+ strains and scored for Gal^- mutants (14), obtaining mutants FF4001 and FF4002, respectively. The *tre* mutation of LCB107 was transferred to MC4100 by cotransduction with *zcf-229*::Tn*10*. First, LCB107 was transduced to Tc^r with P1 lysate prepared from strain CY313. Then, P1 phages grown on a resulting Tc^r Tre^- strain were used to transduce MC4100, selecting Tc^r transductants and scoring for the Tre^- phenotype on eosin-methylene blue agar with trehalose, as described previously (2). From the latter cross, we obtained FF4018. Tn*10* was deleted from FF4011, FF4018, and FF4169 as described previously (3), generating FF4031, FF4019, and FF5169, respectively. A *recA* mutation was introduced into MC4100 and FF5169 by conjugation with JC10240 as described previously (9), generating strains FF1000 and FF6169, respectively.

Molecular cloning and DNA work. Construction of a gene library of CSH7 in the cosmid vector cos4 was carried out as described by Roberts et al. (37). Plasmids were introduced into the osmotically sensitive strain FF6169 by using the standard infection procedure, and an osmotically tolerant clone was selected by direct plating on medium 63–glucose–0.45 M NaCl. Transformation was carried out by using a standard $CaCl_2$ method; isolation of plasmid DNA and restriction cleavage and ligation of DNA were carried out essentially as described previously (28).

Isolation of osmotically sensitive Ots mutants. To generate a culture which generally had transposon Tn*10*, strain L60 was infected with the phage λNK55 (::Tn*10*) essentially as described previously (21). After the transposition, the cells were spread on 100 agar plates containing LB medium, 15 μg of tetracycline per ml, and 25 mM sodium pyrophosphate. About 50,000 Tc^r colonies representing individual transposition events were then collected. Similarly, a culture carrying a random selection of *lacZ* operon fusions was prepared by infecting strain FF1000 with the phage λ p*lac*Mu55 (Km^r) and the helper phage λ pMu507 as described previously (5, 29). After the infected cells were plated on 100 plates with LB medium and 60 μg of kanamycin per ml, about 50,000 Km^r colonies were collected. These collections of Tc^r or Km^r mutants were grown in medium 63 and then inoculated into the same medium with 0.45 M NaCl. The latter cultures were incubated for 2 h at 37°C before 100 μg of ampicillin per ml was added, and the incubation was continued for 5 h. At this stage, the surviving Tc^r cells were plated on medium 63-glucose-agar. The surviving Km^r cells were grown in LB medium overnight, and the whole ampicillin enrichment procedure was repeated once before the cells were plated on medium 63–lactose–0.2 M NaCl–agar. Osmotically sensitive mutants were then isolated by transferring an inoculant of the individual colonies to two sets of agar plates, one

containing medium 63-glucose and one containing the same medium with 0.5 M NaCl.

β-Galactosidase assays. The β-galactosidase activity of *lacZ* fusion mutants was scored on agar plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). This activity was determined quantitatively by using cells treated with chloroform and sodium dodecyl sulfate as described by Miller (31), except that the amount of protein in each sample was determined by the Lowry method and not by measurement of the optical density. The cells were washed once in water in the centrifuge.

Other methods. Natural abundance ^{13}C nuclear magnetic resonance spectroscopy was performed as described previously by us (24). Protein in samples with trehalose-phosphate synthase was determined by the biuret method as modified for whole bacterial cells (18). UV sensitivity caused by *recA* or *uvrC* mutations was scored as described previously (28).

RESULTS

Trehalase activity. Using ^{13}C nuclear magnetic resonance spectroscopy, we have previously shown that *E. coli* strains (e.g., MC4100, CSH7, and K-10) accumulate trehalose, but not glucose, under osmotic stress. We noted, however, that these strains synthesize a trehalase, which may cause the breakdown of trehalose to glucose in vitro (24). Recent reports of Boos and co-workers (4, 10) show that the trehalase is located in the periplasmic space. This is in agreement with our data.

The breakdown of trehalose caused by trehalase precluded a determination of in vitro synthesis of trehalose in toluene-treated cells or crude extracts of wild-type *E. coli*. We found, however, that strain LCB107 (*tre*) was defective in synthesis of trehalase but displayed osmotic stress-dependent accumulation of trehalose. LCB107 was initially isolated by Becerra de Lares et al. (2) for being defective in trehalose utilization. But to our knowledge, the biochemical basis for the Tre^- phenotype of this strain has not been investigated previously. The *tre* mutation of LCB107 was transferred to MC4100 by cotransduction with *zcf-229*::Tn*10* as described above, giving rise to FF4018 and its Tc^s derivative FF4019. Biochemical analysis showed that the two latter strains lacked trehalase activity. The observed cotransduction was 6%, confirming that the *tre* gene encoding trehalase is located near 26 min (1, 2, 4). As also reported previously (2), we found that the Tre mutants of *E. coli* grew readily on minimal agar with trehalose as the only carbon source, but they formed lighter colonies on eosin-methylene blue agar with trehalose.

There appear to be differences between *E. coli* and the closely related *S. typhimurium* with respect to expression and localization of trehalase. Postma et al. (35) have reported that the trehalase of the latter organism is induced by trehalose in the growth medium and that it is localized in the cytoplasm. Thus, in the present biochemical investigation of trehalose synthesis, we have used Tre mutants of *E. coli* (i.e., LCB107 and FF4019) and wild-type *S. typhimurium* LT2.

Properties of trehalose-phosphate synthase of *E. coli*. The present determination of in vitro trehalose synthesis involved the following steps. Cells grown under proper conditions (see below) were permeabilized by toluene and then washed to remove salts and trehalose and its metabolic precursors. After the in vitro synthesis reaction was stopped, the reaction mixture was treated with externally added phosphatase to dephosphorylate trehalose phosphate and then passed through a column packed with mixed bed ion-exchange resin before the amount of trehalose was determined by gas chromatography.

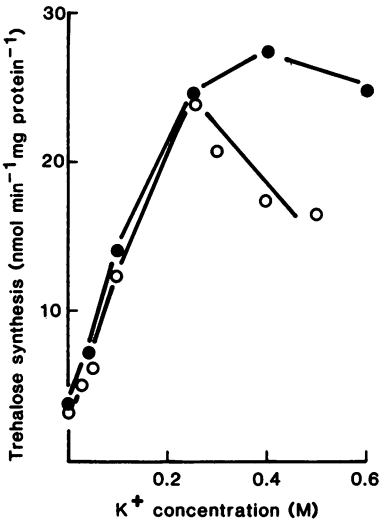

FIG. 1. Effects of potassium salts on the in vitro trehalose-phosphate synthase activity of *E. coli* LCB107. Symbols: ○, KCl; ●, potassium glutamate. The activity was measured in toluene-treated cells.

In the presence of monovalent cations (e.g., 0.25 M KCl), toluene-treated cells catalyzed the synthesis of trehalose from UDP-glucose and glucose 6-phosphate. There was no synthesis of trehalose when one of these metabolites was omitted from the reaction mixture, when ADP-glucose (the precursor of glycogen) was substituted for UDP-glucose, or when glucose was substituted for glucose 6-phosphate. Thus, the substrate requirement indicated that the first step in the pathway was catalyzed by a trehalose-phosphate synthase (EC 2.4.1.15) and that trehalose 6-phosphate was an intermediate metabolite in trehalose synthesis in *E. coli*. The existence of a phosphorylated intermediate was also supported by the finding that when phosphatase treatment of the reaction mixture was omitted, the amount of free trehalose in the effluent of the ion-exchange column was reduced by 50%. Unfortunately, we lacked standard trehalose phosphate which could have helped us to identify the intermediate more precisely. For the same reason, the nature of the endogenous phosphatase(s) which carried out a partial dephosphorylation of trehalose phosphate was not investigated.

The in vitro synthase activity was strongly stimulated by the presence of monovalent cations in the reaction mixture. The synthase activity of toluene-treated cells of LCB107 was very low in the absence of salts, but the activity increased nearly linearly with the KCl or potassium glutamate concentration of the reaction mixture up to 0.25 M (Fig. 1). At higher concentrations of KCl, the in vitro synthase activity diminished. However, with potassium glutamate, the peak activity was reached at about 0.4 M and the activity remained nearly the same up to at least 0.6 M. Within the limit of experimental accuracy, potassium glutamate, KCl, LiCl, and NaCl had the same stimulatory effect at a 0.25 M concentration, whereas $CaCl_2$ had little effect (Table 2). (Strain FF4019 displayed the same properties as LCB107 with respect to salt activation of the synthase).

Induction of trehalose-phosphate synthase and strain vari-

TABLE 2. Influence of cations (0.25 M) on the in vitro activity of trehalose-phosphate synthase of *E. coli* LCB107[a]

Added salt (0.25 M)	Specific synthase activity (U/mg of protein)[b]
None	6 ± 3
KCl	30 ± 6
NaCl	28 ± 2
LiCl	23 ± 3
Potassium glutamate	30 ± 3
$CaCl_2$	11 ± 3

[a] Synthase activity was determined in toluene-treated cells. Each value is an average of at least four independent measurements (± the standard deviation).

[b] The enzyme unit is nanomoles of trehalose synthesized per minute at 37°C.

ations in *E. coli*. Cells of FF4019 grown at steady state in medium 63 with 0.40 M NaCl displayed trehalose-phosphate synthase activity (3 U/mg of protein), whereas cells grown in the same medium without salt did not have any detectable activity (Table 3). When assayed 2 h after transfer from medium 63 without NaCl to the same medium with 0.40 M NaCl, the synthase activity of FF4019 was at the fully induced level. Chloramphenicol (150 μg/ml) completely inhibited the formation of synthase activity after this upshock, indicating that the formation of synthase activity depended on de novo protein synthesis rather than on activation of a precursor. Cells of FF4019 which were grown under osmotic stress (0.40 M NaCl) in the presence of glycine betaine (1 mM) displayed a strongly diminished synthase activity, i.e., only about 1 U/mg of protein, which was close to the detection limit.

Unlike strain FF4019, LCB107 displayed a high constitutive level of trehalose-phosphate synthase activity; i.e., the activity was the same whether the cells were grown at steady state in half-strength medium 63 or in full-strength medium 63 with or without 0.40 M NaCl. Furthermore, its synthase activity was 10-fold higher than the activity of fully induced cells of FF4019 (Table 3).

The differences between FF4019 and LCB107 were also apparent from the following observation. When cells grown at low osmotic strength in LOM-glucose were subjected to osmotic upshock by transfer to the same medium with 0.50 M NaCl, chloramphenicol prevented trehalose accumulation in FF4019 nearly completely but did not influence trehalose accumulation in LCB107. However, in accordance with our previous finding (24), none of the strains accumulated trehalose during growth in the absence of osmotic stress.

Trehalose-phosphate synthase of *S. typhimurium*. In order to determine the wild-type phenotype of synthase expression in enteric bacteria, i.e., a high constitutive or a low inducible activity, we determined the synthase activity of wild-type *S. typhimurium* LT2. We found that LT2 displayed the same osmotic induction and low level of synthase activity as *E. coli* FF4019 (Table 3). Furthermore, the synthase of LT2 was strongly stimulated by monovalent cations, and the osmotic induction of the enzyme was prevented by chloramphenicol.

GalU mutants. The *galU* gene of *E. coli* encodes glucose-1-phosphate uridylyl transferase, which catalyzes the formation of UDP-glucose (1, 14). When known *galU* mutations were introduced into MC4100 by P1 transduction, the resulting strains, FF4001 and FF4002, did not accumulate trehalose and they became osmotically sensitive; i.e., they displayed essentially no growth on agar plates with medium 63–0.50 M NaCl. Glutamic acid was the only osmolyte which could be detected on ^{13}C nuclear magnetic resonance spectra of extracts of osmotically stressed GalU mutants (data not presented). These data confirmed the biochemical data that UDP-glucose was a precursor of trehalose in *E. coli*, and they indicated that a lack of trehalose synthesis resulted in impaired osmotic tolerance. (A preliminary description of this finding has been given elsewhere [38]). It has been reported recently that GlgG mutants which have a defective synthesis of ADP-glucose accumulate trehalose under osmotic stress (10).

Ots (osmoregulatory trehalose synthesis) insertion mutants. A culture of L60 was generally mutagenized with λNK55 to generate Tc^r mutants with Tn*10* insertions, and a culture of FF1000 [Δ(*argF-lac*) *recA*] was generally mutagenized with λ *plac*Mu55 to generate Km^r mutants with *lacZ* operon fusions. These cultures were then subjected to ampicillin enrichment once or twice in succession, respectively, by growth in medium 63–0.45 M NaCl–100 μg of ampicillin per ml. A total of 2% of the Tc^r cells and 70% of the Km^r cells which were recovered on medium 63 agar were osmotically sensitive. (For typical growth characteristics in liquid medium, see Fig. 2).

We selected 50 Tc^r and 250 Km^r osmotically sensitive mutants for further characterization. Colorimetric analysis with the anthrone method revealed that none of the mutants examined accumulated detectable amounts of trehalose when grown in medium 63–0.25 M NaCl; glutamic acid was the only organic osmolyte which was detected by ^{13}C nuclear magnetic resonance spectroscopy. All the mutants became osmotically tolerant in the presence of glycine betaine; i.e., they grew as well as the parental strain on agar plates with medium 63–0.75 M NaCl–1 mM glycine betaine (cf. Fig. 2). An external supply of trehalose, however, did not have any osmoprotective effect on the mutants. Furthermore, only four of the mutants were Gal^-, conceivably $GalU^-$. These mutants were not further characterized.

TABLE 3. Trehalose-phosphate synthase activity of strains of *E. coli* and *S. typhimurium* grown in glucose-mineral medium with various osmotic strengths

Strain	Specific synthase activity (U/mg of protein) when grown in[a]:		
	Half-strength medium 63 (0.15 osmolal)[b]	Full-strength medium 63 (0.28 osmolal)	Full-strength medium 63–0.40 M NaCl (1.01 osmolal)
E. coli LCB107	30 ± 3	30 ± 6	28 ± 6
E. coli FF4019	ND	0	3.3 ± 1.6
S. typhimurium LT2	ND	0	4.0 ± 1.1

[a] Synthase activity was determined in the presence of 0.25 M KCl by using toluene-treated cells. The enzyme unit is nanomoles of trehalose synthesized per minute at 37°C. Each value is an average of eight independent measurements (± the standard deviation).

[b] Values for osmotic strength are from previous studies (22, 24). ND, Not determined.

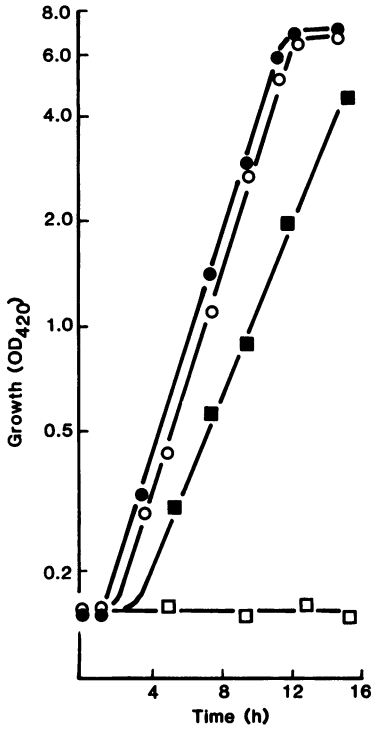

FIG. 2. Growth characteristics of the trehalose-phosphate synthase mutant FF770 (*otsA2*::Tn*10*) and the parental strain CSH7 at elevated osmotic strength. Shown are the growth characteristics of FF770 (□) and CSH7 (■) in medium 63–glucose–0.45 M NaCl and the growth characteristics of FF770 (○) and CSH7 (●) in medium 63–glucose–0.65 M NaCl–1 mM glycine betaine. OD, Optical density.

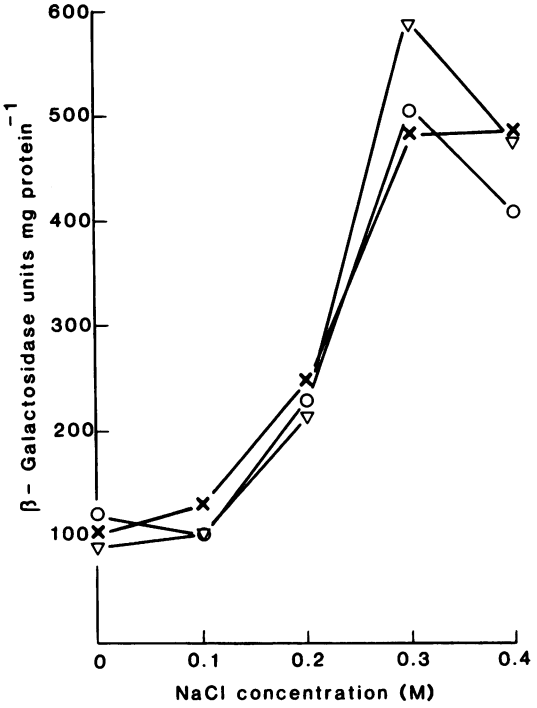

FIG. 3. Osmotic induction of β-galactosidase activities of *otsA-lacZ* and *otsB-lacZ* operon fusion mutants of MC4100 Δ(*argF-lac*). The cells were grown in LOM-glucose with various concentrations of NaCl. Shown are FF2032 Φ(*otsA-lacZ*)*7* (x), FF1112 Φ(*otsB-lacZ*)*8* (○), and FF4011 *otsA1*::Tn*10* Φ(*otsB-lacZ*)*8* (▽). Each point represents the average of three or four independent measurements.

When screened on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside-containing indicator plates with half-strength medium 63 and full-strength medium 63–0.20 M NaCl, the Gal$^+$ *lacZ* fusion mutants displayed a weak osmotic induction of their β-galactosidase activity. For further characterization, we selected the four mutants which were judged to have the stronger induction. Their Km^r markers were transduced with P1 phage into MC4100, giving rise to the osmotically sensitive Lac$^+$ strains FF741, FF1112, FF1608, and FF2032.

Similarly, when the Tc^r markers of the Ots mutants FF69, FF70, FF103, FF108, and FF110 were transduced with P1 phage into trehalose-producing strains (e.g., CSH7, LCB107, and MC4100), all the Tc^r transductants coinherited the Ots$^-$ phenotype. Thus, all Ots mutants listed in Table 1 carried a single insertion of Tn*10* or λ *plac*Mu55.

Cloning analysis of the Ots mutants. From a cosmid gene library of CSH7, we selected a plasmid which complemented the Ots deletion mutant FF6169. Subcloning gave plasmid pFF47, which carried a 2.1-kilobase-pair *Eco*RV-*Hin*dIII chromosomal DNA fragment in the cos4 vector. Plasmid pFF47 restored an osmotically tolerant phenotype and trehalose accumulation in all Ots mutants examined except the *lacZ* fusion mutants FF1112 and FF1608. This finding indicated that the *ots* mutations were located in (at least) two separate loci. For simple identification, we named the mutations which were complemented with pFF47 *otsA* and we named the others *otsB*.

Trehalose-phosphate synthase activity of Ots mutants. The effect of *otsA* and *otsB* mutations on the synthase activity of *E. coli* was investigated by first transducing the mutations into the trehalase-defective mutants LCB107 (*tre*) and FF4019 (*tre*) and then determining the synthase activity of the resulting osmotically sensitive strains. It appeared that the strains constructed (e.g., FF4023, FF4024, FF4025, and FF4029) did not have any detectable synthase activity.

Osmotic induction of β-galactosidase activity in *lacZ* operon fusion mutants. For quantitative determination of β-galactosidase activity of *ots-lacZ* mutants of MC4100 Δ(*argF-lac*), the cells were grown at steady state in LOM-glucose with various concentrations of NaCl. The β-galactosidase activity of FF2032 Φ(*otsA*-lacZ)*7* and FF1112 Φ(*otsB-lacZ*)*8* displayed a fivefold increase under osmotic stress (Fig. 3). The highest β-galactosidase activity was always observed in cells grown with 0.30 M NaCl, whereas 0.10 M NaCl did not increase the β-galactosidase activity above the background value (Fig. 3). [Mutants FF741 Φ(*otsA-lacZ*)*6* and FF1608 Φ(*otsB-lacZ*)*9* had exactly the same properties as the former mutants (data not presented).] It should be noted that the osmotic strength of LOM-glucose with 0.10 M NaCl was similar to that of medium 63 without NaCl, which is 280 mosmol (28). FF4019 and LT2 did not display detectable trehalose-phosphate synthase activity when grown in the latter medium (Table 3).

Mutant FF4011 [*otsA1*::Tn*10* Φ(*otsB-lacZ*)*8*] displayed the same osmotic induction of the β-galactosidase activity as FF1112, which carried the same *otsB-lacZ* fusion but had the wild-type *otsA* gene (Fig. 3). Furthermore, when grown in LOM-glucose containing 0.30 M NaCl and 1 mM glycine betaine, all the *ots-lacZ* fusion mutants displayed 60 to 70% lower β-galactosidase activity than when grown in the same medium without glycine betaine (data not presented).

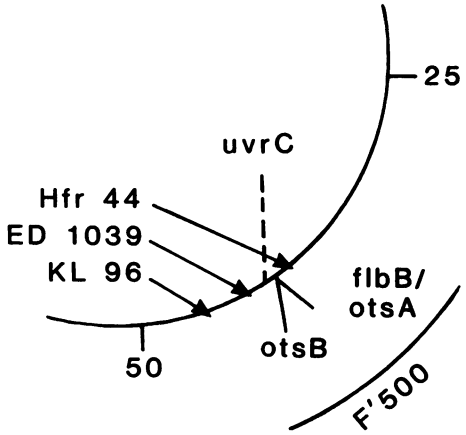

FIG. 4. Extension of chromosomal DNA of F'500, origin of transfer of chromosome of Hfr strains, and localization of some markers used in the mapping of *otsA* and *otsB* mutations.

Mapping of the *otsA* mutation by conjugation. Strains carrying the *otsA*::Tn*10* mutation in an L60 background were HfrC, i.e., transferring the chromosome in counterclockwise direction from about 12 min. They transferred the Tc^r marker with low efficiency. The *otsA1*::Tn*10* allele was therefore transduced with P1 phage into a number of Hfr strains. The most elucidative results were obtained by using the Hfr strains KL96, ED1039, and Hfr44, which transfer the chromosome in a counterclockwise direction from the region between 37 and 47 min (Fig. 4). When FF4020 (constructed from KL96) was crossed with BK2106 (F$^-$ *his*), 37% of the His$^+$ conjugants selected were Tc^r; when FF4021 (constructed from ED1039) was crossed with AB1884 (F$^-$ *uvrC*), 95% of the Tc^r conjugants selected were UvrC$^+$; and when FF4022 (constructed from Hfr44) was crossed with FF4001 (F$^-$ *galU*), none of the Gal$^+$ conjugants selected were Tc^r. These data showed that *otsA* mapped between the origin of transfer of ED1039 and Hfr44 and near the *uvrC* gene at 42 min. The *uvrC* gene appears to be the first known marker which is transferred by ED1039 (41). The markers *his* and *galU* map at 44 and 27 min, respectively (1).

Mapping of the *otsB* mutation by F' complementation. Strain FF4016 [*hisG*::Tn*10* Φ(*otsB-lacZ*)*8*] was complemented with F'500 (Fig. 4), F'148, and F'150 by selecting for His$^+$ exconjugants. When scored for osmotic tolerance, only F'500 complemented the *otsB* mutation. All three F' plasmids carry chromosomal inserts which cover the region from 44 and past 42 min, but F'148 and F'150 are known to carry deletions near 42 min (27; communication from B. J. Bachmann).

Transductional mapping of *otsA* and *otsB* mutations and deletion analysis. When strain AB1884 (*uvrC*) was transduced to Tc^r with a P1 lysate prepared from FF4169 (*otsA1*::Tn*10*), the cotransduction with *uvrC* was 69%. When the same P1 lysate was used to infect FF1112 Φ(*otsB-lacZ*)*8*, the cotransduction with *otsB* was 80%. To determine on which side of *uvrC* the *ots* mutations were located, P1 lysates were prepared from strains BL203 and BL230, which carried Tn*10* insertions located clockwise and counterclockwise from *uvrC*, respectively. These lysates were used to transduce FF2032 Φ(*otsA-lacZ*)*7* and FF1608 Φ(*otsB-lacZ*)*9* to Tc^r, scoring for osmotic tolerance. The *otsA* and *otsB* mutations displayed 30 and 37% cotransduction, respectively, with the clockwise Tn*10* insertion, and they displayed 99.7 and 95% cotransduction, respectively, with the counterclockwise Tn*10* insertion. The Tn*10* marker from BL230 is in or near the *flbB* gene, i.e., displaying 100% cotransduction (B. Lowsky, personal communication).

The data presented above showed that both *otsA* and *otsB* mapped counterclockwise from *uvrC*, and they suggested that the clockwise gene order was *flbB/otsA-otsB-uvrC*. To verify the order of the *ots* genes, a three-factor cross was carried out. P1 lysate prepared from FF4011 [*otsA1*::Tn*10* Φ(*otsB-lacZ*)*8*] was used to transduce AB1884 (*uvrC*) to Tc^r. With Tc^r as the selected marker and 180 transductants scored, four transductant classes were obtained: Tc^r Km^r UV^r (80 mutants), Tc^r Km^s UV^s (65 mutants), Tc^r Km^s UV^r (no mutants), and Tc^r Km^s UV^s (35 mutants). (Tn*10*, λ *plac*Mu55, and *uvrC* conferred the Tc^r, Km^r, and UV^s phenotypes, respectively.) The absence of Tc^r Km^s UV^r transductants confirmed the order of the *ots* genes suggested above. [The Φ(*otsA-lacZ*) mutations were located too close to the Tn*10* mutation of BL230 (i.e., more than 99% cotransduction) to get a sufficient number crossing over in three-factor crosses; thus, the order of these mutations was not determined.]

When Tn*10* was deleted from the double mutant FF4011 [*otsA1*::Tn*10* Φ(*otsB-lacZ*)*8*] by the method of Bochner et al. (3), 4% of the Tc^s mutants obtained were Km^s (e.g., strain FF4031). Obviously, there are no genes located between *otsA* and *otsB*, which are vital for growth in minimal medium of low osmolarity.

DISCUSSION

In the present investigation, we have generated Tn*10* insertion and *lacZ* operon fusion mutations in two genes which govern trehalose-phosphate synthase. We have named these genes *otsA* and *otsB* (osmoregulatory trehalose synthesis). They map near 42 min, and the clockwise gene order is *flbB/otsA-otsB-uvrC*. By biochemical and genetic analyses, we have demonstrated that UDP-glucose is a precursor of trehalose, that trehalose-phosphate synthase is osmotically regulated both at the gene level and at the protein level, and that trehalose accumulation is essential for osmoregulation of *E. coli* in glucose-mineral medium.

The *ots* genes appear to be new examples of osmotically regulated genes in *E. coli* and *S. typhimurium*. The evidence is that only cells of *E. coli* FF4019 (i.e., a *tre* mutant of MC4100) and *S. typhimurium* LT2 which were grown in medium of elevated osmotic strength displayed detectable trehalose-phosphate synthase activity and that mutants of MC4100 Δ(*argF-lac*) with *otsA-lacZ* or *otsB-lacZ* operon fusions displayed fivefold osmotic induction of their β-galactosidase activities. *E. coli* LCB107, which expressed synthase activity at a high constitutive level, appears to be a regulatory mutant, but we do not yet know whether this phenotype is caused by a single mutation or multiple mutations. It is noteworthy that the *otsA* and *otsB* mutations blocked the synthesis of the synthase and the accumulation of trehalose in all strains of *E. coli* examined, regardless of their mode of synthase expression. Further work, however, is needed to decide whether the *otsA* and *otsB* genes are structural or regulatory genes of the synthase and whether they constitute an operon. The *otsA* and *otsB* genes are located sufficiently close to allow generation of deletions which encompass both genes, but the Tn*10* insertion in *otsA* did not have any polar effect on the expression of *otsB-lacZ*. Unfortunately, we have not yet found any mutants with the Tn*10* insertion in *otsB*, which would have allowed us to determine the effect of an *otsB* mutation on the expression of *otsA*.

The in vitro synthase activity of wild-type *E. coli* and *S. typhimurium* was low, i.e., 3 nmol of trehalose synthesized per mg of cell protein per min at 37°C. However, within the limit of experimental accuracy, this is sufficient to explain the in vivo trehalose synthesis of *E. coli*, since the generation time in medium 63–0.45 M NaCl was 2.5 h (Fig. 2) and the trehalose accumulation was shown to be about 0.5 μmol per mg of cell protein (24). The osmotic induction of the *otsA* and *otsB* genes was modest (i.e., fivefold) compared with that of *proU*, which displays more than 100-fold induction (7). The low induction ratio may explain why the *otsA* and *otsB* genes have been overlooked in previous studies of osmotically regulated *lacZ* fusion mutants. The present mutants were initially selected for osmotic sensitivity and not for osmotic induction of β-galactosidase.

It is well known that *E. coli* has two transport systems, Kdp and Trk, which regulate the K^+ concentration of the cytoplasm in response to increases in the osmotic strength of the environment (13). Sutherland et al. (40) have shown that osmotic stress-dependent induction of *proU* is regulated by cytoplasmic K^+ and that accumulation of glycine betaine decreases the accumulation of K^+. The present findings that the presence of glycine betaine reduced the synthase activity of the wild type and the β-galactosidase activity of the *ots-lacZ* fusion mutants indicate that transcription of the *ots* genes is regulated by internal K^+, similar to transcription of the *proU* gene.

The high constitutive synthase activity of LCB107 was convenient for the biochemical studies. Our data showed that trehalose-phosphate synthase itself was stimulated at least fivefold by K^+ and other monovalent cations (Fig. 1; Table 2). At concentrations greater than 0.25 M, KCl partially inhibited the synthase, whereas the enzyme remained stimulated by much higher concentrations of potassium glutamate. Obviously, this reflected that glutamic acid is a major counterion of K^+ in stressed cells of *E. coli* (24, 30). Measures (30) has previously shown that glutamate dehydrogenase of *E. coli* is synthesized constitutively and stimulated by K^+. Thus, the syntheses of the two major endogenously produced organic osmolytes of *E. coli*, glutamic acid and trehalose, are regulated by halophilic enzymes. Since LCB107 did not accumulate trehalose in the absence of osmotic stress, the regulation of trehalose synthesis at the protein level seems to be of biological significance. However, even at low osmolarity the cell must contain sufficient K^+ to give some background activity of trehalose-phosphate synthase. The cascade of reactions determining the level of trehalose in cells may therefore involve additional regulatory steps.

In the present study, we have examined trehalose-phosphate synthase activity in Tre mutants of *E. coli*, i.e., mutants which are blocked in the synthesis of a periplasmic trehalase. We have not found any indication that the *tre* mutation influenced osmoregulatory trehalose synthesis in *E. coli*. This notion is supported by the finding that wild-type *S. typhimurium* lacks a periplasmic trehalase (35).

Mutants defective in trehalose accumulation failed to grow in glucose-mineral medium with an elevated osmotic strength generated with 0.5 M NaCl. However, Ots mutants grew as well as the parental strains did in the same medium, with up to about 0.2 M NaCl (the figures for NaCl tolerance are somewhat strain dependent; data not shown), and they became as osmotically tolerant as the parental strain in the presence of glycine betaine (Fig. 2). Apparently, a hierarchy exists among the osmolytes (compatible solutes) of *E. coli*. K^+ and its counterions (e.g., glutamic acid) can only give protection against a low level of osmotic stress. Trehalose synthesis enables the organism to withstand a moderate level of osmotic stress, whereas an external supply of glycine betaine or its precursors or other osmoprotectants such as proline, proline betaine, and γ-butyrobetaine is needed to withstand a higher level of osmotic stress (6, 7, 22, 24–26).

It is noteworthy that lack of trehalose accumulation was the only lesion found in mutants of *E. coli* selected for osmotic sensitivity in glucose-mineral medium of elevated osmotic strength. This finding indicates that no or few other systems in *E. coli* serve only an osmoregulatory function and are indispensable for osmoregulation under these growth conditions. The other systems known to take part in osmoregulation of the cytoplasm under these conditions have alternate pathways, and they also serve other vital functions in the cell, i.e., K^+ transport (13) and glutamate synthesis (30). Also, the membrane-derived oligosaccharides which constitute the major anionic osmolytes of the periplasmic space are believed to have alternate pathways (32). Furthermore, mutations in many genes which encode systems known to be influenced by osmotic stress do not give an osmotically sensitive phenotype at all (e.g., the genes encoding the outer membrane proteins OmpF and OmpC [17], trehalase [4], and maltose transport [12]), or the mutants display this phenotype only in the presence of an osmoprotective compound (e.g., genes encoding glycine betaine transport [6, 7] or synthesis of glycine betaine from choline [39]). Similarly, mutations in several other genes which are shown by gene fusion techniques to have an osmotic response do not have any known phenotype (15, 16). Although all lethal mutations would have escaped our attention, our data support the notion of Higgins et al. (19) that only a small number of genes are essential for osmoregulation of *E. coli* at elevated osmotic strength.

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