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Transport of Trehalose in *Salmonella typhimurium*

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We have studied trehalose uptake in *Salmonella typhimurium* and the possible involvement of the phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS) in this process. Two transport systems could recognize and transport trehalose, the mannose PTS and the galactose permease. Uptake of trehalose via the latter system required that it be expressed constitutively (due to a *galR* or *galC* mutation). Introduction of a *ptsM* mutation, resulting in a defective II^{Man}/III^{Man} system, in *S. typhimurium* strains that grew on trehalose abolished growth on trehalose. A *ptsG* mutation, eliminating II^{Glc} of the glucose PTS, had no effect. In contrast, a *crr* mutation that resulted in the absence of III^{Glc} of the glucose PTS prevented growth on trehalose. The inability of *crr* and also *cya* mutants to grow on trehalose was due to lowered intracellular cyclic AMP synthesis, since addition of extracellular cyclic AMP restored growth. Subsequent trehalose metabolism could be via a trehalose phosphate hydrolase, if trehalose phosphate was formed via the PTS, or trehalase. Trehalose-grown cells contained trehalase activity, but we could not detect phosphoenolpyruvate-dependent phosphorylation of trehalose in toluenized cells.

The phosphoenolpyruvate (PEP):carbohydrate phosphotransferase system (PTS) catalyzes the transport and concomitant phosphorylation of a large number of carbohydrates in a variety of bacteria. The system has been studied in most detail in the members of the family *Enterobacteriaceae*, e.g., *Escherichia coli* and *Salmonella typhimurium*. More than 10 different sugar-specific, membrane-bound enzymes II of the PTS have been identified. Together with the general, cytoplasmic proteins enzyme I and HPr and (in a number of cases) sugar-specific enzymes III, the enzymes II catalyze carbohydrate uptake and phosphorylation. For instance, glucose (Glc) is taken up and phosphorylated to glucose 6-phosphate via enzyme II^{Glc} and its associated III^{Glc}. Mannitol (Mtl), on the other hand, requires only an enzyme II^{Mtl} (for a review, see reference 14).

Most carbohydrates taken up via the PTS in members of the *Enterobacteriaceae* are C_6 carbohydrates, e.g., glucose, mannose, fructose, and hexitols. Only in a few cases are disaccharides substrates for the PTS. Thus, plasmid-mediated sucrose (Scr) uptake in *E. coli* was shown to be mediated via a plasmid-encoded II^{Scr} and the chromosomally encoded III^{Glc} (8). In *Klebsiella aerogenes*, lactose uptake can be catalyzed both via the PTS and via a non-PTS lactose permease (5). Recently, it was suggested that cellobiose uptake in *Erwinia chrysanthemi* is also PTS mediated (1). Finally, trehalose (1-*O*-α-D-glucopyranosyl-α-D-glucopyranoside) uptake has been studied in *Vibrio parahaemolyticus* and *S. typhimurium*. In *V. parahaemolyticus* in particular it was shown that mutants defective in PTS-mediated glucose uptake due to the absence of a protein that resembles III^{Glc} were unable to take up trehalose as well (6). An enzyme II specific for trehalose was proposed (7). Although trehalose uptake has also been studied in *S. typhimurium*, no such specific proposal was made for this organism (9). Most likely, subsequent metabolism of intracellular trehalose phosphate (trehalose) involves generation of glucose 6-phosphate and glucose (or two glucose molecules in the case of trehalose).

In this paper we report that in *S. typhimurium* trehalose is taken up via the mannose (Man)-specific II^{Man} of the PTS. In mutants that lack a functional PTS due to the absence of enzyme I and HPr, trehalose can still be taken up via the galactose permease.

MATERIALS AND METHODS

Bacterial strains. The *S. typhimurium* strains used in this study are listed in Table 1. *V. parahaemolyticus* 1010 (wild type) and 1050 (*crr*) were obtained from S. Tanaka.

Media and growth conditions. Cells were grown at 37°C on a rotary shaker either in liquid minimal medium A (20), supplemented with a carbon source (0.2%) and 20 μg of the required amino acid per ml, or in Luria broth (1% tryptone, 0.5% yeast extract, and 0.5% NaCl) supplemented with 0.2% of a carbohydrate as indicated. Tetracycline, when present, was added to a final concentration of 25 μg/ml. Generation times were measured by following the change in optical density at 600 nm at 37°C, with 0.2 or 1% of the carbohydrate as a carbon source.

Transport studies. The uptake of labeled substrates in cells growing in the logarithmic phase was measured as described elsewhere (12) and expressed as nanomoles of substrate taken up per minute per milligram (dry weight) of protein at 20°C. Oxygen consumption was measured with a Clark-type electrode in minimal medium A. The oxidation rate was expressed as nanoatoms of oxygen consumed per minute per milligram (dry weight) at 25°C.

Preparation of cell extracts and enzyme measurements. Cell extracts were prepared and PEP-dependent phosphorylation of carbohydrates was measured as described elsewhere (12). Trehalase activity was determined by incubating cell extracts in 120 mM potassium phosphate buffer, pH 7.6, and 10 mM trehalose. Samples were taken at various intervals, and the reaction was stopped with perchloric acid. Glucose was determined in the neutralized samples by the method of Bergmeyer and Bernt (2) with glucose oxidase and peroxidase. PEP-dependent carbohydrate phosphorylation in toluenized cells was determined as described previously (20).

Chemicals. [U-^{14}C]2-deoxyglucose (282 Ci/mol) and [U-^{14}C]galactose (300 Ci/mol) were obtained from New England

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TABLE 1. *S. typhimurium* strains

Strains	Genotype	Construction[a]	Source or reference[b]
SB3507	*trpB223*		15
PP116	Δ(*cysK-ptsHI*)*41 galC1891 trpB223*		10
PP784	*galP283 cysA1539*::Tn*10 trpB223*		15
PP799	*galP283 trpB223*		15
PP800	Δ(*cysK-ptsHI crr*)*49 galP283 trpB223*		15
PP801	Δ(*cysK-ptsHI*)*41 galP283 trpB223*		15
PP994	*crr-307*::Tn*10*		21
PP1373	*crr-307*::Tn*10 galP283 trpB223*	PP784 × P22 (PP994)	A
PP1374	*galP283 trpB223*	PP784 × P22 (PP994)	A
PP1131	*ptsM420 trpB223*, Tn*10* next to *ptsM*		10
PP1139	*ptsG415*::Tn*10 trpB223*		13
PP1419	*galP283 trpB223*, Tn*10* next to *ptsM*$^+$	PP1374 × P22 (PP1131)	A
PP1420	*ptsM420 galP283 trpB223*, Tn*10* next to *ptsM*	PP1374 × P22 (PP1131)	A
PP1455	*trpB223*, Tn*10* next to *ptsM*$^+$	SB3507 × P22 (PP1131)	A
PP1456	*ptsM420 trpB223*, Tn*10* next to *ptsM*	SB3507 × P22 (PP1131)	A
PP1366	*galC2142* Δ(*cysK-ptsHI*)*41 trpB223*	Tre$^+$ PP801	A
PP1436	*ptsG*(?) Δ(*cysK-ptsHI crr*)*49 galP283 trpB223*	Tre$^+$ PP800	A
PP1430	*galP2143 galC2142* Δ(*cysK-ptsHI*)*41 trpB223*	2DGal^r PP1366	A
PP1429	Δ(*cysK-ptsHI*)*41 galC1891 trpB223*	PP801 × P22 (PP116)	A
PP1439	*galC1891 trpB223*	PP1429 × P22 (SB3507)	A
PP1415	*cya-1093 trpB223*	Tn*10* excision from PP1002	A
PP1416	*crp-774 trpB223*	Tn*10* excision from PP1037	3
PP1364	*tre galP283 trpB223*	Tre$^-$ PP799	A
TT1518	*tre-57*::Tn*10*		JR

[a] 2DGal^r, Resistant to 2-deoxygalactose.
[b] A, This paper; JR, J. Roth.

Nuclear Corp. [U-^{14}C]methyl-α-glucoside (291 Ci/mol) and [U-^{14}C]mannose (229 Ci/mol) were obtained from Amersham Corp. Trehalose was purchased from Sigma Chemical Co.

RESULTS AND DISCUSSION

Trehalose uptake and metabolism in *S. typhimurium crr* and *ptsG* mutants. The study of trehalose uptake in *V. parahaemolyticus* suggested that in this organism a cytoplasmic component of the glucose PTS is involved. A protein resembling III^{Glc} of *E. coli* and *S. typhimurium* was characterized preliminarily (6). With an antiserum specific for III^{Glc} from *S. typhimurium* we could detect in *V. parahaemolyticus* a cross-reacting protein with a mobility similar to that of *S. typhimurium* III^{Glc}. This cross-reacting protein was lacking in *V. parahaemolyticus* 1050 (P. W. Postma, unpublished results). It was shown previously that this strain is defective in PEP-dependent glucose and trehalose phosphorylation (6).

These results prompted us to study which PTS, if any, is responsible for trehalose transport in *S. typhimurium*. *crr*::Tn*10* mutants of *S. typhimurium*, which lack III^{Glc} completely, were unable to grow on trehalose (Table 2). Similar results were obtained with *ptsHI* deletion mutants, which lack enzyme I and HPr (Table 2). Although these results suggested that trehalose is taken up via II^{Glc}/III^{Glc} of the PTS, a subsequent observation argued against this conclusion.

It is known that *crr* and *ptsH* and *ptsI* mutations affect adenylate cyclase activity and 3′,5′-cyclic AMP (cAMP) synthesis adversely (14). Conceivably, synthesis of the enzyme(s) involved in trehalose or trehalose phosphate metabolism required cAMP. Addition of cAMP to a *crr*::Tn*10* strain (PP1373) restored growth on trehalose (Table 2). A similar result was obtained by introducing in such a *crr* strain a *crp** mutation, which renders the cAMP-binding protein independent of cAMP (19), either by transduction or by selecting suppressor mutations in a *crr*::Tn*10* mutant strain that restored growth on trehalose, succinate, or citrate (PP1395). These suppressor mutations allowed these mutants, which still lacked III^{Glc} (as measured with a specific antibody), to grow on all three carbon sources.

To investigate further the requirement for cAMP for growth on trehalose, we tested *cya* and *crp* strains (PP1415 and PP1416, respectively). Both were unable to grow on trehalose, but cAMP stimulated growth of the *cya* strain PP1415.

Together, these results ruled out that III^{Glc} of the glucose PTS is essential for trehalose uptake. Rather, the lowered cAMP synthesis in *crr* strains prevented growth on trehalose. This conclusion was strengthened by the observation that introduction of a *ptsG*::Tn*10* mutation, which results in the absence of II^{Glc} (13), also did not affect growth on trehalose (Table 2, PP1139).

Trehalose and enzyme II^{Man}. Glucose can be taken up by the PTS via two systems, II^{Glc}/III^{Glc} and II^{Man}/III^{Man} (4, 14). Since the first system did not seem to be involved in trehalose uptake, we tested the mannose PTS. The presence of a *ptsM* mutation, resulting in defective mannose uptake and phosphorylation, eliminated growth on trehalose (Table 2, PP1456). The corresponding *ptsM*$^+$ strain grew on trehalose and mannose, with generation times of 180 and 90 min, respectively.

Transport studies (Table 3) showed that the absence of mannose and 2-deoxyglucose transport in the *ptsM* strain

TABLE 2. Growth properties of *S. typhimurium* mutants[a]

Strain	Relevant genotype	cAMP added	Growth on:					
			Tre	Glc	Man	Mtl	Cit	Gal
PP799	Wild type	−	+	+	+	+	+	+
PP1373	*crr*	−	−	+	+	+	−	+
PP801	Δ*ptsHI*	−	−	−	−	−	−	+/−
PP1139	*ptsG*::Tn*10*	−	+	+	+	+	+	+
PP1456	*ptsM*	−	−	+	+/−	+	+	+
PP1373	*crr*	+	+	+	+	+	+	+
PP1395	*crr crp**	−	+	+	+	+	+	+
PP1366	Δ*ptsHI galC*	−	+	+	−	−	−	+
PP1430	Δ*ptsHI galC galP*	−	−	−	−	−	−	+/−
PP1364	*tre galP*	−	−	+	+	+	+	+

[a] Growth was monitored on agar plates containing minimal salts medium and 0.2% of the carbon source. Symbols: +, growth after 24 h; +/−, weak growth after 48 h; −, no growth after 48 h. When indicated, 5 mM cAMP was added. Abbreviations: Tre, trehalose; Glc, glucose; Man, mannose; Mtl, mannitol; Cit, citrate; Gal, galactose.

TABLE 3. Transport in mutants of *S. typhimurium*

Strain	Relevant genotype	Carbon source for growth	Uptake[a] (nmol/min per mg)			Growth on trehalose
			αMG	2DG	Man	
SB3507	wild type	Glucose	48	70	30	+
SB3507	wild type	Trehalose	30	30	42	+
PP1455	wild type	Glucose	42	60	36	+
PP1456	*ptsM*	Glucose	35	<1	<1	−
PP1139	*ptsG*	Glucose	<1	65	30	+
PP1364	*tre galP*	Glucose	21	72	16	−

[a] Uptake was measured as described in the text. Results are expressed as nanomoles of substrate taken up per minute per milligram (dry weight) at 20°C. Abbreviations: αMG, methyl α-glucoside; 2DG, 2-deoxyglucose; Man, mannose.

PP1456 correlated with the inability to grow on trehalose. The isogenic *ptsM*$^+$ strain PP1455 exhibited normal transport and grew well on trehalose. Transport via II^{Glc}/III^{Glc}, as measured with methyl-α-glucoside, was normal in these strains.

These results with a *ptsM* strain (lacking II^{Man}) and the *ptsG* strain PP1139 (lacking II^{Glc}) are important for two reasons. First, they eliminated the possibility that trehalose was split in the periplasmic space by a trehalase (see below) and that the resulting glucose was subsequently taken up. PP1456 lacked only the mannose PTS and grew normally on glucose via II^{Glc}/III^{Glc}, but was unable to grow on trehalose. Second, they show that II^{Man} is involved in trehalose uptake in *S. typhimurium*. This is supported by the observation that uptake of ^{14}C-labeled 2-deoxyglucose (which at 0.5 mM is specifically taken up via II^{Man} [22]) was inhibited by approximately 50% in trehalose-grown SB3507 cells by the addition of 5 mM unlabeled trehalose (data not shown).

Isolation of mutants unable to metabolize trehalose. Mutants unable to grow on trehalose (Tre$^-$) were isolated by penicillin selection from PP799. Whereas a number of these mutants showed a pleiotropic phenotype due to mutations in *ptsI* or some glycolytic enzymes such as fructose 1,6-diphosphate aldolase (*fda*), one strain, PP1364, was only defective in growth on trehalose (Table 2). Transport via the glucose PTS and the mannose PTS was intact (Table 3). Possibly this mutant was defective in trehalase (see below and Table 4).

A mutant with a Tn*10* insertion in *tre* was obtained from J. Roth (TT1518), but the Tre$^-$ phenotype of this strain was dependent on the background. After transduction of the *tre*::Tn*10* mutation into SB3507 by selecting for tetracycline resistance, all transductants were able to grow on trehalose. It was also possible to obtain Tre$^+$ revertants of TT1518. In all cases, the strains remained resistant to tetracycline.

Finally, we have noted that various strains of *S. typhimurium* grow very slowly or not at all on trehalose, for instance NK186 (*cysA1539*::Tn*10*). In this case Tre$^+$ revertants could also be obtained easily. We do not know where this mutation is localized, but it is not linked to *ptsM*.

Transport of trehalose via the galactose permease. As mentioned above, *ptsHI* deletion mutants of *S. typhimurium* did not grow on trehalose (Table 2, PP801). Suppressor mutations could be obtained at a low frequency (approximately 10^{-9}) in such *ptsHI* deletion strains (which already contained a *galP*$^+$ allele), however, that restored growth on trehalose (e.g., PP1366, Table 2). The doubling time on 1% trehalose was 174 min; on 0.2% galactose it was 160 min (15). Growth on glucose was restored at the same time. Most strains also became sensitive to 2-deoxygalactose, a toxic analog of galactose that is transported via the galactose permease (10). This suggested a possible involvement of the galactose permease, since it was shown previously that constitutive expression of galactose permease as a consequence of a *galR* or *galC* mutation allows *ptsHI* deletion mutants to grow on glucose (16, 18). Transport studies with labeled galactose showed that PP1366 indeed contained a constitutive galactose permease. Uptake of 0.2 mM [^{14}C]galactose was inhibited approximately 60% by 10 mM trehalose (data not shown). Introduction of a *galP* mutation, resulting in a defective galactose permease, by transduction or selection for resistance to 2-deoxygalactose (10) eliminated growth on trehalose in such a *ptsHI galC* or *ptsHI galR* strain (Table 2, PP1430). Direct transduction of a *galP ptsHI* strain (PP801) to *galP*$^+$ *galC* (with phage P22 grown on a *ptsHI galP*$^+$ *galC* strain) resulted in a strain, PP1429, that regained the ability to grow on trehalose. The low frequency of reversion might be due to the requirement for a second mutation to allow growth on trehalose, most likely a *crp** mutation, which facilitates growth of *ptsHI* deletion strains on substrates that require cAMP for gene expression (19). A similar double mutant has been described before (16). The Tre$^+$ revertants of PP801 regained the ability to grow on maltose, melibiose, and glycerol, non-PTS carbon sources that do not support the growth of a *ptsHI* deletion strain.

The role of galactose permease in trehalose transport could also be demonstrated in *pts*$^+$ strains. Introduction of a *ptsM* mutation (linked to a Tn*10* insertion) by P22 transduction in a recipient containing an inducible galactose permease and selecting for tetracycline resistance resulted in 40% Tre$^+$ and 60% Tre$^-$ transductants. A similar transduction with a *galC* or *galR* strain (e.g., PP1439 *galC1891*) as the recipient yielded only Tre$^+$ transductants (50 of 50).

In a rare case we found Tre$^+$ (and Glc$^+$ at the same time) revertants of PP800 and PP801 (both containing deletions in *ptsHI*) that remained resistant to 2-deoxygalactose (e.g., PP1436), suggesting that these revertants did not acquire a constitutive galactose permease. We have not investigated these mutants in more detail, but they might represent strains in which the II^{Glc} (or II^{Man}) has been altered so that substrates can be transported in the absence of PTS-mediated phosphorylation. Introduction of a *ptsM* mutation in PP1436 did not affect growth on trehalose, but insertion of a *ptsG*::Tn*10* mutation eliminated growth on trehalose (and

TABLE 4. Trehalase activity in *S. typhimurium*[a]

Strain	Relevant genotype	Carbon source for growth	Fraction	Toluene added	Trehalase activity (nmol/min per mg)
SB3507	WT	NB + Tre	Cells	−	0
SB3507	WT	NB + Tre	Cells	+	1
SB3507	WT	NB	CE	−	1.6
SB3507	WT	NB + Tre	CE	−	12.1
SB3507	WT	NB + Tre	CE	+	1.1
PP1419	WT	NB + Tre	CE	−	16.8
PP1420	*ptsM*	NB − Tre	CE	−	17.5
PP1364	*tre galP*	NB + Tre	CE	−	0.5

[a] Trehalase activity was measured as described in the text and expressed as nanomoles of glucose formed per minute per milligram (dry weight) or protein at 37°C in cells and crude extracts (CE), respectively. Toluene (4 μl/ml of cell suspension) was added as indicated. Abbreviations: WT, wild type; NB, nutrient broth; Tre, trehalose.

glucose). These uncoupled II^{Glc} mutants have been described previously (13).

From these results we conclude that growth on and transport of trehalose can occur via the galactose permease in the complete absence of phosphorylation by the PTS enzymes I and HPr if the galactose permease is expressed constitutively.

Trehalose phosphorylation and trehalase activity. To explain how *ptsHI* deletion strains which contain a constitutive galactose permease were able to grow on trehalose in the absence of phosphorylation of trehalase via the PTS, we investigated whether subsequent metabolism of intracellular trehalose involved trehalose activity. Table 4 shows that such a trehalose-splitting activity was detected in SB3507. This trehalase activity was furthermore induced by growth in the presence of trehalose. The Tre^- mutant PP1364 lacked trehalase activity (Table 4). The trehalase activity in wild-type strains was inhibited by more than 90% by toluene (4 μl/ml of cell suspension). Marechal (9) also reported a trehalase activity in *S. typhimurium*, but in that case the activity was the same in glucose-, glycerol-, and trehalose-grown cells.

The induction of trehalase activity by growth in the presence of trehalose was paralleled by the induction of trehalose oxidation by intact cells (^{14}C-labeled trehalose unfortunately is not available commercially anymore). Whereas glycerol-grown SB3507 cells oxidized trehalose at a rate of 6 nanoatoms of oxygen per min per mg (dry weight) (at 25°C), for trehalose-grown cells this rate was 36 nanoatoms per min per mg. For comparison, glucose is oxidized at a rate of 150 to 200 nanoatoms of oxygen per min per mg. The apparent affinity constant for trehalose was 0.4 mM.

In toluenized cells, no trehalose PTS activity could be detected, in contrast to results reported by Marechal (9). However, Marechal (9) reported a rate of trehalose phosphorylation that was quite low, about 4 nmol/min per mg (at 37°C). Whereas toluenized PP799 cells phosphorylated glucose in a PEP-dependent reaction at a rate of 164 and 148 nmol/min per mg (dry weight) (as measured by the formation of pyruvate from PEP) when grown on glycerol and trehalose, respectively, no trehalose-dependent pyruvate formation could be detected in the same cells. Since trehalase activity had been detected, PEP-dependent phosphorylation of glucose which derived from trehalose was expected, even if no trehalose PTS activity existed. The sensitivity of trehalase to toluene might explain this result. Table 4 shows that 4 μl of toluene per ml, as used for permeabilizing cells, inhibited trehalase activity by more than 90%.

In another experiment we measured in cell extracts the formation of glucose 6-phosphate from trehalose and PEP. If trehalose was phosphorylated via the PTS, the subsequent action of trehalose 6-phosphate hydrolase (9) should yield glucose 6-phosphate. Although we found appreciable activity in trehalose-grown cells (8.9 and 1.1 nmol of glucose 6-phosphate formed per min per mg of protein in trehalose- and glycerol-grown cells [PP1374], respectively), one cannot exclude that this was due to trehalase activity and subsequent glucose phosphorylation via glucokinase.

Final conclusions. Trehalose can be transported in at least two different ways in *S. typhimurium*, via II^{Man} of the PTS and via the galactose permease, if the latter transport system is synthesized constitutively. Interestingly, we thus have found yet another substrate that can be recognized by both these transport systems. We pointed out on an earlier occasion that both systems show broad substrate specificity and that all substrates of II^{Man} seem to be at the same time substrates for the galactose permease (17). These substrates include glucose, mannose, fructose, 2-deoxyglucose, galactose, 2-deoxygalactose, and fucose. Now trehalose can be added to this list. The broad specificity differs strongly from the narrow specificity of the complementary glucose and galactose systems, II^{Glc}/III^{Glc} and the galactose-binding protein system, respectively.

It is still not clear whether transport of trehalose via II^{Man} requires phosphorylation via enzyme I and HPr. Although Marechal (9) detected low, PEP-dependent trehalose phosphorylation, we could not find any phosphorylation in toluenized cells. Enzymes II are in general unable to catalyze the uptake of PTS carbohydrates in the absence of concomitant phosphorylation (16), but some exceptions have been noted (for a discussion, see reference 14). Possibly, trehalose is such an exception and is transported via II^{Man} like galactose, i.e., in the absence of phosphorylation (11). Since *ptsM* mutants are unable to grow on trehalose, there is no need to postulate a specific II^{Tre}

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