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Characterization of a Cytoplasmic Trehalase of *Escherichia coli*

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Escherichia coli **can synthesize trehalose in response to osmotic stress and is able to utilize trehalose as a carbon source. The pathway of trehalose utilization is different at low and high osmolarity. At high osmolarity, a periplasmic trehalase (TreA) is induced that hydrolyzes trehalose in the periplasm to glucose. Glucose is then taken up by the phosphotransferase system. At low osmolarity, trehalose is taken up by a trehalose-specific enzyme II of the phosphotransferase system as trehalose-6-phosphate and then is hydrolyzed to glucose and glucose-6-phosphate. Here we report a novel cytoplasmic trehalase that hydrolyzes trehalose to glucose. *treF*, the gene encoding this enzyme, was cloned under *ara* promoter control. The enzyme (TreF) was purified from extracts of an overexpressing strain and characterized biochemically. It is specific for trehalose exhibiting a K_m of 1.9 mM and a V_{max} of 54 μmol of trehalose hydrolyzed per min per mg of protein. The enzyme is monomeric, exhibits a broad pH optimum at 6.0, and shows no metal dependency. TreF has a molecular weight of 63,703 (549 amino acids) and is highly homologous to TreA. The nonidentical amino acids of TreF are more polar and more acidic than those of TreA. The expression of *treF* as studied by the expression of a chromosomal *treF-lacZ* fusion is weakly induced by high osmolarity of the medium and is partially dependent on RpoS, the stationary-phase sigma factor. Mutants producing 17-fold more TreF than does the wild type were isolated.**

In *Escherichia coli*, trehalose is synthesized in response to osmotic stress. The synthesis is mediated by the *otsA*/*B*-encoded enzymes trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase. The first enzyme transfers glucose from UDP-glucose to glucose-6-phosphate, yielding trehalose-6-phosphate; the second enzyme liberates P_i from trehalose-6-phosphate, forming internal trehalose (11, 16). Until now, it was thought that the removal of internal trehalose upon osmotic downshift would occur primarily by its extrusion into the periplasm by an as-yet-unknown mechanism (31) followed by its hydrolysis via a periplasmic trehalase, encoded by *treA* (3, 12), forming glucose. The latter would then again be taken up by the glucose-specific enzyme II of the phosphotransferase system (PTS). This scheme appeared sensible because of the common regulatory features of the *otsB*/*A* operon and the *treA* gene. Both are controlled by RpoS (14), the stationary-phase sigma factor, and neither is induced by the presence of trehalose in the medium. In addition to the function of recapturing extruded trehalose, another role of the periplasmic trehalase (TreA) is to allow *E. coli* the utilization of exogenous trehalose as a carbon source under conditions of high osmolarity (3). This seemed necessary since the trehalose-inducible system for taking up trehalose is not expressed at high osmolarity. This uptake of trehalose works via trehalose-specific enzyme II (encoded by *treB*) of the PTS that transports trehalose-6-phosphate (18) followed by hydrolysis of the latter to glucose and glucose-6-phosphate (catalyzed by an enzyme encoded by *treC* [28]). The osmosensitive *treB*/*C* operon, located at 96 min on the *E. coli* chromosome, is induced by the presence of trehalose in the medium, trehalose-6-phosphate being the active internal inducer (17). The regulation of the *treB*/*C* operon is mediated by TreR. *treR*, the gene encoding TreR, is located directly upstream of *treB* (18). The main reason why the *treB*/*C* operon is not induced at high osmolarity is the osmotic induction of trehalose-6-phosphate phosphatase, the *otsB*-encoded enzyme that effectively hydrolyzes the inducer, thus preventing induction.

In the present publication we report the detection of a new enzyme taking part in the metabolism of trehalose. TreF is a cytoplasmic trehalase that is highly homologous to TreA, the periplasmic trehalase. Similar to the regulation of TreA, the regulation of TreF is partially dependent on the stationary-phase sigma factor RpoS (22) and TreF synthesis is weakly induced by high osmolarity but not by trehalose.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. They were grown under aeration in Luria broth, Tryptone broth, or minimal medium A (MMA) with the appropriate carbon source and different concentrations of NaCl. Strain constructions were done by P1 *vir*-mediated transduction (24).

Molecular biological techniques. Plasmid preparation and transformation were done according to standard methods (30). For restriction and ligation of DNA, the buffer systems of the suppliers were used. DNA fragments were separated in 0.7% agarose gels; the DNA of the appropriate bands was eluted with the QiaQuick gel extraction kit (Quiagen). Chromosomal DNA was prepared with the QiaQuick tissue kit (Qiagen).

Cloning of *treF*. The DNA of Kohara phage 606 (19) was digested with *Eco*RI, and the resulting 5,384-bp fragment was isolated and cloned into plasmid pACYC184 opened with the same restriction enzyme. This *treF*-harboring plasmid was named pRHo600. To overproduce the gene product, *treF* was cloned under the control of the inducible *ara* promoter. pRHo600 was digested with *Bam*HI, filled in with Klenow fragment, and digested with *Nco*I. pBAD22 (13) was digested with *Eco*RI, filled in with Klenow fragment, and digested with *Nco*I. Both fragments were ligated, and strain KU95 was transformed with the ligation mixture. The selection for ampicillin resistance was done. Clones were screened for growth on trehalose in the presence of arabinose. The resulting plasmid was pBAD22*treF*.

Cloning of *otsB*/*otsA* operon under *tac* promoter control (pRHo700). PCR with whole cells was performed with two primers flanking the *otsB*/*A* operon (*ots* *Eco*RI, 5′-GGAATTCTGAAGGAGGAGAACCGGG-3′, and *ots* *Xma*I, 5′-TTAACCCGGGCTACGCAAGCTTTGG-3′). The two oligonucleotides introduced *Eco*RI and *Xma*I restriction sites, respectively. The PCR product was digested with the two enzymes, and the resulting 2,223-bp fragment was cloned into plasmid pTrc99A (1) opened with the same enzymes, yielding plasmid pRHo700.

Isolation of *treF-lacZ* fusion. Plasmid pRHo600 was digested with *Eco*RI and *Xma*I, and the resulting *treF*-containing fragment was ligated in pJL29 (23)

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Known genotype(s) or phenotype(s)	Origin or reference
Strains		
DHB4	F$^-$ *araD139* Δ(*ara-leu*)*7697* Δ*lacX74 pho510 galE15 galK16 relA1 spoT1 rpsL thi phoR68* Δ*phoA Pvu*II Δ*malF*3 (F′ *lacI*^{q} *pro*)	7
HSK42	MC4100 *polA*	*polA* mutation (29)
KU68	DHB4 *treA*::spec *treB treC*	This study
KU95	MC4100 *treA*::spec Φ(*treC-lacZ*)λp*lac*Mu55 kan^r Δ*ara714 leu*::Tn*10*	Δ*ara* from strain LMG194 (35)
MC4100	F$^-$ *araD139* Δ(*argF-lac*)U169 *flbB*5301 *deoC1 relA1 rbsR rpsL150 ptsF25 thi1*	5
RHo14	MC4100 *treA*::spec	18
RHo36	KU68 *trxB*::kan	*trxB*::kan from AD404 (7)
RHo42	MC4100 (*treF*$^+$) carrying *treF-lacZ* (kan) at the λ attachment site	This study
RHo47	RHo42 *otsA*::Tn*10*	This study
RHo48	RHo42 *rpoS*-359::Tn*10*	*rpoS*-359::Tn*10* (21)
RHo58	RHo14 *treF-lacZ* (kan)	This study
RHo59	RHo58 *treR*::Tn*10*(cam)	This study
RHo60	RHo14 *treF*::kan	This study
RHo65	RHo32 exhibiting elevated *treF* expression	This study
Plasmids		
pACYC184	Ap^r Cm^r	6
pAID135	Expresses AP from which amino acids 2 to 22 of the signal sequence are deleted	9
pBAD22	Ap^r p*ara* expression vector	13
pBAD22*treF*$^+$	treF$^+$ in pBAD22	This study
pJL29	Ap^r, vector for *lacZ* protein fusions	23
pRHo600	Tc^r *treF*$^+$	This study
pRHo700	pTrc99A Ap^r *otsB*$^+$ *otsA*$^+$ under *tac* promoter control	This study
pRHo600′ *treF*::kan	Tc^r Kn^r *treF*::kan	This study
pTAC3422	Cm^r *int*$^+$	2
pTAC3588	Ap^r Kn^r *attP*	2
pTrc99A	Ap^r	1
pUC4K	Ap^r Kn^r	Pharmacia
pWK12	Cm^r *treB*$^+$	18

digested with the same enzymes. The resulting plasmid, pJL29*treF′-lacZ*, was digested with *Bsa*AI, and the resulting 5,068-bp fragment containing *treF′-lacZ* was isolated and ligated with the *Sma*I-*Eco*RV fragment of pTAC3588 (2) containing the phage λ attachment site and the kanamycin resistance gene. The circular DNA (lacking an origin of replication) was then transformed into strain MC4100 harboring plasmid pTAC3422 (2) with the λ integrase gene. After selection for Kan^r on 5-bromo-4-chloro-3-indolylgalactopyranoside indicator plates, the clones were screened for Amp^s and Tet^s. The correct integration of the fusion at the λ attachment site was confirmed by P1 cotransduction with a Tn*10* near the λ attachment site. Thus, strain RHo42 carries a *treF-lacZ* fusion.

Isolation of chromosomal *treF*::kan insertion. Plasmid pRHo600 was digested with *Sma*I and *Sna*BI. The deleted part of *treF* was replaced with the kanamycin resistance cassette of pUC4K (Pharmacia) digested with *Pvu*II. The resulting plasmid, pRHo600′*treF*::kan, was transformed into the *polA* strain HSK42 (29). After selection for Kan^r, all clones were pooled, and a P1 *vir* lysate was obtained from the pooled bacteria. This lysate was used to transduce strain MC4100 to kanamycin resistance. A second P1 *vir* lysate was obtained from the pooled kanamycin-resistant transductants and used to transduce strain RHo14 to kanamycin resistance. The colonies were screened for Tet^s and loss of trehalase activity. The correct position of the insertion on the chromosome was verified by Southern blot analysis of chromosomal DNA of a wild-type strain and a *treF*::kan strain digested with *Eco*RI and hybridized with a DNA probe containing *treF*. Thus, strain RHo60 carries a *treF*::kan insertion.

Purification of TreF. Strain KU68 expressing *treF* from pBAD22*treF* was grown at 37°C in 4 liters of MMA (24) containing Casamino Acids (0.4%, final concentration); valine, leucine, and isoleucine (40 μg/ml each); and ampicillin (200 μg/ml). When cells reached an optical density at 600 nm of about 0.5, *treF* expression was induced by the addition of 1 mM arabinose to the growth medium, and incubation was continued for another 2 h.

Cells were harvested by centrifugation (20 min at 4,200 × *g*), resuspended in 20 ml of buffer B (20 mM potassium phosphate [pH 6.0]), and broken in a French pressure cell at 16,000 lb/in^2. Remaining intact cells were removed by centrifugation (30 min at 27,000 × *g*). Nucleic acids were precipitated with 2% streptomycin sulfate and pelleted by centrifugation (30 min at 27,000 × *g*). Subsequently, the cell extract was subjected to 40% saturated ammonium sulfate, and precipitated proteins were dissolved in 8 ml of buffer A (20 mM potassium phosphate [pH 6.0], 10% saturated with ammonium sulfate). Nonsoluble proteins were removed by centrifugation (30 min at 27,000 × *g*).

The resulting supernatant was loaded on a 80-ml phenyl-Sepharose column (fast flow, low substituted; Pharmacia) which had been equilibrated with buffer A. Noninteracting proteins were washed off with 400 ml of buffer A, and the remaining proteins were eluted with a linear gradient with a 10 to 0% saturation of ammonium sulfate in a total volume of 650 ml at a flow rate of 5 ml/min. TreF was eluted at about 6% saturation of ammonium sulfate. Fractions containing TreF were pooled, dialyzed against buffer B, and loaded on a 5-ml MonoQ column equilibrated with buffer B. A linear gradient (20 ml) of 0 to 0.5 M NaCl was applied in buffer B. The flow rate was 1 ml/min. TreF eluted at about 250 mM NaCl.

Trehalase assay. Trehalase assays were performed in 100 μl of 20 mM potassium phosphate buffer (pH 6.0) containing about 20 ng (or less) of TreF. The reaction was started by the addition of 10 or 100 mM trehalose, and the mixture was incubated for 5 to 15 min at room temperature. The reaction was stopped by boiling the samples for 5 min. TreF-dependent cleavage of trehalose was assayed by determining the glucose concentration with the glucose test kit from Merck. Conditions were varied as indicated in the text according to the intention of the assay.

Gel filtration assay. Purified TreF (56 μg) in 150 μl of 20 mM potassium phosphate buffer (pH 6.0) containing 150 mM NaCl was applied to a Superose 12 column (Pharmacia) and eluted with a flow rate of 0.5 ml/min. The molecular weight was determined by comparison with standard proteins.

Thin-layer chromatography (TLC). About 1 μg of purified enzyme was incubated for 16 h with 0.6 μmol of trehalose, lactose, maltose, or sucrose in 30 μl of 20 mM potassium phosphate buffer (pH 6.0) at room temperature. Samples were boiled and centrifuged. The supernatant (5 μl) was applied to a silica gel TLC plate (0.25 mm; Merck) and run overnight in 2-propanol-ethanol-water (5:3:2 [vol/vol/vol]). The saccharide spots were visualized by dipping the plate into methanol containing 2% H_2SO_4, drying it, and charring it for 10 min at 180°C.

RESULTS

Cloning of TreF. From a GenBank entry (accession no. U00039), we learned that a gene whose deduced amino acid sequence (Fig. 1) exhibits high homology to the periplasmic trehalase encoded by *treA* had been sequenced (12). From the position of *treF* on the *E. coli* chromosome at about 78 min, the Kohara phage 606 (19) was chosen to clone *treF* (Fig. 2). Phage

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TreA            --EETPVTPQPPDIL--------LGP------------------------
TreF            MLNQKIQNPNPDELMIEVDLCYELDPYELKLDEMIEAEPEPEMIEGLPAS
                  ..    *.* ...        *.*

TreA            ----------LFNDVQNAKLFPDQKTFADAVPNSDPLMILADYRMQQNQ
TreF            DALTPADRYLELFEHVQSAKIFPDSKTFPDCAPKMDPLDILIRYRKVRRH
                        **..**.**.*** ***.*  *. *** **  **  . .

TreA            SGFDLRHFVNVNFTLPKE-GEKYVPPEGQSLREHIDGLWPVLTRSTENTE
TreF            RDFDLRKFVENHFWLPEVYSSEYVSDPQNSLKEHIDQLWPVLTREPQDHI
                 .**** **. .* **   .  **.   .**.**** *******  ..

                          PGGRFxExYxWDxY
TreA            KWDSLLPLPEPYVVPGGRFREVYYWDSYFTMLGLAESGHWDKVADMVANF
TreF            PWSSLLALPQSYIVPGGRFSETYYWDSYFTMLGLAESGREDLLKCMADNF
                 * ***.**..*.****** * ****************. * .  *  **

TreA            AHEIDTYGHIPNGNRSYYLSRSQPPFFALMVELLAQHEGDAALKQYLPQM
TreF            AWMIENYGHIPNGNRTYYLSRSQPPVFALMVELF-EEDGVRGARRYLDHL
                *  *. *********.********* *******. ...*  . ..** ..

TreA            QKEYAYWMDGVENLQAGQQEKRVVKLQDGTLLNRYWDDRDTPRPESWVED
TreF            KMEYAFWMDGAESLIPNQAYRHVVRMPDGSLLNRYWDDRDTPRDESWLED
                . ***.**** *.* . *  ..**.. **.************ ***.**

TreA            IATAKSNPNRPATEIYRDLRSAAASGWDFSSRWMDNPQQLNTLRTTSIVP
TreF            VETAK-HSGRPPNEVYRDLRAGAASGWDYSSRWLRDTGRLASIRTTQFIP
                . *** .. **. *.*****..******.****. .  .* ..*** ..*

TreA            VDLNSLMFKMEKILARASKAAGDNAMANQYETLANARQKGIEKYLWNDQQ
TreF            IDLNAFLFKLESAIANISALKGEKETEALFRQKASARRDAVNRYLWDDEN
                .***...**.*  .*  *   *..      .   *.**. ....***.*..

TreA            GWYADYDLKSHKVRNQLTAAALFPLYVNAAAKDRANKMATATKTHLLQPG
TreF            GIYRDYDWRREQL-ALFSAAAIVPLYVGMANHEQADRLANAVRSRLLTPG
                * * *** . ...   ..***. **** *  ..*...* * ...** **

                        QWDxPxGAWPAF
TreA            GLNTTSVKSGQQWDAPNGWAPLQWVATEGLQNYGQKEVAMDISWHFLTNV
TreF            GILASEYETGEQWDKPNGWAPLQWMAIQGFKMYGDDLLGDEIARSWLKTV
                *. ..   .*.*** *********.* .*.. **.  .. .*..  *  *

TreA            QHTYDREKKLVEKYDVSTTGT-GGGGGEYPLQDGFGWTNGVTLKMLDLIC
TreF            NQFYLEQHKLIEKYHIADGVPREGGGGEYPLQDGFGWTNGVVRRLIGLY-
                .. *  . **.***...      ****************  ....*

TreA            PKEQPCDNVPATRPTVKSATTQPSTKEAQPTP
TreF            ----------------------------GEP
                                           *
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FIG. 1. Sequence comparison of TreF and TreA. An asterisk indicates amino acid identity; a dot indicates the exchange with a homologous amino acid. The two boxed sequences are homologous to consensus sequences (shown above the TreA sequence) typical for glucosyl hydrolases (15).

DNA was digested with *Eco*RI, and the 5,384-bp-long chromosomal fragment was ligated into plasmid pACYC184, resulting in pRHo600. Strain KU68 is *treA treB treC* and cannot grow on trehalose. The transformation of KU68 with pWK12(*treB*$^+$) allowed transport of trehalose as trehalose-6-phosphate and formation of internal trehalose by the low levels of trehalose-6-phosphate phosphatase, the *otsB* gene product. It still did not allow the strain to grow on trehalose. Only the additional transformation with pRHo600 allowed growth on trehalose, presumably by the TreF-mediated hydrolysis of internal trehalose.

For the overproduction of TreF, we subcloned the *treF*-containing portion of pRHo600 into plasmid pBAD22 (13), bringing the expression of *treF* under arabinose-inducible control. Strain KU95 (*treA treC* Δ*ara*) harboring pBAD22*treF* produced large amounts of TreF only when arabinose was present in the growth medium (see Fig. 6). Concomitantly, growth on trehalose was also only possible in the presence of arabinose. Figure 2 shows the different plasmids and their origin from Kohara phage 606.

Isolation of a mutant exhibiting an elevated level of chromosomally encoded TreF. Strain KU68 was made *trxB*::kan, allowing the formation of disulfide bridges in the cytoplasm (8), and yielding strain RHo36. This strain was transformed with two plasmids, pWK12 (*treB*$^+$) and pAID135. The first one allows the uptake of trehalose as trehalose-6-phosphate (without its hydrolysis to glucose and glucose-6-phosphate). The second one harbors *phoA* (the structural gene for alkaline phosphatase) without its signal sequence, thus allowing the fast hydrolysis of trehalose-6-phosphate to trehalose. This strain does not grow on trehalose, but mutants that allow growth can easily be isolated (Fig. 3). An extract of one of these mutants contains 17 times more trehalose-hydrolyzing activity than the corresponding extract from the wild-type strain (0.071 U/mg of protein versus 0.004 U/mg of protein, respectively). The pres-

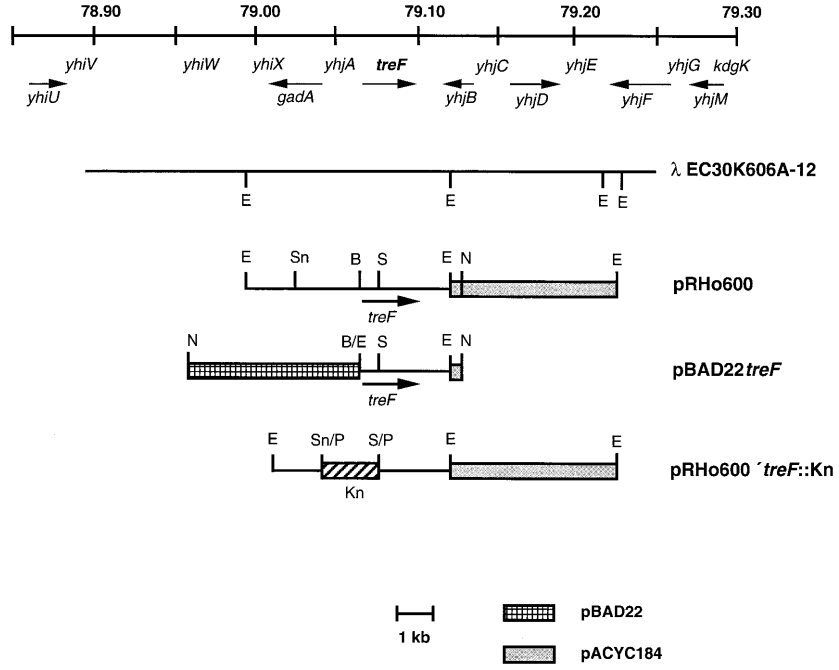

FIG. 2. Position of *treF* on the chromosome and the Kohara phage and the plasmids used. The numbers on the first line indicate the minutes on the genetic map of *E. coli*. The second line indicates the Kohara phage from which *treF* was isolated. The following restriction enzymes were used: *Bam*HI (B), *Eco*RI (E), *Nco*I (N), *Pvu*II (P), *Sma*I (S), and *Sna*BI (Sn).

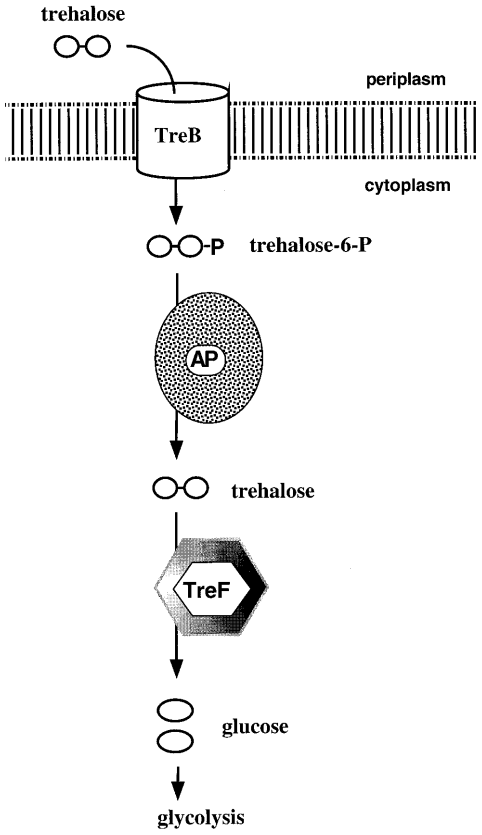

FIG. 3. Genetic selection for elevated TreF activity. Trehalose is transported into the cytoplasm via TreB and is converted to trehalose-6-phosphate. Trehalose-6-phosphate is dephosphorylated by alkaline phosphatase to trehalose. AP was expressed without its signal sequence in a thioredoxin reductase (*trxB*) mutant, allowing proper folding of AP in the cytoplasm. Cytoplasmic trehalose is converted to glucose by TreF. The activity of chromosomally encoded wild-type TreF was not sufficient to allow growth in minimal medium with trehalose. Thus, mutants exhibiting elevated trehalase activity could be isolated.

ence of 300 mM NaCl in the growth medium increases the expression of TreF in the wild type as well as in the mutant (0.044 U/mg of protein in the wild type versus 0.245 U/mg of protein in the mutant). The higher activity in the mutant can also be visualized by TLC analysis with 10 mM trehalose as the substrate. Whereas very little trehalose is hydrolyzed by the wild-type extract, the extracts of the mutants are quite effective (Fig. 4). As shown by Western blotting with specific anti-TreF antibodies, these mutations resulted in an increase of the amount of the TreF protein but not in the removal of an inhibitor (data not shown). From a pool of random Tn*10* insertions, one that was equally cotransducible with all four tested mutations was selected. This insertion was highly cotransducible with the *treF*::kan insertion described below. Thus, the mutations yielding elevated expression of *treF* must be closely linked to *treF* itself.

Isolation of a *treF-lacZ* fusion and a *treF*::kan insertion. From plasmid pRHo600 an *Eco*RI-*Xma*I fragment harboring the 5′ end of the *treF* gene was isolated. This fragment was ligated into the appropriately digested plasmid, pJL29 (23), creating an in-frame fusion with *lacZ*. A *Bsa*AI fragment containing the *treF-lacZ* fusion was obtained and combined with plasmid pTAC3588 harboring the phage λ attachment site (2). By transforming strain MC4100(pTAC3422*int*$^+$), integration of *treF-lacZ* into the λ attachment site of the chromosome occurred and was verified by P1 cotransduction. Thus, strain RHo42 carries a chromosomal *treF-lacZ* fusion in addition to an intact copy of *treF*.

For the construction of a chromosomal *treF*::kan insertion, the kanamycin cassette of pUC4K was isolated and ligated into the *treF* gene of pRHo600. To transfer *treF*::kan into the chromosome, the *polA* strain HSK42 (29) was transformed with pRHo600′ *treF*::kan. The correct chromosomal *treF*::kan insertion in MC4100 was then obtained by P1 transduction with the use of the transformants as P1 donors. The correct position of the insertion was verified by Southern blot analysis (data not shown). Extracts of strains containing *treA*::spec and *treF*::kan showed no residual trehalose-hydrolyzing activity. Strains carrying *treF*::kan grow discernibly faster than *treF*$^+$ strains on MMA plates with glycerol as carbon source in the presence of 300 mM NaCl. An additional *treA* mutation had no effect on growth.

The expression of *treF-lacZ* responds to turgor and is partially dependent on *rpoS*. Strain RHo42 (*treF-lacZ*) was grown in MMA with 0.4% glucose as the carbon source. Specific β-galactosidase activity was monitored along the growth curve. Figure 5 shows that during entry into stationary phase there is a weak temporal induction. However, the addition of 300 mM NaCl during the logarithmic phase of growth led to a fourfold increase in activity, which remained elevated after the initial overshoot (Fig. 5; Table 2). On the other hand, when the massive synthesis of internal trehalose is initiated by the isopropyl-β-D-thiogalactopyranoside (IPTG)-dependent induction of the plasmid-encoded *otsA*/*B* genes (pRHo700) without concomitant osmotic shock, the expression of *treF-lacZ* is decreased by a factor of 6 (Table 2). The introduction of *rpoS*::Tn*10* into strain RHo42 (strain RHo48) also led to a fivefold reduction in *treF-lacZ* expression, which was, however, still increased by high osmolarity (Table 2). The strain carrying the *treF* mutations, described above, also had a four- to fivefold increase in TreF activity after growth in the presence of 300 mM NaCl. Thus, the mutation does not impinge on the osmoregulation of *treF*. When the *treF* mutation was transduced into

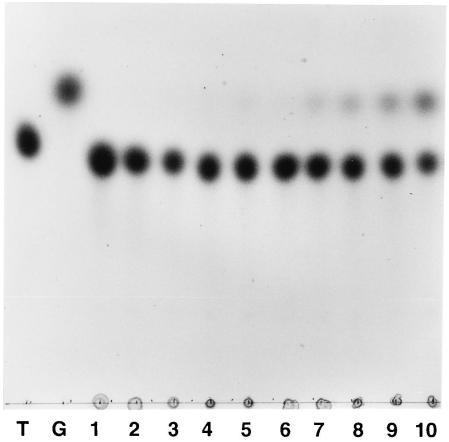

FIG. 4. Pattern of trehalose hydrolysis in a mutant derepressed for TreF activity analyzed by TLC. Extracts (100 μg of protein) of strain RHo36 (*treF* wild type) (lanes 1 to 5) and strain RHo65 (*treF* mutant) (lanes 6 to 10) were incubated with 10 mM trehalose. Samples (5 μl) were taken at 0 (lanes 1 and 6), 10 (lanes 2 and 7), 30 (lanes 3 and 8), 60 (lanes 4 and 9), and 180 (lanes 5, 10) min and spotted on a TLC plate. Controls (5 μl of 10 mM solutions) were trehalose (lane T) and glucose (lane G). The plate was developed in 2-propanol–ethanol–water (5:3:2 [vol/vol/vol]). The saccharide spots were visualized by dipping the plate into methanol containing 2% H_2SO_4, dried, and charred for 10 min at 180°C.

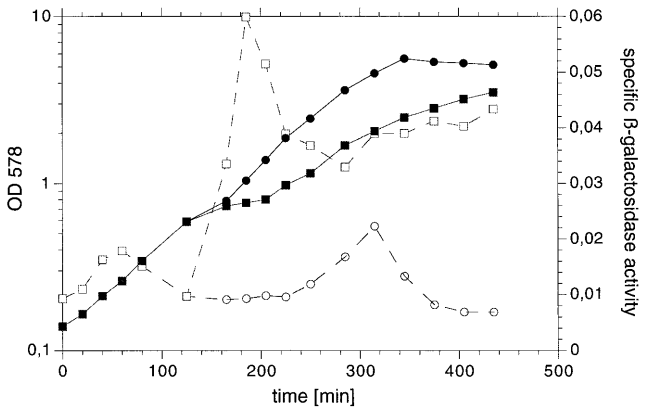

FIG. 5. TreF-LacZ activity during growth on glucose and after upshift with 300 mM NaCl. Strain RHo58 (*treF-lacZ*) was grown aerobically at 37°C in MMA and 0.4% glycerol as the carbon source (circles). After 2 h, the culture was divided, and 300 mM NaCl was added to one portion (squares). Specific β-galactosidase activity (in units per milligram of protein) (open symbols) and the optical density of the culture at 578 nm (OD 578) (filled symbols) were monitored.

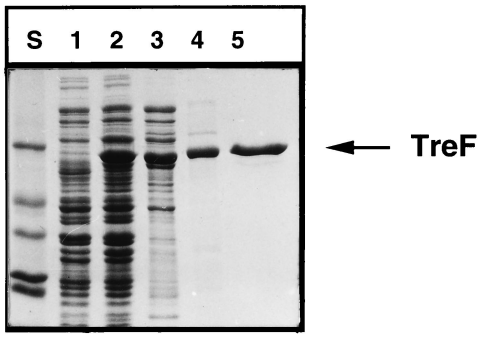

FIG. 6. SDS-PAGE of TreF after overproduction and during purification. Lanes: 1, crude extract from KU68 harboring pBAD22*treF* grown in MMA containing Casamino Acids (0.4%, final concentration); 2, crude extract from KU68 harboring pBAD22*treF* grown in the same medium plus 1 mM L-arabinose; 3, fraction containing 40% saturated ammonium sulfate; 4, pooled fractions after phenyl-Sepharose column chromatography; 5, pooled fractions after MonoQ column ion-exchange chromatography; S, molecular mass standards (66, 45, 36, 29, and 20.1 kDa). Protein (10 μg) was applied to each slot.

the strain carrying the *treF-lacZ* fusion, no increase in the *treF-lacZ* activity was observed. Thus, the *treF* mutation is most likely affecting *treF* expression in *cis* and might be a promoter mutation.

Purification and properties of TreF. TreF activity was present in the crude cellular extracts but was not released to a significant extent by the classical cold osmotic shock procedure (25). This is consistent with the lack of a typical signal sequence for exported proteins. TreF was purified from cellular extracts by $(NH_4)_2SO_4$ precipitation, phenyl-Sepharose chromatography, and ion-exchange chromatography. The protein profiles of the different purification steps as well as their activities are shown in Fig. 6 and Table 3. Purified TreF migrated on a sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS–10% PAGE) gel in accordance with a molecular mass of about 64 kDa. In gel filtration experiments, it behaved as a protein with a molecular mass of 74 kDa, indicating that TreF is monomeric (data not shown). The N-terminal amino acid sequence was determined, confirming the open reading frame proposed from DNA sequencing. The comparison of the deduced amino acid sequence of TreF (549 amino acids with a molecular weight of 63,703) with the periplasmic TreA (535 amino acids with a molecular weight of 60,425, excluding the signal sequence) is shown in Fig. 1. TreA shows a 61-amino-acid extension at the N terminus, and TreF shows a 41-amino-acid extension at the C terminus with no apparent homology. There is 48.9% sequence identity (75% conserved amino acids) over a core of 478 (TreA) and 477 (TreF) consecutive amino acids between the two proteins. Two short sequences that are thought to be conserved in glucosyl hydrolases can be identified in both polypeptides (15).

To determine optimal conditions for the assay of TreF, several parameters were investigated. The pH optimum of TreF for the hydrolysis of trehalose was pH 6.0 (Table 4). Therefore, we used 20 mM potassium phosphate buffer (pH 6.0) in most of the subsequent assays. We did not detect any stimulating effects of divalent cations on trehalase activity. Accordingly, the addition of 1 and 10 mM EDTA to the reaction buffer had no effect on TreF activity (Table 5).

The kinetic parameters of purified TreF were determined by varying substrate concentrations between 0.5 and 20 mM. The K_m was 1.9 mM, the V_{max} was 54 μmol min^{-1} mg of $protein^{-1}$. In comparison, for TreA, the periplasmic trehalase, a K_m of 0.78 mM and a V_{max} of 87 μmol min^{-1} mg of $protein^{-1}$ had been reported for the same reaction (32).

To determine the substrate specificity of TreF, we incubated TreF with 10 mM trehalose, lactose, maltose, and sucrose and analyzed the reaction products by TLC analysis. Of the four disaccharides, only trehalose was hydrolyzed.

Since trehalose accumulates in the cytoplasm of *E. coli* under conditions of high osmolarity and since *treF* is induced under these conditions, we tested whether high concentrations of NaCl, KCl, potassium glutamate, or glycine betain would affect TreF activity. We found that TreF activity was about

TABLE 2. Expression of *treF-lacZ* in different backgrounds

Strain	Relevant genotype	Activity (U/mg of protein)	
		Without NaCl	With 300 mM NaCl
RHo42		0.014	0.038
RHo47	*otsA*	0.012	0.042[a]
RHo48	*rpoS*	0.002	0.013[a]
RHo58	*treA*	0.018	0.026
RHo59	*treR*	0.018	0.038
RHo42 pTrc99A	Vector	0.019	
		0.017[b]	
RHo42 pRHo700	$potsB^+$ $otsA^+$	0.010	
		0.003[b]	

[a] These values were obtained at 200 mM NaCl since the *otsA* and *rpoS* mutations restrict growth at 300 mM NaCl.
[b] IPTG was added to the medium.

TABLE 3. Purification of TreF

Sample	Total protein (mg)	Sp act (U/mg of protein)[a]
French press extract	1,020	2.5
Ammonium sulfate (10–40%)	92	22.7
Phenyl-Sepharose	44	37.4
MonoQ	3.8	59.9

[a] Trehalase activity was determined by assaying the release of free glucose from trehalose. Assays were done in 20 mM potassium phosphate buffer (pH 6.0) in the presence of 100 mM trehalose.

TABLE 4. pH dependence of TreF activity

pH[a]	Sp act (U/mg of TreF)[b]
4.0	3.1
4.5	8.0
5.0	13.1
5.5	28.6
6.0	38.6
6.5	36.1
7.0	36.1
7.5	32.1
8.0	28.6
8.5	27.6
9.0	14.3

[a] At pH 4.0 to 5.5, pH 5.5 to 7.5, and pH 7.5 to 9.0, 100 mM sodium citrate, potassium phosphate, and sodium carbonate, respectively, were used as buffers. At pH 5.5, trehalase activity was identical in sodium citrate buffer and potassium phosphate buffer, and at pH 7.5 it was identical in potassium phosphate buffer and sodium carbonate buffer.

[b] Specific TreF activity was determined by assaying the release of free glucose from trehalose. Assays were done in the presence of 100 mM trehalose.

TABLE 5. Effect of ions on activity of TreF

Ion	% Sp act of TreF assayed with indicated ions at[a]:	
	1 mM	10 mM
$CaCl_2$	100	24
$MgCl_2$	100	65
$MnCl_2$	103	44
$ZnCl_2$	55	24
$CoCl_2$	90	13
NH_4Cl_2	105	110
EDTA	110	113

[a] Specific activity of 100% corresponded to 45 μmol of trehalose per min per mg of TreF and was determined in 100 mM Tris-HCl (pH 7.0).

twofold lower in the presence of 0.5 M NaCl, KCl, or potassium glutamate. TreF activity further decreased with increasing salt concentrations. Interestingly, equal concentrations of the osmoprotectant glycine betaine had no effect on TreF activity (Fig. 7). TreA, the periplasmic trehalase, was more tolerant to conditions of high osmolarity than was TreF.

DISCUSSION

Here we report a second trehalase in *E. coli*. This enzyme is located in the cytoplasm. Previously, we had reported a periplasmic trehalase whose function, in our view, was the utilization of external trehalose under conditions of high osmolarity where the PTS-mediated uptake of trehalose was strongly repressed (3, 4). This seems logical since *treA* is strongly induced under high-osmolarity growth conditions and *treA* mutants can no longer grow on trehalose under these conditions. An additional function of TreA is that of a recapture system for trehalose which is being synthesized in the cytoplasm under conditions of high osmolarity and may exit the cell (31).

TreF, the cytoplasmic trehalase with 549 amino acids, very closely resembles TreA, with 535 amino acids. The sequence homology is lost at both ends of the two polypeptide chains. TreF carries a nonhomologous 61-amino-acid extension at its N terminus while mature TreA carries a nonhomologous 41-amino-acid extension at the C terminus. Not counting the N- and C-terminal extensions, the remaining core polypeptide of 478 amino acids exhibits 48.9% identity (Fig. 1) and a close structural correlation in the Kyte-Doolittle plot (Fig. 8). Curiously, even though the core protein of TreA is larger by 1 amino acid than that of TreF, its molecular mass is smaller by 1,065 Da. Thus, the 51% nonidentical amino acids of TreA are, on average, smaller than those of TreF. Looking more closely at the differences in the nonidentical amino acids of the core protein, TreF contains more acidic amino acids (16 Asp, 23 Glu, and 10 His residues) than TreA (12 Asp, 14 Glu, and 8 His residues) as well as more basic amino acids (23 Arg and 12 Lys residues for TreF versus 7 Arg and 20 Lys residues for TreA). In addition, TreF has two cysteines that are not present in TreA. Other significant differences are the low content of Gln and Asn residues in TreF (11 of each) versus 24 Gln and 22 Asn residues in TreA. One is tempted to speculate that these differences that apparently do not affect the enzymatic

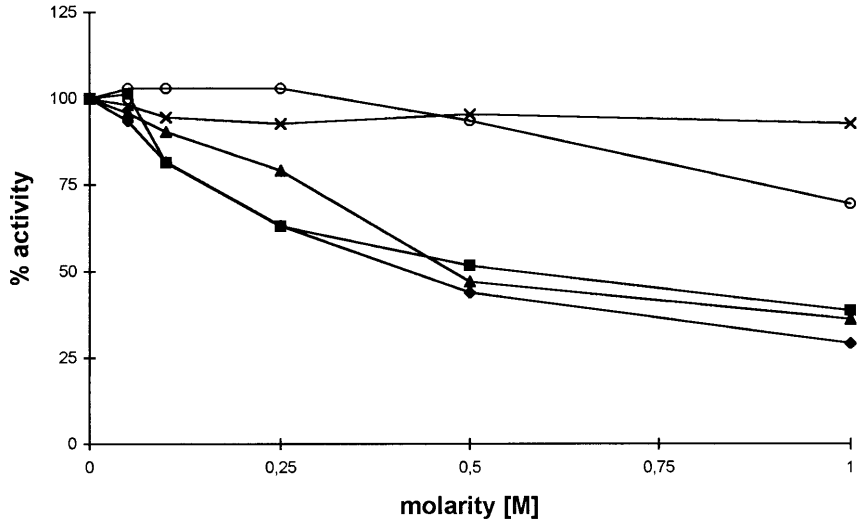

FIG. 7. Inhibition of TreF by salt. Trehalase assays were performed under substrate saturation in 20 mM potassium phosphate buffer (pH 6.0) containing the indicated concentrations of potassium chloride (diamonds), sodium chloride (squares), potassium glutamate (triangles), and glycine betain (crosses). For comparison, the assay was also done with periplasmic trehalase and sodium chloride as an inhibitor (circles).

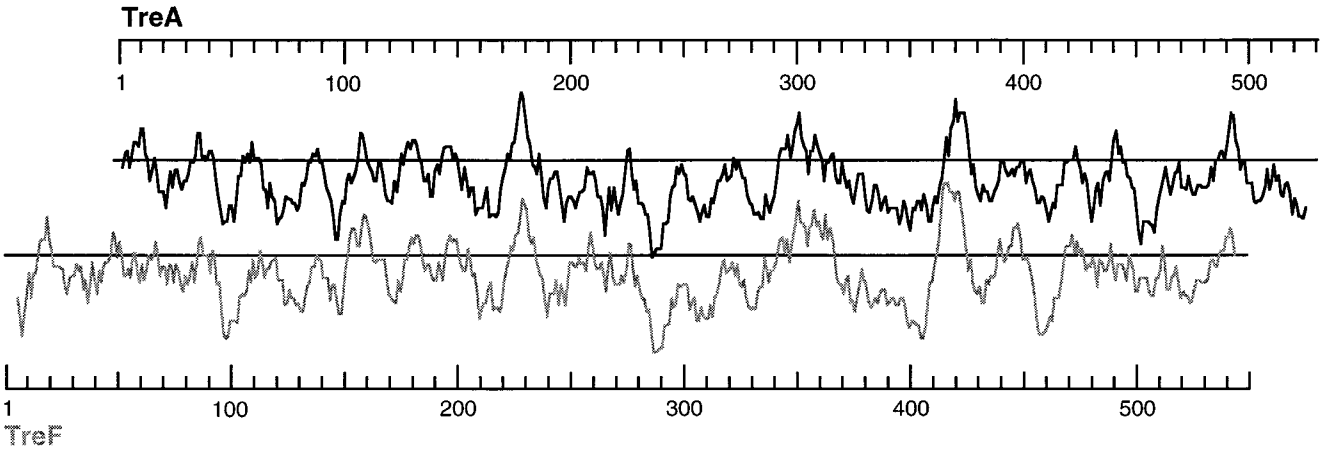

FIG. 8. Hydropathy plot of TreF and TreA. The hydropathy plot was done according to the method of Kyte and Doolittle (20). The heavy line is for TreA and the light line is for TreF. The numbers indicate the amino acid position starting at the N terminus. For TreA, the mature protein without its signal sequence is given.

activity were brought about by a divergent evolution of the same protein in the two different cellular compartments.

Both enzymes contain two sequence motifs that are characteristic for glucosyl hydrolases (15), and both enzymes hydrolyze only trehalose. The V_{max} values of both enzymes are rather close (54 U/mg for TreF and 87 U/mg for TreA). Also, the regulation for the expression of both enzymes appears to be related. Both are under the control of RpoS (14), the stationary-phase sigma factor, even though the dependency in both cases is not complete. Both TreA and TreF are regulated by osmolarity, but their osmoregulation is not dependent only on *rpoS* (27). It was interesting that the expression of *treF* was decreased about sixfold when massive internal trehalose synthesis was induced in the absence of an osmotic shock. We interpret this phenomenon by the inverse of the osmotic shock, i.e., an increase of turgor by the synthesis of internal trehalose.

We isolated mutants with elevated TreF activity by using a genetic selection scheme involving alkaline phosphatase (AP). Cytoplasmically expressed AP was previously used to study reduction and formation of disulfide bonds in the cytoplasm (8), the determinants of membrane protein topology (33), and the translocation kinetics of periplasmic domains of integral inner membrane proteins (34). We show here that it can also be used in genetic selections for isolating mutants with elevated enzymatic activity. One mutant isolated exhibited a 17-fold increase in trehalase activity.

The enzymatic capacity of TreA in the periplasm even under low osmolarity growth conditions is significantly larger than that of the cytoplasmic TreF. The activity of the latter had been noticed previously only as low background activity in mutants lacking TreA (4, 28). What is the function of TreF in the cytoplasm? At high osmolarity the cell synthesizes large amounts of internal trehalose. One could argue that the enzyme functions as a safety valve in case the internal production of trehalose becomes too high. This would make sense only if the K_m of TreF would be on the order of the concentration of trehalose that is being reached under high-osmolarity growth conditions, estimated to be around 400 mM (10). Yet, TreF exhibits a K_m of 1.9 mM for trehalose, far too low for the function of an enzyme controlling its activity under physiological conditions by substrate concentration. Also, we did not find evidence for the existence of a trehalase inhibitor in extracts of *treA treF* mutants that would control the activity of TreF under different osmolarity growth conditions. It seems then that the function of TreF is the removal and utilization of internal trehalose after the cells return from high osmolarity to low osmolarity. The enzymatic activity of TreF is low enough not to compromise the trehalose-synthesizing machinery at high osmolarity but high enough to ensure the utilization of the accumulated trehalose after the return to low osmolarity when the synthesizing machinery has been turned off. It has been noticed that the cellular trehalose content as well as the TreA activity is elevated when cells are growing at submicromolar concentrations of glucose even in the absence of high osmolarity (26). It would be interesting to see whether TreF follows the same pattern or whether it is reduced under these conditions. It would be difficult to understand why the cells should establish a futile cycle under conditions of extreme starvation.

With the discovery of TreF, we believe that the description of the metabolic reactions of trehalose in *E. coli* is now complete. Thus, trehalose utilization in *E. coli* occurs by PTS-mediated uptake as trehalose-6-phosphate followed by its internal hydrolysis to glucose and glucose-6-phosphate. In addition, trehalose can be hydrolyzed to glucose by TreA and TreF on both sides of the membrane. *treA treF* double mutants are entirely devoid of any trehalose-hydrolyzing activity.

The unique situation of having two nearly identical enzymes optimized for their routing and location on both sides of the membrane offers the opportunity to study the intrinsic requirements of protein secretion through the cytoplasmic membrane versus protein folding in the cytoplasm with a pair of proteins that most likely have evolved from the same ancestral gene.

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