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Fosfomycin Resistance: Selection Method for Internal and Extended Deletions of the Phosphoenolpyruvate:Sugar Phosphotransferase Genes of *Salmonella typhimurium*[1]

J. CHRISTOPHER CORDARO,[2] THOYD MELTON,[3] JOHN P. STRATIS,[4] MEHTAP ATAGÜN,[5] CHRISTINE GLADDING, PHILIP E. HARTMAN,* AND SAUL ROSEMAN

Department of Biology and McCollum-Pratt Institute, The Johns Hopkins University, Baltimore, Maryland 21218*

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Selection for resistance to the antibiotic fosfomycin (FOS; L-*cis*1,2-epoxypropylphosphonic acid, a structural analogue of phosphoenolpyruvate) was used to isolate mutants carrying internal and extended deletions of varying lengths within the *ptsHI* operon of *Salmonella typhimurium*. Strains carrying "tight" *ptsI* point mutations and all mutants in which some or all of the *ptsI* gene was deleted were FOS resistant. In contrast, strains carrying *ptsH* point mutations were sensitive to FOS. Resistance to FOS appeared to result indirectly from catabolite repression of an FOS transport system, probably the *sn*-glycerol-3-phosphate transport system. Resistant *ptsI* mutants became sensitive to FOS when grown on D-glucose-6-phosphate, which induces an alternate transport system for FOS, or when grown in the presence of cyclic adenosine 3',5'-monophosphate. A detailed fine-structure map of the *pts* gene region is presented.

The antibiotic fosfomycin (FOS), or phosphonomycin (L-*cis*-1,2-epoxypropylphosphonic acid), is known to enter the bacterial cell by at least two inducible transport systems, the *sn*-glycerol-3-phosphate transport system (*glpT*) and the hexose phosphate transport system (*uhp*) (9). Intracellularly, FOS inhibits irreversibly the activity of phosphoenolpyruvate (PEP):uridine diphospho-*N*-acetylglucosamine enolpyruvyl transferase, and some mutant strains showing increased resistance to FOS produce a transferase enzyme with a reduced affinity for FOS (25, 26).

During a search for resistant mutants of *Salmonella typhimurium* by direct selection on FOS-containing medium with lactate as the sole carbon source, many *glpT* mutants were found, suggesting that the *glpT* system is partially constitutive. In addition, *ptsI* point mutants lacking enzyme I of the PEP:glycose phosphotransferase system (PTS) were recovered (T. Melton, unpublished data). Consequently we tested the *pts* mutants existing in our collection (6) with the following results: (i) growth of all *ptsH* mutants and of "leaky" *ptsI* mutants was inhibited by FOS, whereas (ii) all "tight" *ptsI* point mutants and those strains carrying *pts* deletions encompassing part or all of the *ptsI* gene were FOS resistant. From these observations followed the design of a selection method leading to the recovery of internal and extended deletions of the *pts* operon.

The known enzymatic composition (2, 10-13) and the physiological roles of the PTS have been extensively reviewed (19-21) and will be presented here only in summary form (Fig. 1). In *S. typhimurium* and *Escherichia coli*, the following sugars (D configuration) are concomitantly phosphorylated and transported across the cell membrane by the PTS: glucose, mannose, fructose, the hexitols, *N*-acetylglucosamine, *N*-acetylmannosamine, glucosamine, mannosamine, and β-glucosides. The overall reaction, the transfer of the phosphoryl group from PEP to sugar, involves the transfer of the phosphoryl group down the chain of PTS proteins, the sequence being PEP to enzyme I, HPr, a sugar-specific protein (III or II-A), and finally to the sugar, the last step being catalyzed by another sugar-specific protein, II-B (Fig. 1). The general proteins, enzyme I and HPr, are found in the soluble fraction of cell-

[1] Contribution no. 877 of the McCollum Pratt Institute and the Department of Biology, The Johns Hopkins University.

[2] Present address: National Science Foundation, Washington, DC 20550.

[3] Present address: Department of Microbiology, North Carolina State University at Raleigh, Raleigh, NC 27607.

[4] Present address: Department of Physiology, Fairleigh Dickinson University, Hackensack, NJ 07601.

[5] Present address: University of Maryland, School of Medicine, Baltimore, MD 21201.

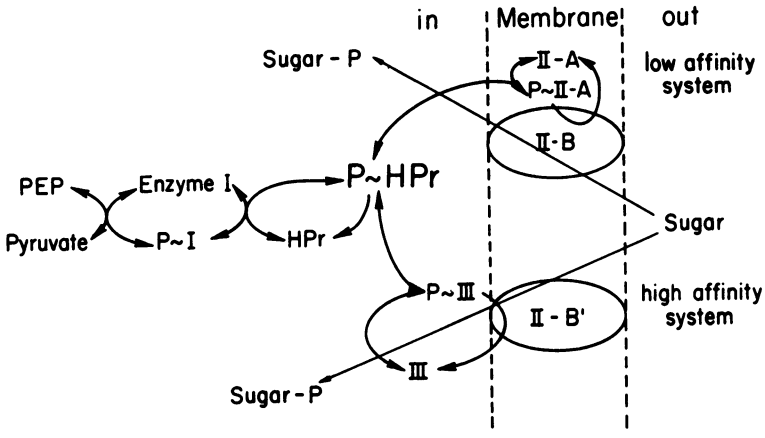

FIG. 1. *PTS and its biochemical reactions: phosphate transfer and sugar transport via the PTS.*

free homogenates whereas a sugar-specific pair may be entirely membrane bound (II-A/II-B), or one component may be soluble (III) whereas the other is an integral membrane protein (II-B'). In some cases, there are two transport systems for a given sugar; for example, glucose is transported and phosphorylated by a $II\text{-}A^{Glc}$/II-B and by a III^{Glc}/$II\text{-}B'^{Glc}$ system.

Since, as mentioned earlier, strains carrying "tight" *ptsI* point mutations and all previously isolated *pts* deletion mutants (6) are FOS resistant, mutagenesis under conditions that are known to produce PTS deletions (5, 6) and selection for FOS resistance should yield strains with *pts* multisite mutations encompassing at least a portion of the *ptsI* gene and permit fine-structure mapping of this region.

MATERIALS AND METHODS

Bacterial strains and media. Table 1 lists the derivation and relevant genotype of each strain used in this study. The preparation of growth and indicator media, the propagation of phage lysates, techniques for transduction and reversion analyses, and nitrous acid mutagenesis were performed as previously described (5, 6). After nitrous acid mutagenesis of strain SB3507 (*trpB223*) and overnight growth in nutrient broth (Difco) at 37°C, samples of each culture were plated on medium A (7) agar plates containing 20 μg of L-tryptophan and 40 μg of FOS per ml with either 0.2% lactate or 0.2% maltose as sole carbon source. Master plates of the same composition were prepared, incubated overnight at 37°C, and replica plated onto eosin-methylene blue plates containing 1% D-mannitol followed by overnight incubation at 37°C. D-Mannitol-nonfermenting clones were purified through two single-colony isolations and tested for their ability to ferment D-maltose. Strains carrying putative *pts* multisite mutations that are crr^+ fail to ferment D-maltose, whereas those that are crr^- do ferment D-maltose (6).

PTS assays. Cell-free extract preparations separating the soluble and particulate PTS proteins and the in vitro PTS assays were performed as previously described (5, 6, 12, 13) such that the particular PTS activity being examined was made rate limiting in the presence of added quantities of the remaining PTS components. Homogeneous HPr, highly purified enzyme I, and factor III were used where appropriate. Strain SB2950, carrying a complete deletion of the *pts* region (6), was used as the source of membranes containing the enzymes II. Membrane preparations from this strain have elevated enzyme II levels when compared with wild type (6) and contain none of the soluble PTS components that are sometimes trapped inside such vesicle preparations. In each case, the specific activity is expressed as micromoles of sugar phosphate formed in 30 min at 37°C per milligram of protein.

RESULTS

FOS resistance: pts deletion isolation. Examination of the *Salmonella pts* mutants in our collection (see Table 1) showed that all strains carrying *ptsH* point mutations were FOS sensitive. In contrast, most strains carrying *ptsI* point mutations, and all strains carrying *pts* deletions that included at least a portion of *ptsI*, were FOS resistant. This observation led to the design of a selection method for isolation of internal and extended deletions of the *pts* gene region. Nitrous acid mutagenesis was followed by selection for FOS resistance under conditions where the presence of functional *pts* genes is unselected (e.g., lactate or, when deletions extend into the *crr* gene region, maltose as the sole carbon source). Subsequent screening on eosin-methylene blue medium containing 1% D-mannitol allowed the detection of strains carrying *pts* point mutations and deletions. After examination of mutant isolates for their inability to revert to prototrophy on media that select for the pts^+ phenotype (5, 6), strains carrying

putative new *pts* deletions were retained for future reversion, transduction, transport, and PTS enzyme analyses.

Deletion mapping. Figure 2 presents a deletion map of the *Salmonella pts* region determined by transduction analyses with phage KB1 (3) or P22 HT *int-4* (John Roth, personal communication). Phage were grown on strains carrying *pts* point and deletion mutations as donors and various strains carrying *pts* deletions as recipients. Selection was made for *pts*$^+$ prototrophic recombinants, and the data were used to determine the termini of the various *pts* deletions (6).

TABLE 1. *Strains of S. typhimurium used for genetic tests and physiological studies*

SB no.[a]	Genotype[b]	PTS defect	Mutagen[c]	Source
3507	*pts*$^+$ *trpB223*	None		E. Balbinder
761	*ptsI5*	Enzyme I	AP	M. Saier, unpublished data
762	*ptsH6*	HPr	AP	M. Saier, unpublished data
840	*ptsI10*	Enzyme I	NG	M. Saier, unpublished data
1470	*ptsI11*	Enzyme I	AP	M. Saier, unpublished data
1476	*ptsI17*	Enzyme I	AP	(21)
1477	*ptsI18*	Enzyme I	AP	(21)
1681	*ptsI16*	Enzyme I	AP	M. Saier, unpublished data
1685	*ptsH24*	HPr	AP	M. Saier, unpublished data
1690	*ptsI34 trpB223*	Enzyme I	DES	(6)
1740	*ptsI8*	Enzyme I	AP	M. Saier, unpublished data
2227	*ptsI39 trpB223*	Enzyme I	DES	(6)
2309	*cysK-ptsPHIΔ41 trpB223*	Promoter, HPr, enzyme I deletion	NA	(6)
2310	*cysK-ptsPHIΔ42 trpB223*	Promoter, HPr, enzyme I deletion	NA	(6)
2311	*cysK-ptsPHIΔ43 trpB223*	Promoter, HPr, enzyme I deletion	NA	(6)
2313	*cysK-ptsPHIΔ44 trpB223*	Promoter, HPr, enzyme I deletion	NA	(6)
2314	*cysK-ptsPHIΔ45 trpB223*	Promoter, HPr, enzyme I deletion	NA	(6)
2348	*cysK-ptsPHIΔ46 trpB223*	Promoter, HPr, enzyme I deletion	NA	(6)
2349	*cysK-ptsPHIΔ47 trpB223*	Promoter, HPr, enzyme I deletion	NA	(6)
2357	*cysK-ptsPHIΔ48 trpB223*	Promoter, HPr, enzyme I deletion	NA	(6)
2696	*ptsI152 trpB223 mem-1*	Enzyme I	DES	Cordaro et al.[d]
2698	*ptsI154 trpB223 mem-1*	Enzyme I	DES	Cordaro et al.[d]
2728	*cysK-ptsPHIcrrΔ155 trpB223*	Promoter, HPr, enzyme I, factor III deletion	NA	J. C. Cordaro, unpublished data
2732	*cysK-ptsPHIcrrΔ157 trpB223*	Promoter, HPr, enzyme I, factor III deletion	NA	J. C. Cardaro, unpublished data
2950	*cysK-ptsPHIcrrΔ49 trpB223*	Promoter, HPr, enzyme I, factor III deletion	NA	(6)
3678	*ptsI168 trpB223*	Enzyme I	Spontaneous	This paper[e]
3680	*ptsIΔ163 trpB223*	Enzyme I deletion	NA	This paper[e]
3681	*ptsI164 trpB223*	Enzyme I	NA	This paper[e]
3686	*ptsPHIcrrΔ166 trpB223*	Promoter, HPr, enzyme I, factor III deletion	NA	This paper[f]
3687	*ptsIcrrΔ167 trpB223*	Enzyme I, factor III deletion	NA	This paper[e]
3688	*ptsI169 trpB223*	Enzyme I	NA	This paper[f]
3689	*ptsI189 trpB223*	Enzyme I	NA	This paper[f]
3696	*ptsI183 trpB223*	Enzyme I	NA	This paper[f]
3727	*ptsI172 trpB223*	Enzyme I	NA	This paper[f]
3728	*ptsI173 trpB223*	Enzyme I	NA	This paper[e]
3729	*ptsI174 trpB223*	Enzyme I	NA	This paper[e]
3737	*ptsH196 trpB223*	HPr	Spontaneous	(16)
3749	*ptsHIcrrΔ180 trpB223*	HPr, enzyme I, factor III deletion	NA	This paper[f]
3750	*ptsIΔ181 trpB223*	Enzyme I deletion	NA	This paper[f]
3751	*cysK-ptsPHIΔ182 trpB223*	Promoter, HPr, enzyme I deletion	NA	This paper[f]
3769	*ptsH197 trpB223*	HPr	NA	(16)
3770	*ptsI184 trpB223 ptsG217*	Enzyme I	Spontaneous	(16)

TABLE 1.—*Continued*

SB no.[a]	Genotype[b]	PTS defect	Mutagen[c]	Source
3778	*ptsI185 trpB223*	Enzyme I	Spontaneous	This paper[f]
3786	*ptsI186 trpB223*	Enzyme I	ICR 191	This paper[e]
3787	*ptsI187 trpB223*	Enzyme I	NA	This paper[e]
3788	*ptsI188 trpB223*	Enzyme I	NA	This paper[e]
3789	*ptsI189 trpB223*	Enzyme I	NA	This paper[e]
3790	*ptsI190 trpB223*	Enzyme I	NA	This paper[f]
3791	*ptsI191 trpB223*	Enzyme I	NA	This paper[f]
3798	*ptsH198 trpB223*	HPr	NA	(16)
3799	*ptsI199 trpB223*	Enzyme I	NA	(16)
3800	*ptsH192 trpB223*	HPr	NA	(16)
3802	*ptsIΔ194 trpB223*	Enzyme I deletion	NA	(16)

[a] All strains carrying *pts* point mutations and *pts* deletion mutations that include at least a portion of the *ptsI* gene are resistant to FOS on medium A agar plates containing 0.2% lactate as sole carbon source and 40 μg of FOS per ml with the exception of the following "leaky" strains: SB1470 (*ptsI11*), SB1476 (*ptsI17*), and SB1740 (*ptsI8*).

[b] The two *pts*$^+$ parents for the isolation of all *pts* point and deletion mutants are SB3507 (*trpB223*) and the wild-type strain, LT-2.

[c] The mutagens used to induce the *pts* mutants listed in Table 1 are: nitrous acid (NA); 2-aminopurine (AP); diethyl sulfate (DES); and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NG).

[d] J. C. Cordaro, P. W., Postma, and S. Roseman, Fed. Proc., p. 1326, abstr. 580, 1974.

[e] Isolated as FOS resistant on medium A agar plates containing 20 μg of L-tryptophan per ml, 40 μg of FOS per ml, and 0.2% D-maltose as the sole carbon source. Bacteria in about 2% of FOS-resistant colonies carried *pts*$^-$ mutations; about 25% of the *pts* mutations were deletions.

[f] Same as in *e* above except the sole carbon source was 0.2% lactate. It should be noted that strain SB3507 yields a large number of spontaneously resistant cells when plated on FOS at the concentration used. In the cases where mutagens were used for the induction of deletions, the mutagenized cultures were diluted to sufficiently minimize the presence of spontaneous mutants.

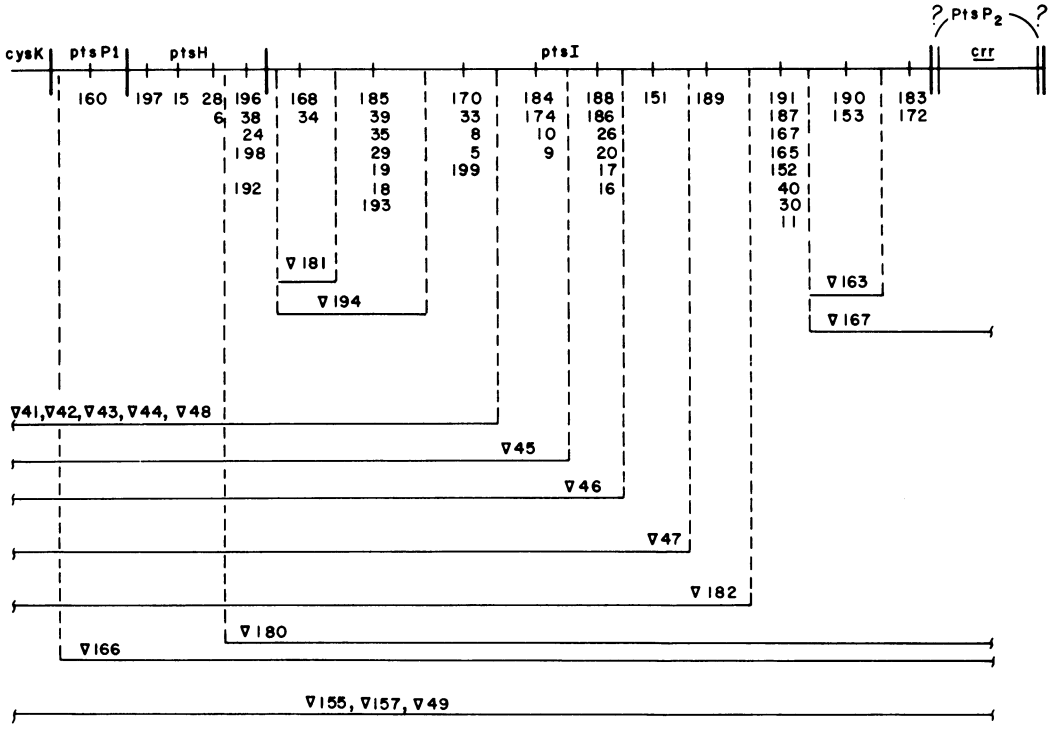

FIG. 2. *Deletion map of the ptsHI gene region of S. typhimurium (not drawn to scale). Heavy vertical lines indicate gene boundaries. The cysK gene is closely linked, but not necessarily adjacent, to ptsP1, the promoter region for the ptsH and ptsI genes (6). The crr gene appears to be regulated separately from the ptsHI operon, and the site of its promoter (ptsP2) has not been established (6).*

PTS assays of new deletion strains. Table 2 shows the specific activities of the various PTS components measured in the presumptive *pts* deletions isolated as FOS resistant and determined to be D-mannitol-nonfermenting strains. Three classes of *pts* deletion strains are evident from the in vitro PTS assays presented in Table 2. The phenotypic classes reflect the extents of the deletions, as shown on the genetic map (Fig. 2). One class of strains, represented by strains SB3680 (*ptsIΔ163*) and SB3750 (*ptsIΔ181*), completely lacked detectable enzyme I activity but contained both HPr and factor III^{Glc} activities. The presence of factor III^{Glc} (i.e., *crr*$^+$) also was suggested by the inability of these strains to ferment and grow on two non-PTS compounds, maltose and glycerol. These strains also were triazole sensitive (*cysK*$^+$).

A second class of strains is represented by strain SB3751, containing the *cysK-ptsPHIΔ182* deletion. This strain lacked detectable Hpr and enzyme I activity and was resistant to 1,2,4-triazole, yet it produced normal levels of factor III^{Glc} (i.e., it was *crr*$^+$). Again, this result agrees with the genetic map (Fig. 2). This type of *cysK-ptsPHI* deletion has been isolated previously by selection for 1,2,4-triazole-resistant mutants that are D-mannitol nonfermenters (6).

TABLE 2. *Specific activities of pts components in fosfomycin-resistant pts deletion mutants*[a]

SB no.	Relevant genotype	PTS components (sp act)		
		HPr	Enzyme I	Factor III^{Glc}
3507	*pts*$^+$	1.1	5.0	0.24
3680	*ptsIΔ163*	4.0	<0.01	0.29
3750	*ptsIΔ181*	1.0	<0.01	0.29
3751	*cysK-ptsPHIΔ182*	<0.01	<0.01	0.19
3687	*ptsIcrrΔ167*	2.0	<0.01	<0.02
3749	*ptsHIcrrΔ180*	<0.01	<0.01	<0.03
3686	*ptsPHIcrrΔ166*	<0.01	<0.01	<0.02

[a] Strains analyzed for levels of the soluble PTS components were grown to saturation in medium A containing 20 μg of L-tryptophan per ml and 0.2% lactate as sole carbon source, harvested, washed in 0.9% NaCl, suspended in 10 mM tris(hydroxymethyl)aminomethane buffer, pH 7.5, containing 1 mM ethylenediaminetetraacetic acid and 0.2 mM dithiothreitol, and disrupted in the French press. The cell-free extract was clarified in the Sorvall centrifuge at 16,000 × *g* for 10 min and then in the Spinco centrifuge at 200,000 × *g* for 2 h to separate the soluble and membrane-bound PTS components. The specific activity of each PTS component was determined as described in Materials and Methods. Methyl-α-D-[U-^{14}C]glucopyranoside (3.0 × 10^5 cpm/mol) was the substrate for the HPr and enzyme I determinations, whereas methyl-β-D-[*methyl*-^{14}C]thiogalactoside was the substrate in the assays for factor III^{Glc} activity. All sugar substrates were used at 10 mM final concentration. Protein determinations were performed according to the biuret procedure of Layne (14).

Deletions in a third class of *pts* mutants included a part or all of both the *ptsI* and *crr* genes. Consequently, these strains could grow on a non-PTS sugar such as maltose in the absence of a functional *ptsI* gene (*crr*$^-$ phenotype). Three separate deletions, each sensitive to 1,2,4-triazole, comprise the members of this class. Strain SB3687 containing *ptsIcrrΔ167* lacked both enzyme I and factor III^{Glc} activities but produced HPr, in agreement with Fig. 2, showing the leftward terminus of this deletion lying within the *ptsI* gene. Strains SB3749 (*ptsHIcrrΔ180*) and SB3686 (*ptsPHIcrrΔ166*) lacked detectable levels of all three soluble PTS components, HPr, enzyme I, and factor III^{Glc}. Genetically, each of these two *pts* deletions had a different leftward terminus (Fig. 2). Mutation *ptsHIcrrΔ180* recombined with *ptsP160*, *ptsH15*, and *ptsH28* but not with *ptsH38*, suggesting that its leftward terminus lies within the *ptsH* gene. The *ptsPHIcrrΔ166* deletion, by contrast, was unable to recombine with any *ptsH* mutation or with *ptsP160*, suggesting that its leftward terminus is located in the *pts* operator-promoter region (*ptsP*) or between *ptsP1* and *cysK*.

Failure of FOS to inhibit PTS activities. One possible mechanism to explain the recovery of *pts* deletions as a consequence of FOS resistance is that one or more of the PTS components is inhibited by FOS. To test this notion, 0.1 to 1 mM FOS was added before, during, or after the transport of methyl-α-glucoside into *Salmonella* strain SB3507 (*trpB223 pts*$^+$). No inhibition of the initial transport rate of the glycoside was seen, nor did FOS increase the efflux of methyl-α-glucoside from the cells after transport had occurred. In addition, FOS was added to in vitro PTS assays under a variety of conditions, and no inhibition of PTS activity was observed. These findings suggest that the involvement of enzyme I in conferring sensitivity to FOS must occur by a mechanism that does not depend directly on the presence of catalytically active enzyme I.

Mechanism of FOS resistance of enzyme I mutants. Several experiments suggest that the resistance of enzyme I (*ptsI*) mutants is indirect and a consequence of extreme PTS-mediated repression (19) operative in these mutants. Two transport systems, the *sn*-glycerol-3-phosphate transport system and the hexose phosphate transport system, both of which are inducible, are known to transport FOS (8, 9). The data presented in Fig. 3 show that the growth of a *pts* deletion mutant strain, SB2309, was resistant to 40 μg of FOS per ml when grown with 0.2% lactate but was inhibited by the same

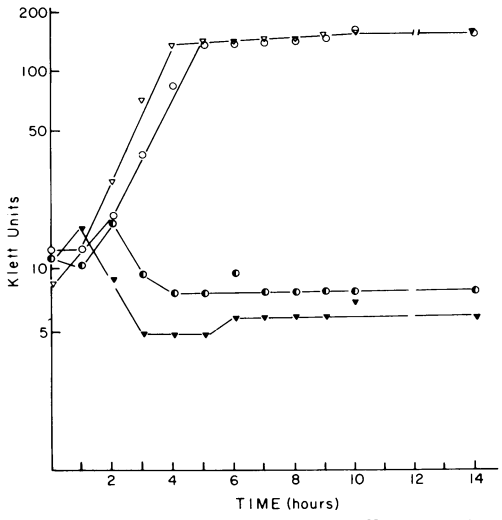

FIG. 3. *Effects of fosfomycin on cells grown in* D-*glucose-6-phosphate. Bacteria were grown overnight in 0.2%* D-*glucose-6-phosphate (medium A minus citrate) at 37°C with aeration. These preadapted cultures were used to inoculate fresh medium (medium A minus citrate) containing 0.2%* D-*glucose-6-phosphate (10 ml) in bubbler tubes. All tubes contain 20 μg of* L-*tryptophan per ml, with and without 40 μg of fosfomycin per ml. Turbidity was measured at intervals in a Klett-Summerson photoelectric colorimeter using filter no. 54. Strain SB3507 (trpB223) with (▼) and without (▽) fosfomycin. Strain SB2309 (HI deletion) with (◐) and without (○) fosfomycin.*

concentration of FOS when grown with 0.2% D-glucose-6-phosphate as the sole carbon source. The data represent results obtained with strains SB3678 (*ptsI* point mutant), SB3680 (internal *ptsI* deletion), and SB3686 (*ptsPHIcrr* deletion), all of which are resistant to the drug on lactate-minimal salts medium. Under the same conditions as those used in the experiment depicted in Fig. 3, one cannot stimulate sensitivity to FOS by using *sn*-glycerol-3-phosphate as the sole carbon source. These results may be taken as evidence that the resistance of these mutants to FOS is not due to the lack of the enzyme I protein directly, but is a secondary consequence of the effect of the mutation on the transport system(s) responsible for the uptake of the FOS when cells are grown in lactate as the sole carbon source.

Table 3 shows the growth of *pts* deletion mutants on minimal agar plates with various carbon sources in the presence and absence of added FOS. All of the deletion mutant strains were FOS resistant when grown on lactate and 40 μg of FOS per ml, but retained sensitivity to the drug in the presence of D-glucose-6-phosphate. This agrees with the inability of these strains to grow in liquid medium containing 40 μg of FOS per ml and glucose-6-phosphate (cf. Fig. 3). With the exception of strains SB2349 and SB3686, growth of these deletion mutant strains on *sn*-glycerol-3-phosphate plus FOS did

TABLE 3. *Growth of pts deletion strains on minimal plates with different carbon sources with and without fosfomycin*[a]

Strain	*pts* lesion	Carbon Source[b]							
		Lac	Lac/FOS	G6-P	G6-P/FOS	G3P	G3P/FOS	Gly	Gly/FOS
SB3507	*pts*$^+$	++	0	++	0	++	0	++	0
SB2309	*HI*	++	++	++	0	++	++	0	ND
SB2310	*HI*	++	++	++	0	++	++	0	ND
SB2311	*HI*	++	++	++	0	++	++	0	ND
SB2314	*HI*	++	++	++	0	+	+	0	ND
SB2348	*HI*	++	++	++	0	+	+	0	ND
SB2349	*HI*	++	++	++	0	++	+	0	ND
SB2357	*HI*	++	++	++	0	+	+	+	0
SB2728	*HIcrr*	++	++	++	0	+	+	+	0
SB2732	*HIcrr*	++	++	++	0	+	+	+	0
SB2950	*HIcrr*	++	++	++	0	++	++	++	0
SB3686	*HIcrr*	++	++	++	0	++	+	++	0
SB3687	*Icrr*	++	++	++	0	++	++	++	0
SB3749	*HIcrr*	++	++	++	0	++	++	++	0
SB3750	*I*	++	++	++	0	++	++	0	ND
SB3751	*HI*	++	++	++	0	+	+	0	ND

[a] Cultures were aerated overnight in nutrient broth at 37°C. Bacteria were harvested, washed in 0.9% saline, and then streaked onto the various minimal agar plates. All agar plates contained 20 μg of L-tryptophan per ml and the indicated carbon sources at 0.2% with and without 40 μg of fosfomycin (FOS) per ml. The plates were allowed to incubate overnight and then scored for growth. Symbols: (++) strong growth; (+) weak growth; (0) no growth.

[b] Media contain lactate (Lac), glucose-6-phosphate (G6-P), *sn*-glycerol-3-phosphate (G3P), or glycerol (Gly) without and with fosfomycin (FOS).

not produce the pattern of sensitivity observed with D-glucose-6-phosphate (G3P columns, Table 3). Those *pts* deletion strains SB2728, SB2732, SB2950, SB3686, SB3687, and SB3749 that included the *crr* (catabolite repression resistance) gene were sensitive to the antibiotic when grown on glycerol as the sole carbon source. In contrast, most strains carrying internal *ptsI* deletions and *ptsHI* deletions were not able to grow on glycerol or on a number of other non-PTS sugars such as melibiose and maltose (not shown in table). The results indicate that the selection for *pts*$^-$ mutants by FOS resistance on lactate as sole carbon source involves indirect effects of the *pts* mutations on other systems, probably on one or more transport systems capable of FOS uptake.

The majority of the *pts*$^-$ FOS-resistant mutants (growth on lactate + FOS) became sensitive to FOS (no growth) when cyclic 3',5'-adenosine monophosphate (cAMP) was added to the medium (Table 4). Addition of cAMP also enhanced growth of a number of *ptsI* (enzyme I) and *ptsHI* (HPr and enzyme I) strains on glycerol and on *sn*-glycerol-3-phosphate (Table 5). Of the strains that did not respond to cAMP (Tables 4 and 5), strains SB2696 and SB2698 carried a mutation (*mem-1*) that affected the *Salmonella* membrane and the uptake of a number of compounds, one of which may have been cAMP (see Table 5; J. C. Cordaro, P. W. Postma, and S. Roseman, in preparation). The lack of return of FOS sensitivity to strains SB1690 and SB1477 in the presence of cAMP (Table 4) may indicate either the inability of these cells to take up the nucleotide or the lack of some intracellular component necessary to interact with the nucleotide once taken up. The latter seems more likely for strain SB1477 since cAMP stimulated the growth of this strain on maltose (Table 5).

TABLE 4. *cAMP sensitization of pts FOS-resistant mutants of S. typhimurium*

PTS FOS^r mutants	Medium[a]		Diam of S-zone (mm)[b]
	Lactate/ FOS	Lactate/ FOS/ cAMP	
Point *ptsI* mutants			
Selected with FOS SB3678, SB3681, SB3727, SB3728	++	0	2.5
SB3696, SB3729	++	0	2.0
Not selected with FOS SB1477, SB1690	++	++	NS
SB2227	++	0	2.5
SB2696 (*mem-1*), SB2698 (*mem-1*)	++	++	NS
Deletion mutants			
ptsI deletions			
SB3680, SB3750	++	0	3.0
ptsHI deletions			
SB2309, SB2314	++	0	2.5
SB2310	++	0	2.3
SB2311	++	0	2.7
SB2313, SB2349, SB2357	++	0	2.0
SB2348, SB3751	++	0	3.0
ptsHIcrr deletions			
SB2732	++	0	2.5
SB2950, SB3686, SB3749	++	0	3.0
ptsIcrr deletion			
SB3687	++	0	2.5

[a] Cultures were treated as in the legend to Table 3, and 0.1 ml of the washed cultures was spread on minimal 0.2% lactate (medium A minus citrate) agar plates (1.5% BBL agar). The plates contained 20 μg of L-tryptophan and 40 μg of fosfomycin (FOS) per ml. To some of the plates a sterile disk was added, and 0.1 ml of a 0.1 M solution of cAMP (sodium salt) was applied to the disk. The plates were allowed to incubate at 37°C overnight and were scored for growth the next morning. Symbols: ++, Growth; 0, no growth; NS, not sensitive.

[b] S-zone refers to the area around the disk containing cAMP where no growth could be detected due to sensitization to fosfomycin (sensitization zone).

DISCUSSION

We have demonstrated that recovery of internal and extended deletions of the *pts* gene region in *S. typhimurium* is possible by selection for resistance to FOS, a structural analogue of phosphoenolpyruvate. Selection for resistance to FOS with lactate as the sole carbon source allows the recovery of *ptsI* deletions, some of which extend from *ptsI* in either direction, e.g., leftward toward *ptsP1* and *cysK* and rightward into or through the *crr* gene region (Fig. 2). In contrast, selection for FOS resistance on maltose allows the exclusive recovery of *ptsI* deletions, which must include the *crr* gene in addition to portions of either or both *ptsH* and *ptsP1* (cf. reference 23).

Our studies indicate that the resistance of these mutants to FOS is a result of their inability to take up the antibiotic. Growth on D-glucose-6-phosphate, which induces an alternate uptake system for FOS (9), leads to FOS sensitivity among the *ptsI* mutants. In addition, supplementation of the medium with cAMP restores FOS sensitivity. We propose that the *sn*-glycerol-3-phosphate uptake system (*glpT*) is slightly constitutive or that there exists another FOS uptake system (either constitutive or induced by FOS) that is expressed in lactate medium by wild-type bacteria but hypersensitive to PTS-mediated repression (19) in "tight" *ptsI* mutants grown on lactate.

Presently at least three biological activities have been described that may be responsible for regulating the levels of cAMP in bacterial cells. The first activity may be the excretion of the

TABLE 5. *Growth pattern of pts fosfomycin-resistant mutants on glycerol, sn-glycerol-3-phosphate, maltose, and succinate with and without cAMP*

Strain	Carbon source[a]							
	Glycerol		G3P[b]		Maltose		Succinate	
	-cAMP	+cAMP	-cAMP	+cAMP	-cAMP	+cAMP	-cAMP	+cAMP
ptsI mutants								
SB1477	+	+	++	++	0	++	0	0
SB2227	+	++	+	++	+	++	0	0
SB2696 (mem-1)	0	0	0	0	0	0	0	0
SB2698 (mem-1)	0	0	0	0	0	0	0	0
SB3680	0	+	++	+	0	+	0	0
SB3681	0	++	+	+	0	0	0	0
SB3727	+	++	+	++	0	0	0	0
SB3729	+	++	0	++	0	0	0	0
SB3750 (Δ)	0	++	++	++	0	+	0	+
SB3770	0	0	0	+	0	0	0	0
HIcrr deletions								
SB2728	++	++	+	+	++	++	+	+
SB2950	++	++	++	++	++	++	+	++
SB3686	++	++	++	++	+	++	0	0
SB3749	++	++	+	+	++	++	0	0
Icrr deletion								
SB3687	++	++	++	++	++	++	0	+

[a] Cultures were grown in nutrient broth overnight with aeration at 37°C. The cells were washed with 0.9% saline, and 0.1 ml of this culture was spread on minimal agar plates with different carbon sources (0.2% sugar) and 20 μg of L-tryptophan per ml. To some of the plates a sterile disk (1.5-cm diameter) was added and 0.1 ml of a sterile 0.1 M solution of cAMP (sodium salt) was applied to the disk. The plates were allowed to incubate overnight and were scored for growth. Symbols: ++, Strong growth; +, weak growth; 0, no growth.

[b] G3P, *sn*-glycerol-3-phosphate.

nucleotide from the bacterial cell into the medium (15). Another activity that may control the intracellular levels of cAMP is the cAMP phosphodiesterase, an enzyme that catalyzes the cleavage of cAMP to 5'-adenosine monophosphate (1). Finally, there is the adenylate cyclase activity, which is responsible for the synthesis of cAMP from adenosine triphosphate (4, 17, 24). Recent studies suggest that enzyme I mutations influence both adenylate cyclase activity (18, 22) and excretion of cAMP (22).

Strains carrying deletions that extend into the *crr* gene region (cf. reference 23) permit growth on some non-PTS carbon sources, particularly glycerol (Table 5), but do not at the same time condition to FOS sensitivity (Table 5), itself mediated by exogenous cAMP (Table 4). In addition, the *crr* deletion strains are heterogeneous with regard to their ability to grow on succinate (Table 5). These dichotomies in response may be due to a hierarchy of cAMP response mechanisms (1), coupled to differing sensitivities of the various detection methods we have used. The PTS strains described here, contained in a standard genetic background, implicate absolute enzyme I deficiency in the pleiotropic physiological effects observed. The strains should prove of value in further analyses of PTS-mediated repression (19), as they have in analyses of *Salmonella* chemotaxis receptors for sugars (T. Melton, P. E. Hartman, J. P. Stratis, T. L. Lee, and A. T. Davis, in preparation).

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