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Regulation of competence development and sugar utilization in *Haemophilus influenzae* Rd by a phosphoenolpyruvate:fructose phosphotransferase system

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Summary

Changes in intracellular cAMP concentration play important roles in *Haemophilus influenzae*, regulating both sugar utilization and competence for natural transformation. In enteric bacteria, cAMP levels are controlled by the phosphoenolpyruvate:glycose phosphotransferase system (PTS) in response to changes in availability of the preferred sugars it transports. We have demonstrated the existence of a simple PTS in *H. influenzae* by several methods. We have cloned the *H. influenzae ptsI* gene, encoding PTS Enzyme I; genome analysis locates it in a *pts* operon structurally homologous to those of enteric bacteria. *In vitro* phosphorylation assays confirmed the presence of functional PTS components. A *ptsI* null mutation reduced fructose uptake to 1% of the wild-type rate, and abolished fructose fermentation even when exogenous cAMP was provided. The *ptsI* mutation also prevented fermentation of ribose and galactose, but utilization of these cAMP-dependent sugars was restored by addition of cAMP. In wild-type cells the non-metabolizable fructose analogue xylitol prevented fermentation of these sugars, confirming that the fructose PTS regulates cAMP levels. Development of competence under standard inducing conditions was reduced 250-fold by the *ptsI* mutation, unless cells were provided with exogenous cAMP. Competence is thus shown to be under direct nutritional control by a fructose-specific PTS.

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Introduction

All bacteria have mechanisms for regulating the acquisition of metabolites and the conservation of intracellular energy resources. Central to these are sensors that detect the scarcity or depletion of preferred energy sources, and signal pathways that transduce the resulting information into appropriate alterations in gene expression. One well-characterized enteric bacterial sensor is the phosphoenolpyruvate:sugar phosphotransferase system (PTS), an enzyme complex that takes up preferred sugars and detects changes in their availability. The sugar-availability signal is then transmitted via PTS-induced changes in the activity of adenylate cyclase and thus in the intracellular concentration of cAMP. Lack of PTS sugar causes cAMP levels to rise, and formation of its active complex with the transcription factor CRP (cAMP receptor protein) results in transcriptional activation of genes for uptake and catabolism of available but less-preferred sugars.

Little is known about metabolic control in *Haemophilus influenzae*, a small Gram-negative bacterium in the family Pasteurellaceae. Both cAMP and CRP are present, and are known to be required for fermentation of some sugars, but prior to the release of the *H. influenzae* genome sequence, the presence of a PTS was not established. Analysis of the genome revealed open reading frames with significant homology to the *Escherichia coli* PTS genes *ptsH*, *ptsI*, *crr*, *fruA* and *fruB* (Fleischmann *et al*., 1995), but the role of this predicted PTS was unknown. We are particularly interested in an *H. influenzae* PTS because of its possible role in regulation of natural competence – the ability of cells to take up DNA fragments from the environment.

Competent *H. influenzae* cells can take up a total of several hundred kilobases of DNA fragments from their environment. Some of this DNA may then be homologously recombined into the chromosome, while the rest is degraded and the nucleotides recycled (Stewart and Carlson, 1986). Development of the competent state by *H. influenzae* is tightly regulated. It is undetectable during exponential growth and occurs spontaneously at the onset of stationary phase in rich medium. Competence reaches maximal levels when exponential-phase cells

are transferred to a 'starvation' medium called MIV (Herriott *et al*., 1970), which lacks the nucleotide precursors, cofactors and carbon source required for normal growth.

Induction of competence requires a cAMP–CRP complex, suggesting that competence development may be controlled by a PTS. Addition of exogenous cAMP to exponential-phase cells induces competence development, giving a transformation frequency of 10^{-4} (Wise *et al*., 1973), the same level that develops at the onset of stationary phase in rich medium. Moreover, *H. influenzae* contains homologues of the cAMP receptor protein (CRP) of *E. coli* (Chandler, 1992) and adenylate cyclase (Dorocicz *et al*., 1993), and mutations in the corresponding genes (*crp* and *cya*) totally abolish competence; competence development is restored to the *cya* strain by addition of exogenous cAMP (Dorocicz *et al*., 1993).

E. coli and *H. influenzae* adenylate cyclases are homologous throughout their regulatory and catalytic domains (Dorocicz *et al*., 1993), implying that they participate in similar regulatory interactions. As regulation of adenylate cyclase is PTS mediated in *E. coli*, a PTS may also regulate the *H. influenzae* adenylate cyclase. This would place competence under direct nutritional control. Such regulation would suggest that DNA uptake by competent cells, widely considered to function primarily for horizontal gene transfer, might more accurately be considered to be a nutrient-scavenging process (Redfield, 1993).

To assess whether *E. coli* and *H. influenzae* adenylate cyclases are in fact similarly regulated, we have examined complementation of an *H. influenzae cya* strain by the *E. coli cya* gene. We have demonstrated the presence of a fructose-specific adenylate cyclase-regulating PTS in *H. influenzae* by cloning and mutagenising the *ptsI* gene, and by assessing the sugar uptake and fermentation capabilities of wild-type and *ptsI* strains. *In vitro* phosphorylation assays confirmed the presence (predicted by sequence analysis (Fleischmann *et al*., 1995)) of functional homologues of the *E. coli* PTS components Enzyme I, HPr, $EIIA^{Glc}$ and the fructose-specific PTS Enzyme II complex. To examine whether the PTS regulates competence development in *H. influenzae* we have constructed a *ptsI* mutant strain and characterized its competence development.

Results

Adenylate cyclase complementation

The *H. influenzae* adenylate cyclase gene was originally isolated by its ability to complement an *E. coli cya* deletion mutant (Dorocicz *et al*., 1993). Although this showed that the *H. influenzae* gene is active in *E. coli*, it did not address its regulation. Attempts to measure intracellular *H. influenzae* cAMP levels directly have been unsuccessful. However, placing the *E. coli cya* gene in *cya H. influenzae* allows regulation of cAMP levels to be assayed indirectly, by following the development of *H. influenzae* competence in response to changed growth conditions.

The *H. influenzae cya* mutant RR668 is unable to develop competence unless cAMP is provided (Dorocicz *et al*., 1993). Figure 1 shows that normal competence development was restored by both the *H. influenzae* and *E. coli cya* genes on the moderate-copy plasmid pSU18; timing and rate were both indistinguishable from the standard cya^+ strain KW20. Importantly, neither strain showed competence during exponential growth (indicating that cAMP levels were low during this phase), and both developed competence at the onset of stationary phase. The simplest interpretation is that competence in *H. influenzae* is controlled by cAMP, and that adenylate cyclase is regulated by a PTS in a fashion similar to that in *E. coli.*

Cloning, mapping and mutagenesis of ptsI

The existence of an *H. influenzae* PTS was confirmed by cloning the *ptsI* gene, chosen because of its central role in PTS-dependent processes (*ptsI*-encoded Enzyme I is always the initial phosphate acceptor in PTS-mediated

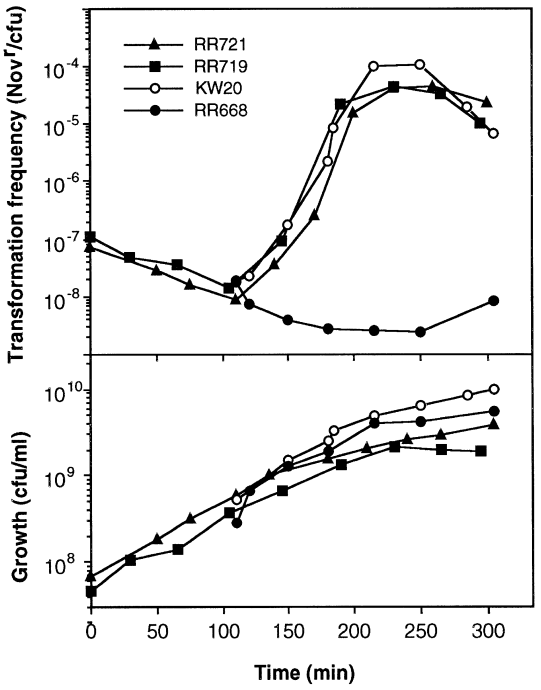

Fig. 1. Complementation of the RR668 (*cya*) transformation deficiency by plasmid-borne *cya* genes from *E. coli* and *H. influenzae*. Complemented strains RR719 (*cya*, pID10) and RR721 (*cya*, pID11) were inoculated into sBHI; aliquots were removed at intervals and incubated with 1 μg ml^{-1} MAP7 DNA at 37°C for 30 min. Competence development was assessed by selecting for novobiocin-resistant (Nov^r) transformants.

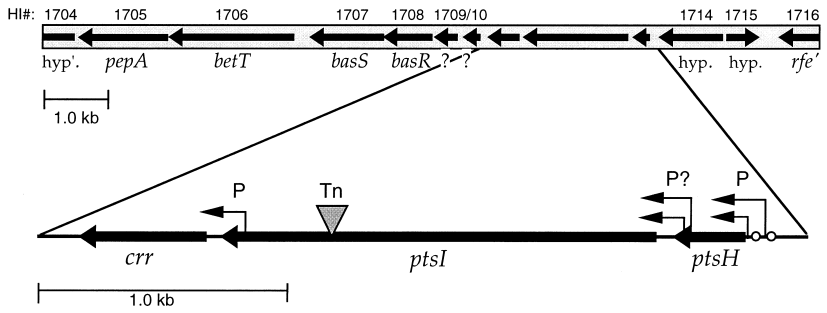

Fig. 2. Schematic diagram of the 12.5 kb *H. influenzae* insert of plasmid pLPM1, and the *pts* operon. Genes identified in the TIGR database are indicated by arrows, with the *H. influenzae* gene number above each gene and its name below. Genes with no known homologues are indicated as '?'; genes homologous to *E. coli* genes of unknown function are indicated as hyp (Fleischmann *et al*., 1995). *rfe′* and *hyp′* indicate that only the 3′ and 5′ ends of these genes, respectively, are contained in plasmid pLPM1. P, putative promoter site; open circles, putative CRP-binding sites; inverted triangle, approximate position of the mini-Tn*10kan* insertion in the *ptsI* gene of RR745.

phosphotransfer (for a review, see Postma *et al*., 1993)). An *H. influenzae* genomic library was transformed into the *E. coli ptsI* temperature-sensitive strain LJ176, and complementing clones were identified by growth at 42°C on minimal salts agar supplemented with 1% mannitol (an *E. coli* PTS sugar). A complementing clone with a 12.5 kb DNA insert was chosen for further analysis (pLPM1, Fig. 2).

To locate the *ptsI* gene within this insert, and to disrupt it for later construction of chromosomal mutants, pLPM1 was mutagenised with mini-Tn*10kan* and transformed into fresh competent LJ176. Loss of complementation of the *ptsI* phenotype identified plasmids carrying insertions in the putative *ptsI* gene. Restriction analysis of five independent isolates showed that all carried insertions in the same 0.6 kb *Pst*I–*Eco*RI fragment of the pLPM1 insert. Most of the subsequent analysis was done with one of these, pLPM1::E2; results with the others were similar.

Sequencing and gene analysis of the pts *operon*

Preliminary sequencing of pLPM1::E2 gave approx. 500 bp of *ptsI* sequence, sufficient to identify homologies with *E. coli* and *Salmonella typhimurium ptsI* genes from GenBank. At this time, the complete genome sequence of *H. influenzae* KW20 became available, and a homologue of the *E. coli ptsHIcrr* operon was identified (*H. influenzae* gene numbers HI#1711-1713) (Fleischmann *et al*., 1995). Comparison of this with the pLPM1 restriction map confirmed that the insert contained this operon, and that all five pLPM1 transposon insertions lay within *ptsI*, a few hundred bp from its 3′ end. Analysis of sequences flanking the *pts* operon indicated that pLPM1 also contained genes HI#1705-1710 and HI#1714-1715 (Fig. 2); these comprise genes for an aminopeptidase, a choline-transport protein (BetT homologue), and homologues of the BasR–BasS two-component system (function unknown), and four other genes of unknown function (two homologous to previously sequenced *E. coli* genes, and two novel genes) (Fleischmann *et al*., 1995).

The full-length 1725 bp *ptsI* coding region is preceded by a putative ribosome-binding site initiating at bp −14 (7/12 matches to the 3′ end of 16 s rRNA; data not shown), and is predicted to encode a 575 amino acid polypeptide with 84.3% similarity (70.2% identity) to *S. typhimurium* Enzyme I and 82.8% similarity (70.4% identity) to *E. coli* Enzyme I. The active-site histidine, His-189, which accepts a phosphate residue from phosphoenolpyruvate, and surrounding active-site amino acids, are identical to those of all homologous Enzymes I (Reizer *et al*., 1993a; L. P. Macfadyen and R. J. Redfield, unpublished data).

The *ptsI* gene lies downstream from *ptsH* and upstream from *crr*, as in the *pts* operons of enteric bacteria. Sequence comparison with promoter consensus sequences identified two potential *ptsH* promoters upstream of the ATG initiation codon, each one preceded by a putative CRP-binding site identified by comparison with the *E. coli* consensus (Botsford and Harman, 1992) (not shown). As in *E. coli*, a potential independent *crr* promoter is found within the 3′ end of *ptsI*. Thus, *H. influenzae* contains a *pts* operon structurally similar to those of enteric bacteria. A GCG 'findpatterns' search also identified two novel potential *ptsI* promoters, beginning 90 bp and 111 bp upstream of the ATG initiation codon, within the 3′ end of *ptsH*. (Postulated regulatory sites are indicated in Fig. 2; their precise locations and sequences can be found on the WorldWide Web, at http://www.zoology.ubc.ca/~redfield.)

Sugar-fermentation assays of wild-type cells

Before investigating the role of the *H. influenzae* PTS in sugar uptake and regulation, we tested wild-type cells for the ability to ferment a broad range of sugars, using a qualitative phenol-red broth (PRB) assay. Fructose, fucose, sialic acid and glycerol gave positive results, as did the previously tested glucose, ribose, galactose, and xylose (Kilian and Frederiksen, 1981) (Table 1). A large number of other sugars gave negative results (deoxyribose, maltose, mannose, arabinose, sucrose, glucose-6-phosphate, sorbitol,

Table 1. Sugar fermentation by strains of *H. influenzae* Rd.

	KW20	RR745 (*ptsI*)		RR668 (*cya*)		RR540 (*crp*)	
Sugar	without cAMP	without cAMP	with cAMP	without cAMP	with cAMP	without cAMP	with cAMP
Fructose	+	−	−	+	+	+	+
Fucose	+	+	+	−	+	−	−
Xylose	+	+	+	−	+	−	−
Ribose	+	−	+	−	+	−	−
Galactose	+	−	+	±	+	±	±
Glycerol	+	+	+	+	+	+	+
Sialic acid	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+

Fifty microlitres of an overnight culture was inoculated into 2 ml phenol red assay broth (PRB) (Difco) supplemented with 10 mg ml^{-1} haemin, 2 μg ml^{-1} NAD, 10% brain–heart infusion (Difco) and 0.5% sugar. Results were scored after at least 8 h of growth in rolled, loosely capped tubes. +, yellow; ±, orange; −, red.

mannitol, inositol, erythritol, galactosamine, glucosamine, mannosamine, *N*-acetylgalactosamine, *N*-acetylglucosamine, gluconic acid, glucoronic acid, methyl-α-glucoside and methyl-β-glucoside). Analysis of the *H. influenzae* genome sequence (Macfadyen and Redfield, 1996) confirmed that the necessary genetic information is present for use of the fermented sugars, and absent for the others. *H. influenzae* ferments far fewer sugars than most enteric bacteria, but this is consistent with its small genome and restricted niche.

Construction of H. influenzae ptsI *mutants and assessment of sugar-fermenting capability*

A *ptsI* mutant strain was constructed by replacing the chromosomal *ptsI* gene with the mutagenised allele via homologous recombination. The wild-type *H. influenzae* Rd strain KW20 was transformed with linearized plasmid pLPM1::E2, and a kanamycin-resistant strain was isolated (RR745). Southern blotting of RR745 genomic DNA with a mini-Tn*10kan* probe confirmed that it contained a single transposon insertion in the identified *pts* locus (data not shown). Blotting of the same filter with a pLPM1 probe confirmed that only the desired recombination event had occurred.

We used PRB assays to investigate the role of the *H. influenzae* PTS in sugar uptake and regulation. Fermentation of fucose, xylose, ribose, and, to a lesser extent, galactose is known to be blocked in *H. influenzae cya* and *crp* mutants, and to be restored in *cya* mutants by added cAMP (Table 1; Dorocicz *et al*., 1993). Similarly, we found that the *ptsI* strain RR745 did not ferment ribose or galactose (Table 1). This suggests that the *H. influenzae* PTS is required to raise intracellular cAMP to a level permitting transcription from CRP-dependent promoters controlling sugar uptake and catabolic genes. Moreover, because addition of exogenous cAMP to the *ptsI* mutant restored fermentation of these sugars, it can be concluded that ribose and galactose are not transported by the PTS itself.

The collected data shown in Table 1 imply that xylose, ribose, fucose and galactose enter *H. influenzae* via cAMP-dependent non-PTS uptake systems. Analysis of the *H. influenzae* genome sequence identified potential CRP sites with between 11 and 17 matches to the 22 bp *E. coli* CRP consensus-binding site near promoters for some genes of related sugar-utilization operons (upstream from *xylA* (HI#1112), *xylT* (HI#1111), *rbsD* (HI#0501), *mglB* (HI#0822) and *fucR* (HI#0615)) (not shown; see WorldWide Web site http://www.zoology.ubc.ca/~redfield).

Fermentation of fructose and glucose is not affected by cya or *crp* mutations (Table 1, Dorocicz *et al*., 1993). This is also seen in *E. coli*, where both sugars are taken up directly by the PTS. The transposon insertion in *H. influenzae ptsI* completely prevented fermentation of fructose (Table 1), and exogenously supplied cAMP did not correct the defect, suggesting that fructose is directly transported by the PTS. This is supported by the observation that the *H. influenzae* genome contains homologues of the two fructose-PTS proteins of *E. coli* (Fleischmann *et al*., 1995). The fructose permease EIIB′BC^{Fru} is encoded by *fruA* (HI#0446), while *fruB* (HI#0448) encodes a multidomain EIIA^{Fru}/FPR protein (Reizer *et al*., 1996) expected to transfer phosphate from EI to EIIB′BC^{Fru}. Together with *ptsI*, these genes specify a complete fructose PTS. Although the EI, HPr and EIIA^{Glc} components of a glucose PTS are specified by the *pts* operon, no glucose PTS permease (EIICB^{Glc}) gene has been identified (Fleischmann *et al*., 1995), and fermentation of glucose was unaffected by Enzyme I disruption. This suggests that, unlike *E. coli*, *H. influenzae* takes up glucose via a cAMP-independent non-PTS permease.

Fermentation of glycerol and sialic acid was not altered by the *ptsI* mutation; homologues of the *nanT* sialic acid permease (Martinez *et al*., 1995; Casari *et al*., 1995) and *glpF* glycerol-uptake facilitator genes (Reizer

Table 2. Identification of PTS enzymes in extracts of fructose-grown cells of *H. influenzae*.

Experiment/Sugar	Components in Reaction				Sugar–P formed (nmole h^{-1})
Experiment A[a]	*E. coli* TP2811 Δ(*ptsHIcrr*) extract	Enzyme I	HPr	*H. influenzae* extract	
[^{14}C]-Mannitol	+	−	−	−	0.03
	+	+	−	−	0.06
	+	−	+	−	0.04
	+	+	+	−	3.2
	+	+	−	+	1.2
	+	−	+	+	3.0
Experiment B[b]	*E. coli* TP2819 Δ(*ptsIcrr*) extract	Enzyme I	Enzyme IIA^{Glc}	*H. influenzae* extract	
[^{14}C]-Methyl-α-glucoside	+	−	−	−	0.03
	+	+	−	−	0.3
	+	−	+	−	0.03
	−	+	+	+	0.02
	+	+	+	−	0.85
	+	+	−	+	0.8
Experiment C[c]	Enzyme I	HPr	DTP	*H. influenzae* membranes	
[^{14}C]-Fructose	+	+	−	−	0.03
	+	−	+	−	0.03
	+	+	−	+	0.05
	+	−	+	+	0.35

a. Assay mixtures (50 μl) contained an extract of *E. coli* TP2811 (10 μg) as the source of Enzyme II^{Mtl}, purified *E. coli* Enzyme I (2.8 μg), purified *E. coli* HPr (2.5 μg), and *H. influenzae* extract (37.5 μg) as indicated.
b. Assay mixtures (50 μl) contained an extract of *E. coli* TP2819 (8 μg) as the source of Enzyme II^{Glc}, purified *E. coli* Enzyme I (2.8 μg), purified *E. coli* Enzyme IIA^{Glc} (3.2 μg), and *H. influenzae* extract (37.5 μg) as indicated.
c. Assay mixtures (50 μl) contained purified Enzyme I (2.8 μg) and HPr (2.5 μg), both from *E. coli*, purified DTP (6 μg) of *S. typhimurium*, and washed membranes of *H. influenzae* (5 μg protein) as indicated.

et al., 1993b; Fleischmann *et al*., 1995) probably account for the observed cAMP-independent PTS-independent uptake of sialic acid and glycerol, respectively.

PTS-mediated regulation of non-PTS sugar utilization

Enteric bacterial PTS systems only activate adenylate cyclase in the absence of glucose or other preferred PTS sugars, and thus cAMP–CRP-dependent operons are not expressed when preferred sugars are available. This, in combination with EIIA^{Glc}-mediated exclusion of certain catabolic operon inducers, comprises the phenomenon known as 'PTS-mediated repression'. The PTS-mediated regulatory effects of the fructose analogue xylitol and the glucose analogue methyl-α-glucoside are well-characterized in enteric bacteria. These analogues are taken up and phosphorylated by their respective PTS permeases, but are not metabolized. We have found that these analogues are also not fermented by *H. influenzae* (data not shown). To determine whether fructose or glucose cause PTS-mediated repression in *H. influenzae*, PRB assays of wild-type cells were carried out using 1–13% of the analogue xylitol or methyl-α-glucoside in combination with the sugars glucose, fucose, xylose, ribose or galactose. Lactose (1–13%), a non-metabolizable sugar in *H. influenzae*, replaced the analogues in negative-control assays. (Although these analogue concentrations are high, culture growth was not affected.) Sugar utilization by KW20 was unaffected by the presence of up to 13% methyl-α-glucoside or lactose. In contrast, 5% and 13% xylitol prevented utilization of all the above sugars except glucose (data not shown). These findings suggest that the *H. influenzae* PTS, similarly to its *E. coli* counterpart, has two functions: in addition to directly transporting sugar (in this case fructose), it regulates cAMP levels to control expression of other sugar-utilization operons (in this case ribose and galactose). Moreover, these data strengthen the argument that glucose must be a non-PTS sugar in this organism.

In vitro *analyses of sugar phosphorylation: identification of PTS components*

The following experiments were carried out to determine whether the PTS genes identified in the *H. influenzae* genome express PTS proteins with functions identical to their *E. coli* homologues. Using [^{14}C]-mannitol as the sugar substrate, complementation assays in which *E. coli* Enzyme II^{Mtl} and either purified Enzyme I or HPr were added in excess demonstrated that *H. influenzae* expresses functional HPr and Enzyme I (Table 2, Experiment A). Using [^{14}C]-methyl-α-glucoside as the substrate

Table 3. Presence of galactokinase and glucokinase in *H. influenzae* extracts.[a]

Sugar	Phosphoryl donor	Sugar–P formed (nmole $mg^{-1} h^{-1}$)
[^{14}C]-Glucose	None	0.004
	PEP	0.15
	ATP	2.2
[^{14}C]-Galactose	None	0.08
	PEP	0.1
	ATP	1.9

a. Sugar phosphorylation was measured as described in the text.

(Table 2, Experiment B), active $EIIA^{Glc}$ was demonstrated in the *H. influenzae* extract. Finally, using [^{14}C]-fructose as the sugar substrate and purified Enzyme I and diphosphoryl transfer protein (DTP) of *E. coli* and *S. typhimurium*, respectively (Table 2, Experiment C), it was established that membranes from fructose-grown *H. influenzae* cells exhibited fructose Enzyme II activity. These complementation data confirm the functionality of all of the *H. influenzae* PTS proteins except DTP, which was not tested.

In vitro *analyses of sugar phosphorylation: identification of non-PTS sugar-transport system components*

Sugars taken up by the PTS are concomitantly phosphorylated with phosphate derived from phosphoenolpyruvate (PEP). In contrast, non-PTS sugars are phosphorylated only after uptake, and by ATP-dependent sugar-specific kinases. The sugar-phosphorylation assay data summarized in Table 3 established the presence of ATP-dependent galactokinase activity in galactose-grown cells, and the presence of ATP-dependent glucokinase activity in glucose-grown cells. This is in agreement with the identification of genes specifying galactokinase (*galK*; HI#0819) and glucokinase (*glk*; HI#0144). Moreover, significant PEP-dependent activity was not observed, consistent with the absence of the PTS glucose permease EII^{Glc}. The non-PTS sugar, galactose, served as a control. ATP-dependent phosphorylation of glucose was also demonstrated in the *H. influenzae ptsI* mutant (data not shown). This confirmed the conclusion that phosphate from ATP was not being transferred to PEP and thence to glucose via the PTS.

Sugar uptake by intact cells: characterization of galactose, fructose and glucose permeases

The above genetic and biochemical data imply that fructose enters *H. influenzae* via a PTS permease, and that ribose, xylose, fucose, galactose, glycerol and sialic acid are transported by PTS-independent systems. However, no glucose permease has been identified. Glucose may enter via an as yet unidentified permease, or may share a permease specific to some other sugar. In an attempt to account for glucose uptake, we have characterized induction and inhibition of the transport systems for glucose, fructose and galactose (Fig. 3).

Fructose-uptake activity was low unless the cells had been grown in medium supplemented with fructose (Fig. 3A); the *ptsI* mutation reduced this fructose-induced uptake to less than 1% of the rate observed for wild-type cells (data not shown). This confirms that fructose enters *H. influenzae* cells via a fructose-inducible PTS permease. In inhibition studies, glucose competed poorly with labelled fructose for this permease, causing only 60% inhibition when present in 100-fold excess, suggesting that the PTS is fructose specific. No other tested sugar showed significant inhibition (data not shown). The observed fructose-specific induction could be due to the action of a fructose-specific transcriptional regulator protein. Several *H. influenzae* genes show some sequence similarity to

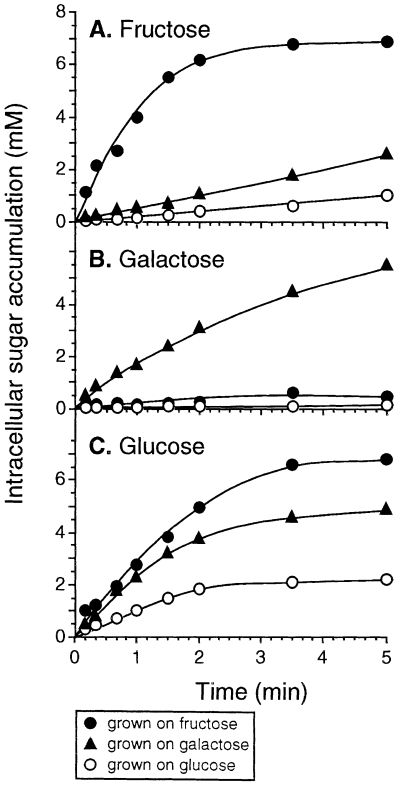

Fig. 3. Uptake of [^{14}C]-fructose (A), [^{14}C]-galactose (B), and [^{14}C]-glucose (C) after growth in the presence of fructose (black circles), galactose (black triangles), or glucose (open circles). Growth conditions and transport assays with 20 μM substrate were conducted as described in the text. Transport experiments were conducted three times with three independent cultures, and each experiment gave essentially similar results (± 15%). Representative results are shown.

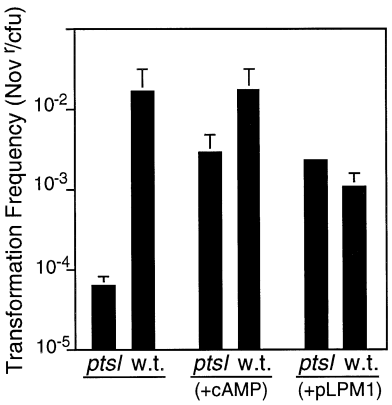

Fig. 4. Competence development of RR745 (*ptsI*) and KW20 in MIV starvation medium, ± 1 mM cAMP, and after transformation with pLPM1. Competence was induced by transfer of cells to MIV medium (with and without 1 mM cAMP), and cells were incubated with 1 μg ml^{-1} MAP7 DNA for 30 min. Transformation frequency was calculated as the number of Nov^r transformants divided by the total number of cells. Median values are shown, with error bars showing the range.

the *E. coli* fructose operon repressor FruR. However, none are sufficiently similar that they would be expected to have the same regulatory function as FruR.

Galactose uptake was only observed after growth in galactose (Fig. 3B), implying that galactose is taken up via a galactose-inducible permease. This is consistent with the presence of a galactose-operon repressor, GalS (HI#0821), whose *E. coli* homologue is galactose induced.

In contrast to the substrate-specific induction of fructose and galactose permeases, uptake of glucose was found to be lowest after growth in glucose (Fig. 3C). Glucose may either be repressing an as yet unidentified glucose-specific permease, or fructose or galactose may induce it. Glucose is known to enter *E. coli* cells via the galactose permease (Henderson, 1990), and our data suggest that this might be the case in *H. influenzae*: cells pregrown in galactose show high glucose-uptake activity (Fig. 3B). On the other hand, unlabelled glucose or galactose (but no other tested sugar) in 100-fold excess strongly (95%) inhibited uptake of ^{14}C-labelled glucose in glucose-grown cells (in which the galactose permease is poorly expressed) (data not shown). This finding might imply that galactose competes with glucose for entry via the putative glucose-specific permease, but does not account for glucose uptake by fructose-grown cells.

In conclusion, uptake of glucose by *H. influenzae* remains perplexing. It has yet to be determined whether this pattern of glucose uptake is general to the family Pasteurellaceae or specific to the species or even the strain.

Competence development

Development of natural competence for DNA uptake is known to be absolutely dependent on cAMP and CRP (Dorocicz *et al.*, 1993; Chandler, 1992). This might mean that DNA uptake is, similarly to sugar uptake and utilization, activated by the PTS when fructose is scarce. Alternatively, it could result from some other cAMP-dependent regulatory process specific to competence. To clarify this point we assessed competence induction in a *ptsI* mutant strain.

After standard competence-inducing treatment by transfer to MIV starvation medium, the *ptsI* mutant RR745 transformed with a frequency of 6×10^{-5}, ~250-fold lower than the wild-type KW20. Near-normal competence was restored by addition of 1 mM cAMP to the medium during competence induction. Plasmid pLPM1 also complemented this transformation deficiency, confirming that the transposon insertion in *ptsI* is the mutation preventing competence development (Fig. 4). These results confirm that the competence-triggering rise in cAMP is controlled by the PTS. Surprisingly, a wild-type competence level (transformation frequency of 10^{-4}) was reached by RR745 at onset of stationary phase in rich medium (Fig. 5), although a delay of approximately one generation was observed.

Discussion

The genetic and biochemical data described above complement our analysis of the *H. influenzae* genome

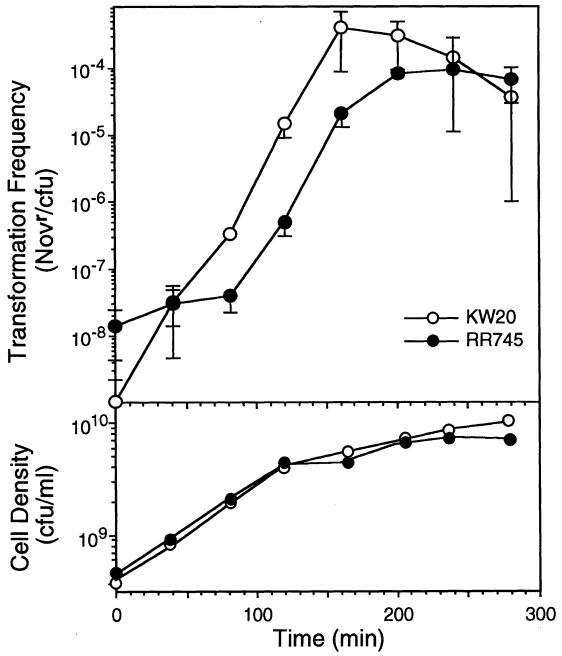

Fig. 5. Spontaneous competence development by RR745 (*ptsI*) and KW20 in sBHI. Transformation was assayed as in the legend to Fig. 4. Mean values and standard errors for triplicate data are shown.

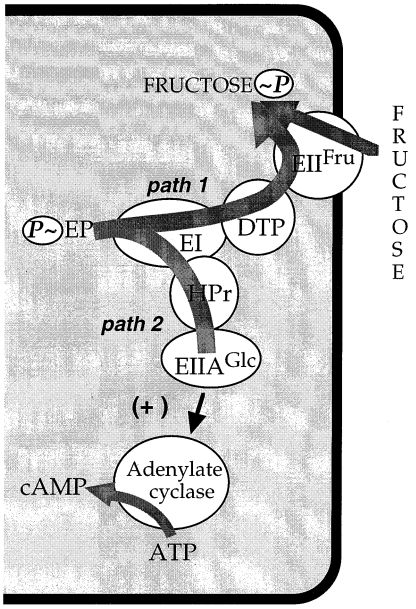

Fig. 6. A model for activation of adenylate cyclase by the *H. influenzae* PTS. The *H. influenzae* PTS comprises two general energy-coupling proteins, Enzyme I (EI) and HPr; the 'glucose-specific' EIIA ($EIIA^{Glc}$) regulatory component; and the fructose-specific components DTP and Enzyme II (EII) permease. 'Path 1' consists of EI, DTP and EII^{Fru}, and catalyses phosphorylation of fructose (by phosphotransfer from PEP) and concomitant release of fructose-1-phosphate in the cytoplasm. In the absence of fructose, phosphotransfer to fructose halts, and 'Path 2', consisting of EI, HPr and $EIIA^{Glc}$, causes phosphate to accumulate on $EIIA^{Glc}$, whose phosphorylated form activates adenylate cyclase (for a review, see Postma *et al*., 1993).

(Macfadyen and Redfield, 1996) and give the following picture of the *H. influenzae* PTS and its activities (summarized in Fig. 6).

H. influenzae *has a simple but complete PTS with two identified functions: uptake of fructose and control of cAMP levels*

Uptake and phosphorylation of fructose. Both genetic and biochemical evidence indicate that the *H. influenzae* PTS transports only fructose. No other fermentable sugars showed this absolute PTS dependence, and no other PTS permease genes have been found. The position of fructose as the 'preferred' carbon source in the *H. influenzae* sugar hierarchy was confirmed by PTS-mediated repression data: only the fructose analogue, xylitol, prevented use of non-PTS sugars. Based on models of PTS-mediated fructose uptake in enteric bacteria (Charbit *et al*., 1996; Postma *et al*., 1993), we propose that this PTS transfers high-energy phosphate from PEP to Enzyme I, and then via the two domains of DTP (Reizer *et al*., 1996) to the EIIB′BC fructose permease and finally to incoming fructose (Fig. 6, 'path 1').

Regulation of cAMP levels. As a glucose-specific PTS permease is absent, HPr and the glucose-specific regulatory component $EIIA^{Glc}$ appear to have no role in sugar uptake. Because competence development by *H. influenzae* was regulated by the *E. coli* adenylate cyclase, and as *H. influenzae* processes known to be cAMP dependent were also PTS dependent, the orthologous adenylate cyclases of *H. influenzae* and *E. coli* are probably similarly regulated. Thus, we propose that in the absence of fructose, phosphotransfer from PEP to fructose is blocked, and that this blockage allows some phosphotransfer via HPr to $EIIA^{Glc}$ (Fig. 6, 'path 2'). As in enteric bacteria, the phosphorylated form of $EIIA^{Glc}$ can then activate adenylate cyclase, causing cAMP levels to rise and CRP-dependent promoters to be induced.

Regulation of the pts *operon*

By comparing promoter consensus sequences with the *H. influenzae pts* operon sequence we have identified putative promoters upstream of the *ptsH*, *ptsI* and *crr* genes (Fig. 2). The presence of an independent *ptsI* promoter might allow the fructose-uptake function of the PTS (which requires EI, DTP and $EIIB'BC^{Fru}$) to be regulated independently of its cAMP-regulating function (which requires EI, HPr and $EIIA^{Glc}$). Expression of the *pts* operon in enteric bacteria is positively controlled by both extracellular glucose and the intracellular cAMP concentration (Rephaeli and Saier, 1980; De Reuse and Danchin, 1988). These regulatory mechanisms are alternate, never acting simultaneously in a cell, and keep *pts* expression at a similar level under conditions of both high and low catabolite repression (Postma *et al*., 1993). Glucose-mediated transcriptional activation is controlled by the enteric $EIICB^{Glc}$ permease (De Reuse and Danchin, 1988). As *H. influenzae* lacks this PTS component, glucose availability may not regulate *pts* operon expression in this organism. However, we have identified two putative CRP sites upstream of the *pts* operon (not shown), suggesting that expression of the *H. influenzae pts* operon may nevertheless be positively regulated by cAMP–CRP.

Utilization of non-PTS sugars by H. influenzae

Our composite data (Table 1) suggest that in *H. influenzae*, xylose, ribose, galactose and fucose are cAMP-dependent sugars transported by non-PTS permeases. Expression of catabolic operons specific to these sugars is expected to be dependent on transcriptional activation by the cAMP–CRP complex. This is supported by identification of putative CRP-binding sites that could control expression of the *H. influenzae xyl*, *rbs*, *mgl* and *fuc* operons (not shown).

Evolution of the PTS

The presence of glucose PTS permeases in diverse bacterial groups suggests that the gene for EIICB^{Glc} may have been lost by an ancestor of *H. influenzae* Rd. The ability of the *H. influenzae* EIIA^{Glc} to support PTS-dependent phosphorylation of methyl-α-glucoside (a glucose analogue) by the *E. coli* EIICB^{Glc} (Table 2, Experiment B) suggests that this gene has been lost only very recently, perhaps even during laboratory passage of the Rd strain. It will be interesting to examine glucose uptake by other *H. influenzae* isolates and *Haemophilus* species.

Biological significance of the H. influenzae *sugar diet*

H. influenzae is an obligate commensal which inhabits the mucus of the human respiratory tract. Unlike enteric bacteria, which encounter a wide range of carbohydrates in the human gut, *H. influenzae* has no direct contact with the bulk of sugars in the host diet. Effectively, only sugars that cross the mucosal cell membrane are available to the mucosal flora. The monosaccharides glucose, galactose and fructose are the only dietary sugars which can enter the circulation. Glycerol also enters the circulation, primarily from breakdown of dietary and stored fats (Gibney *et al*., 1995). These may well be the major carbon sources in the mucosal niche. Fucose and sialic acid (*N*-acetyl neuraminic acid), in conjugated form, comprise 30–35% of mucous glycoprotein oligosaccharides, and are a further potential carbon source. Clearly, *H. influenzae* has maintained genes which allow it to exploit the major sugars that are probably available to it in the mucosal niche. (For a more detailed review, see Macfadyen and Redfield, 1996.)

Role of the PTS in competence

As competence development is reduced 250-fold by the *ptsI* mutation, but restored to near-normal if cAMP is added to the medium, the PTS must be responsible for the rise in cAMP required for competence. A small fraction of *ptsI* mutant cells do become competent, as revealed by the residual competence upon starvation and the essentially normal response to late-log conditions. This is possibly because the mutant continues to produce some cAMP (various *E. coli ptsI* mutants retain 5–15% of wild-type adenylate cyclase activity (Peterkofsky *et al*., 1989)). Consistent with this suggestion, some cAMP-dependent sugar fermentation also persists in the *ptsI* mutant (Table 1); we have observed that this cAMP-dependent PTS-independent fermentation (e.g. of xylose) can be induced, in a *cya* mutant, by lower concentrations of added cAMP than are needed to induce the PTS-dependent fermentations (e.g. of ribose) (data not shown).

Adenylate cyclase is known to be transcriptionally regulated by cAMP and CRP, and cAMP levels may be modulated by active degradation and excretion (Peterkofsky *et al*., 1993). We are currently developing a *lacZ*-based cAMP reporter, which will allow us to determine whether alternate mechanisms of adenylate cyclase activation are indeed significant under different growth conditions, and whether these play significant roles in competence.

Systems regulating competence development probably evolved to maximize the benefits and minimize the costs of DNA uptake. We have chosen to study this regulation, because an understanding of the nature of the signals that induce competence will highlight the primary benefits of this process. It is known that a medium lacking nucleotides, cofactors, sugars and certain amino acids induces competence, and that cAMP, a common bacterial indicator of nutritional requirement, is essential for development of competence. We have now shown that the *H. influenzae* PTS, an enzyme complex which monitors carbon-source availability and directs efficient catabolic gene expression, also regulates competence development. These data strengthen the proposition that cells take up DNA primarily to obtain the nucleotides it contains. Indeed, competence development might be viewed as a beneficial adaptation by *H. influenzae* to its environment: in the mucus of the human respiratory tract it may encounter several hundred micrograms of DNA per ml (Matthews *et al*., 1963). We propose a model in which cells preferentially obtain nucleosides by uptake. If externally available nucleosides are insufficient, availability of energy resources will determine whether nucleotides are synthesised *de novo*, or whether cells become competent in order to take up any free DNA in the environment.

Experimental procedures

Bacterial strains and plasmids

The wild-type Rd *H. influenzae* strains KW20 (Alexander and Leidy, 1951) and the following derivatives were used: MAP7 (*str nal kan nov stv spc vio*) (Barcak *et al*., 1991), RR668 (*cya*::mini-Tn*10kan*) (Dorocicz *et al*., 1993), RR540 (*crp*::mini-Tn*10kan)* (Chandler, 1992). *E. coli* strains used were: DH5α (obtained from D. Hanahan), NM554 (obtained from N. Murray), LJ176 (*cpd ptsI313*^{ts}) (Isaacs *et al*., 1994), CA8306 (Δ*cya*) (Kiely and O'Gara, 1983), TP2811 (Δ(*ptsHIcrr*)) and TP2819 (Δ(*ptsIcrr*)) (Levy *et al*., 1990). The *H. influenzae* shuttle vectors pSU18, pSU20 and pSU40 were obtained from Bartolome *et al*. (1991). Plasmid pEC1 was obtained from Koop *et al*. (1984). Construction of pRJR124 has been described (Dorocicz *et al*., 1993). λ1316 (vector for mini-Tn*10kan*) was obtained from Kleckner (1991).

Culture conditions and competence induction

E. coli and *H. influenzae* cells were grown aerobically at 37°C in appropriate media, usually Luria–Bertani (LB) for *E. coli* (Ausubel *et al*., 1994) and brain–heart infusion medium supplemented with haemin and NAD (sBHI) for *H. influenzae* (Barcak *et al*., 1991), with the recommended concentrations of antibiotics. Additional haemin was applied to sBHI plates greater than 24 h old. Spontaneous competence development was followed during exponential growth in sBHI medium, while maximal competence was induced in wild-type *H. influenzae* by transfer to MIV starvation medium (Herriott *et al*., 1970), as described by Barcak *et al*. (1991). Plasmids were transformed into *H. influenzae* strains by a glycerol-aided transformation method (Stuy, 1986). *E. coli* cells were made competent by transfer to cold $CaCl_2$, and transformed by standard procedures (Ausubel *et al*., 1994).

Molecular techniques

A *Pst*I–*Eco*RI fragment of pEC1 carrying the *E. coli cya* gene was subcloned into shuttle vector pSU18 to give pID11. A *Sac*I fragment of pRJR124 carrying the *H. influenzae cya* gene was subcloned into pSU18 to give pID10. In each case, ligation products were transformed into *E. coli* strain CA8306 (Δ*cya*) and transformants containing the desired plasmid selected as red colonies on MacConkey lactose plates supplemented with chloramphenicol. pID10 and pID11 were each used to transform the *cya* strain RR668 to give strains RR719 and RR721, respectively. Restriction mapping of pID10 prepared from RR719 confirmed that there had been no recombination between the chromosomal *cya* mutant gene and the wild-type copy of *cya* contained in pID10.

A *H. influenzae* genomic library was constructed by partial *Sau*3AI digestion of *H. influenzae* MAP7 genomic DNA and ligation of the resultant fragments into the *E. coli*/*H. influenzae* shuttle vector pSU20, pre-cut with *Bam*HI. The library was amplified by transformation into *E. coli* DH5α, and chloramphenicol-resistant colonies were pooled for preparation of plasmid DNA. The *H. influenzae ptsI*$^+$ plasmid, pLPM1, was selected by complementation of LJ176 (see the *Results*), transformed into NM554, and mutagenized by mini-Tn*10kan* insertion as described by Kleckner (1991). Insertions in *ptsI* were identified (see the *Results*) and the mutation was transferred to the KW20 chromosome by homologous recombination; transposon insertion in the chromosome of the new *ptsI*::mini-Tn*10kan* mutant strain RR745 was confirmed by Southern blotting. Downward alkaline blotting of DNA from agarose gel to nitrocellulose (Koetsier *et al*., 1993) was followed by non-radioactive probe labelling and signal visualization using the 'DIG DNA Labeling and Detection Kit – Non-radioactive' (Boehringer Mannheim). In preparation for sequencing, pLPM1 was cut with *Sac*I and *Xho*I to yield two large fragments each carrying half of the mini-Tn*10kan* transposon. These were gel purified and subcloned into pSU40. Sequencing from the Tn*10* end of each subcloned fragment was carried out by the University of British Columbia (UBC) Nucleic Acid Protein Service Unit, using ABI AmpliTaq Dye-Deoxy Terminator Cycle Sequencing Chemistry and a primer complementary to the ends of mini-Tn*10kan* (Chandler, 1992). The complete nucleotide sequence of the KW20 *pts* operon region, predicted polypeptide sequences and results of comparisons with *E. coli* and *S. typhimurium* homologues were obtained from The Institute for Genomic Research (TIGR) WorldWide Web site (http://www.tigr.org) (Fleischmann *et al*., 1995). Further sequence analysis was carried out using programs developed by the Wisconsin Genetics Computer Group (GCG) (Devereux *et al*., 1984).

Transport studies

Cells were cultured in 100–200 ml of heart infusion (Difco) supplemented with haemin and NAD and with sugars as specified in Fig. 3, and harvested in the midlogarithmic phase of growth by centrifugation (12000 × *g* for 5 min) at 4°C. Cells were then washed twice with 50 mM potassium phosphate buffer (pH 7.4) containing 0.2% $(NH_4)_2SO_4$ and 0.02% $MgSO_4.7H_2O$, and resuspended in the same buffer at a concentration that had an OD_{600} of 0.5–0.6. Cell suspensions containing 0.5 μg ml^{-1} NAD and 0.2% casamino acids were prewarmed to 37°C for 5 min before addition of [^{14}C]-fructose, [^{14}C]-glucose, or [^{14}C]-galactose (20 μM; specific activity, 5 μCi μmol^{-1}). The transport reaction was terminated by withdrawing samples at appropriate intervals and collecting the cells by filtration on 25 mm membrane filters (0.45 μm pore size; Millipore Corp.). The cells were washed with two 3 ml volumes of transport buffer, and filters with cells thereon were dried under an infrared lamp and placed in vials containing 10 ml of scintillation fluid, for determination of radioactivity. An intracellular volume of 2.15 μl per OD_{600} of 1.2 was used to determined the concentration of substrate that accumulated (R. C. Goldman, personal communication; J. Reizer, unpublished data).

Preparation of cell extracts and membrane fractions

Cells from 500 ml of culture were harvested by centrifugation (12 000 × *g* for 5 min), washed twice and suspended in 10 ml of 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM dithiothreitol (DTT) and 0.1 mM phenylmethylsulphonyl fluoride (TDP buffer). Cells were ruptured by two passages through a French pressure cell at 10 000 lb in^{-2}, and cell extracts were centrifuged for 15 min at 10 000 × *g* to remove whole cells and cell debris. Membranes were then collected by centrifugation at 100 000 × *g* for 180 min at 4°C, washed twice with TDP buffer and resuspended in the same buffer at a protein concentration of 5–10 mg ml^{-1}.

Sugar phosphorylation assays

ATP- or PEP-dependent sugar phosphorylation assays were performed as previously described (Reizer, 1989). Assay mixtures contained 50 mM potassium phosphate buffer (pH 7.4), 25 mM KF, 12.5 mM $MgCl_2$, 2.5 mM DTT, 5 mM PEP or ATP, 20 μM of the [^{14}C]-sugar substrate (specific activity, 5 μCi μmol^{-1}), and either crude extracts or purified protein constituents of the PTS and washed membranes as indicated. Reaction mixtures were incubated at 37°C for 20–60 min and assayed for [^{14}C]-sugar–phosphate using ion-exchange columns to separate phosphorylated from free sugar (Kundig and Roseman, 1971).

Protein purification

Enzyme I, HPr and Enzyme IIA^{Glc} of *E. coli* were overproduced and purified as previously described (Reizer *et al*., 1992). Overproduction and purification of the diphosphoryl transfer protein (DTP) of *S. typhimurium* have also been described (Charbit *et al*., 1996).

Other methods

Protein concentration was determined by the bicinchoninic acid (BCA) method (Pierce Chemical Co.) using bovine serum albumin as standard.

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