Inducer expulsion and the occurrence of an HPr(Ser-P)-activated sugar-phosphate phosphatase in *Enterococcus faecalis* and *Streptococcus pyogenes*

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Inducer expulsion, a phenomenon in which rapidly metabolizable sugars cause cytoplasmic dephosphorylation and efflux of pre-accumulated sugar-phosphates (sugar-P), has been documented for *Streptococcus pyogenes*, *Streptococcus bovis*, and *Lactococcus lactis*, but not for other Gram-positive bacteria. Using intact cells and membrane vesicles, we show that *Enterococcus faecalis* exhibits both inducer exclusion and inducer expulsion, and that the latter phenomenon is dependent on the metabolite-activated ATP-dependent HPr(Ser) kinase that phosphorylates Ser-46 in HPr of the phosphotransferase system. A small, heat-stable, membrane-associated, HPr(Ser-P)-activated sugar-P phosphatase (Pase II), previously identified only in *Lc. lactis*, is shown to be present in extracts of *Enterococcus faecalis* and *Streptococcus pyogenes* but not in those of *Staphylococcus aureus*, *Streptococcus mutans*, *Streptococcus salivarius*, or *Bacillus subtilis*, organisms that do not exhibit the inducer expulsion phenomenon. Further, *Lactobacillus brevis*, an organism that exhibits inducer expulsion by a different mechanism, also apparently lacks Pase II. The results reveal that Pase II is present in those organisms that exhibit the coupled sugar-P hydrolysis/expulsion mechanism but not those that lack this mechanism. They provide correlative evidence that Pase II initiates inducer expulsion in species of enterococci, streptococci and lactococci.

Keywords: carbohydrate metabolism, transport, sugar-phosphate phosphatase, Gram-positive bacteria, HPr, phosphotransferase system

INTRODUCTION

Inducer expulsion is the term given to the phenomenon wherein the addition of a metabolizable carbohydrate to the growth medium of a micro-organism elicits the rapid efflux of a pre-accumulated sugar. Two types of such mechanisms have been demonstrated in Gram-positive bacteria. In *Lactobacillus brevis*, the lactose and glucose permeases function by H⁺ symport and accumulate their non-metabolizable substrates as the free (non-phosphorylated) cytoplasmic sugars. Addition of a metabolizable sugar uncouples sugar transport from H⁺ symport, and efflux consequently occurs (Romano *et al.*, 1987; Ye *et al.*, 1994a, b). In contrast, in *Streptococcus pyogenes* and *Lacto-*

coccus lactis, lactose and glucose are accumulated via the phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS)-mediated group translocation mechanism, and consequently their non-metabolizable substrates accumulate in the cytoplasm as the phosphorylated derivatives (Reizer & Panos, 1980; Thompson & Saier, 1981). In this case inducer expulsion is a two-step process in which cytoplasmic phosphohydrolysis precedes efflux (Reizer *et al.*, 1983).

Both types of efflux processes are mediated by a metabolite-activated ATP-dependent protein kinase that specifically phosphorylates Ser-46 in HPr of the PTS (Ye *et al.*, 1994a–d), and HPr(Ser-P) then binds to various targets of regulation such as the lactose:H⁺ and glucose:H⁺ symporters of *Lb. brevis* (Ye & Saier, 1995a, b) and a small, heat-stable sugar-P phosphatase of *Lc. lactis* (Ye & Saier, 1995c). The phosphatase has been solubilized from the membranes of *Lc. lactis*, purified to near homogeneity and

Abbreviations: 2DG, 2-deoxyglucose; FBP, fructose-1,6-bisphosphate; Pase I & II, phosphatase I & II; PEP, phosphoenolpyruvate; PTS, phosphotransferase system; TMG, thiomethyl β -D-galactopyranoside.

characterized (Ye & Saier, 1995c). It is a monomeric enzyme (approx. 9000 Da) which apparently forms a 1:1 stoichiometric complex with HPr(Ser-P), and this complex is more than 10 times more active than the free enzyme. Like the efflux process, the enzyme is dependent on Mg^{2+} but insensitive to fluoride inhibition. It has been designated phosphatase II (Pase II) to distinguish it from a different, larger, heat-sensitive phosphatase (Pase I) of *Lc. lactis* that is inhibited by fluoride and insensitive to HPr(Ser-P) activation (Thompson & Chassy, 1983; Ye & Saier, 1995c).

Little is known about the distribution of Pase II in Gram-positive bacteria. Inducer expulsion has been demonstrated for *Lc. lactis* (Thompson & Saier, 1981), *Strep. pyogenes* (Reizer & Panos, 1980) and *Strep. bovis* (Cook *et al.*, 1995), but attempts to demonstrate this phenomenon in *Staphylococcus aureus* (Saier & Simoni, 1976) and *Bacillus subtilis* (Deutscher *et al.*, 1994; Fujita & Miwa, 1994) have given consistently negative results. Moreover, although *Lb. brevis* exhibits the sugar:H⁺ symport uncoupling mechanism of inducer expulsion, its potential for catalysing the expulsion of pre-accumulated cytoplasmic sugar-Ps has not been tested. All of these Gram-positive bacteria and others are known to possess HPr and the HPr(Ser) kinase (Reizer *et al.*, 1988).

In this paper we report comparative studies which demonstrate that *Enterococcus faecalis* cells and membrane vesicles accumulate the lactose analogue thiomethyl β -D-galactopyranoside (TMG) as the 6-phosphate ester (TMG-6-P) and that they exhibit both inducer exclusion and inducer expulsion under conditions that result in the generation of HPr(Ser-P) intracellularly or intravesicularly. We have further identified Pase II in extracts of both *E. faecalis* and *Strep. pyogenes*, and show that no such activity can be demonstrated in extracts of *Lb. brevis*, *B. subtilis*, *Strep. mutans*, *Strep. salivarius* and *Staph. aureus*. Those bacteria that lack Pase II do not exhibit sugar-P phosphatase-dependent inducer expulsion *in vivo*. These results provide clear evidence to suggest that Pase II is required for inducer expulsion and occurs in a limited subgroup of the low GC Gram-positive bacteria. They lead to the conclusion that the regulatory targets of HPr(Ser-P) action are species-specific.

METHODS

Many of the methods used have been described in our previous publications (Ye *et al.*, 1994a-d; Ye & Saier, 1995a-c) and are repeated here only where substantially different from the published procedures.

Enzyme assay. Sugar-P phosphatase activity was routinely determined by a modification of an earlier method (Parvin & Smith, 1969; see Ye & Saier, 1995c). Briefly, 10–25 μ l enzyme (5–10 μ g) was added to an assay solution containing 50 mM MES buffer (pH 7.0), 20 mM $MgSO_4$ and 10–20 mM sugar-P. The assay solutions were incubated at 40 °C for 10–30 min before 1 ml isobutanol and 0.3 ml 4.25% (w/v) ammonium molybdate in 0.43 M sulfuric acid were added. Phosphate was extracted into the organic phase by vigorous mixing, and when the organic phase had cleared, 1 ml was removed and mixed with 1 μ l 4% (w/v) $SnCl_2$ in concentrated HCl before measure-

ment of OD₇₆₀. For TMG-6-P phosphatase activity measurements, 1.5 mM [¹⁴C]TMG-6-P [specific activity, 0.5 mCi mmol⁻¹, prepared as described previously (Ye *et al.*, 1994c)] was added and incubated for up to 2 h before measuring the ratio of [¹⁴C]TMG-P to free [¹⁴C]TMG by ion-exchange chromatography (5 × 0.5 cm column size) as outlined by Kundig & Roseman (1971). Protein concentration was determined following the procedure of Lowry.

Organisms and growth conditions. *Streptococcus pyogenes* (Reizer *et al.*, 1983), *Streptococcus salivarius* (ATCC 25975) (Waygood *et al.*, 1986; Vadeboncoeur *et al.*, 1991), *Streptococcus mutans* (ATCC 25175) (Mimura *et al.*, 1987), *Lactobacillus brevis* (Romano *et al.*, 1987; Ye *et al.*, 1994a), *Staphylococcus aureus* (Saier & Simoni, 1976) and *Bacillus subtilis* (Reizer *et al.*, 1989) have all been described. All bacteria except *Lb. brevis* were grown at 37 °C for 16 h in LB medium containing 25 mM galactose (4 l for a typical preparation) as described by Ye *et al.* (1994c). *Lb. brevis* was grown as described by Ye *et al.* (1994a) or Saier *et al.* (1995).

Preparation of cell extracts, membranes and partially purified enzymes. The same procedures were used for all of the bacterial species examined. Washed cell pellets, each from 4 l growth medium, were resuspended to a volume of 60 ml in 100 mM HEPES buffer (pH 7.3) containing 1 mM EDTA, 1 mM dithiothreitol and 5 mM $MnSO_4$ (HEDM buffer) as well as 0.1 mM PMSF. Cells were broken by three passages through a French pressure cell [11000 p.s.i. (76 MPa)] and debris was removed by centrifugation at 10000 g for 20 min. The membranes were collected by ultracentrifugation at 200000 g for 90 min.

Membranes were resuspended in HEDM buffer and treated with 8 M urea and 4% (v/v) 1-butanol at 0 °C for 30 min (Kundig & Roseman, 1971; Saier *et al.*, 1977). They were then centrifuged (200000 g for 90 min) and the supernatants were collected and dialysed overnight against a 4 l volume of 20 mM Tris buffer (pH 7.0) at 4 °C.

The dialysed urea-butanol-treated supernatants (40 ml each) were transferred (at a flow rate of 0.5 ml min⁻¹) on to a DEAE-Sephacel anion-exchange column (3 × 20 cm) previously equilibrated with HEDM buffer. Proteins were eluted with a linear gradient of 0.1–0.4 M NaCl in HEDM buffer.

Transport in *E. faecalis* cells and vesicles. Transport of [¹⁴C]TMG into *E. faecalis* cells was conducted and membrane vesicles of *E. faecalis* were prepared as described previously for *Lc. lactis* (Ye *et al.*, 1994c, d). The electroporation procedure allowing entry of proteins and metabolites into the vesicles was as described previously (Ye *et al.*, 1994a–d).

Reagents. [¹⁴C]TMG (58 μ Ci μ mol⁻¹) was from New England Nuclear (β -D-[methyl-¹⁴C]thiogalactopyranoside). [¹⁴C]TMG-6-P was prepared by using starved cells of *Lc. lactis* as described by Thompson & Saier (1981). Hydrofluor scintillation cocktail was purchased from Research Products International. Analytical grade anion-exchange resin AG1-X2 was obtained from Bio-Rad. DEAE-Sephacel anion-exchange resin was obtained from Pharmacia. All phosphorylated compounds and other reagents were obtained from Sigma.

RESULTS

Inducer exclusion and expulsion in *Enterococcus faecalis* cells

Previous studies have established the phenomena of glucose-promoted inducer exclusion and expulsion in *Strep. pyogenes*, *Strep. bovis*, and *Lc. lactis*, but attempts to

Fig. 1. Inducer expulsion in cells (a) and vesicles (b) of *E. faecalis*. (a) Time course for the expulsion of TMG-6-P from intact cells of *E. faecalis*. Cells were grown, washed, resuspended and exposed to 60 μ M [14 C]TMG (58 μ Ci μ mol⁻¹) for 10 min at 30 °C as described in Methods and in previous reports (Ye *et al.*, 1994c, d). At $t = 0$, glucose (20 mM) was added to one culture (■) but not to the other (●), and efflux of the radioactive sugar was followed as a function of time. (b) Time course for the expulsion of TMG-6-P accumulated as the phosphate ester from vesicles derived from *E. faecalis* cells as described in the legend to Table 1 and in previous reports (Ye *et al.*, 1994c, d). Retention of vesicular radioactivity was followed as a function of time following electroporation in the absence (●) or presence (■) of S46D HPr (50 μ M).

demonstrate comparable behaviour in *B. subtilis* and *Staph. aureus* were unsuccessful (see Introduction). *E. faecalis* has not been examined for this phenomenon. This organism accumulates TMG as the phosphate ester (TMG-6-P) as a result of the activity of the lactose/galactose PTS. Fig. 1(a) shows a time course for glucose-promoted expulsion of TMG from *E. faecalis* cells. While whole cells retained [14 C]TMG-P with a very low turnover rate in the absence of glucose, the presence of glucose promoted efflux with a half life of less than 30 s. Efflux was preceded by intracellular TMG-P hydrolysis as reported previously for *Strep. pyogenes* (Reizer *et al.*, 1983).

As shown in Table 1, column 2, several sugars, present in excess (20 mM inhibiting sugar as compared with 60 μ M TMG), strongly inhibited TMG uptake (inducer exclusion). These sugars included glucose, mannose and galactose as well as the glucose analogue, 2-deoxyglucose (2DG). Other sugars (fructose and ribose) inhibited TMG

Table 1. TMG exclusion and expulsion in *E. faecalis* cells

For measurement of exclusion, cell suspensions (16 μ g dry wt ml⁻¹) in 50 mM Tris/maleate buffer, pH 7.0, were preincubated with or without 20 mM of the sugar indicated below for 2 min before addition of 60 μ M [14 C]TMG (58 μ Ci μ mol⁻¹). Uptake was measured after a 10 min incubation period at 30 °C. A value of 1.0 corresponds to 35.6 nmol [14 C]TMG-6-P accumulated (mg protein)⁻¹. For measurement of expulsion, the cell suspensions described above were allowed to accumulate radioactive TMG (60 μ M) for 10 min before addition of the sugar (20 mM) indicated below. Retention of radioactivity was measured after an additional 10 min incubation period. A value of 1.0 corresponds to 32.1 nmol [14 C]TMG-6-P remaining in the cells (mg protein)⁻¹. Values are the means of three experiments $\pm$ SD.

Sugar added (20 mM)	Relative TMG accumulation	
	Exclusion assay	Expulsion assay
None	1.00	1.00
Glucose	0.06 $\pm$ 0.02	0.04 $\pm$ 0.02
Mannose	0.07 $\pm$ 0.01	0.23 $\pm$ 0.07
2DG	0.03 $\pm$ 0.005	0.25 $\pm$ 0.09
Galactose	0.05 $\pm$ 0.01	0.34 $\pm$ 0.11
Fructose	0.31 $\pm$ 0.12	0.44 $\pm$ 0.10
Ribose	0.36 $\pm$ 0.11	0.56 $\pm$ 0.13
Glucitol	0.89 $\pm$ 0.23	1.10 $\pm$ 0.15
Mannitol	1.04 $\pm$ 0.18	1.12 $\pm$ 0.13

uptake to a lesser extent while glucitol and mannitol were not inhibitory. In contrast, of these sugars, only glucose caused essentially all of the pre-accumulated TMG to efflux from galactose-grown cells, although several other sugars were partially effective (Table 1, column 3).

Comparable experiments were conducted with radioactive, non-metabolizable 2DG (data not shown). Uptake of the sugar analogue was strongly inhibited by glucose and mannose but not by galactose, fructose or glucitol. Similarly, glucose stimulated rapid expulsion of pre-accumulated 2DG-P, but galactose, fructose, ribose and glucitol were either substantially less effective or without effect. The results establish the occurrence of selective exclusion and expulsion of both TMG and 2DG in *E. faecalis* in a fashion that resembles these phenomena reported previously for *Lc. lactis*, *Strep. pyogenes* and *Strep. bovis*.

TMG expulsion in vesicles of *E. faecalis*

Previous communications from this laboratory have demonstrated the inducer expulsion phenomenon in membrane vesicles from *Lc. lactis* (Ye *et al.*, 1994b, d). Efflux was not observed when either HPr or fructose-1,6-bisphosphate (FBP) was electroporated into the vesicles, but the two together promoted rapid efflux. Moreover, when the S46D mutant HPr derivative, in which the phosphorylatable Ser-46 was replaced by Asp, thereby introducing a permanent negative charge at that position, was introduced into the vesicles by electroporation, efflux

Table 2. Regulation of TMG efflux from *E. faecalis* vesicles

The effects of HPr and its mutant derivatives on TMG-6-P hydrolysis-dependent intravesicular expulsion were studied in the presence or absence of FBP, glucose-6-P or fructose-6-P. [14 C]TMG (60 μ M; specific activity, 58 μ Ci μ mol $^{-1}$) uptake was allowed to occur for 10 min at 30 $^{\circ}$ C before vesicle suspensions were chilled to 0 $^{\circ}$ C. HPr, or one of its mutant derivatives (100 μ M), with or without 20 mM FBP, glucose-6-P or fructose-6-P, as indicated below, was electroporated into the vesicles at 0 $^{\circ}$ C. The samples were rapidly brought to 30 $^{\circ}$ C and incubated at this temperature for an additional 10 min during which time efflux occurred. Remaining intravesicular TMG was then measured. Values are the means of three experiments $\pm$ SD.

Addition*	[14 C]TMG accumulation [nmol (mg protein) $^{-1}$]
None	0.56 $\pm$ 0.03
FBP	0.49 $\pm$ 0.13
HPr	0.62 $\pm$ 0.14
HPr, FBP	0.15 $\pm$ 0.04
HPr, fructose-6-P	0.28 $\pm$ 0.02
HPr, glucose-6-P	0.33 $\pm$ 0.03
H15A HPr	0.57 $\pm$ 0.12
H15A HPr, FBP	0.16 $\pm$ 0.02
S46D HPr	0.14 $\pm$ 0.03
S46D HPr, FBP	0.13 $\pm$ 0.05
S46A HPr	0.66 $\pm$ 0.18
S46A HPr, FBP	0.58 $\pm$ 0.16
H15A S46D HPr	0.17 $\pm$ 0.01
H15A S46D HPr, FBP	0.16 $\pm$ 0.05

* H15A is a mutant HPr in which His-15 is replaced by Ala; in S46D, Ser-46 is replaced by Asp; in S46A, Ser-46 is replaced by Ala.

was dependent only on the intravesicular presence of the protein (Ye *et al.*, 1994b, d).

Fig. 1(b) shows time courses for TMG expulsion from *E. faecalis* membrane vesicles. Little efflux was observed when vesicles were incubated at 30 $^{\circ}$ C, but electroporation into the vesicles of the HPr(Ser-P) analogue, S46D HPr, promoted rapid efflux of the pre-accumulated TMG-6-P.

Table 2 summarizes the results of TMG efflux experiments in which several different intravesicular agents were examined. Extravesicular HPr did not stimulate TMG efflux, in either the presence or the absence of a metabolite such as FBP (data not shown). Similarly, electroporation in the presence of each of these agents alone had little effect (Table 2). However, when HPr and FBP were simultaneously electroporated into the vesicles, rapid efflux was observed (Table 2). When the mutant HPr, in which Ser-46 was replaced by Ala (S46A HPr), replaced wild-type HPr, no stimulation of efflux was observed, even when FBP was present. S46A HPr cannot be phosphorylated by the metabolite-activated HPr kinase. In contrast, when S46D HPr was electroporated into the vesicles, efflux occurred even in the absence of FBP, as also documented in Fig. 1(b). H15A HPr, in which the

Fig. 2. Elution of phosphatase activities from DEAE-Sephacel. In both cases, extracts were prepared and membranes were extracted with urea-butanol using the procedure described in Methods. The extracted material from *E. faecalis* (a) and *Strep. pyogenes* (b) was applied to and then eluted from the DEAE-Sephacel column with a linear salt gradient (0.1–0.4 M NaCl) and activity peaks were assayed for stimulation by S46D HPr. In both cases, stimulation was observed only for the second peak (designated Pase II; see Table 3). Similar chromatographic separation of the activities obtained from *B. subtilis*, *Staph. aureus*, *Strep. mutans* and *Strep. salivarius* each yielded a single peak of phosphatase activity which eluted before Pase II of *Lc. lactis*, *Strep. pyogenes* or *E. faecalis*. None of these activity peaks exhibited S46D HPr stimulation. *Lb. brevis* extracts gave two activity peaks, but neither exhibited appreciable stimulation by S46D HPr.

active site His-15 was replaced by Ala (Reizer *et al.*, 1989), behaved like wild-type HPr in this respect, while H15A S46D HPr behaved like S46D HPr. In the presence of wild-type HPr, glucose-6-P and fructose-6-P were marginally effective in promoting TMG efflux (Table 2). These results lead to the conclusion that phosphorylation of Ser-46 in HPr mediates inducer expulsion in *E. faecalis* as in *Lc. lactis*.

Presence of Pase II in *E. faecalis* and *Strep. pyogenes*, but not in *B. subtilis*, *Staph. aureus*, *Lb. brevis*, *Strep. mutans* or *Strep. salivarius*

The S46D-activated sugar-P phosphatase (Pase II) of *Lc. lactis* can be solubilized from the membrane by urea-

Table 3. Activation of sugar-P phosphatase activities in various preparations from *Strep. pyogenes*, *E. faecalis* and *Lb. brevis*

Extracts and derived protein fractions were prepared and assayed for fructose-6-P phosphatase activity with (+) and without (–) 50 µM S46D HPr as described in Methods. Extracts and derived protein fractions from *B. subtilis* and *Staph. aureus* were similarly assayed, but like *Lb. brevis* fractions, they did not exhibit measurable stimulation by S46D HPr.

Purification step	Phosphatase activity [nmol (mg protein) ⁻¹ min ⁻¹]		Activation (-fold)
	– S46D HPr	+ S46D HPr	
<i>E. faecalis</i>			
Crude extract	25 ± 4	90 ± 18	3.8
Supernatant from urea–butanol- treated membranes	49 ± 13	162 ± 42	3.4
DEAE-Sephacel			
Fraction I	190 ± 25	210 ± 30	1.1
Fraction II	66 ± 18	444 ± 120	6.7
<i>Strep. pyogenes</i>			
Crude extract	30 ± 1	60 ± 12	2.0
Supernatant from urea–butanol- treated membranes	73 ± 13	190 ± 48	2.7
DEAE-Sephacel			
Fraction I	320 ± 66	310 ± 90	1.0
Fraction II	127 ± 25	570 ± 130	4.5
<i>Lb. brevis</i>			
Crude extract	6.2 ± 0.7	6.6 ± 1.2	1.0
Supernatant from urea–butanol- treated membranes	19 ± 2	19 ± 5	1.0
DEAE-Sephacel			
Fraction I	72 ± 18	67 ± 24	0.9
Fraction II	42 ± 12	48 ± 6	1.0

butanol treatment (Ye & Saier, 1995c) and the resultant protein elutes from DEAE-Sephacel at a high salt concentration, well after Pase I (Ye & Saier, 1995c). As shown in Fig. 2, two peaks of phosphatase activity eluted from the column when an extract was prepared either from *E. faecalis* cells (Fig. 2a) or from *Strep. pyogenes* cells (Fig. 2b). The activity in peak I was not stimulated by S46D HPr in either case, but that in peak II was stimulated about tenfold by S46D HPr in both cases. Peak II therefore corresponds to Pase II.

Using our standard assay procedure, urea–butanol-extracted material from membranes of *B. subtilis*, *Staph. aureus*, *Strep. mutans* and *Strep. salivarius* each exhibited only one peak of phosphatase activity while that of *Lb. brevis* exhibited two peaks (data not shown). These activities were not stimulated by S46D HPr. Activities found in crude extracts, in membranes, and in urea–butanol-extracted membranes from these organisms did not exhibit appreciable stimulation by S46D HPr. The activities from all of these organisms eluted at lower salt concentration than was observed for Pase II from *Lc.*

lactis, *Strep. pyogenes* or *E. faecalis* (see legend to Fig. 2 and Ye & Saier, 1995c).

The various fractions obtained from *E. faecalis*, *Strep. pyogenes* and *Lb. brevis* were assayed for stimulation of sugar-P phosphatase activity by S46D HPr. The results are summarized in Table 3. Crude extracts derived from *E. faecalis* or *Strep. pyogenes* cells exhibited sugar-P phosphatase activity that was weakly stimulated by S46D HPr. In both cases, solubilization of the membrane-associated activities and assay of the two DEAE-Sephacel activity peaks revealed that the first peak (Fraction I) was not stimulated by S46D HPr but that the second peak (Fraction II) was stimulated several-fold (Table 3). The results suggest that the first peak corresponds to Pase I from *Lc. lactis* (Thompson & Chassy, 1983), while the second peak corresponds to Pase II (Ye & Saier, 1995c).

In a recent report we demonstrated that *Lb. brevis* possesses a complete fructose-specific PTS as well as the initial enzymes of glycolysis that act on fructose-1-P, fructose 1-P kinase and FBP aldolase (Saier *et al.*, 1995). Synthesis of the enzymes responsible for fructose ca-

tabolism were induced under anaerobic conditions in the presence of fructose. *Lb. brevis* cells were therefore grown under these conditions, and an extract was prepared for assay of sugar-P phosphatase activities. Membranes were extracted with butanol-urea, and the resultant extract was passed through a DEAE-Sephacel column as outlined in the legend to Fig. 2. Fractions with phosphatase activities were assayed with and without 50 μ M S46D HPr as indicated in the legend to Table 3. Less than twofold activation of fructose-1-P phosphatase activity was observed.

DISCUSSION

The results reported in this paper and previous communications (Saier & Simoni, 1976; Deutscher *et al.*, 1994; Ye *et al.*, 1994a-d; Cook *et al.*, 1995; Ye & Saier, 1995a-c) reveal a perfect correlation between the occurrence of glucose-promoted, cytoplasmic, sugar-P hydrolysis-dependent inducer expulsion in intact cells of several low GC Gram-positive bacteria and the presence of S46D HPr-stimulated Pase II in extracts derived from these cells (Table 4). Within the low GC Gram-positive bacteria, cytoplasmic sugar-P hydrolysis-dependent inducer expulsion has been easily demonstrated in species of lactococci and enterococci as well as some streptococcal species, but not in species of lactobacilli, bacilli or staphylococci or in some species of streptococci. Our attempts to measure TMG expulsion *in vivo* in *Strep. mutans* and *Strep. salivarius* were negative although *Strep. pyogenes* and *Strep. bovis* did exhibit this phenomenon. Moreover, as reported here, those organisms that exhibit S46D-stimulated Pase II are the same ones that exhibit the expulsion phenomenon (Table 4). Therefore, it can be postulated that it is the presence of Pase II, in addition to the low GC Gram-positive HPr(Ser) kinase, that determines the propensity of any one of these organisms for the expulsion phenomenon.

All low GC Gram-positive bacteria so far assayed for HPr

and the ATP-dependent, metabolite-activated HPr(Ser) kinase have been found to exhibit these activities. In addition to the organisms listed in Table 4, these organisms include *Acholeplasma laidlawii* (Hoischen *et al.*, 1993) and *Listeria monocytogenes* (Mitchell *et al.*, 1993). The latter two organisms have not yet been assayed either for inducer expulsion or the presence of Pase II. Other low GC Gram-positive bacteria, some of which are known to possess the PTS (Romano & Saier, 1992), have not yet been assayed for the HPr(Ser) kinase.

In contrast, high GC Gram-positive bacteria, including species of *Corynebacterium*, *Arthrobacter*, *Brevibacterium* and *Streptomyces*, all of which possess a PTS, do not exhibit demonstrable HPr(Ser) kinase activity when assayed *in vitro* (Titgemeyer *et al.*, 1995; J. Reizer & M. H. Saier, Jr, unpublished results). *Escherichia coli* and other enteric bacteria also lack this enzyme (J. Reizer, J. Deutscher & M. H. Saier, Jr, unpublished results). It therefore appears that the presence of the HPr(Ser) kinase may be a characteristic feature of low GC Gram-positive bacteria.

A revealing experiment can be performed once the gene encoding Pase II has been cloned. This gene, incorporated into an appropriate plasmid, can be transferred to an organism which lacks the expulsion phenomenon (e.g., *Staph. aureus* or *B. subtilis*), and it can be determined if the presence of the plasmid allows expulsion to occur. If it does, then the presence of Pase II alone must determine sensitivity to this phenomenon. However, if rapid, glucose-promoted, cytoplasmic sugar-P phosphohydrolysis is observed without efflux, then an additional component of the efflux apparatus, a specific sugar efflux system characteristic of the organisms that naturally exhibit the phenomenon of inducer expulsion, may be required. This possibility is not unreasonable as many substrates have been shown to be taken up via transport systems that differ from their efflux counterparts (Krämer, 1994a, b). As the route of sugar efflux involved in the inducer exclusion phenomenon has not yet been defined, these experiments are likely to prove informative.

Table 4. Correlation of the occurrence of inducer expulsion in cells with the presence of S46D-stimulated Pase II in various low GC Gram-positive bacteria

Organism	Presence of HPr and HPr(Ser) kinase	Occurrence of inducer expulsion	Presence of HPr(Ser-P)-stimulated Pase II
<i>Lc. lactis</i>	+	+	+
<i>Strep. pyogenes</i>	+	+	+
<i>Strep. bovis</i>	+	+	+
<i>E. faecalis</i>	+	+	+
<i>Lb. brevis</i>	+	—	—
<i>Staph. aureus</i>	+	—	—
<i>B. subtilis</i>	+	—	—
<i>Strep. mutans</i>	+	—	—
<i>Strep. salivarius</i>	+	—	—

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REFERENCES

- Cook, G. M., Kearns, D. B., Russell, J. B., Reizer, J. & Saier, M. H., Jr (1995). Regulation of the lactose phosphotransferase system of *Streptococcus bovis* by glucose: independence of inducer exclusion and expulsion mechanisms. *Microbiology* **141**, 2261–2269.
- Deutscher, J., Sauerwald, H., Reizer, J., Saier, M. H., Jr, Zwacka, R. & Steinmetz, M. (1994). Loss of protein kinase catalysed phosphorylation of HPr, a phospho-carrier protein of the phosphotransferase system, by mutation of the *ptsH* gene confers catabolite repression resistance to several catabolic genes of *Bacillus subtilis*. *J Bacteriol* **178**, 3338–3344.
- Fujita, Y. & Miwa, Y. (1994). Catabolite repression of the *Bacillus subtilis* *gnt* operon mediated by the CcpA protein. *J Bacteriol* **176**, 511–513.
- Hoischen, C., Reizer, J., Dijkstra, A., Rottem, S. & Saier, M. H., Jr (1993). Presence of protein constituents of the Gram-positive bacterial phosphotransferase regulatory system in *Acholeplasma laidlawii*. *J Bacteriol* **175**, 6599–6604.
- Krämer, R. (1994a). Secretion of amino acids by bacteria: physiology and mechanism. *FEMS Microbiol Rev* **13**, 75–94.
- Krämer, R. (1994b). Systems and mechanisms of amino acid uptake and excretion in prokaryotes. *Arch Microbiol* **162**, 1–13.
- Kundig, W. & Roseman, S. (1971). Sugar transport. II. Characterization of constitutive membrane-bound Enzymes II of the *Escherichia coli* phosphotransferase system. *J Biol Chem* **246**, 1407–1418.
- Mimura, C. S., Poy, F. & Jacobson, G. R. (1987). ATP-dependent protein kinase activities in the oral pathogen *Streptococcus mutans*. *J Cell Biochem* **33**, 161–171.
- Mitchell, W. J., Reizer, J., Herring, C., Hoischen, C. & Saier, M. H., Jr (1993). Identification of a phosphoenolpyruvate:fructose phosphotransferase system (fructose-1-P forming) in *Listeria monocytogenes*. *J Bacteriol* **175**, 2756–2761.
- Parvin, R. & Smith, R. A. (1969). Determination of inorganic phosphate in the presence of labile organic phosphates. *Anal Biochem* **27**, 65–72.
- Reizer, J. & Panos, C. (1980). Regulation of β -galactoside phosphate accumulation in *Streptococcus pyogenes* by an expulsion mechanism. *Proc Natl Acad Sci USA* **77**, 5497–5501.
- Reizer, J., Novotny, M. H., Panos, C. & Saier, M. H., Jr (1983). The mechanism of inducer expulsion in *Streptococcus pyogenes*: a two step process activated by ATP. *J Bacteriol* **156**, 354–361.
- Reizer, J., Saier, M. H., Jr, Deutscher, J., Grenier, F., Thompson, J. & Hengstenberg, W. (1988). The phosphoenolpyruvate:sugar phosphotransferase system in Gram-positive bacteria: properties, mechanism and regulation. *CRC Crit Rev Microbiol* **15**, 297–338.
- Reizer, J., Sutrina, S. L., Saier, M. H., Jr, Stewart, G. C., Peterkofsky, A. & Reddy, P. (1989). Mechanistic and physiological consequences of HPr(Ser) phosphorylation on the activities of the phosphoenolpyruvate:sugar phosphotransferase system in Gram-positive bacteria: studies with site-specific mutants of HPr. *EMBO J* **8**, 2111–2120.
- Romano, A. H. & Saier, M. H., Jr (1992). Evolution of the bacterial phosphoenolpyruvate:sugar phosphotransferase system. I. Physiologic and organismic considerations. In *The Evolution of Metabolic Function*, pp. 143–170. Edited by R. P. Mortlock. Boca Raton, FL: CRC Press.
- Romano, A. H., Brino, G., Peterkofsky, A. & Reizer, J. (1987). Regulation of β -galactoside transport and accumulation in heterofermentative lactic acid bacteria. *J Bacteriol* **169**, 5589–5596.
- Saier, M. H., Jr & Simoni, R. D. (1976). Regulation of carbohydrate uptake in Gram-positive bacteria. *J Biol Chem* **251**, 893–894.
- Saier, M. H., Jr, Feucht, B. U. & Mora, W. K. (1977). Sugar-P:sugar transphosphorylation and exchange group translocation catalysed by the Enzyme II complexes of the bacterial phosphoenolpyruvate:sugar phosphotransferase system. *J Biol Chem* **252**, 8899–8907.
- Saier, M. H., Jr, Ye, J.-J., Klinke, S. & Nino, E. (1995). Identification of an anaerobically-induced phosphoenolpyruvate-dependent fructose-specific phosphotransferase system and evidence for the Embden–Meyerhof glycolytic pathway in the heterofermentative bacterium, *Lactobacillus brevis*. *J Bacteriol* **178**, 314–316.
- Thompson, J. & Chassy, B. M. (1983). Intracellular hexose-6-phosphate:phosphohydrolase from *Streptococcus lactis*: purification, properties, and function. *J Bacteriol* **156**, 70–80.
- Thompson, J. & Saier, M. H., Jr (1981). Regulation of methyl- β -D-thiogalactopyranoside-6-phosphate accumulation in *Streptococcus lactis* by exclusion and expulsion mechanisms. *J Bacteriol* **146**, 885–894.
- Titgemeyer, F., Walkenhorst, J., Reizer, J., Stuijver, M. H., Cui, X. & Saier, M. H., Jr (1995). Identification and characterization of phosphoenolpyruvate:fructose phosphotransferase systems in three *Streptomyces* species. *Microbiology* **141**, 51–58.
- Vadeboncoeur, C., Brochu, D. & Reizer, J. (1991). Quantitative determination of the intracellular concentration of the various forms of HPr, a phosphocarryer protein of the phosphoenolpyruvate:sugar phosphotransferase system in growing cells of oral streptococci. *Anal Biochem* **196**, 24–30.
- Waygood, E. B., Mattoo, R. L., Erickson, E. & Vadeboncoeur, C. (1986). Phosphoproteins and the phosphoenolpyruvate:sugar phosphotransferase system of *Streptococcus salivarius*. Detection of two different ATP-dependent phosphorylations of the phosphocarryer protein HPr. *Can J Microbiol* **32**, 310–318.
- Ye, J.-J. & Saier, M. H., Jr (1995a). Cooperative binding of lactose and HPr(SerP) to the lactose:H⁺ permease of *Lactobacillus brevis*. *Proc Natl Acad Sci USA* **92**, 417–421.
- Ye, J.-J. & Saier, M. H., Jr (1995b). Allosteric regulation of the glucose:H⁺symporter of *Lactobacillus brevis*: cooperative binding of glucose and HPr(Ser-P). *J Bacteriol* **177**, 1900–1902.
- Ye, J.-J. & Saier, M. H., Jr (1995c). Purification and characterization of a small membrane-associated sugar-phosphate phosphatase that is allosterically activated by HPr(Ser-P) of the phosphotransferase system in *Lactococcus lactis*. *J Biol Chem* **270**, 16740–16744.
- Ye, J. J., Reizer, J., Cui, X. & Saier, M. H., Jr (1994a). ATP-dependent phosphorylation of serine in HPr regulates lactose:H⁺ symport in *Lactobacillus brevis*. *Proc Natl Acad Sci USA* **91**, 3102–3106.
- Ye, J. J., Neal, J. W., Cui, X., Reizer, J. & Saier, M. H., Jr (1994b). Regulation of the glucose:H⁺ symporter by metabolite-activated ATP-dependent phosphorylation of HPr in *Lactobacillus brevis*. *J Bacteriol* **176**, 3484–3492.
- Ye, J. J., Reizer, J., Cui, X. & Saier, M. H., Jr (1994c). Inhibition of

the phosphoenolpyruvate:lactose phosphotransferase system and activation of a cytoplasmic sugar-phosphate phosphatase in *Lactococcus lactis* by ATP-dependent metabolite-activated phosphorylation of serine-46 in the phosphocarrier protein, HPr. *J Biol Chem* **269**, 11837–11844.

Ye, J. J., Reizer, J. & Saier, M. H., Jr (1994d). Regulation of 2-deoxyglucose phosphate accumulation in *Lactococcus lactis* vesicles

by metabolite-activated, ATP-dependent phosphorylation of serine-46 in HPr of the phosphotransferase system. *Microbiology* **140**, 3421–3429.

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