Phosphotransferase System of *Staphylococcus aureus*: Its Requirement for the Accumulation and Metabolism of Galactosides

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The phosphotransferase system of *Staphylococcus aureus* was characterized. Mutants defective in enzyme I and heat-stable (HPr) protein as well as in the two components specific to lactose accumulation, factor III and enzyme II, were isolated. Colorimetric assays for each of the components are presented based on the formation of *o*-nitrophenyl- β -D-galactoside-6-phosphate by the system and its hydrolysis by the staphylococcal 6-phospho- β -galactosidase. The components were partially purified and their molecular weights were estimated: enzyme I, 100,000 $\pm$ 15%; HPr, 10,000 $\pm$ 15%; factor III, 30,000 $\pm$ 15%; 6-phospho- β -galactosidase, 45,000. Enzyme II is a membrane-bound protein.

The metabolism and accumulation of galactosides in *Staphylococcus aureus* require a phosphoenolpyruvate (PEP)-dependent phosphotransferase system (4, 5, 8, 11, 12, 16) which can be outlined as follows:

HPr is a heat-stable protein; EI (enzyme I) is a soluble protein; both are produced constitutively by the staphylococcal cell. EII (enzyme II) is a membrane-bound component, presumably protein, which is carbohydrate-specific. EII specific for galactosides is produced after induction by growth in galactose or galactose-6-P (15). More recently Simoni et al. (16) described additional components of the staphylococcal PEP-dependent kinase system for both galactosides and mannitol. These components probably act in the second reaction of the above scheme, since both are induced by their respective substrates. The new factor for lactose metabolism, designated FIII lac, is found in the soluble protein fraction of *S. aureus*. Recently, we isolated a mutant which is missing FIII lac (7).

In this paper we describe the partial purification of the staphylococcal EI, HPr, EII lac, and FIII lac. The assay procedures used are colorimetric and dependent upon the ability of the staphylo-

coccal 6-phospho- β -galactosidase to hydrolyze 6-phospho- β -galactosides (4). Omitting the first step, the reaction sequence using *o*-nitrophenyl- β -D-galactopyranoside (ONPG) as substrate is:

By limiting the amount of one of the four components in the phosphorylating system (EI, HPr, EII, FIII) and keeping the others in excess, it is feasible to construct an assay system for any component desired. There are two possibilities to limit the concentration of one component.

(i) Isolate and purify the components of the system and remix them with one component in limiting amount. This prodigious task has been accomplished by Roseman and collaborators for the phosphotransferase system of *Escherichia coli*, an approach which they have also applied to the study of the staphylococcal system (9, 10; Anderson et al., Fed. Proc., p. 643, 1968). The colorimetric procedures described below permit an easy method to analyze the staphylococcal PEP-dependent phosphotransferase system. They should provide a more rapid evaluation of the phosphotransferase system in relation to transport and metabolism. (ii) Isolate bacterial mutants

lacking single components of the phosphotransferase system.

We utilized the second approach and isolated mutants defective in each of the steps of the phosphotransferase system (EI and HPr) and in EII and FIII. With such an approach, the isolation and partial characterization of all the components were accomplished.

MATERIALS AND METHODS

Isolation and characterization of staphylococcal mutants. In isolating mutants in the phosphotransferase system for a specific carbohydrate the components of which are inducible, it is desirable that the other components are synthesized in the absence of an inducer. Therefore, the primary choice is of a bacterial strain with a constitutive metabolism of the particular carbohydrate. The synthesis of EI and HPr has been shown to be constitutive, but the carbohydrate-specific steps of accumulation and hydrolysis are inducible (2, 3). Mutants which are defective in FIII lac and EII lac were derived from a strain producing both constitutively.

Mutations were induced with either ultraviolet (UV) light or with N-methyl-N'-nitro-N-nitrosoguanidine (nitrosoguanidine). The mutants were detected on the dehydrocholate-neutral red medium (C medium; 13). The mutagenic treatment was as follows. (i) Suspensions of staphylococcal cells in phosphate buffer or saline were irradiated to an approximate 0.1% survival (90 sec) with a 15-w General Electric germicidal lamp at a distance of 90 cm. Samples containing approximately 5×10^4 survivors were plated on C medium with selected carbohydrates. On this medium the wild-type cell produces red colonies. White to pink colonies indicate nonfermenters of the respective carbohydrate. After treatment with UV, approximately one colony per 10^8 to 10^4 survivors is mutant. The pleiotropic *car* mutants described earlier were isolated on C medium containing sucrose and fructose. (ii) In more recent experiments, we used nitrosoguanidine as a mutagen, since it produces a much higher yield of mutants to survivors. Cells were treated with nitrosoguanidine (5 μ g/ml, pH 6.3) for 180 min (around 1.0% survival) and plated on C medium. For the isolation of mutants lacking HPr, we used C medium containing lactose and mannitol since both substances must be utilized via the PEP-transferase system. The EII and FIII mutants were isolated on C medium containing lactose and galactose, and the EI mutant was isolated on C medium containing sucrose and fructose or lactose and mannitol. The mutants in the phosphotransferase system are described in Table 1. All were derived from a lactose-constitutive derivative of strain NCTC 8511 (PS 53). All mutant strains are lysogenic for phage 53 of the staphylococcal typing phage system. This phage is UV-inducible in most strains and useful for genetic analysis by transduction (7, 14, 15).

Biochemical characterization of the mutants. For the preliminary identification of mutants, mutants yielding white colonies on C medium containing lactose

TABLE 1. *Mutants of Staphylococcus aureus defective in the phosphotransferase system and in the components specific for the utilization of lactose*

Strain	Phenotype	Factors present ^a				
		EI	HPr	EII lac	FIII lac	6-Phosphogalactosidase
S305A	Wild type	+	+	+	+	+
S306A	Wild type	+	+	+	+	+
S710A	Pleiotropic ^b	0	+	+	+	+
S797A	Pleiotropic	+	0	+	+	+
S797B	Pleiotropic	+	0	+	+	+
S714G	Lac ⁻ Gal ⁻	+	+	+	0	+
S714B	Lac ⁻ Gal ⁻	+	+	0	+	+

^a Factor present, +; factor absent, 0.

^b Pleiotropic = unable to utilize lactose, sucrose, maltose, galactose, mannitol, mannose, fructose, trehalose, and glycerol. Mutants affected in EII lac and FIII lac have identical phenotypes and must be identified by complementation experiments; i.e., washed membranes of S714G complement extracts of S714B, and a soluble component from S714B complements sonic extracts of S714G. All mutations were induced in strains which produced each component constitutively. S797A and S797B are both derived from S306A, a constitutive strain independent of S305A, which is the parent of S710A, S714B, and S714G.

and mannitol were picked, purified by sequential streaking on the same medium, and then grown to saturation in 125 ml of 2% peptone broth. The cells were harvested from these cultures, suspended in 1 ml of 0.1 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 7.6), 0.05 M NaCl, 0.05 M MgCl₂ buffer (optical density = ca. 200, 60 mg., dry weight), and disrupted with 0.5 g of glass beads (Minnesota Mining and Manufacturing Co., Class IV, Type 4000 size) with a Branson Sonifier for 2 min (setting 8). After separation of the beads and debris, the extracts were first tested for phosphogalactosidase activity by the addition of ONPG-6P (6). Extracts of mutants producing phosphogalactosidase constitutively were then supplemented with purified EI to ascertain which mutants were EI-defective. The purified EI was obtained by using an extract of mutant S710A as an assay tool. Extracts of mutants which did not respond to EI were next tested with partially purified HPr which had been purified by using an assay system composed of purified EI, FIII lac, EII, and the phosphogalactosidase. This purification was achieved prior to the isolation of the HPr mutant.

EII lac and FIII lac mutants have the same phenotype. They were so distinguished on the basis of whether the defect is in a soluble or a membrane-bound component. Thus, isolated membranes of a constitutive wild-type strain complement EII mutants, and a soluble component from EII lac mutants complements sonic extracts of FIII lac mutants.

Screening procedures. For the rapid screening of mutants for EI and HPr production, the procedures of the following sections were used.

EI assay. To assay for EI, 2 g of S710A cells (wet paste) and 1 g of glass beads were suspended in 2 ml of 0.1 M Tris buffer (pH 7.6) containing 0.05 M NaCl, 0.05 M MgCl₂, and 0.001 M dithiothreitol. The mixture was exposed to a Branson Sonifier for 4 min at maximal power output. Remaining cells and cell debris were separated by centrifugation at 10,000 × *g*. To 0.05 ml of the turbid extract, varying amounts of EI were added. The reaction was started by adding PEP (3.1 μmoles) and ONPG (2 μmoles) in 0.1 ml of Tris buffer. After 10 min of incubation at 37 C, 2 ml of 0.25 M Na₂CO₃ was added to stop the reaction. The turbid solution was centrifuged for 10 min at 40,000 × *g*. *o*-Nitrophenol (ONP) was determined at 415 nm in a Beckman DB spectrophotometer. The extinction coefficient of ONP in sodium carbonate is 4.8 × 10⁶ cm²/M. EI activity is expressed in micromoles of ONP released in 10 min; 0.05 ml of S710A extract had a 6-phosphogalactosidase activity of 3.4 units. The specific activity of the extract was 0.56 units per mg of protein per min.

HPr assay. A 2-g amount of S797A wet paste was sonically treated and centrifuged as in the EI assay. Instead of EI, varying amounts of HPr were added. An 0.05-ml amount of S797A extract had a 6-phospho-β-galactosidase activity of 5.93 units. The specific activity was 1.2 units per mg of protein per min.

EII assay. EII was assayed by employing strain S714B and supplementing with washed membranes of S710A, S305A, S714G, or S797A. An 0.05-ml amount of S714B extract had a 6-phospho-β-galactosidase activity of 4.14 units. The specific activity of the extract was 0.68 units per mg per min.

FIII lac assay. FIII was assayed as above, employing extracts of S714G; 0.05 ml of the extract was supplemented with FIII lac from S305A. An 0.05-ml amount of the S714G extract had a 6-phospho-β-galactosidase activity of 1.86 units. The specific activity was 0.4 units per mg per min.

6-Phospho-β-galactosidase assay. A measured amount of the enzyme was brought to a volume of 0.9 ml with 0.1 M Tris-hydrochloride buffer (pH 7.6) containing 0.05 M NaCl and 0.05 M MgCl₂. The mixture was incubated at 30 C in a 1-ml cuvette. The reaction was started by adding 3 μmoles of ONPG-6-P in 0.1 ml to the enzyme solution. The increasing absorbance was recorded at 405 nm (17), in a Beckman DB spectrophotometer attached to a model 43 Photovolt Recorder. One unit of 6-phospho-β-galactosidase is equal to 1 μmole × min⁻¹ of ONP released at 30 C.

Assay for an ONPG-6-P-specific phosphatase. An 0.6-μmole amount of ONPG-6-P, crystalline *E. coli* β-galactosidase, and 3.1 μmoles of PEP were added to a sonic extract of a 6-phospho-β-galactosidaseless mutant as a test for ONPG-6-P-specific phosphatase activity. No detectable ONP formation was observed during the assay time of 10 min.

Cell cultivation. Large amounts of cells of *S. aureus* were grown at 37 C in a model F-14 New Brunswick fermentor in 2% petpone medium. Maximal cell densities of 10¹⁰ cells per ml were obtained after over-

night growth. Stirring was at 400 rev/min, and aeration was achieved by applying suction to the fermentor vessel to avoid aerosol buildup. The cells were harvested by using a Lourdes centrifuge with a continuous-flow head. To avoid aerosols, the collecting vessel for the effluent of the centrifuge was kept under reduced pressure.

Cell disruption. A 10-g amount of cells was suspended in 20 ml of 0.1 M Tris-hydrochloride buffer (pH 7.6) containing 0.05 M NaCl, 0.05 M MgCl₂, and 0.001 M dithiothreitol. The resulting slurry was ground in a Bronwill cell disruption apparatus together with 50 g of glass beads (0.11 to 0.012 mm in diameter) for 6 min at 4 C. Glass beads and cell fragments were removed by centrifugation at 10,000 × *g*. The supernatant fluid, containing about 30 mg of protein per ml was used for further purification procedures.

(NH₄)₂SO₄ fractionation. Fractionations were carried out at room temperature (ca. 23 C) by using neutralized saturated solutions.

Chromatography. Diethylaminoethyl (DEAE) cellulose (Cellex D; Biorad), at 0.7 meq/g, was converted to the chloride form. Two columns were used, 2 by 12 cm and 2 by 34 cm. The former was eluted with 300 ml of fluid, the latter with 700 ml. Samples of the (NH₄)₂SO₄ fractionations were desalted with a Canalco EFC apparatus and applied to the column top. A linear elution gradient, ranging from 0.1 to 0.5 M NaCl in 0.01 M sodium phosphate buffer (pH 7.5) containing 0.001 M dithiothreitol, was applied and fractions were collected. The NaCl concentrations in the tubes were determined refractometrically. Fractions containing the desired enzyme activity were concentrated in the Canalco apparatus.

Enzyme I purification. The dissolved precipitate of the (NH₄)₂SO₄ fraction (S305A) at 40 to 55% saturation was desalted and applied to a DEAE cellulose column. EI was eluted at an NaCl concentration of 0.32 M at 4 C as assayed by the use of an S710A extract. The fractions containing EI were pooled and concentrated.

HPr purification. S305A extract was heated to 93 C for 10 min, and the precipitate was removed by centrifugation at 40,000 × *g*. The supernatant fluid was concentrated and chromatographed on a DEAE cellulose column at 4 C. HPr was eluted with 0.12 M NaCl. Additional purification prior to chromatography was achieved by acid precipitation (HCl) at pH 3.7 performed at 0 C (16), followed by heating and concentrating the HPr which remained in the supernatant fluid.

FIII lac purification. FIII lac was isolated at the same time as EI from DEAE cellulose by using the assay described. FIII lac eluted at 0.27 M NaCl.

Preparation of membrane fractions. A 20-g amount of S305A wet paste was disrupted with the Bronwill apparatus, and cell debris was removed at 10,000 × *g*. The pellet, resuspended in buffer by stirring and mild sonic oscillation, was subjected to a second centrifugation at 100,000 × *g* for 30 min. This washing procedure was repeated once. Membranes prepared in this way contained only trace amounts of the other components of the phosphotransferase system.

Purification of the 6-phospho-β-galactosidase. Most

of the activity in S305A extracts precipitates in the 55 to 75% $(\text{NH}_4)_2\text{SO}_4$ fraction. A desalted fraction was chromatographed on DEAE cellulose. Tubes with activity were pooled and concentrated. Details of this procedure and characteristics of the enzyme will be reported elsewhere.

Sephadex molecular weight estimation. Columns (1.5 by 54 cm) with G-200 and G-75 were used. The G-200 column was calibrated with the following proteins: cytochrome *c* (horse heart), molecular weight 12,400; ovalbumin, molecular weight 45,000; intestinal phosphatase, molecular weight 130,000; and *E. coli* β -galactosidase, molecular weight 540,000, at 4 C. The G-75 was calibrated with cytochrome *c*, molecular weight 12,400, and ovalbumin, molecular weight 44,000 to 46,000. The elution buffer was 0.05 M Tris-hydrochloride (pH 7.6) containing 0.1 M NaCl. Elution volumes (V_e) for the different proteins were determined gravimetrically. The void volume (V_0) was estimated by using Dextran blue 2000. The ratio, V_e/V_0 , for the calibration proteins was plotted against the logarithm of the molecular weight and results in a straight line (1, 18). The molecular weights of the unknown proteins were read from this standard curve.

Uptake experiments. Cells were suspended to an optical density of about 0.16 (48 μg , dry weight, per ml) in Davis minimal medium containing 0.4% succinate and 25 μg of chloramphenicol per ml. A 5-ml amount of suspension was warmed to 37 C and made 10^{-8} M with ^{14}C -isopropylthiogalactoside (IPTG) (Calbiochem, 25 mc/mmol). One-milliliter samples were removed at 0, 10, 20, and 30 min and filtered through membrane filters (0.45 μm pore size; Millipore Corp., Bedford, Mass.); the collected cells were washed with 2 ml of cold Davis minimal medium. Dried filters were counted in a Beckman CPM-100 scintillation counter using 2,5-diphenyloxazole (PPO) in toluene as a counting fluid. Counts per minute were plotted against time, the initial rate was estimated graphically, and uptake was expressed as minutes^{-1} per 10^9 cells.

RESULTS

Assay systems. By using the mutants and procedures outlined above, assay systems were designed for each of the components of the phosphotransferase system, EI and HPr, and for FIII and EII lac. The assays are linear over the following ranges: EI, 30-fold; HPr, 300-fold; FIII lac, 90-fold; EII lac, 1,700-fold. No ONPG-6-P specific phosphatase activity was noted under the standard assay conditions. The sensitivity of the assays for EI, HPr, and factor III is shown in Table 2 by the specific activities of the partially purified components. In the enzyme II assay, the specific activity of washed membranes was 0.33 μM per 10 min per mg of protein.

Purification of the components. The components of the system were partially purified from sonic extracts of the wild-type strain, S305A, as follows.

TABLE 2 *Purification of enzyme I and FIII lac from extracts of S305A*

Source	Enzyme I		FIII Lac	
	Specific activity ^a	Purification	Specific activity ^a	Purification
Crude extract	0.105		0.062	
40-55% ammonium sulfate fraction	0.349		0.119	
DEAE column fraction	4.26	40×	1.73	28×

^a Specific activity is given in micromoles of ONP per 10 min per mg of protein.

TABLE 3. *Purification of HPr*

Source	Specific activity ^a	Purification
Crude extract	0.054	
Heated extract	0.23	
Acid precipitation, and heating	0.623	
Concentration	1.197	
DEAE column fractions	18.2	317×

^a Specific activity is given in micromoles of ONP per 10 min per mg of protein.

(i) Enzyme I and FIII lac, from the protein fraction precipitating between 40 and 55% saturation with ammonium sulfate, were separated on DEAE cellulose. The purification achieved is given in Table 2.

(ii) HPr was purified by heat and acid treatments (16) followed by concentration and chromatography on DEAE; the purification achieved is shown in Table 3.

Need for all the components for hydrolysis of ONPG. To obtain hydrolysis of ONPG, all of the components of the phosphotransferase system and the 6-phosphogalactosidase are necessary. The results (Table 4) indicate that the omission of one component diminishes by at least 70% the activity of the system. The residual activity reflects incomplete purification.

Need for the intact system for galactoside accumulation in the whole cell. It was shown previously (2) that mutants missing enzyme I (*car* mutants) cannot concentrate carbohydrates. The effect of the other mutations on the ability of the cell to concentrate is demonstrated in Table 5. The mutations greatly decrease concentration of galactosides by the cells. Chromatographic analysis of the IPTG accumulated by mutant 710A revealed the phosphorylated derivative.

TABLE 4. Dependence of ONPG hydrolysis in vitro on the four components of the system^a

Omission	Activity ^b	Per cent activity
None	0.323	100
EII lac	0.01	3
EI	0.048	15
HPr	0.075	23
FIII lac	0.093	28

^a The complete mixture contained: EI, 240 μ g; HPr, 550 μ g; FIII lac, 120 μ g; EII lac, 0.05 ml; ONPG, 2 μ moles; PEP, 3.1 μ moles; β -galactosidase, 20 μ liters (1 unit); in 0.1 M Tris buffer containing 0.05 M NaCl, 0.05 M MgCl₂, and 0.001 M dithiothreitol.

^b Expressed as micromoles of ONP per 10 min.

TABLE 5. Galactoside accumulation by the phosphotransferase mutants

Mutant	Missing component	Rate of accumulation ^a
S305A	None	27,360
S710A	EI	507
S797B	HPr	290
S714G	FIII lac	Not detected
S714B	EII	Not detected

^a Counts per minute per 10⁹ cells over background. The substrate was 10⁻⁶ M IPTG (isopropylthiogalactoside-2-¹⁴C, Calbiochem, Los Angeles, Calif.), 25 μ c per μ mole. Counting efficiency is about 93%.

Mutant 710A, therefore, has residual enzyme I activity.

Preliminary estimations of the molecular weights of the proteins involved in lactose metabolism. The purified proteins of the phosphotransferase system have been co-chromatographed on Sephadex with substances of known molecular weight in order to obtain estimations of their molecular weight (Table 6).

DISCUSSION

The galactoside-specific phosphotransferase system of *S. aureus* has been characterized. It involves four components, two of which are sugar-specific. No evidence was found for an additional factor, such as the *K_m* factor described by Hanson and Anderson (Fed. Proc., p. 644, 1968) for fructose accumulation by cells of *Aerobacter aerogenes*. These results confirm and extend the preliminary results of Simoni et al. (16) on the components of the staphylococcal phosphotransferase system. In addition, partial purifications and estimations of the molecular weights of the

TABLE 6. Molecular weights of the components of the phosphotransferase system and the components specific for the utilization of lactose^a

Component	Molecular weight	Elution by NaCl from DEAE
EI	100,000 $\pm$ 15%	0.32
HPr	10,000 $\pm$ 15%	0.12
FIII lac	30,000 $\pm$ 15%	0.27
EII	Membrane bound	
6-phospho- β -galactosidase	45,000	0.29

^a The components were chromatographed separately with substances with known molecular weights: cytochrome *c* (molecular weight 12,400); ovalbumin (molecular weight 44,000 to 46,000); β -galactosidase (*E. coli*, molecular weight 510,000 to 530,000); alkaline phosphatase (bovine intestinal, molecular weight 130,000).

factors, including the new factor, factor III, are presented. Mutants lacking each of the factors have been isolated and provide systems of assay for each component. The mutant cells described may provide better sources for purification of each of the components.

All the components of the system are necessary for the synthesis of galactoside-6-phosphates. In addition, the importance of the specific components in the accumulation of galactosides has been shown. The absence of the galactoside-specific factors, EII and FIII, completely prevent accumulation and phosphorylation of galactosides.

The results presented indicate that the phosphotransferase system is necessary for accumulation and metabolism of galactosides. The question of whether the phosphotransferase system is necessary for membrane passage requires the measurement of the rate of entry into the cell by simple diffusion, facilitated diffusion catalyzed by the galactoside specific factors, and by the phosphorylation reaction. Measurement of the first two of these processes is difficult to perform because it is often not possible to determine whether a galactoside molecule has entered the cell or has been adsorbed to the cell surface.

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