

Plasmid-Mediated Uptake and Metabolism of Sucrose by *Escherichia coli* K-12

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The conjugative plasmid pUR400 determines tetracycline resistance and enables cells of *Escherichia coli* K-12 to utilize sucrose as the sole carbon source. Three types of mutants affecting sucrose metabolism were derived from pUR400. One type lacked a specific transport system (*srcA*); another lacked sucrose-6-phosphate hydrolase (*scrB*); and the third, a regulatory mutant, expressed both of these functions constitutively (*scrR*). In a strain harboring pUR400, both transport and sucrose-6-phosphate hydrolase were inducible by fructose, sucrose, and raffinose; if a *scrB* mutant was used, fructose was the only inducer. These data suggested that fructose or a derivative acted as an endogenous inducer. Sucrose transport and sucrose-6-phosphate hydrolase were subject to catabolite repression; these two functions were not expressed in an *E. coli* host (of pUR400) deficient in the adenosine 3',5'-phosphate receptor protein. Sucrose uptake (apparent $K_m = 10 \mu\text{M}$) was dependent on the *scrA* gene product and on the phosphoenolpyruvate-dependent sugar:phosphotransferase system (PTS) of the host. The product of sucrose uptake (via group translocation) was identified as sucrose-6-phosphate, phosphorylated at C₆ of the glucose moiety. Intracellular sucrose-6-phosphate hydrolase catalyzed the hydrolysis of sucrose-6-phosphate ($K_m = 0.17 \text{ mM}$), sucrose ($K_m = 60 \text{ mM}$), and raffinose ($K_m = 150 \text{ mM}$). The active enzyme was shown to be a dimer of M_r 110,000.

Plasmids conferring to their hosts the ability to utilize sucrose as the sole carbon source have been isolated from strains of *Salmonella* and *Escherichia coli* (13, 35, 41). Previous investigators have concentrated on the epidemiology, transmissibility, and molecular nature of various sucrose plasmids. However, little is known about the genetic determinants (*scr* genes) and the plasmid-encoded functions involved in sucrose metabolism and about their regulation and interaction with host metabolic enzymes.

Known metabolic plasmids that mediate the utilization of lactose (4) or raffinose (29, 31) by *E. coli* were found to encode specific transport and hydrolyzing enzymes responsible for the "peripheral" metabolism of these sugars. To analyze the role played by plasmid-encoded functions in the metabolism of sucrose, we used the 52-megadalton conjugative plasmid *scr-53* (41) in the present study. To avoid confusion with symbols for genetic loci that control the utilization of sucrose and in accordance with the proposed plasmid nomenclature (21), we will refer to the plasmid as pUR400. This article describes (i) the isolation of *scr* mutants, (ii) the characterization of plasmid- and host-determined functions required for sucrose utilization, and (iii) the identification of sucrose-6-phosphate as the primary product of the phospho-

enolpyruvate (PEP)-dependent sucrose transport.

MATERIALS AND METHODS

Chemicals. [¹⁴C]glucose, [¹⁴C]glucose-6-phosphate, [¹⁴C]fructose, [³H]raffinose, and [^{U-14}C]sucrose were purchased from NEN Chemicals GmbH (Dreieichenhain) and [¹⁴C]lactose was purchased from Amersham Buchler (Braunschweig). [³H]melibiose was prepared from [³H]raffinose by hydrolysis with commercial invertase (Serva, Heidelberg) and subsequently purified by gel filtration on Sephadex G-15. *N*-acetylglucosamine, streptomycin, PEP, cAMP, and glucose-6-phosphate were obtained from Sigma Chemical Co. (München); ovalbumin, aldolase, catalase, *E. coli* RNA polymerase, bovine serum albumin, trypsin inhibitor, NADP, and tetracycline hydrochloride were obtained from Boehringer (Mannheim), and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine were obtained from Serva. All other chemicals were purchased from E. Merck AG (Darmstadt).

Strains, plasmids, and mating procedure. Table 1 lists the derivatives of *E. coli* K-12 and plasmids used. For conjugal transfer of sucrose plasmids, 0.5-ml portions of parental broth cultures (5×10^8 cells/ml) were mixed, mated for at least 2 h, diluted appropriately, and spread on selective agar media. *Scr*⁺ exconjugants were selected on minimal agar plates (30) containing 0.2% sucrose or on complete medium (39) supplemented with tetracycline (10 $\mu\text{g/ml}$). Selection for tetracycline resistance was based on the finding that pUR400 contains a tetracycline resistance deter-

minant in addition to sucrose markers (K. Schmid, J. Altenbuchner, and R. Schmitt, unpublished data). Donor cells were counterselected either by the omission of required amino acids from minimal medium or by supplementing complete medium with streptomycin (200 µg/ml). Transduction with phage P1 followed the procedure of Rothman (26).

Selection of *scr* mutants by streptozotocin treatment. Exponentially growing cells of DS402(pUR400) were harvested, suspended in minimal medium without carbon source at 10^9 cells/ml, and treated with nitroso-guanidine (100 µg/ml) at 37°C for 30 min. The mutagen was removed by centrifugation, and cells were transferred to minimal medium supplemented with *N*-acetylglucosamine to allow for segregation and for induction of the streptozotocin uptake system (14). After 16 h of incubation at 37°C, cells were harvested, suspended at 10^8 cells/ml in minimal medium supplemented with 0.2% sucrose, and once more incubated at 37°C. Streptozotocin was added at a concentration of 50 µg/ml after one cell division, and growth was allowed to proceed for 3 h. This procedure selects for non-growing cells and increases the proportion of mutants unable to utilize sucrose as a carbon source. Cells were collected, washed, and grown overnight in minimal glucose medium. *Scr*⁻ colonies were identified on MacConkey indicator plates (19) supplemented with 1% sucrose instead of lactose.

Synthesis and isolation of sucrose-6-phosphate. Sucrose-6-phosphate was prepared by incubating a cell suspension of fructose-induced strain DS420 [about 10^{13} cells in 100 ml of 100 mM bis(2-hydroxymethyl)-imino-tris(hydroxymethyl)methane (Sigma), pH 7.0] with 10 ml of [¹⁴C]sucrose (9 mM; 1.1 mCi/mmol) at 37°C for 10 min. Cells were sedimented at $23,000 \times g$ for 10 min, suspended in 100 ml of distilled water, and boiled for 5 min. After removal of cell debris by centrifugation at $23,000 \times g$ for 15 min, the clear supernatant was concentrated to 4 ml under vacuum, using P₂O₅ as a desiccant. The concentrated extract

was fractionated on Sephadex G-15, using distilled water for elution, and fractions containing ¹⁴C-labeled material were pooled and again concentrated under vacuum to 200 µl (final volume). [¹⁴C]sucrose-6-phosphate was separated from contaminating material by thin-layer chromatography on DC-cellulose and monitored by autoradiography (see below), isolated from the plate together with the adsorbant, and eluted with distilled water. After a final concentration in vacuo, 1 ml of a 1.7 mM solution of [¹⁴C]sucrose-6-phosphate was recovered, corresponding to 2% final yield. Throughout the procedure, sucrose-6-phosphate was monitored by determining the radioactivity.

Enzyme assays. Sucrose-6-phosphate hydrolase activity was determined in bacterial cells exponentially grown in minimal glycerol medium with or without inducer (0.2%) treated with toluene (2 drops/5 × 10⁸ cells at 25°C for 10 min). Alternatively, crude extracts from cell suspensions passed through a French pressure cell at 15,000 lb/in² and freed from debris by centrifugation (37,000 × *g* for 15 min) were used. Suspensions in 100 mM phosphate buffer, pH 6.6, were incubated with 50 mM sucrose at 25°C for 30 min. Enzymatic cleavage of sucrose was stopped by heating the reaction mixture at 100°C for 1 min. After removal of denatured protein by centrifugation, the liberated glucose was determined in 0.1-ml samples by using a commercially available test solution (Merck) containing glucose dehydrogenase (5.2 U/ml), mutarotase (0.11 U/ml), and NAD (1.1 mM) in phosphate buffer (12 mM, pH 7.6) and following the extinction at 365 nm. The reaction was complete after 20 min at 25°C. Activities of sucrose-6-phosphate hydrolase are expressed as nanomoles of glucose (or glucose-6-phosphate) per minute per milligram of protein (1 mg of protein corresponds to 4 × 10⁹ cells).

The hydrolysis of sucrose-6-phosphate was determined by the amount of glucose-6-phosphate released, using the glucose-6-phosphate dehydrogenase- and NADP-coupled assay (37). Contaminating glucose-6-

TABLE 1. Bacterial strains and plasmids

Strain	Plasmid	Plasmid markers ^a	Chromosomal markers	Source
WR3026 <i>Scr</i> ⁺	pUR400	<i>Scr</i> ⁺ <i>Tc</i> ^r	<i>lac thi his ile val met arg rpsL</i>	L. S. Baron (41)
K-12 CA8404 L191			Wild type <i>cya crp-45 rpsL</i> <i>lacY galT, K malA</i> <i>mil(PO)50</i> <i>gut(PO)50 gat(PO)50 thi</i> <i>argG his metB ptsI, H</i> <i>rpsL</i>	E. Lederberg 28; obtained from J. Lengeler J. Lengeler
DS402	pUR400	<i>Scr</i> ⁺ <i>Tc</i> ^r	Prototroph	WR3026 <i>Scr</i> ⁺ × K-12
DS402-1	pUR400	<i>Scr</i> ⁺ <i>Tc</i> ^r	<i>crp-45 rpsL</i>	P1 transduction of <i>crp-45 rpsL</i> from CA8404 into DS402
DS405	pUR401	<i>scrA</i> (EII ^{scr}) <i>Tc</i> ^r	Prototroph	This paper
DS409	pUR404	<i>scrR</i> (<i>Scr</i> ^{const}) <i>Tc</i> ^r	Prototroph	This paper
DS415	pUR400	<i>Scr</i> ⁺ <i>Tc</i> ^r	<i>ptsI, H rpsL</i>	DS402 × L191
DS420	pUR406	<i>scrB</i> (<i>Hyd</i> ⁻) <i>Tc</i> ^r	Prototroph	This paper

^a The symbol *scr* stands for plasmid-encoded mutant loci that control the utilization of sucrose. "Scr" and "Hyd" denote phenotypic traits concerning the ability to utilize sucrose and to synthesize hydrolase, respectively; "const" denotes constitutive expression of sucrose functions, and "Tc" denotes tetracycline resistance. Other genetic symbols are those of Bachmann and Low (2).

phosphate (approximately 4% in our preparation) was consumed by preincubation of the reagents before the reaction was initiated by adding the extract containing sucrose-6-phosphate hydrolase. Glucose-6-phosphate was assayed in 100 mM Tris, pH 7.6, by incubation at 20°C with 1 U of glucose-6-phosphate dehydrogenase (Sigma) in the presence of 5 mM NADP. The amount of NADPH generated by the reduction of glucose-6-phosphate was determined by the extinction at 340 nm.

Transport assay. The substrate, [U - ^{14}C]sucrose, was freed from contaminating sugars by gel filtration on Sephadex G-15, and its purity was tested by thin-layer chromatography on DC-cellulose. Bacteria were grown at 37°C in minimal medium supplemented with 0.2% of the carbon source indicated to a density of 1.5×10^9 cells/ml. Mutants unable to utilize the inducing sugar as carbon source were grown in Luria broth (10 g of tryptone, 5 g of yeast extract, and 5 g of sodium chloride per liter). Cells were harvested at room temperature, washed three times, and suspended in 100 mM phosphate buffer, pH 6.6, to a final density of 5×10^8 cells/ml. Routinely, 0.6 ml of cell suspension was added to 100 μ l of [^{14}C]sucrose, rapidly mixed, and incubated at 25°C. Samples (0.2 ml) were taken after 20, 40, and 60 s, collected on cellulose nitrate disks (0.6- μ m pore size; Sartorius, Göttingen) by filtration, and washed twice with 1 ml of buffer. Filters were dried at 80°C for 30 min, and their radioactivity was determined in a Beckman LS-335 scintillation counter, using 5 ml of PermaBlend II (Packard Instruments GmbH, Frankfurt). The nonspecific adsorption of radioactivity was determined by using a plasmid-free strain deficient in sucrose transport. Transport activities are expressed in nanomoles per minute per milligram of protein.

In vitro phosphorylation of [^{14}C]sucrose by EII scr . The preparation of cell-free extracts and the assay of sucrose-specific enzyme II (EII scr) were essentially conducted as described by Lengeler and Lin (15). Extracts of wild-type *E. coli* K-12 served as the source of PEP-dependent sugar:phosphotransferase system (PTS) enzymes I (EI) and HPr. Extracts containing membrane vesicles of fructose-induced cells of DS420 provided the specific EII scr . Extracts (20 to 30 mg of protein/ml) were obtained by sonication (3 min at 75 W, 4°C; Branson Sonifier; Branson Instruments Co., Stamford, Conn.) and subsequent centrifugation ($37,000 \times g$ for 20 min). The reaction mixture consisted of 20 μ l of PEP (50 mM), 20 μ l of $MgCl_2$ (0.5 mM), 40 μ l of Tris (100 mM, pH 7.6), 100 μ l of *E. coli* K-12 extract, and 20 μ l of [^{14}C]sucrose (4.3 mCi/mmol; 0.36 mM); 20 μ l of strain DS420 extract was added to start the reaction (25°C). After 1, 5, and 10 min, 50- μ l samples were transferred to DEAE filters (DE81; Whatman), placed into 80% ethanol for 10 min, and washed three times with distilled water. Filters were dried at 80°C for 30 min, and the radioactivity retained was determined.

Thin-layer chromatography. Ten microliters of ^{14}C -labeled carbohydrate was separated by cellulose thin-layer chromatography (DC-cellulose; Merck), using a mixture of *n*-butanol, acetic acid, and water (5:3:2). Plates were dried, and autoradiographs were taken in a spark chamber (Beta-Kamera LB290B; Berthold, Wildbad).

RESULTS

General properties of the pUR400-encoded sucrose system. The conjugative sucrose plasmid pUR400 enables cells of *E. coli* K-12 to utilize sucrose as the sole carbon source (41). In addition to the *scr*-specific genetic loci, we have detected a plasmid-borne tetracycline resistance determinant. Preliminary experiments indicated that at least two inducible plasmid-encoded functions were involved in sucrose metabolism: (i) a sucrose-specific transport system; and (ii) a hydrolyzing enzyme, β -D-fructofuranoside fructohydrolase (EC 3.2.1.26), referred to as sucrose-6-phosphate hydrolase (3, 37). These functions were not detectable in the plasmid-free *E. coli* K-12. The plasmid-containing derivative DS402(pUR400) expressed low levels of sucrose transport and sucrose-6-phosphate hydrolase if grown on glycerol (no induction). If grown on inducing substrates such as fructose, sucrose, or raffinose, both sucrose transport and hydrolase activities increased up to 70-fold (Table 2). Induction by raffinose was dependent on a fully induced or constitutively expressed *lac* operon, indicating that raffinose required *lacY*-encoded transport to enter the cells (S. Schaeffler, *Bacteriol. Proc.*, 1967, p. 54; 28). Glucose, galactose, lactose, or melibiose was not an inducer of sucrose metabolic enzymes. The simultaneous induction of sucrose transport and sucrose-6-phosphate hydrolase by three different carbohydrates suggested that the two functions were under common genetic control. This notion was supported by the properties of a constitutive mutant (DS409), which expressed high-level activities of sucrose transport and hydrolase without induction (Table 2). In sucrose-induced cells of DS402, the expression of both sucrose transport and hydrolase was about 12-fold reduced if glucose had been added to the growth medium. This together with the observation that addition of cAMP to the culture led to maximal expression suggested that the sucrose system was also subject to catabolite repression (20).

Isolation and analysis of *scr* mutants. Screening of mutagenized and streptozotocin-treated cultures of DS402(pUR400) on MacConkey sucrose plates produced two classes of mutants unable to metabolize sucrose. One class, DS405(pUR401), was deficient in sucrose transport (*scrA*), and the other class, DS420(pUR406), was deficient in sucrose-6-phosphate hydrolase (*scrB*). The mutational blocks were identified by determining sucrose transport and sucrose-6-phosphate hydrolase activities in induced and noninduced cells (Table 2).

The glucose effect exerted on sucrose utilization by DS402 (Table 2) was used to select constitutive mutants. Since systems sensitive to

catabolite repression depend on cAMP and cAMP receptor protein (CRP) for full expression (23, 42), a deficiency in either of these compounds leads to a negative phenotype. If *crp-45*, a deletion in the structural gene of CRP (27) and cotransducible with streptomycin resistance (2), was transferred into DS402 by P1 transduction, *Scr⁻* streptomycin-resistant transductants were isolated, which formed pale colonies on Mac-

Conkey sucrose plates. Spontaneous revertants detectable by their pink color were isolated after 3 days at 37°C. When their plasmids were transferred to the *crp⁺* host strain K-12, resulting transconjugants [e.g., DS409(pUR404)] showed high-level constitutive expression of sucrose transport and sucrose-6-phosphate hydrolase. Their expression was still subject to catabolite repression by sucrose and glucose, which was relieved by cAMP (Table 2), indicating that the mutation leading to constitutivity did not produce a CRP-independent *scr* promoter (5). Conceivably, the mutant locus resides in a plasmid-borne regulatory gene, designated *scrR*.

An analysis of *scrA* and *scrB* mutants revealed certain features of the sucrose system concerning inducer and substrate specificities. In the hydrolase-deficient strain DS420, sucrose transport was inducible by fructose, but not by sucrose and raffinose, suggesting that fructose or a derivative (fructose phosphate?) was the actual inducer of the *scr* genes. Hence, sucrose and raffinose required hydrolysis by sucrose-6-phosphate hydrolase to become inducers. In the transport mutant DS405, hydrolase was inducible by raffinose and fructose, but not by sucrose. Whereas fructose (6) and raffinose (Schaeffler, *Bacteriol. Proc.*, 1967, p. 54; 28) could enter the cells by host transport systems, sucrose could not. The plasmid-encoded product of *scrA* was the only uptake system specific for sucrose and was therefore absolutely required for the utilization of this sugar by *E. coli* K-12. As shown below, sucrose transport was dependent on the PTS of the host bacterium (8, 11). The *scrA* gene encodes the membrane-bound EII^{scr} of the PTS.

Properties of sucrose transport. Transport activities were determined by the initial rates of uptake, using [¹⁴C]sucrose as the substrate. The transport activity of fructose-induced DS402 (pUR400) was 0.4 nmol/min per mg of protein (Fig. 1A); it was low in uninduced DS402, and it was not detectable in the plasmid-free *E. coli* K-12. The constitutive mutant DS409 exhibited a threefold higher initial uptake (Fig. 1B) than induced cells of DS402 (Fig. 1A). However, the rate of sucrose accumulation decreased rapidly in the former and reached a plateau after 60 s. At this level, the influx of [¹⁴C]sucrose and the efflux of ¹⁴C-labeled metabolites are thought to be at equilibrium, an interpretation supported by the hydrolase-deficient mutant DS420 showing linear uptake kinetics for at least 80 s (Fig. 1B). Linearity was extended in DS420 to several minutes by increasing the external substrate concentration (data not shown), which indicated that substrate was continuously accumulated (in the form of sucrose phosphate), but could not be released from a strain unable to further metabo-

TABLE 2. Characterization of *scr* mutants by activities of sucrose transport and sucrose-6-phosphate hydrolase

Strain ^a	Inducing substrate (0.2%)	Uptake of sucrose ^b (pmol/min per mg of protein)	Sucrose-6-phosphate hydrolase (nmol/min per mg of protein)
K-12	None	<1	<1
	Fructose	<1	<1
DS402 (<i>scr⁺</i>)	None	5	4
	Fructose	320	140
	Sucrose	280	190
	Sucrose + glucose	24	14
	Sucrose + cAMP (6 mM)	450	360
	Raffinose ^c	380	240
DS405 (<i>scrA</i>)	None	<1	6
	Fructose	<1	170
	Sucrose	<1	10
	Raffinose ^c	<1	120
DS420 (<i>scrB</i>)	None	5	<1
	Fructose	360	<1
	Sucrose	4	<1
	Raffinose ^c	5	<1
DS409 (<i>scrR</i>)	None	1,230	620
	Sucrose	250	90
	Glucose	220	90
	Sucrose + cAMP (6 mM)	1,100	500
DS415 (<i>ptsI, H</i>)	None	<1	<1
	Fructose	<1	20
	Fructose + cAMP (6 mM)	<1	150

^a Cells were grown at 37°C in minimal medium (30) containing 0.2% glycerol (no inducer). Cells of the *pts* mutant DS415 were grown in Luria broth. Inducers and cAMP were added as indicated. Activities were assayed in exponentially growing cells at maximal induction. Values represent averages of three experiments, the deviations being ±50%.

^b Uptake was determined at 60 nM [¹⁴C]sucrose (673 mCi/mmol).

^c Raffinose enters the cells via *lacY*-encoded permease fully induced by 100 μM isopropylthiogalactoside.

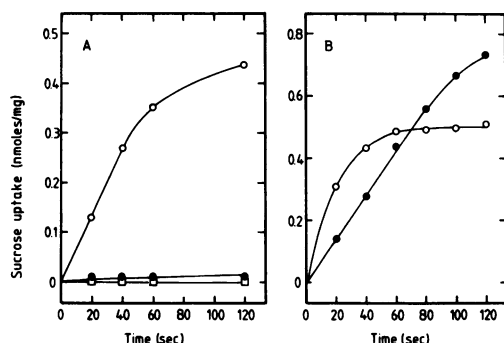


FIG. 1. Sucrose uptake by the plasmid-encoded sucrose transport system. [¹⁴C]sucrose (673 mCi/mmol; 0.130 μ M) was used. (A) Sucrose uptake by *E. coli* K-12 (Scr⁻) grown on 0.2% fructose (\square) and by DS402 (Scr⁺) grown on 0.2% glycerol (\bullet ; no induction) or 0.2% fructose (\circ ; induction). (B) Sucrose uptake by DS409 (scrR) grown on 0.2% glycerol (\circ) and by DS420 (scrB) grown on 0.2% fructose (\bullet).

lize it. Assuming a cell water volume of 1.3×10^{-12} ml per cell (40), we calculated that the intracellular substrate concentration of mutant strain DS420 was 700-fold above the external concentration after 1 min (Fig. 1B).

Sucrose was the preferred substrate of the sucrose transport system. Glucose or fructose at a 10 mM concentration (1,000-fold excess) led to only 50% inhibition of sucrose uptake. The high concentrations of inhibitor needed suggested that both glucose and fructose were poor substrates. No uptake of [³H]raffinose, [³H]melibiose, and [¹⁴C]lactose could be detected in appropriate mutant hosts of pUR404 lacking melibiose (30) and lactose (9) permease (data not shown).

Sucrose transport is PTS dependent. When pUR400 was conjugally transferred into the *pts* mutant L191 (by selection for plasmid-borne tetracycline resistance), the resulting transconjugant DS415(pUR400) had a Scr⁻ phenotype. Upon induction with fructose, the strain showed no detectable sucrose uptake, but sucrose-6-phosphate hydrolase activity. The latter was about sevenfold reduced relative to the fructose-induced wild-type DS402 (Table 2). The low hydrolase activity in DS415 has been ascribed to reduced levels of cAMP observed in PTS⁻ strains (22, 24). The inhibition of plasmid *scrB* gene expression owing to catabolite repression (23, 42) was released by the addition of 6 mM cAMP to growing cells of DS415, which increased the hydrolase activity nearly to the level of wild-type cells (Table 2). However, cAMP did not at all stimulate sucrose uptake, suggesting that an intact PTS was required for the transport of sucrose.

The presence of an intact *scrA* gene in the *pts* mutant DS415 has been demonstrated by outcrossing and expression in a PTS⁺ strain. Expression of the *scrA* gene in the PTS⁻ background of strain L191 containing pUR404 (*scrR*) grown in Luria broth was shown after *in vitro* reconstitution (15) of membrane vesicles of this strain with cell extracts of *E. coli* K-12 containing the PTS proteins (Lengeler, personal communication). It has been concluded that the plasmid-borne *scrA* gene encodes a membrane-bound EII^{scr} acting in concert with the soluble PTS functions, EI and HPr, provided by the host (11). As shown in the accompanying paper (16), sucrose uptake is exceptional in requiring an additional host function, termed enzyme III^{glc,scr} (EIII^{glc,scr}). Since PTS-mediated transport involves vectorial phosphorylation (8, 11), sucrose uptake should lead to the accumulation of sucrose phosphate.

***In vitro* phosphorylation of sucrose.** Substrate-specific phosphorylation can be assayed *in vitro* if cell extracts containing the soluble components (EI and HPr) and membrane vesicles containing the specific transport protein (EII) are used (15). Typically, this phosphorylation is dependent on PEP. The phosphorylation of sucrose in extracts of *E. coli* K-12 (containing excess EI, HPr, and EIII^{glc,scr}; 15) mixed with membrane vesicles of fructose-induced DS420 (containing EII^{scr}) strictly depended on the presence of PEP in the mixture (Fig. 2). PEP could not be replaced by ATP.

Sucrose was phosphorylated by the system with a K_m of $12 \pm 5 \mu$ M (Fig. 3). This value compares with an apparent $K_{m,scr}$ of $10 \pm 3 \mu$ M of *in vivo* transport (Fig. 3), calculated from the initial rate of sucrose uptake by fructose-induced cells of DS420 (Fig. 1A). This close similarity of values suggested that we did, in fact, measure the same reaction *in vivo* and *in vitro*. The observed specificities of *in vitro* phosphorylation corroborated this statement: raffinose, melibiose, lactose, and lactulose (4-O- β -D-galactosyl-D-fructose) did not inhibit the reaction, and glucose and fructose led to 40% inhibition if added at a 10 mM concentration (1,000-fold excess). Differences in V_{max} values (for transport, $V_{max} = 100$ nmol/min per mg; for phosphorylation, $V_{max} = 0.5$ nmol/min per mg; Fig. 3) were ascribed to the low activity of the *in vitro* system. In our hands, the latter could not be improved by other methods of cell extraction such as toluene treatment (7). Conceivably, EII^{scr} itself or its interaction with host functions was especially sensitive to extraction, resulting in the low specific activity observed *in vitro*.

Identification of sucrose-6-phosphate as the primary metabolite accumulated by Scr⁺ cells. Fructose-induced cells of the *scrB* mutant DS420

were incubated with [U - ^{14}C]sucrose and subsequently extracted with boiling water (see Materials and Methods). Thin-layer chromatography of extracts revealed a slowly migrating radioactive compound different from sucrose (not shown). The new compound was isolated from plates and, upon hydrolysis by sucrose-6-phosphate hydrolase (from DS409) or boiling hydrochloric acid, produced two species comigrating with fructose and glucose-6-phosphate, respectively, in cellulose thin-layer chromatography (Fig. 4). The putative glucose-6-phosphate was isolated from the chromatogram, and its chemical nature was verified by the reaction with glucose-6-phosphate dehydrogenase. Based on the specific radioactivity of the substrate and the turnover of NADP, more than 90% (9.6 nmol) of the isolated compound had been converted (to 6-phosphogluconate) after 15 min by the highly specific enzyme reaction. From the assignment of glucose-6-phosphate, we deduced that the immediate product of PTS-dependent sucrose uptake was sucrose-6-phosphate phosphorylated at glucose- C_6 .

Sucrose-6-phosphate hydrolase: enzymatic activity, substrate specificity, and molecular weight. The enzyme hydrolyzing sucrose-6-phosphate is located intracellularly; no activity was found in the membrane fraction or in periplasmic cold-shock preparations. The hydrolysis of sucrose-

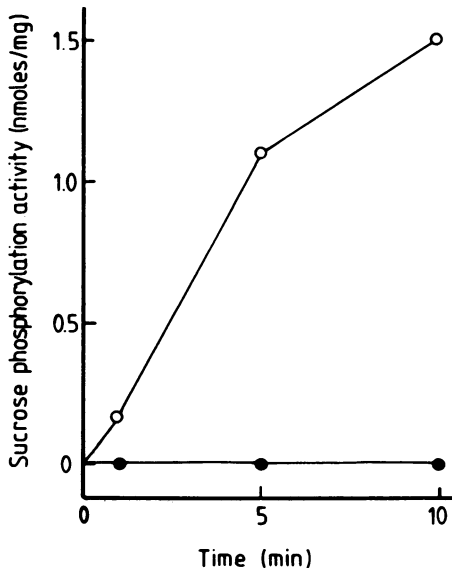


FIG. 2. Sucrose phosphorylation in vitro and dependence on PEP. Soluble extract containing EI and HPr from strain K-12 and membranes of DS420 were dialyzed against 100 mM Tris, pH 7.0, before the assay (to remove PEP and ATP; see Materials and Methods). Addition of 2.5 mM ATP (●) or 2.5 mM PEP (○) to the reaction mixture.

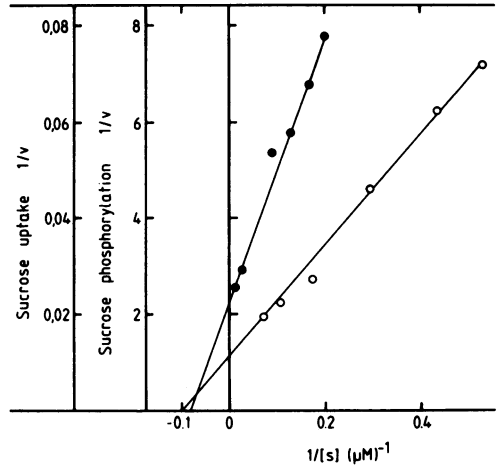


FIG. 3. Lineweaver-Burk plot of initial velocities of sucrose uptake in vivo (○) and sucrose phosphorylation in vitro (●). To cells of DS420 grown on 0.2% fructose, [^{14}C]sucrose was added at concentrations ranging between 1.9 and 14 μM , and samples were taken 10 s after the addition of substrate. The phosphorylation of [^{14}C]sucrose was assayed at substrate concentrations from 5 to 100 μM , and the reaction was stopped after 3 min. $1/v$ is expressed in nanomoles per minute per milligram of protein.

6-phosphate and of sucrose was optimal in 100 mM phosphate at pH 6.6. The enzyme was stable for at least 2 h at 37°C or for 3 days at 4°C. Enzyme preparations showed a rapid loss of activity at low ionic strength. After 24 h of dialysis against 10 mM phosphate buffer, less than 1% of the initial activity was recovered.

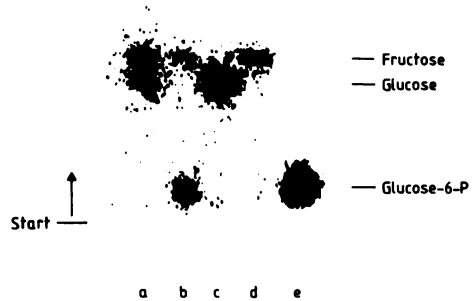


FIG. 4. Autoradiogram of ^{14}C -labeled sugars separated on a thin-layer cellulose plate. The original product of sucrose transport was extracted from fructose-induced cells of DS420 incubated with [^{14}C]sucrose (see Materials and Methods). Before chromatography, all samples were boiled in 0.1 N HCl for 10 min (to hydrolyze disaccharides) and then neutralized with NaOH. Lane a, Acid-hydrolyzed [^{14}C]sucrose; lane b, acid-hydrolyzed PTS reaction product; lane c, [^{14}C]glucose; lane d, [^{14}C]fructose; lane e, [^{14}C]glucose-6-phosphate.

Additions such as dithiothreitol, metal ions, glycerol, or sucrose did not significantly stabilize the enzyme. Heavy losses in enzymatic activity occurred when purification by conventional methods was attempted. Therefore, cell-free extracts of *E. coli* DS409 were routinely used without further fractionation to assay the enzyme.

Sucrose-6-phosphate hydrolase catalyzed the hydrolysis of β -D-fructosides, such as sucrose-6-phosphate, sucrose, and raffinose. The Michaelis constants for these substrates were 0.17 ± 0.05 , 60 ± 10 , and 150 ± 10 mM, respectively. The results suggested that sucrose-6-phosphate was the essential substrate of the hydrolyzing enzyme. α -D-Glucosides such as *p*-nitrophenyl- α -D-glucoside and galactosides such as melibiose and lactose were not hydrolyzed. This specificity classifies the enzyme as a β -D-fructofuranoside fructohydrolase (EC 3.2.1.26).

Enzymatically active sucrose-6-phosphate hydrolase had an M_r of approximately 110,000 as determined by gel filtration on Sephadex G-200, using ovalbumin, aldolase, and catalase for calibration (1; data not shown). A partially purified enzyme preparation (using ammonium sulfate precipitation and fractionation on octyl-Sepharose CL-4B and on hydroxyapatite) was analyzed by sodium dodecyl sulfate-gel electrophoresis, using trypsin inhibitor, bovine serum albumin, and RNA polymerase for calibration (32). The mobility of the major protein band corresponded to M_r 55,000 (data not shown), suggesting that the active enzyme was a 110,000-dalton dimer consisting of identical subunits.

DISCUSSION

Plasmid pUR400 mediates the "peripheral" metabolism of sucrose in *E. coli* K-12 by furnishing the sucrose-specific, membrane-bound compound EII^{scr} of the PTS and the hydrolyzing enzyme sucrose-6-phosphate hydrolase. The host contributes the general PTS proteins, including EI and HPr (8, 11), and, as shown in the accompanying paper (16), an additional factor, $EIII^{glc,scr}$, which is required for the activity of the sucrose transport system. Figure 5 illustrates the interplay of plasmid- and chromosome-encoded functions as currently understood. This concept is a result of the genetic and biochemical studies presented in this paper.

The analysis of *scr* mutants revealed the existence of at least three plasmid-encoded genetic loci distinguished by the biochemical alterations they produced. These pertain to sucrose transport (*scrA*, strain DS405), sucrose-6-phosphate hydrolase (*scrB*, strain DS420), and constitutive expression of the two functions (*scrR*, strain DS409). Regulatory mutants such as DS409 suggest that the *scr* functions are under common

genetic control, a notion also supported by the simultaneous induction of sucrose transport and sucrose-6-phosphate hydrolase by fructose, sucrose, and raffinose (DS402; Table 2). Results obtained with the hydrolase-deficient mutant DS420 revealed that neither sucrose nor raffinose, but only fructose was capable of inducing the *scr* system.

This is reminiscent of the *lac* system, which requires the conversion of lactose into allolactose by β -galactosidase for induction (10). The mechanism may ensure that the disaccharide can be broken down by the cell before wasting energy for its uptake. With the available data we cannot determine whether fructose or a metabolite of fructose functions as the endogenous inducer. Externally supplied fructose is translocated (predominantly) via two fructose PTSs as fructose-1-phosphate and fructose-6-phosphate, whereas the primary intracellular product of sucrose-6-phosphate hydrolysis is fructose (Fig. 5), which is converted to fructose-6-phosphate by an ATP-dependent kinase (6). In an analogy to the sucrose system, Lengeler and Steinberger (17) observed that the PTS-dependent mannitol and glucitol systems were inducible by the intracellular unphosphorylated substrates. These authors favor a model in which the unphosphorylated EII complex of the PTS functions as a bidirectional carrier for the free substrate, whereas the phosphorylated complex catalyzes vectorial phosphorylation. This concept allows for minor intracellular concentrations of unphosphorylated substrate sufficient for induction. It also accounts for our observation that the *pts* mutant DS415 was inducible by fructose (Table 2). The nature of the endogenous inducer of the

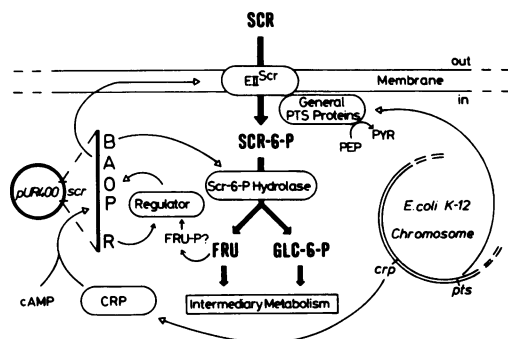


FIG. 5. Proposed gene-enzyme relationships in the sucrose metabolism of *E. coli* DS402. Plasmid pUR400 (left) and a portion of the host chromosome (right) with relevant genetic loci are shown together with plasmid-mediated steps in sucrose metabolism (SCR-6-P, sucrose-6-phosphate; GLC-6-P, glucose-6-phosphate; FRU, fructose; FRU-P, fructose phosphate). General PTS proteins refer to EI, HPr, and $EIII^{glc,scr}$ (16).

sucrose system can unequivocally be determined in an in vitro system, which is currently being developed.

Our results are compatible with a plasmid-encoded "scr operon" or "scr regulon" controlling the synthesis of the specific transport component EII^{scr} and of sucrose-6-phosphate hydrolase. Although none of the mutants has been shown to lie in the structural genes, newly isolated polar insertions are in favor of an scr operon with the gene order *scr(O,P) scrA scrB* (K. Schmid, J. Altenbuchner, and R. Schmitt, to be published). The data available are not sufficient for clearly defining the nature of the regulatory mutant DS409. Since it was isolated as a Scr^+ revertant from a *crp*⁻ background, it was expected to overcome catabolite repression. However, the constitutive expression of sucrose transport and sucrose-6-phosphate hydrolase (in the *crp*⁺ background of DS415) was sevenfold decreased by sucrose or glucose, and the repression was released by cAMP (Table 2). Instead of the expected CRP-independent promoter mutation (5, 42), the isolated sucrose-constitutive mutant DS409 (pUR404) is likely to represent a regulatory gene mutation, such as the *araC*^C (activator gene constitutive) mutation of the arabinose system (33) or the *lacI* (repressor negative) mutation of the lactose system (9). Preliminary data on a newly isolated sucrose-constitutive mutant obtained after transposon mutagenesis favor negative control exerted by the product of *scrR* on the expression of *scr* structural genes (Schmid et al., to be published). The current concept, as illustrated in Fig. 5, involves a twofold control by (i) the *scrR* product interacting with the *scr* operator and with fructose or a derivative (inducer) and (ii) catabolite activation by the cAMP-CRP complex interacting with the *scr* promoter (5, 42). This model is presently being tested and refined by experiments that include transposon mutagenesis, physical mapping, and complementation.

PEP-dependent EII^{scr} activity was demonstrated by in vitro reconstitution (Fig. 2) and by the inability of a *pts* mutant to transport sucrose (Table 2). The product of vectorial phosphorylation was isolated and characterized as sucrose-6-phosphate by hydrolysis into fructose and glucose-6-phosphate (Fig. 4). The hydrolyzing enzyme, sucrose-6-phosphate hydrolase, is a 110,000-dalton dimer which in molecular size and substrate specificity closely resembles an "invertase" encoded by the plasmid-borne raffinose operon (31). The possible relationship between these hydrolases from different genetic systems will be analyzed by serology and DNA hybridization.

Known PTS sugars of *E. coli* were previously

restricted to monosaccharides and polyols (17). The *scr* system is novel in that it represents the first PTS-dependent disaccharide transport linked to a plasmid. Other possible candidates for PTS-dependent disaccharide transport in *E. coli* are the *bgl* system, which controls the uptake and metabolism of β -glucosides such as arbutin, salicin (25), and possibly cellobiose, and the trehalose system mentioned by Lengeler et al. (16). It is conceivable that the *scr* genetic system of pUR400 originated from another bacterial species. Similar routes of sucrose metabolism have been described in the gram-positive bacteria *Bacillus subtilis* (18), *Streptococcus lactis* (12, 38), and *Streptococcus mutans* (3, 34, 37). Though related in the overall reaction scheme and in the specificity of their sucrose functions, these systems are inducible by sucrose (and some analogs), but not by fructose. Moreover, the molecular weights reported for the enzymes hydrolyzing sucrose-6-phosphate in *S. lactis* (M_r 28,000; 38) and *S. mutans* (M_r 42,000; 3) are considerably smaller than M_r 110,000 found for the pUR400-encoded dimeric enzyme. A sucrose metabolic system similar to the one described here has been recently identified in the gram-negative bacterium *Klebsiella pneumoniae* (16), which therefore may be considered a potential ancestor of the pUR400-encoded sucrose functions. The possible origin of the *scr* genes and relationships among various sucrose plasmids could be determined either by Southern hybridization (36) or by the immunological cross-reaction of their hydrolases. The latter technique has been successfully applied to establish relationships among the raffinose genes of 39 plasmids (28). Although various sucrose plasmids differ in size, transmissibility, and incompatibility (13, 35, 41), their *scr* determinants may well be related. Experiments are in progress to investigate whether inverted repeats or insertion elements flanking the *scr* genes may be responsible for their spread between plasmids and chromosomes, as has been shown for plasmid-borne lactose (4) and raffinose genes (31).

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LITERATURE CITED

1. Andrews, P. 1964. Estimation of the molecular weight of proteins by Sephadex gel-filtration. *Biochem. J.* **91**:222-233.
2. Bachmann, B. J., and K. B. Low. 1980. Linkage map of *Escherichia coli* K-12, edition 6. *Microbiol. Rev.* **44**:1-56.
3. Chassy, B. M., and E. V. Porter. 1979. Initial characterization of sucrose-6-phosphate hydrolase from *Streptococcus mutans* and its apparent identity with intracellular invertase. *Biochem. Biophys. Res. Commun.* **89**:307-314.

4. Cornelis, G., H. Sommer, and H. Saedler. 1981. Transposon Tn951 (TnLac) is defective and related to Tn3. *Mol. Gen. Genet.* 184:241-248.
5. Dickson, R. C., J. Abelson, W. M. Barnes, and W. S. Reznikoff. 1975. Genetic regulation: the *lac* control region. *Science* 187:27-35.
6. Ferenci, T., and H. L. Kornberg. 1974. The role of phosphotransferase-mediated synthesis of fructose-1-phosphate and fructose-6-phosphate in the growth of *Escherichia coli* on fructose. *Proc. R. Soc. London Sev. B* 187:105-119.
7. Gachelin, G. 1970. Studies on the α -methylglucoside permease of *Escherichia coli*. *Eur. J. Biochem.* 16:342-357.
8. Hays, J. B. 1978. Group translocation transport systems, p. 43-102. In B. P. Rosen (ed.) *Bacterial transport*. Marcel Dekker, New York.
9. Jacob, F., and J. Monod. 1961. Genetic regulator mechanisms in the synthesis of proteins. *J. Mol. Biol.* 3:318-356.
10. Jobe, A., and S. Bourgeois. 1972. *lac* repressor-operator interaction. VI. The natural inducer of the *lac*-operon. *J. Mol. Biol.* 69:397-408.
11. Kundig, W., S. Ghosh, and S. Roseman. 1964. Phosphate bound to histidine in a protein as an intermediate in a novel phosphotransferase system. *Proc. Natl. Acad. Sci. U.S.A.* 52:1067-1074.
12. LeBlanc, D. J., V. L. Crow, and L. N. Lee. 1980. Plasmid mediated carbohydrate catabolic enzymes among strains of *Streptococcus lactis*, p. 31-41. In C. Stuttard and K. R. Rooze (ed.), *Plasmids and transposons—environmental effects and maintenance metabolism*. Academic Press, Inc., New York.
13. LeMinor, L., C. Coynault, R. Rhode, B. Rowe, and S. Aleksic. 1973. Localisation plasmidique du déterminant génétique du caractère atypique 'saccharose⁺⁺' des *Salmonella*. *Ann. Microbiol. (Inst. Pasteur)* 124B:295-306.
14. Lengeler, J. 1979. Streptozotocin, an antibiotic superior to penicillin in the selection of rare bacterial mutations. *FEMS Microbiol. Lett.* 5:417-419.
15. Lengeler, J., and E. C. C. Lin. 1972. Reversal of the mannitol-sorbitol diauxie in *Escherichia coli*. *J. Bacteriol.* 112:840-848.
16. Lengeler, J., R. Mayer, and K. Schmid. 1982. Phosphoenol-pyruvate-dependent phosphotransferase system enzyme III and the plasmid-encoded sucrose transport in *Escherichia coli* K-12. *J. Bacteriol.* 151:468-471.
17. Lengeler, J., and H. Steinberger. 1978. Analysis of the regulatory mechanisms controlling the synthesis of the hexitol transport systems in *Escherichia coli* K12. *Mol. Gen. Genet.* 164:163-169.
18. Lepesant, J.-A., F. Kunst, M. Pascal, J. Kejzlarova-Lepesant, M. Steinmetz, and R. Dedonder. 1976. Specific and pleiotropic regulatory mechanisms in the sucrose systems of *Bacillus subtilis* 168, p. 58-69. In D. Schlessinger (ed.), *Microbiology—1976*. American Society for Microbiology, Washington, D.C.
19. MacConkey, A. 1905. Lactose-fermenting bacteria in faeces. *J. Hyg.* 8:333-379.
20. Magasanik, B. 1970. Glucose effects: inducer exclusion and repression, p. 189-219. In J. R. Beckwith and D. Zipser (ed.), *The Lactose operon*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
21. Novick, R. P., R. C. Clowes, S. N. Cohen, R. Curtiss III, N. Datta, and S. Falkow. 1976. Uniform nomenclature for bacterial plasmids: a proposal. *Bacteriol. Rev.* 40:168-189.
22. Pastan, I., and R. L. Perlman. 1969. Repression of β -galactosidase synthesis by glucose in phosphotransferase mutants of *Escherichia coli*. *J. Biol. Chem.* 244:5836-5842.
23. Perlman, R., and I. Pastan. 1968. Cyclic 3'5'-AMP: stimulation of β -galactosidase and tryptophanase induction in *E. coli*. *Biochem. Biophys. Res. Commun.* 30:656-664.
24. Peterkofsky, A., and C. Gazdar. 1978. The *Escherichia coli* adenylate cyclase complex: activation by phosphoenolpyruvate. *J. Supramol. Struct.* 6:495-502.
25. Prasad, I., and S. Schaefer. 1974. Regulation of the β -glucoside system in *Escherichia coli* K-12. *J. Bacteriol.* 120:638-650.
26. Rothman, J. L. 1965. Transduction studies on the relation between prophage and host chromosome. *J. Mol. Biol.* 12:892-912.
27. Sabourin, D., and J. Beckwith. 1975. Deletion of the *Escherichia coli* *crp* gene. *J. Bacteriol.* 122:338-340.
28. Schmid, K., R. Ritschewald, and R. Schmitt. 1979. Relationships among raffinose plasmids determined by the immunochemical cross-reaction of their α -galactosidases. *J. Gen. Microbiol.* 114:477-481.
29. Schmid, K., and R. Schmitt. 1976. Raffinose metabolism in *Escherichia coli* K12. Purification and properties of a new α -galactosidase specified by a transmissible plasmid. *Eur. J. Biochem.* 67:95-104.
30. Schmitt, R. 1968. Analysis of melibiose mutants deficient in α -galactosidase and thiomethyl-galactoside permease II in *Escherichia coli* K-12. *J. Bacteriol.* 96:462-471.
31. Schmitt, R., R. Mattes, K. Schmid, and J. Altenbuchner. 1979. Raf plasmids in strains of *Escherichia coli* and their possible role in enteropathogeny, p. 199-210. In K. N. Timmis and A. Pühler (ed.), *Plasmids of medical, environmental and commercial importance*. Elsevier/North Holland Biomedical Press, Amsterdam.
32. Shapiro, A. L., E. Vinuela, and J. V. Maizel. 1967. Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrylamide gels. *Biochem. Biophys. Res. Commun.* 28:815-820.
33. Sheppard, D. E., and E. Englesberg. 1967. Further evidence for positive control of the L-arabinose system by gene *arcC*. *J. Mol. Biol.* 21:443-454.
34. Slee A. M., and J. M. Tanzer. 1979. Phosphoenolpyruvate-dependent sucrose phosphotransferase activity in *Streptococcus mutans* NCTC 10449. *Infect. Immun.* 24:821-828.
35. Smith, H. W., and Z. Parsell. 1975. Transmissible substrate-utilizing ability in enterobacteria. *J. Gen. Microbiol.* 87:129-140.
36. Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98:503-517.
37. St. Martin, E. J., and C. L. Wittenberger. 1979. Regulation and function of sucrose-6-phosphate hydrolase in *Streptococcus mutans*. *Infect. Immun.* 26:487-491.
38. Thompson, J., and B. M. Chassy. 1981. Uptake and metabolism of sucrose by *Streptococcus lactis*. *J. Bacteriol.* 147:543-551.
39. Wiebauer, K., S. Schraml, S. W. Shales, and R. Schmitt. 1981. Tetracycline resistance transposon Tn1721: *recA*-dependent gene amplification and expression of tetracycline resistance. *J. Bacteriol.* 147:851-859.
40. Winkler, H. H., and T. H. Wilson. 1966. The role of energy coupling in the transport of β -galactosides by *Escherichia coli*. *J. Biol. Chem.* 241:2200-2211.
41. Wohlhüter, J. A., J. R. Lazere, N. J. Snellings, E. M. Johnson, R. M. Syrenki, and L. S. Baron. 1975. Characterization of transmissible genetic elements from sucrose-fermenting *Salmonella* strains. *J. Bacteriol.* 122:401-406.
42. Zubay, G., D. Schwartz, and J. Beckwith. 1970. Mechanism of activation of catabolite-sensitive genes: a positive control system. *Proc. Natl. Acad. Sci. U.S.A.* 66:104-110.