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Trehalase of *Escherichia coli*

MAPPING AND CLONING OF ITS STRUCTURAL GENE AND IDENTIFICATION OF THE ENZYME AS A PERIPLASMIC PROTEIN INDUCED UNDER HIGH OSMOLARITY GROWTH CONDITIONS*

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***Escherichia coli* can use the nonreducing disaccharide trehalose as a sole source of carbon and energy. Trehalose transport into the cell is mediated via the phosphotransferase system, and a mutant depleted in the nonspecific proteins enzyme I, HPr, and enzyme III^{Glc} of this system was not only unable to grow on glucose or mannitol but also was strongly reduced in its ability to grow on trehalose. A pseudorevertant (PPA69) of such a deletion mutant was isolated that could again grow on glucose but not on mannitol. This revertant could now also use trehalose as a carbon source due to a constitutive galactose permease. PPA69 was subjected to Tn*10* insertional mutagenesis, and a mutant (UE5) was isolated that no longer could use trehalose as a carbon source but could still grow on glucose. UE5 lacked a periplasmic trehalase that was present in PPA69. P1-mediated transduction of this Tn*10* insertion (*treA*::Tn*10*) into a *pts*$^+$ wild-type strain (MC4100) had no effect on the ability of MC4100 to grow on trehalose but resulted in loss of the periplasmic trehalase activity. The Tn*10* insertion was mapped at 26 min on the *E. coli* linkage map and was 3% cotransducible with *trp*, in the order *treA*::Tn*10*, *trp*, *cys*. Trehalase activity in MC4100 was not induced by growth in the presence of trehalose but increased by about 10-fold when 0.6 M sucrose was added to minimal growth medium. Using the *in vivo* mini-Mu cloning system and growth on trehalose as selection, we cloned the *treA* gene. A 9-kilobase *Eco*RI fragment containing *treA* was subcloned into pBR322. Strains carrying this plasmid (pTRE5) contained about 100-fold higher periplasmic trehalase activity than PPA69 or MC4100. Using polyacrylamide gel electrophoresis, we found a protein of molecular weight 58,000 among the periplasmic proteins of the pTRE5-carrying strain that was absent in UE5. This protein was purified by ammonium sulfate precipitation and DEAE-Sepharose ion-exchange chromatography and contained all the trehalase activity. Minicells containing the *treA*$^+$ plasmid produced, in addition to three other proteins, the 58,000-dalton protein. Thus, the plasmid carries the structural gene for the periplasmic trehalase and not just a gene involved in the regulation of the enzyme.**

Our interest in trehalose metabolism in *Escherichia coli* originates from our work on maltose transport, chemotaxis, and metabolism in this organism (1–3). Previous studies on the induction of the *mal* regulon (4) showed that trehalose is an inducer (5, 6), indicating a possible involvement of the maltose system in trehalose metabolism. However, *malT* mutants that do not express any maltose genes still grow well on trehalose. Also, trehalose does not interfere with maltose transport, nor is it recognized by the maltose-binding protein (7). Nevertheless, there seemed to be a direct connection of trehalose recognition to the maltose system. *E. coli* exhibits chemotaxis towards trehalose (8), which is abolished in a *malT* mutant. The search for the responsible maltose gene indicated that at least *lamB*, which codes for the λ receptor in the outer membrane, was necessary for trehalose chemotaxis. Thus, LamB provides the facilitated entry of trehalose into the periplasm without being directly involved in the chemotactic signal transduction.[1] This is in line with the observation that trehalose is a good substrate for LamB in the liposome swelling assay (9).

To search for a periplasmic trehalose chemoreceptor (possibly also linked to the control by *malT*), analogous to the maltose-binding protein (10), we tested periplasmic shock fluids by equilibrium dialysis for binding affinity towards trehalose. Our attempts were frustrated by the presence of a highly active enzyme that splits trehalose into glucose. Therefore, we decided to elucidate the origin of this enzyme and its relationship to trehalose utilization.

The study of trehalose transport and metabolism in *E. coli* has not advanced to any great extent. Mutants have been isolated that are unable to grow on trehalose, and all of these have been mapped at 26 min on the *E. coli* linkage map (11). Recently, it was found that trehalose is transported under simultaneous phosphorylation, which is indicative of phosphotransferase-mediated transport. In addition, the enzymatic activity of a trehalose-6-phosphate hydrolase was found in the cytoplasmic extracts of cells grown in the presence of trehalose. Thus, metabolism of trehalose would be rather simple: uptake via a specific enzyme II under simultaneous phosphorylation, followed by its subsequent hydrolysis to glucose and glucose 6-phosphate. Besides the trehalose-6-phosphate hydrolase, the extracts also contained a trehalase activity. It is not clear whether this activity is caused by a separate enzyme or represents a side reaction of the trehalose-6-phosphate hydrolase (12).

In *Salmonella typhimurium*, trehalose is also transported via a phosphotransferase-mediated transport. In this case, the enzyme II for mannose (II^{Man}) appears to be responsible for the recognition of trehalose. Surprisingly, trehalose seems not

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[1] W. Boos and M. Manson, unpublished results.

TABLE I
Bacterial strains and plasmids

Strain	Known genotype	Source
Bacterial strains		
MC4100	F⁻Δ(*argF-lac*)*U169 araD139 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR*	Casadaban (15)
UE17	MC4100, Δ(*ptsHI-crr*) *zfc-706*::Tn*10*	
UE14	MC4100, *treA*::Tn*10*	
GM160	F⁻ *cysB93 trp75 tfr-8* Δ(*argF-lac*) *U169*	Derivative of CGSC5783
BW7622	Hfr (PO44), *relA1 spoT1 thi1 trpB-114*::Tn*10*	Wanner (16)
CLG1	MC4100 Δ(*brnQ-phoA-proC*) *osmA-134*::Tn*phoA*	Gutierrez *et al.* (17)
DS410T	*minB ara lacY malA mtl xyl rpsL thi fhuA azi* Δ(*glpT-glpA*)593	Refs. 18 and 19
PPA69	Hfr KL16, *thi* Δ(*ptsHI-crr*) *galR*	Postma
UE7	PPA69, *galP*::Tn*10*	Henderson (20)
UE5	PPA69, *treA*::Tn*10*	
UM6	PPA69, *treA*	
UE12	PPA69, F⁻ *treA rpsL 150*	
BRE2099	PPA69, (Mu *cts 62* Ap)	
BRE2100	UE5, (Mu *cts 62* Ap)	
UE18	UM6, *treA*⁺ *zch-754*::Tn*10*	
UE20	UM6, *treA*⁺ *zcg-755*::Tn*10*	
UE22	UM6, *treA*⁺ *zcg-756*::Tn*10*	
UE24	UM6, *treA*⁺ *zcg-757*::Tn*10*	
Plasmids		
pBR322		Bolivar *et al.* (21)
pEG5005	Mini-Mu element	Groisman and Casadaban (22)
pTRE1	Mini-Mu containing *treA*⁺	
pTRE5	pBR322 carrying *treA*⁺ on a 9-kilobase *Eco*RI fragment	

to be phospshorylated during transport (13). Mutants lacking enzyme I and HPr, the general components of the phosphotransferase system, no longer grow on trehalose. Revertants, that have regained the ability to grow on trehalose remain Δ*pts* but carry a mutation in *galR* that render galactose permease (*galP*) constitutive. From this observation, it was concluded that trehalose can enter *S. typhimurium* not only via phosphotransferase-mediated transport but also via the proton-motive-force-driven galactose permease (13).

Independent of the presence of trehalose in the growth medium, large amounts of this disaccharide are accumulated during growth of *E. coli* on any carbon source when the osmolarity of the medium is high, and, presumably, it acts as an intracellular osmoprotectant (14).

To learn more about trehalose transport and metabolism, its connection to maltose utilization, and its osmoprotective function under osmotic stress, we mapped the structural gene for trehalase, cloned it, and identified its product as a periplasmic protein.

MATERIALS AND METHODS

Bacterial Strains, Growth Conditions, and Genetic Methods—All strains are derivatives of *E. coli* K12 and are listed in Table I. Minimal medium A (MMA)[2] with 0.2% carbon source as well as Luria broth were used as growth media (23). To measure the growth rate on trehalose in liquid culture, strains were pregrown overnight in MMA containing 0.2% trehalose and 1% casamino acids. They were washed three times at room temperature in MMA without a carbon source and resuspended in MMA containing 0.2% trehalose to an optical density (OD) of 0.1 at 578 nm. Growth at 37 °C under aeration was monitored by following the OD at 578 nm.

Standard genetic methods such as phage P1-mediated transduction or Hfr × F⁻ crosses were done as described by Miller (23). Random Tn*10* insertions in MC4100 were obtained using phage λ NK55 as the Tn*10* donor as described (24). 50,000 colonies were pooled and a P1 lysate was obtained from the resulting culture. This lysate was used to transduce PPA69 to tetracycline resistance and the transductants were then screened for the inability to take up trehalose. For this purpose, transductants (about 400/plate) were selected on MMA/maltose plates containing 5 μg/ml tetracycline and 0.1 μM [^{14}C] trehalose (150 mCi/mmol from Amersham Corp.) and were transferred onto filter paper (Whatman No. 1). The filters were then dried, autoradiographed, and searched for lightly stained colonies. In this way the *treA*::Tn*10* strain UE5 was found.

To isolate Tn*10* insertions next to *treA*, the Tn*10* insertion of UE5 was removed using a procedure described by Bochner *et al.* (25) yielding strain UM6. This strain was made *rpsL* by P1-mediated transduction using a lysate of MC4100. The resulting streptomycin-resistant strain was grown in succession three times overnight in Luria broth containing 10% sodium dodecyl sulfate (26), each time diluting 1:10^4, and plated for single colonies. By plate crosses, colonies were screened for their ability to serve as recipients in Hfr-mediated crosses. One strain, UE12, was chosen for crosses with several Hfr strains. It still lacked trehalase activity (*treA*), did not grow on mannitol (Δ*pts*), and was an effective recipient (F⁻).

UM6 was transduced with the above described P1 lysate of pooled Tn*10* insertions. Tetracycline-resistant colonies were selected and screened for their ability to grow on trehalose as well as on mannitol in the presence of tetracycline. P1 lysates were grown on the Tre⁺ Mtl⁻ transductants, and the P1 cotransduction frequency of *treA* and the particular Tn*10* insertion was tested using UM6 as recipient.

For the transfer of the Δ(*ptsHI-crr*) mutation, the P1 lysate of

[2] The abbreviations used are: MMA, minimal medium A; SDS, sodium dodecyl sulfate.

pooled Tn*10* insertions described above was used to isolate Tn*10* insertions next to the deletion. PPA69 was transduced to tetracycline resistance and screened for growth on mannitol. One insertion, *zfc-706*::Tn*10*, exhibited a cotransduction frequency of 60% with the deletion. It was used to construct UE17, the Δ(*ptsHI-crr*) derivative of MC4100. UE17 is able to grow on maltose, which is not a substrate for the phosphotransferase system.

For the *in vivo* cloning of the *treA* gene, the mini-Mu element pEG5005 was used. The protocol of Groisman and Casadaban (22) was followed to obtain the gene bank.

Minicell preparation and labeling was done with strain DS410T (18, 19). It was transformed with pTRE5 and grown overnight in Luria broth containing 50 μg of ampicillin/ml. Minicells were prepared according to Maegher *et al.* (27) with the modification suggested by Reeve (28). Labeling with 10 μCi of [^{35}S]methionine (1000 mCi/mmol, from Amersham Corp.) was for 1 h at 37 °C.

SDS gel electrophoresis was carried out on 12% polyacrylamide slab gels using the buffer system of Laemmli (29). The samples were routinely heated at 100 °C for 4 min before loading onto the gel.

Osmotic Shock and Trehalase Activity—To test strains for trehalase activity, they were grown overnight in 100-ml cultures. The cold osmotic shock procedure was done according to Neu and Heppel (30) using 20-ml volumes for Tris conditioning, sucrose EDTA treatment, and shock solution. The final shock solution was lyophilized, resuspended in 1 ml of 10 mM Tris-HCl, pH 7.2, and dialyzed against the same buffer. The resulting solution was clarified by centrifugation and used for enzymatic assays and gel electrophoretic analysis. It routinely contained 1.5–2 mg of protein/ml of crude osmotic shock fluid. To test for trehalase that remained cell bound after the osmotic shock, cells were resuspended in 10 ml of 10 mM Tris-HCl, pH 7.2, and lysed in the French pressure cell using 1000 p.s.i. Cellular debris were removed by centrifugation (40,000 × *g* for 30 min); the supernatant was dialyzed against the same buffer and used for the trehalase assay.

100-ml cultures grown in the presence of 0.6 M sucrose were slowly brought to low osmolarity prior to osmotic shock by first adding the same volume of MMA over 30 min. The cultures were then centrifuged, the cells were resuspended in 10 ml of supernatant, and 90 ml of MMA was added slowly over 30 min. The resulting suspensions were then subjected to the normal osmotic shock procedure.

Trehalase activity was assayed by incubating 50 μl of 20 mM trehalose in 10 mM Tris-HCl, pH 7.2, with 50 μl of crude osmotic shock fluid, dilutions thereof, or other solutions to be tested. At time intervals, 10-μl samples were removed and spotted on silica-coated thin-layer plates (from Merck). The plates were developed with 1-propanol/water (6:1) (v/v) and charred at 120 °C for 10 min after spraying with 20% sulfuric acid. The units of enzymatic activity in micromoles of trehalose hydrolyzed per minute at room temperature were determined by the time at which 50% of the trehalose was split to glucose. The identity of the split product of trehalose action with glucose was confirmed by assaying the incubation mixture with glucose oxidase and by using the reduction of alkaline Cu^{2+} tartarate (Fehling's solution) to Cu_2O as test for the reducing sugar activity.

To assay for trehalase activity *in vivo*, strain UE7 was grown overnight in 50 ml of MMA with maltose as carbon source (to induce the LamB protein). After washing three times in 50 ml of MMA, the cells were resuspended in 1 ml of MMA (to about 10^{11} cells/ml), and 1 ml of 20 mM trehalose in MMA was added. After different time intervals, 50-μl samples were added to 50 μl of mixed bed ion exchange resin V from Merck, the suspension was centrifuged in an Eppendorf centrifuge, and 10 μl of the supernatant was subjected to thin-layer chromatography as described above. Under these conditions 50% of the initial trehalose was hydrolyzed to glucose after about 20–30 min. After 1 h, trehalose was completely hydrolyzed to glucose. The latter was then slowly used up by the dense bacterial culture. It disappeared to 50% from the supernatant after about 20 h.

Purification of Periplasmic Trehalase—BRE2100 (pTRE1) containing *treA*$^+$ on a mini-Mu plasmid was grown at 35 °C to an OD (578 nm) of 1.2 in six 1-liter aerated cultures of MMA containing 0.2% trehalose. The osmotic shock procedure was done in six separate batches using 100-ml solutions for Tris conditioning, EDTA sucrose, and shock solution. The shock solution was lyophilized and resuspended in a total of 100 ml of 10 mM Tris-HCl, pH 7.2, and RNA was precipitated by adding streptomycin (0.1 g/ml) at 0 °C until no further precipitation occurred (about 6 ml). The supernatant was made 100% saturated with ammonium sulfate at 0 °C. The precipitate was resuspended in 15 ml of 100 mM Tris-HCl, pH 7.2, clarified from insoluble material by centrifugation, and dialyzed against the same buffer. The resulting solution was separated by ammonium sulfate precipitation into three fractions: 0–45, 45–65, and 65–100% saturation. Each precipitate was resuspended in 5 ml of 10 mM Tris-HCl, pH 6.0, and dialyzed extensively against the same buffer. The 45–65% fraction was loaded onto a 50-ml DEAE-Sepharose CL-6B column equilibrated with the same buffer. The column was first washed with 150 ml of the same buffer. Then the column was eluted with 300 ml of a linear (0–300 mM) NaCl gradient. All of the trehalase activity was eluted in a single peak at about 160 mM NaCl. The enzyme was precipitated with 100% ammonium sulfate, dialyzed against 100 mM Tris-HCl, pH 7.2, and stored frozen at a protein concentration of 0.5 mg/ml.

TABLE II

Growth of different strains in liquid minimal medium with trehalose as carbon source

Strain	Relevant genotype	Generation time
MC4100	pts$^+$	57 min
UE14	pts$^+$ *treA*::Tn*10*	57 min
UE17	Δ(*ptsH ptsI-crr*)	14 h
PPA69	Δ(*ptsH ptsI-crr*) *galR*	260 min
UE7	Δ(*ptsH ptsI-crr*) *galR galP*::Tn*10*	>30 h
UE5	Δ(*ptsH ptsI-crr*) *galR treA*::Tn*10*	∞
UE5/pTRE5	*treA*$^+$ on multicopy plasmid	260 min[a]
CLG1	*osmA-phoA*	15 h
CLG1/pTRE5	*tre*$^+$ on multicopy plasmid	160 min[a]

[a] The plasmid-containing strains were biphasic in their growth pattern: up to an OD of 0.4 (578 nm) they grew with the indicated generation time; then they grew much slower but reached a final OD of 1.2 and 1.5, respectively.

RESULTS

The Function of Galactose Permease in the Utilization of Trehalose—E. coli strain DG102 carries a *ptsHI-crr* deletion[3] that results in the loss of enzyme I, HPr, and enzyme III^{glc} of the phosphotransferase system. This strain is unable to grow on any carbohydrate transported by the phosphotransferase system but can grow on other carbohydrates, including maltose and glycerol. DG102 could not grow on glucose (which is transported by the phosphotransferase system), but the introduction of a mutation in *galR* that caused constitutivity in the galactose permease (20) allowed growth of the resulting strain PPA69 on glucose via glucokinase. When a *galP*::Tn*10* mutation was introduced into PPA69, the resulting strain, UE7, was defective in galactose permease and exhibited only residual growth on glucose.

Growth of PPA69 on trehalose (Table II) exhibited the same pattern as growth on glucose. Growth on trehalose was rather slow in comparison to a *pts*$^+$ strain (MC4100), but again it depended on a constitutive galactose permease. The *galP*::Tn*10* derivative of PPA69 is Tre$^-$. Thus, one could conclude that in absence of functional genes for the general enzymes of the phosphotransferase system, glucose and trehalose are able to enter *E. coli* via the galactose permease, as has been found for *S. typhimurium* (13). The discovery of a periplasmic trehalase in *E. coli*, described in this publication, offers another explanation: trehalose, after entering the periplasm, is split into glucose, which in turn enters the cell via the galactose permease.

Periplasmic Shock Fluid of E. coli Contains an Enzyme That Efficiently Hydrolyzes Trehalose—Using the osmotic shock procedure of Neu and Heppel (30), we isolated the periplasmic proteins of strains PPA69 and MC4100 after growth in glucose, maltose, trehalose, and glycerol. We found that, irrespective of the carbon source and the strain used, all the preparations of periplasmic proteins were able to hydrolyze

[3] W. Epstein, personal communication.

trehalose at 10 mM concentration with a rate of about 0.3 nmol of trehalose split per min and per shock protein from 10^9 cells, or 0.02 μmol/min × mg of protein. The enzyme appears only partially shockable, since cellular extracts of shocked cells contained routinely five to 10 times more of the total enzymatic activity than the proteins released by osmotic shock. However, it is clear that the nonshockable enzyme also has a periplasmic location: we incubated UE7, the *galP*::Tn*10*-carrying derivative of PPA69, with 10 mM trehalose. Even though the mutant could not use trehalose as carbon source, it could degrade it quantitatively to glucose that was then released into the medium. The rate by which trehalose was hydrolyzed to glucose by whole cells (3 nmol/min and 10^9 cells) was approximately the same as the rate catalyzed by the total amount of trehalase extracted from the cell. Thus, trehalase must be localized outside the osmotic barrier of the cell, and GalP-mediated growth on trehalose in PPA69 must occur via degradation in the periplasm of trehalose to glucose and its subsequent uptake into the cell via GalP.

A Tn10 Insertion in treA Results in the Loss of Periplasmic Trehalase—Strain PPA69 was transduced by phage P1 to tetracycline resistance with a pooled lysate of about 50,000 independent Tn*10* insertions in strain MC4100. About 12,000 tetracycline-resistant transductants were plated on minimal maltose plates containing 0.1 μM [^{14}C]trehalose. The plates were replicated on filter paper that was subsequently autoradiographed and searched for lightly stained colonies. One mutant (UE5) that had lost the ability to grow on trehalose was found in this way. P1-mediated transduction of this Tn*10* insertion into PPA69 showed a 100% linkage between the tetracycline resistance and the Tre$^-$ phenotype (300 transductants were tested). Osmotic shock fluids prepared from UE5 did not contain trehalase activity nor did the cellular extracts of the shocked cells. We conclude that the Tn*10* insertion in UE5 had occurred in a gene necessary for the production of the periplasmic trehalase and we termed the gene *treA*. It is clear that an intact *treA* gene is not necessary for growth of a *pts*$^+$ wild-type strain on trehalose because transduction of the *treA*::Tn*10* into MC4100 did not alter its ability to grow on trehalose (Table II), even though it had lost its periplasmic trehalase.

Mapping of treA—We first constructed a recipient strain that contained a *treA* mutation in the appropriate genetic background of UE5. By treatment according to Bochner *et al.* (25) we selected UM6, a tetracycline-sensitive derivative of UE5 that was still unable to grow on trehalose. By P1 transduction we introduced the *rpsL* mutation of MC4100, and by growth in the presence of SDS (25) we selected an F$^-$ derivative of the original Hfr mating type. The resulting strain, UE12, was used as recipient in Hfr-mediated crosses with a series of donor strains that have their start of transfer at different points on the genetic linkage map and carry a Tn*10* insertion in close proximity to their origin of transfer (32). After crossing for 2 h we selected exconjugants that were resistant to tetracycline and streptomycin. These were screened for growth on trehalose and mannitol. Of 12 different crosses, only one showed significant coinheritance (60%) of tetracycline resistance with a Tre$^+$, Mtl$^-$ phenotype. BW7622, the donor strain used in this cross, transfers its DNA counterclockwise starting at about 44 min and carries its Tn*10* insertion in *trp* between 27 and 28 min on the *E. coli* linkage map. This indicated that *treA* was located in the vicinity of *trp*. P1-mediated transductions using BW 7622 as donor and UE12 as recipient revealed a 3% cotransduction of *treA* and the *trp*::Tn*10* insertion.

To more closely define the position of *treA*, we isolated a series of Tn*10* insertions in its vicinity. For this purpose, we transduced UM6 with a P1 lysate of 50,000 pooled Tn*10* insertions in MC4100, selected for tetracycline resistance, and screened for growth on trehalose. From about 4000 transductants, 45 were found that were able to grow on trehalose. Approximately half of these were also able to grow on mannitol, indicating their cotransduction with Δ(*ptsHI-crr*). Ten of the Tre$^+$, Mtl$^-$ transductants were further analyzed. P1 lysates prepared from these transductants were used to transduce UM6 to tetracycline resistance, and the linkage to *treA* was determined. Cotransduction frequencies from 12 to 95% were obtained (Table III). To further determine their position clockwise or counterclockwise to *treA*, P1-mediated three-factor crosses were done using the above lysates as donors and strain GM160 (*cysB trp*) as recipient. Two donors revealed a significant cotransduction between their Tn*10* insertion and *cysB* and *trp*. The data are shown in Table III and their interpretation is given in Fig. 1. With the position of *cysB* and *trp* given on the *E. coli* linkage map (31), the order of markers in clockwise direction was: *zcg-755*::Tn*10*, *treA*, *zcg-756*::Tn*10*, *zch-754*::Tn*10*, *trp*, *cysB*. This places *treA* at 26 min on the linkage map. A similar location had been reported previously for mutations leading to a Tre$^-$ phenotype (11).

Cloning of treA and Its Expression in Minicells—To clone *treA*, we used the mini-Mu system developed by Croisman and Casadaban (22). A gene bank was prepared *in vivo* after heat induction of strain BRE2099 ((Δ*ptsHI-crr*) *treA*$^+$ Mu *cts* Ap) carrying the mini-Mu element pEG5005. The resulting lysate was used to infect strain BRE2100 (Δ(ptsHI-crr) treA::Tn*10*Mu *cts* Ap), and selection was made for growth on trehalose in the presence of tetracycline and kanamycin. One strain, containing the mini-Mu plasmid pTRE1, was chosen for further studies. It contained about 100–150 times more shockable trehalase than PPA69. As described below, BRE2100 (pTRE1) was used to isolate and purify the trehalase. Since the mini-Mu plasmids are somewhat unstable due to secondary transposition, DNA from pTRE1 (*treA*$^+$) was digested with several restriction endonucleases and the resulting fragments were subcloned into plasmid pBR322 (21). In this way a 9-kilobase *Eco*RI fragment was identified that expressed trehalase activity in UE5 (*treA*::Tn*10*) after transformation with the corresponding plasmid pTRE5. Restriction analysis of this plasmid demonstrated that the cloned *Eco*RI fragment carries only chromosomal DNA (data not shown).

To demonstrate that pTRE5 carries *treA*$^+$ itself and not just a gene coding for a positive regulator of *treA*, we expressed pTRE5 in the minicell system (27, 28). Fig. 3 shows the autoradiogram of the SDS-polyacrylamide gel electrophoretic analysis of the [^{35}S]methionine-labeled proteins, the synthesis of which was directed by pTRE5. A protein of 58,000 molecular weight was observed that was identical in its electrophoretic mobility to purified trehalase. Three additional proteins of lower molecular weight that are not encoded by the pBR322 vector were also synthesized, but their relation to *treA* is unclear. As will be shown below, the synthesis of trehalase in wild-type cells is increased after growth in media of high osmolarity. In the minicell system this osmodependency of *treA* expression was not observed, even though this system is able to respond[4] to the osmoregulation of proteins encoded by *proU*, another osmoregulated operon in *E. coli* (32).

Purification of Periplasmic Trehalase—When periplasmic proteins from strain PPA69 and UE5 (lacking trehalase) were analyzed by SDS-polyacrylamide gel electrophoresis, no ob-

[4] E. Bremer, unpublished results.

TABLE III

P1-mediated cotransduction of several Tn10 insertions with treA, cys, and trp

Cross[a]	Donor	Recipient	Selection	Recombinants	% of total
1	BW7622 *trp*::Tn*10*	UE12 *treA*	Tet^R	$TreA^+$	3
2	UE20 *zcg-755*	UM6 *treA*	Tet^R	$TreA^+$	51
3	UE20 *zcg-755*	GM160 *trp, cysB*	Tet^R	Trp^+	0
4	UE24 *zcg-757*	UM6 *treA*	Tet^R	$TreA^+$	95
5	UE24 *zcg-757*	GM160 *trp, cysB*	Tet^R	Trp^+	1
6	UE22 *zcg-756*	UM6 *treA*	Tet^R	$TreA^+$	62
7	UE22 *zcg-756*	GM160 *trp, cysB*	Tet^R	Trp^-, $CysB^-$	81
				Trp^+, $CysB^-$	10
				Trp^+, $CysB^+$	9
				Trp^-, $CysB^+$	0
			Trp^+	Tet^R, $CysB^+$	10
				Tet^R, $CysB^-$	6
				Tet^S	84
			$CysB^+$	Tet^R, Trp^+	8
				Tet^R, Trp^-	2
				Tet^S	90
9	UE18 *zch-754*	UM6 *treA*	Tet^R	$TreA^+$	12
10	UE18 *zch-754*	GM160 *trp, cysB*	Tet^R	Trp^-, $CysB^-$	64
				Trp^+, $CysB^-$	22
				Trp^+, $CysB^+$	14
				Trp^-, $CysB^+$	0
			Trp^+	Tet^R, $CysB^-$	20
				Tet^R, $CysB^+$	16
				Tet^S	64
			$CysB^+$	Tet^R, Trp^+	7
				Tet^R, Trp^-	1
				Tet^S	92

[a] 300 transductants were screened in crosses 1 and 3, while 100 were screened in all others.

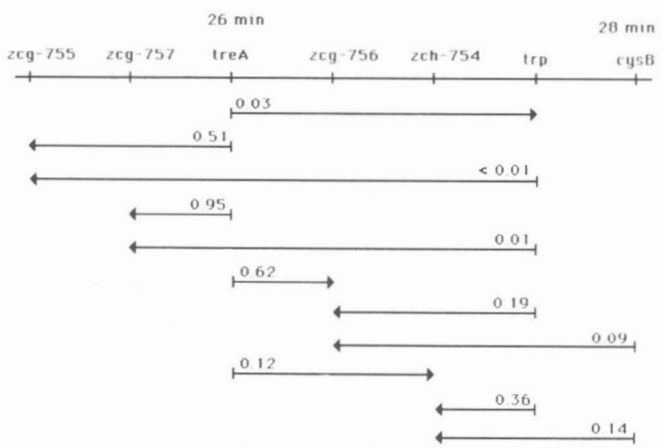

FIG. 1. **Cotransduction frequencies of *treA* to nearby markers by P1 transduction.** The numbers are given in fractions of 1 (equal to 100%). The *arrows* indicate the selected marker. The cotransduction frequencies are taken from the data given in Table III. *zcg-757* could be positioned on either side of *treA*.

TABLE IV

Purification of periplasmic trehalase

Crude shock fluid was obtained by the cold osmotic shock procedure (30) from a 6-liter culture of strain BRE2100 (pTRE1). Units are given in μmol of trehalose split/min at room temperature.

Fraction	Total activity	Total protein	Specific activity
	units	*mg*	*units/mg*
Crude shock fluid	345	70.4	4.9
45–65% ammonium sulfate	324	19.3	16.8
DEAE-Sepharose	279	4.2	66.4

vious difference in the protein pattern could be observed (Fig. 2). However, periplasmic proteins from strains harboring the $treA^+$ gene on a multicopy plasmid exhibited a 100–150-fold increased trehalase activity and an increase in the amount of

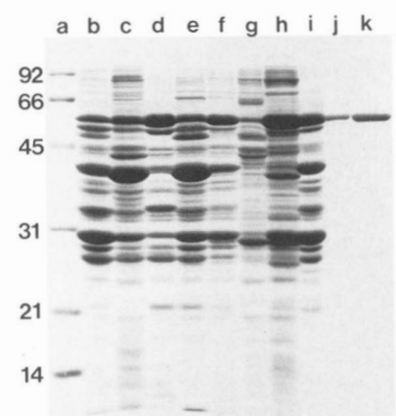

FIG. 2. **Polyacrylamide gel electrophoresis of trehalase preparations.** *Lane a*, molecular weight standards. Crude periplasmic shock fluid from the different strains are shown in the following lanes: *lane b*, PPA69 ($treA^+$); *lane c*, MC4100 ($treA^+$); *lane d*, MC4100 grown in the presence of 0.6 M sucrose; *lane e*, UE5 (*treA*); and *lane f*, BRE2100 (pTRE1) containing 100-fold more trehalase activity than PPA69. Ammonium sulfate fractions of shock fluid from strain BRE2100 (pTRE1) are shown in the following lanes: *lane g*, 0–45%; *lane h*, 45–65%; and *lane i*, 65–100%. 94% of the trehalase activity was contained in the 45–65% fraction. Purified trehalase after DEAE-Sepharose chromatography is shown in *lanes j* and *k*. 7 and 14 μg of protein were applied. The molecular weight of the marker proteins are given in kilodaltons. The gel contained 12% acrylamide.

a protein band with an apparent molecular weight of 58,000 (Fig. 2). It is clear that this gel position is occupied by more than one protein. To prove that trehalase was among these proteins, we purified it from a derivative of UE5 carrying the $treA^+$ gene on the mini-Mu plasmid. Six-liter cultures grown on trehalose were subjected to the osmotic shock procedure of Neu and Heppel (30). The concentrated shock fluid, after treatment with streptomycin sulfate, was fractionated by ammonium sulfate precipitation, and 94% of trehalase activity was found in the 45–65% fraction (Table IV). This purifica-

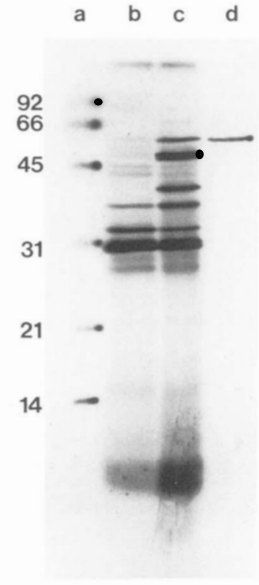

FIG. 3. **pTRE5-directed protein synthesis in minicells.** Minicells transformed with pBR322 (*lane b*) or pTRE5 (*lane c*) were labeled with [^{35}S]methionine, dissolved in sodium dodecyl sulfate, and analyzed by polyacrylamide gel electrophoresis. *Lane a*, molecular weight standards; *lane d*, purified trehalase. The gel was stained with Coomassie Blue and dried. The protein bands in *lanes a* and *d* were marked with radioactive ink and the gel was autoradiographed. The synthesis of four proteins was observed that are encoded by the *treA*$^+$-containing *Eco*RI fragment of pTRE5. One of them exhibits the same electrophoretic mobility as trehalase. The *numbers* indicate the molecular weight of the marker proteins in kilodaltons.

tion step removed the comigrating protein band in the electrophoresis gels. Subsequently, ion-exchange chromatography through DEAE-Sepharose in 10 mM Tris-HCl, pH 6.0, with a linear NaCl gradient (0–300 mM) was applied. The protein eluted in a single peak at 160 mM NaCl, contained all the trehalase activity, and was essentially free of other proteins (Fig. 2). The total yield from a 6-liter culture after a 13-fold purification was 4.2 mg, representing 81% of the activity present in the crude shock fluid (Table IV). The protein was stored frozen in 100 mM Tris-HCl, pH 7.2, at a protein concentration of 0.5 mg/ml. The specific activity of this preparation in 10 mM Tris-HCl, pH 7.2, was 66.4 μmol of trehalose hydrolyzed/min × mg of protein (Table IV). The enzyme hydrolyzed trehalose, but not maltose, maltooligosaccharides, sucrose, or lactose. When tested by fast protein liquid chromatography, using a sizing column, trehalase eluted with an apparent molecular weight of 60,000. Thus, the molecular weight determined by SDS-polyacrylamide gels must represent the molecular weight of the active enzyme that is composed of only one polypeptide chain.

treA Is Contained in an Operon That Is Osmoregulated—Growth on any carbon source in the presence of high osmolarity results in the accumulation of trehalose in the cytoplasm (14). One would therefore expect degradation of trehalose and thus trehalase activity or synthesis to be stimulated at low osmolarity. Gutierrez *et al.* (17) have recently isolated in *E. coli* a number of gene fusions to *phoA*, the structural gene of alkaline phosphatase. Provided that the reading frame is correct, the resulting hybrid proteins will exhibit phosphatase activity only when the fusion has occurred to a gene coding for a periplasmic protein or for the portion of a membrane protein that is exposed to the periplasmic side of the membrane (33). Several *phoA* fusions isolated by Gutierrez *et al.* (17) are highly induced after growth in medium of high osmolarity. One of them, called *osmA-phoA*, maps at 26 min and results in a reduced growth rate on trehalose. Its growth properties resemble that of the Δ(*ptsHI-crr*) strain UE17 (Table II). We tested the osmotic shock fluid of CLG1, the strain carrying *osmA-phoA*, and found it to be lacking trehalase activity. Since the loss of trehalase activity in a *pts*$^+$ strain does not result in reduced growth on trehalose (Table II), the *phoA* fusion in CLG1 has most likely not occurred in *treA* but in a gene proximal to it, in the same operon. Transformation of CLG1 with the *treA*$^+$ plasmid pTRE5 restored growth on trehalose (Table II). This indicates that the plasmid contains additional *tre* genes, possibly related to transport and metabolism of trehalose.

Since the expression of the *osmA-phoA* fusion was induced after growth in high osmolarity medium and since the fusion simultaneously abolished the appearance of trehalase in the periplasm, it appeared likely that *treA* itself is osmoregulated. Indeed, osmotic shock fluids of MC4100 grown in minimal medium A with maltose as carbon source plus 0.6 M sucrose exhibited about 10 times more trehalase activity than shock fluids of cells grown in the same way but without sucrose. This osmotic regulation of trehalase is also shown by SDS-polyacrylamide gel electrophoresis of the periplasmic proteins (Fig. 2). A protein band at the position of 58,000 daltons (composed of at least two proteins) increased in amount after growth at high osmolarity. Interestingly, the same analysis also reveals that the periplasmic maltose-binding protein, an essential component of the maltose transport system, was strongly reduced after growth in the presence of 0.6 M sucrose. Most likely this is due to the presence of contaminating glucose in the high concentration of sucrose, exerting catabolite repression on the synthesis of maltose-binding protein.

DISCUSSION

In this paper we describe a periplasmic enzyme from *E. coli* that hydrolyzes the nonreducing sugar trehalose. Its structural gene has been cloned, and the enzyme has been overproduced and purified. The enzyme is composed of one polypeptide chain of 58,000 molecular weight. Its specific activity is 66.4 units/mg of protein or 240 molecules of trehalose split/s/molecule of enzyme at room temperature in 10 mM Tris-HCl, pH 7.2. The amount of trehalase in the periplasm of cells grown at low osmolarity (MMA) represents a minimal trehalose-hydrolyzing capacity of about 3 nmol/min/10^9 cells, hardly enough for its utilization as a carbon source (in a fully induced wild-type strain, maltose is used up at an approximate rate of 20 nmol/min/10^9 cells (34).

In wild-type strains, trehalase appears unnecessary for growth on trehalose since *treA* mutants lacking this enzyme are not impaired in the utilization of trehalose as a carbon source. The enzyme is not induced by growth in the presence of this sugar, in contrast to the situation in *S. typhimurium* (13), and it is not subjected to catabolite repression by glucose. However, its synthesis is controlled by the osmolarity of the medium. The addition of 0.6 M sucrose to MMA increased the amount of trehalase in the periplasm by a factor of 10.

treA, the structural gene of the enzyme is located at 26 min on the *E. coli* chromosome, apparently in an operon together with *osmA*, a gene that has been identified purely on its property of increased expression at high osmolarity (17). Surprisingly, when trehalase was expressed from the *treA*$^+$-carrying plasmid pTRE5 in minicells, no dependency on high osmolarity medium could be observed, even though this system responds well in the osmodependent expression of *proU*.[4] Either the induction ratio in the *tre* operon is too small for the minicell system to respond or the type of osmoregulation is different in both systems; conceivably, it is the high accu-

mulation of trehalose that acts as an endogenous inducer under conditions of high osmolarity.

The synthesis of large amounts of trehalose in the cytoplasm has been observed when cells are grown at high osmolarity (14). It is clear that cytoplasmic synthesis of trehalose occurs independently of the carbon source present in the medium, in particular of trehalose itself. Also, the gene coding for the trehalose-synthesizing enzyme does not map in the vicinity of *treA*. The synthesis of the osmoprotective cytoplasmic trehalose appears not to be coupled to its uptake from the medium. Uptake of external trehalose is not osmoprotectant (14), presumably because trehalose is taken up as trehalose 6-phosphate (12) and is subsequently hydrolyzed internally to glucose and glucose 6-phosphate.

The synthesis of the enzyme involved in the production of internal trehalose at high osmolarity is in itself not under osmsotic control.[5] This is surprising in view of our finding that periplasmic trehalase and presumably additional trehalose-metabolizing enzymes are indeed turned on under these conditions. The role of this osmodependency and its connection to the internal accumulation of trehalose, the role of a periplasmic trehalase itself, as well as the documented relationship of trehalose to the maltose regulon are at the moment entirely unclear.

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