Synthesis and Physicochemical Characterization of D-Tagatose-1-Phosphate: The Substrate of the Tagatose-1-Phosphate Kinase in the Phosphotransferase System-Mediated D-Tagatose Catabolic Pathway of *Bacillus licheniformis*

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Key Words

Tagatose · Phosphoenolpyruvate:sugar phosphotransferase system · Tagatose-1-phosphate kinase · *Bacillus licheniformis* · *Klebsiella pneumoniae* · ³¹P and ¹H nuclear magnetic resonance · Nuclear magnetic resonance characterization of tagatose phosphate

Abstract

We report the first enzymatic synthesis of D-tagatose-1-phosphate (Tag-1P) by the multicomponent phosphoenolpyruvate:sugar phosphotransferase system (PEP-PTS) present in tagatose-grown cells of *Klebsiella pneumoniae*. Physicochemical characterization by ³¹P and ¹H nuclear magnetic resonance spectroscopy reveals that, in solution, this derivative is primarily in the pyranose form. Tag-1P was used to characterize the putative tagatose-1-phosphate kinase (TagK) of the *Bacillus licheniformis* PTS-mediated D-tagatose catabolic pathway (Bli-TagP). For this purpose, a soluble protein fusion was obtained with the 6 His-tagged trigger factor

(TF^{His6}) of *Escherichia coli*. The active fusion enzyme was named TagK-TF^{His6}. Tag-1P and D-fructose-1-phosphate are substrates for the TagK-TF^{His6} enzyme, whereas the isomeric derivatives D-tagatose-6-phosphate and D-fructose-6-phosphate are inhibitors. Studies of catalytic efficiency (k_{cat}/K_m) reveal that the enzyme specificity is markedly in favor of Tag-1P as the substrate. Importantly, we show in vivo that the transfer of the phosphate moiety from PEP to the *B. licheniformis* tagatose-specific Enzyme II in *E. coli* is inefficient. The capability of the PTS general cytoplasmic components of *B. subtilis*, HPr and Enzyme I to restore the phosphate transfer is demonstrated.

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Introduction

In many bacterial species, from both Gram-positive [Reizer et al., 1988; Thompson, 1987] and Gram-negative genera [Meadow et al., 1990; Postma et al., 1993], glycans

Fig. 1. a Organization of the *B. licheniformis* ATCC 14580 tagatose gene cluster coding for *Bli*-TagP. **b** Transport and concomitant phosphorylation of tagatose by the *B. licheniformis* or *K. pneumoniae* PTS components. In the cell, Tag-1P is phosphorylated by the ATP-dependent TagK in Tag 1,6-BP, which is cleaved by the class II Tag 1,6-BP aldolase GatY. Genbank protein accession numbers of the tagatose gene cluster products in *B. licheniformis* are YP_006714841 to YP_006714845.

are phosphorylated simultaneously with vectorial translocation via sugar-specific phosphoenolpyruvate:sugar phosphotransferase systems (PEP-PTS) [Deutscher et al., 2006]. In this multicomponent complex (fig. 1b), the high-energy phosphoryl moiety of PEP is transferred sequentially to two general cytoplasmic proteins, Enzyme I (EI) and HPr (a histidine-containing phosphocarrier protein). Subsequently, the phosphate moiety is transferred by group translocation to the incoming sugar via multidomain sugar-specific membrane proteins designated Enzymes II (EII). For a discussion and nomenclature of PTS components, see Saier and Reizer [1992]. Recent nuclear magnetic resonance (NMR) studies by Clore and colleagues [Cai et al., 2003; Clore and Venditti, 2013] have provided molecular-level insight to the structure, dynamics and biophysical interactions of the protein-protein complexes comprising the bacterial PEP-PTS.

The lactose PEP-PTS is present in and has been studied in many microorganisms, including: industrially important Group N streptococci [Bissett and Anderson, 1974; Thompson, 1979], *Lactobacillus casei* [Chassy and Thompson, 1983], oral streptococci [Hamilton and Lebtag, 1979; Hamilton and Lo, 1978] and, significantly, *Staphylococcus aureus* [Bissett and Anderson, 1980a, b; Bissett et al., 1980]. The multicistronic genes encoding the proteins of the lactose (*lac*)-operon may have a plasmid or chromosomal locus. The product of translocation via the *lac*-PTS is lactose-6'-phosphate. Intracellularly, the phosphorylated disaccharide is cleaved by β -D-

phosphogalactoside galactohydrolase EC 3.2.1.85 (P- β -gal) to yield galactose-6-phosphate (Gal-6P) and glucose. After ATP-dependent phosphorylation, the latter hexose (glucose-6P) may directly enter the glycolytic pathway. Conversely, Gal-6P must first be converted to D-tagatose-6-phosphate (Tag-6P) by the D-tagatose pathway prior to glycolytic fermentation. First reported by Bissett and Anderson in 1974, the three-stage D-tagatose pathway comprises: Gal-6P isomerase, EC 5.3.1.26 [Bissett et al., 1980], Tag-6P kinase, EC 2.7.1.144 [Bissett and Anderson, 1980a] and class I D-ketohexose 1,6-bisphosphate (1,6-BP) aldolase, EC 4.1.2.40 [Bissett and Anderson, 1980b]. The structural genes comprising the *lac*-operon and Tag-6P pathway have been characterized, cloned and sequenced from several bacterial species [Rosey et al., 1991; Rosey and Stewart, 1992; van Rooijen et al., 1991].

For many years, the Tag-6P pathway was considered to be the primary (if not the sole) route for the dissimilation of tagatose by microorganisms. However, in 2004, studies by Shakeri-Garakani et al. [2004] revealed an unsuspected PTS-mediated D-tagatose catabolic pathway in certain species of Enterobacteriaceae, including *Klebsiella oxytoca*, *K. pneumoniae* and *Salmonella enterica*. For these Gram-negative bacteria, it seemed that sugar-specific EIIA/EIIBC^{Tag} proteins catalyzed the PEP-dependent translocation and phosphorylation of tagatose into the cell. Together with the PTS genes, the putative *tag*-operon encoded additional genes, designated *tagK* and *gatYZ*. Comparative sequence alignment of amino acids encoded by *tagK* revealed sig-

Fig. 2. **a** The hydroxyl region of an HSQC-TOCSY experiment of 0.2 M tagatose, pH 7.5 at -10°C , with a 10-ms DIPSI mixing time. **b** Overlaid HSQC-TOCSY spectra, collected as in **a**, of tagatose (blue) and tagatose phosphate (red). Labels indicate the anomeric

configuration followed by the corresponding nuclei. Arabic numerals indicate the atom position. P indicates the phosphorylated compound.

nificant homology with enzymes assigned to the large ribokinase family, particularly with D-fructose-1-phosphate (Fru-1P) kinases and Tag-6P kinases [Bork et al., 1993]. The protein encoded by *gatYZ* possessed the characteristics of a hetero-dimeric class II tagatose 1,6-BP (Tag 1,6-BP) aldolase. Based largely on the functional and sequence relatedness of PTS proteins and metabolic enzymes, Shakeri-Garakani et al. [2004] suggested that: (i) D-tagatose-1-phosphate (Tag-1P; not Tag-6P) was the likely product of PTS activity, (ii) Tag-1P was phosphorylated by the *tagK*-encoded enzyme (tagatose-1-phosphate kinase; TagK) to form Tag 1,6-BP and (iii) that the bisphosphate derivative was hydrolyzed by Tag 1,6-BP aldolase to yield the glycolytic metabolites, glyceraldehyde 3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP).

In 2013, we reported the existence of a closely related PTS-mediated D-tagatose catabolic pathway in the Gram-positive bacterium *Bacillus licheniformis* (Bli-TagP; fig. 1) [Van der Heiden et al., 2013]. Although consistent with experimental data, in neither of the reports from Shakeri-

Garakani et al. [2004] or Van der Heiden et al. [2013] was unequivocal evidence presented to support two crucial hypotheses. First, that the tagatose-PTS product was in fact Tag-1P and not the isomeric (and more common) Tag-6P derivative, and, second, no data were presented to establish the specificity of the putative TagK, nor was it verified that Tag-1P was a specific substrate for this enzyme. The purpose of our investigation was to obtain experimental evidence to validate (or refute) these hypotheses. To this end, we report the first PEP-PTS catalyzed synthesis and physicochemical characterization of Tag-1P. Importantly, we describe the purification and substrate specificity of the putative TagK from *B. licheniformis*.

Results

Tag-6P and Tag 1,6-BP have previously been synthesized and characterized spectroscopically by Eyrisch et al. [1993]. However, to our knowledge, this is the first report

Table 1. Chemical shift assignments and coupling constant values for tagatose, Tag-1P and Tag-6P anomers

		Tag-1P		Tagatose		Tag-6P	
		–10°C	25°C	–10°C		–10°C	
Atom position							
1	¹ H _{axial}	3.61	3.63	3.48	3.48		
	¹ H _{equatorial}	3.98	3.98	3.72	3.70		
	¹ HO	NA	NA	5.88	5.86	–	–
	¹³ C	65.98	66.05	63.93	63.40	62.29	62.35
2	¹ H	–	–	–	–	–	–
	¹³ C	98.34	98.41	98.39	98.53	102.92	105.39
	¹ HO	NA	NA	NA	NA	NA	NA
3	¹ H	3.97	3.97	3.89	3.85	4.29	4.30
	¹ HO	NA	NA	6.08		NA	NA
	¹³ C	69.52	69.82	69.76	69.49	70.31	70.32
4	¹ H	3.86	3.86	3.85	4.02	4.40	4.43
	¹ HO	NA	NA	6.01	6.11	NA	NA
	¹³ C	70.67	70.76	71.07	71.07	70.98	71.45
5	¹ H	3.84	3.84	3.85	3.92	4.19	4.43
	¹ HO	NA	NA	6.25	6.34	–	–
	¹³ C	66.50	66.55	66.51	66.51	79.44	78.59
6	¹ Ha	3.64	3.63	3.62	3.59	3.96	3.92
	¹ Hb	3.74	3.75	3.74	4.18	4.11	4.03
	¹ HO	–	–	–	–	NA	NA
	¹³ C	62.29	64.39	62.26	60.08	63.23	63.07
Coupling constants, Hz	² J _{CP}	4.00	4.34	–	–	4.20	5.00
	³ J _{CP}	7.56	6.58	–	–	7.74	7.76
	³ J _{H6aH5}	9.94					
	³ J _{H6eH5}	5.5					
	³ J _{H3H4}	3.14					
	³ J _{H4H5}	–					

The data were collected on samples at pH 6.5, 90:10 H₂O:D₂O, 0.1% DSS at the indicated temperature on a Bruker Avance II 700 MHz instrument. ‘–’ = Not observed/measured; NA = not applicable.

of the synthesis and physicochemical characterization of Tag-1P. Furthermore, the tagatose hydroxyl proton signals have not been assigned, nor have their observation and chemical shifts been utilized for structural characterization. We assigned the hydroxyl protons of tagatose because they enabled us to easily distinguish between α - and β -anomers due to their characteristic chemical shifts [Battistel et al., 2014] and because their absence can be used to infer functionalization (fig. 2). Additionally, hydroxyl signals can be used to uncover substitution pattern in glycans. As explained in Materials and Methods, the absence of a hydroxyl hydrogen signal may indicate the formation of a glycosidic linkage or a ring, when its absence is considered in conjunction with other data. In the case of tagatose, the molecular formula is known and the hydroxyl group positions were determined. Therefore,

we can infer that the absence of a hydrogen signal for the group at C6 and the presence of a hydroxyl hydrogen signal at C5 indicates that tagatose exists primarily in the pyranose rather than the furanose form in solution at –10°C. Conversely, the furanose form would show a hydroxyl signal at C6 but not at C5. Both α - and β -anomers were detected, in agreement with Freimund et al. [1996]; however, no furanose or open form signals were observed under the experimental conditions tested. The hydroxyl assignments for tagatose are presented in table 1. Additionally, tagatose serves as a chemical shift reference and helps establish the phosphorylation site. Phosphorylation produces chemical shift changes at the site of phosphorylation and upon neighboring atoms with respect to its unphosphorylated form. Thus, this reference enables us to easily establish the phosphorylation locus (see below).

Fig. 3. a Tagatose chemical structures in cyclic form (major forms in solution) [Freimund et al., 1996]. **b** Most likely phosphorylation positions (indicated in red) of the α -conformers.

Characterization of Enzymatically Prepared Tag-1P

Previously, phosphorylation of tagatose has been shown [Eyrisch et al., 1993] to occur at the C1 (when C6 is already phosphorylated) and C6 positions (fig. 3a). Determination of the phosphorylation position is only the first step because one has also to establish the glycan's major conformation in solution (e.g. open form, or 5 or 6 member ring formation in either the α or β configuration; fig. 3b). Three lines of evidence lead to the conclusion that biosynthesized tagatose phosphate is phosphorylated at C1. First, comparison between heteronuclear single quantum coherence (HSQC) for tagatose and tagatose phosphate (fig. 2b) shows a pronounced change for H1s,C1 cross peaks, but not for H6s,C6. One would expect a pronounced chemical shift change at the site of phosphorylation. This result alone strongly indicates that the phosphate group is at position 1. Second, a ^{31}P - ^{13}C coupling between C1-P (4 Hz); and C2-P (8 Hz) indicates that the phosphate is either attached to C1 or C2 (online suppl. fig. S1A; for all online suppl. material, see www.karger.com/doi/10.1159/000370115). The ^1H , ^{31}P long-range HSQC experiment (online suppl. fig. S1B) shows

that both ^1H 's at position 1 are coupled to phosphorus as well, which further supports the incorporation of a phosphate at either C1 or C2, and not C6. However, the chemical shift change observed upon phosphorylation for ^{13}C signals at C1 and not at C2 or C6 indicates that the phosphate is at C1. Larger values for vicinal versus geminal ^{13}C - ^{31}P coupling constants have been reported and agree with the values presented in this manuscript [Eyrisch et al., 1993; Thompson et al., 1999]. Finally, comparison of the deuterium isotope effect observed on ^{13}C signals for tagatose and tagatose phosphate showed that there is no hydroxyl group at C6 and at C1 and C6, respectively. Figure 4 shows the deuterium two-bond isotope effect observed in the overlaid ^{13}C NMR spectra of tagatose phosphate in 50:50 $\text{H}_2\text{O}:\text{D}_2\text{O}$ and 10% D_2O . The isotope effect is most significant at C4 and C5 (fig. 4a and c, respectively), indicating that OH exchange is slow and that long-range isotope effects from neighboring OH groups may also be present. It is evident from the spectra that no isotope shift is observed at C6 (fig. 4d). As explained earlier in the text, the absence of isotope shift is expected for substituted carbon atoms bearing no exchangeable H's, such as phosphate. Since we demonstrated that there is no phosphate group at C6 of phosphorylated tagatose, the exchangeable proton in the hydroxyl group at C6 must therefore have been lost while forming a pyranose ring. Thus, this result supports the conclusion that the compound in question is in the pyranose form. It may be argued that another possibility is that a phosphate group has been incorporated at this position; however, there is no ^{31}P - ^{13}C coupling involving C6. Line broadening and small chemical shift changes are observed at C2 and C3, indicating that OH at C2 and C3 are in fast exchange with solvent (fig. 4b and c, respectively).

Moreover, ^1H ^{13}C chemical shifts more closely match those of the α -anomer of tagatose (table 1; fig. 2) than the β -anomer. Homonuclear ^1H , ^1H coupling constants are also consistent with a pyranose form (table 1), where H3,H4 coupling is small, i.e. ~ 3 Hz (2.6, 3.5, 4.8 and 4.7 Hz for the α -pyranose, β -pyranose, α -furanose and β -furanose, respectively; fig. 2a) [Freimund et al., 1996]. A small coupling constant (0–3 Hz) for H3,H4 is expected if the coupled hydrogen atoms are oriented roughly 90 degrees from each other (pyranose forms, online suppl. fig. S2A) and larger if the angle between them is ~ 0 (furanose forms, online suppl. fig. S2B). Thus, the above results indicate that the PEP:PTS-generated product is Tag-1P, and that the compound exists primarily in a pyranose form with ^1H and ^{13}C chemical shifts indicative of the α -anomer.

Fig. 4. Overlaid ^{13}C 1D spectra for phosphorylated tagatose in 10% (red) and 50% (green) D_2O in H_2O . **a–d** Different spectral regions for C1 and C5 (a), C2 (b), C3 and C4 (c) and C6 (d), respectively. The data were collected under the same conditions as in figure 4.

Additionally, as a control, we collected NMR experiments on Tag-6P under the same conditions as for tagatose and Tag-1P. As expected, ^{31}P - ^{13}C coupling constants are observed for C6 and C5, and not for C1 and C2 (table 1).

TagK-TF^{His6} Enzyme Specificity and Inhibition Assays

Enzyme activity was tested with Tag-1P, Fru-1P, Tag-6P and D-fructose-6-phosphate (Fru-6P) as potential substrates. The specificity of TagK is clearly in favor of the Tag-1P substrate compared to the Fru-1P substrate. When the activity of TagK is fixed to 100% with 2 mM of Tag-1P as the substrate, the relative activity calculated for the enzyme with 10 mM of Fru-1P as the substrate is 31%.

As no significant activities on Tag-6P and Fru-6P were detected (fig. 5), it was of interest to test the inhibitory effects of these compounds on the activity of TagK with Tag-1P 2 mM as the substrate. As expected, Tag-6P and Fru-6P are inhibitors of TagK but not Fru-1P. The effect is particularly significant with 10 mM of Tag-6P or Fru-6P with a residual activity of 43 and 69% in the assay condition, respectively. The relative activities and standard deviation are depicted in figure 5.

Kinetic Parameters of TagK-TF^{His6}

From the nonlinear fit of the Michaelis-Menten curve, the apparent K_m and k_{cat} values for Tag-1P, at a constant level of ATP (2 mM), were determined to be 3.2 ± 0.4 mM

Fig. 5. Specificity (left) and inhibition (right) assays of the TagK-TF^{H6} enzyme. Specificity assays were performed by comparing the activities of TagK-TF^{H6} with the sugar phosphates Tag-1P, Fru-1P, Tag-6P and Fru-6P as substrates. The inhibition assays were performed with Fru-1P, Tag-6P and Fru-6P in the presence of Tag-1P (2 mM) as a substrate.

and $0.44 \pm 0.01 \text{ s}^{-1}$, respectively. For Fru-1P, the apparent K_m was $6.2 \pm 0.6 \text{ mM}$ and the apparent k_{cat} was $0.021 \pm 0.001 \text{ s}^{-1}$. The catalytic efficiency (apparent $k_{cat}/\text{apparent } K_m$) of the enzyme for Tag-1P ($137.5 \text{ M}^{-1}\text{s}^{-1}$) was 40-fold higher than that for Fru-1P ($3.4 \text{ M}^{-1}\text{s}^{-1}$), confirming the enzyme as a Tag-1P-specific kinase.

Complementation of *Escherichia coli* DH5 α Harboring the *Bli*-TagP with HPr and EI from *B. subtilis*

In a previous report, we showed that *E. coli* DH5 α (initially a Tag-negative phenotype) metabolized tagatose after transformation with the pDG TAG vector harboring the *Bli*-TagP (Tag-positive phenotype; fig. 1a) [Van der Heiden et al., 2013]. However, growth of the transformant was poor, and we suspected that HPr from *E. coli* might be unable to transfer the phosphate group to the EI^{tag} of *B. licheniformis* (fig. 1b). To test the hypothesis, DH5 α was then transformed with a second plasmid expressing the *ptsHI* operon of *B. subtilis* (pPtsHI plasmid). The *E. coli* DH5 α strain harboring both pDG TAG and pLysS grows considerably more slowly in the defined medium (DM) supplemented with tagatose than with glucose. This observation is consistent with our previous results (fig. 6a) [Van der Heiden et al., 2013]. As we hypothesized, the growth rates of *E. coli* DH5 α harboring both pDG TAG and pPtsHI plasmids were essentially the same in DM supplemented with either tagatose or glucose as carbohydrate sources (fig. 6b).

Discussion

Results from the present investigation augment our knowledge of the mechanisms of glycan transport and metabolism in microorganisms. Importantly, by enzymatic synthesis and physicochemical analyses, we have validated the first of the hypotheses presented in the introduction, by confirming that Tag-1P is the product of the PEP-dependent PTS transport of tagatose by cells of *B. licheniformis* and *K. pneumoniae*. Physicochemical analysis involved primarily conventional ^1H , ^{13}C and ^{31}P NMR experiments with the unprecedented incorporation of a hydroxyl signal and $^2\text{H}/^1\text{H}$ isotope effects for the determination of the ring size and phosphorylation position. Validation of the second hypothesis required the synthesis of Tag-1P in the substrate amount, and expression in the active form of the protein YP_006714842 encoded by the *fruK2* gene (renamed *tagK* hereafter) of the tagatose gene cluster from *B. licheniformis* (fig. 1a).

Initial attempts to obtain the wild-type TagK were unsuccessful due to the production of insoluble protein inclusion bodies. However, fusion of YP_006714842 with the ‘trigger factor’ (TF) from *E. coli* plus a His⁶-tag enabled the expression and purification of a soluble and enzymatically active fusion protein, TagK-TF^{His6}. The second hypothesis (see Introduction) was confirmed by the finding that Tag-1P is a substrate phosphorylated by the ATP and Mg²⁺-dependent TagK (TagK-TF^{His6}). In addition, specificity and inhibition assays reveal that the 1P derivatives

Fig. 6. Growth curves of *E. coli* DH5α transformed with pDGTAG (*Bli*-TagP) and either with pLysS as a negative control (**a**) or with the pPtsHI plasmid (*B. subtilis* *ptsHI* operon; **b**). Growth was performed at 28°C in a DM supplemented with D-glucose (20 mM, Glu), D-tagatose (20 mM, Tag) or H₂O (sugar-negative control). Growth curves were confirmed by two independent experiments.

of tagatose and fructose are substrates of the TagK-TF^{His6} enzyme, whereas the corresponding 6P-isomers (Tag-6P and Fru-6P) are inhibitors. Since the catalytic efficiency with Tag-1P as the substrate is 40-fold higher than that with Fru-1P, it is clear that the specificity of the TagK-TF^{His6} enzyme is in favor of Tag-1P. For this reason, and because of the significant amino acid sequence identity (39%) between the *B. licheniformis* *fruK2* product (YP_006714842) and the *K. oxytoca* M5a1 *tagK* product (AAL60166), we propose renaming the *fruK2* gene of *B. licheniformis* as *tagK*, and its product FruK2 (TagK).

Our NMR analyses show that, in solution, Tag-1P exists primarily in the pyranose form. However, we cannot discount the presence of minor concentrations of the furanose, open-chain or other tautomeric forms of the hexose phosphate that were undetected by NMR. Since there are no reported structures for the TagK complex, the molecular structure of the compound that actually binds to TagK is uncertain. If, as seems reasonable, the pyranose form of Tag-1P is recognized (see fig. 7), then this ring must open to assume a furanose conformation in order to permit ATP-dependent phosphorylation to occur at C6, thus yielding Tag 1,6-BP. A binding preference for a pyranose as opposed to the furanose ring is consistent with the higher catalytic efficiency observed for Tag-1P versus Fru-1P. Presently, there is no evidence to suggest the presence of a single catalytic site, or whether separate domains facilitate the sequential binding, ring-opening and phosphorylation of Tag-1P. Solution of the X-ray structure of TagK and co-crystallization of the enzyme with its ligands (Mg²⁺, ATP and Tag-1P) may clarify these issues and provide insight to the catalytic mechanism.

As noted earlier (see Introduction), the PTS-mediated D-tagatose catabolic pathway of *B. licheniformis* is closely related to that of some enteric bacteria [Shakeri-Garakani et al., 2004; Van der Heiden et al., 2013]. The tagatose-specific PTS protein constituents of *B. licheniformis* are EIIBC^{Tag} and EIIA^{Tag}, which belong to the PTS fructose-mannitol (Fru) family [Saier, 2000]. As EIIBC^{Tag} of *K. oxytoca* is encoded by *tagT* (EIIBC^{Tag,Kox} or TagT, AAL60167), EIIBC^{Tag} of *B. licheniformis* encoded by the *fruA2* gene (EIIBC^{Tag,Bli}, YP_006714843) consists of two domains: IIB and IIC. Both EIIBC proteins exhibit 54% identity. In contrast, EIIA^{Tag,Bli} encoded by the orf 51 (Genbank accession number for the *B. licheniformis* ATCC 14580 genome: NC-006322) has no homology with the EIIA^{Tag,Kox} (TagH), encoded by the *tagH* gene, which is a EIIA-pseudoHPr-like protein. This means that the EIIA^{Tag,Kox} is constituted of a EIIA domain (from the Fru family) followed by an HPr-like domain also called TPr [Shakeri-Garakani et al., 2004]. This domain is absent from the EIIA^{Tag,Bli} component. Genes encoding for the anionic cell wall polymers synthesis are already named *tagT* and *tagH* in *B. subtilis* [Kawai et al., 2011] and are also found in the *B. licheniformis* genome. For these reasons, we suggest renaming *orf 51* encoding EIIA^{Tag,Bli} to *tagM*, and the gene product itself to TagM. In addition, the *fruA2* gene should be renamed *tagL* and its product, the EIIBC^{Tag,Bli}, TagL. A gene (*orf 48*) encoding for a regulator from the RpiR family is also present in the tagatose gene cluster (fig. 1a). The RpiR family regulators contain

Fig. 7. In silico model of TagK bound to tagatose phosphate. Since the structure of the TagK is not yet known, we used fructose-1P kinase (PDB ID: 2ABQ) as a model. The TagK model is shown in a translucent gray surface representation. Different conformations of the tagatose phosphate(s) are shown in stick and space-filling representations. We speculate that the TagK may recognize Tag-1P in the pyranose form. The pyranose 1-phosphate form must undergo a change into the furanose 1-phosphate conformation in order permit phosphorylation at C6.

Fig. 8. Coupled enzyme assay used to measure the activity of TagK. Tag-1P or Fru-1P are phosphorylated by TagK in Tag 1,6-BP or Fru 1,6-BP, respectively. The second reaction is the cleavage by the class I ketohexose 1,6-BP aldolase LacD of either Tag 1,6-BP or Fru 1,6-BP into the glycolytic intermediates DHAP and G3P. G3P is isomerized in DHAP by the TPI. DHAP is subsequently reduced by NADH to glycerol-3P by G3P dehydrogenase (G3PDH). The rate of reaction is measured by the decrease in the NADH absorbance at 340 nm.

an N-terminal helix-turn-helix DNA binding motif and a C-terminal SIS (sugar isomerase) domain, which is found in many phosphosugar isomerases and phosphosugar-binding proteins [Yamamoto et al., 2001]. We also propose renaming this *orf* 48 in *tagR*.

The class I ketohexose 1,6-BP aldolase (LacD) from *S. aureus* was chosen as the second enzyme in the coupled enzyme assay (fig. 8) because this class of enzyme is non-specific with respect to the configuration of hydroxyl groups at carbon atoms 3 and 4. LacD is thus able to cleave both Tag 1,6-BP and fructose 1,6-BP (Fru 1,6-BP; C4-isomers) into DHAP and G3P [Bissett and Anderson, 1980b]. This enzyme allowed us to test fructose and tag-

atose-derivatives in the assays. However, if LacD was replaced by the *B. licheniformis* Zn²⁺-requiring class II Tag 1,6-BP aldolase GatY^{His6} (5.4 μM) in the coupled enzyme assay, then a decrease of NADH absorbance was also measured with Tag-1P (2 mM) used as the substrate for TagK-TF^{His6} (with a slope similar to that measured with LacD). No NADH absorbance decrease was detected with Fru-1P (10 mM) used as the substrate for the TagK-TF^{His6} enzyme (data not shown). Our results show that whilst GatY^{H6} is highly active on Tag 1,6-BP, the enzyme exhibits poor to negligible activity upon Fru 1,6-BP. Similarly, the high specificity of class II Tag 1,6-BP aldolases from enteric bacteria for Tag 1,6-BP and a low specificity for Fru 1,6-BP have been highlighted in previous papers [Brinkkotter et al., 2002; Eyrisch et al., 1993]. In this context, our results establish that GatY is the functional enzyme that cleaves Tag 1,6-BP (to DHAP and G3P) in the PTS-mediated D-tagatose catabolic pathway of *B. licheniformis* (fig. 1b).

In our previous paper, we obtained evidence for a function role of *Bli*-TagP during supplementation and transformation experiments with *E. coli* DH5α and *B. subtilis* 168 that were initially unable to use tagatose as a carbohydrate source (Tag⁻ phenotype) [Van der Heiden et al., 2013]. Interestingly, the growth rates of a stable *Bli*-TagP-transformed *B. subtilis* 168 (Tag⁺ phenotype) in the presence of D-tagatose or the PTS sugar D-glucose were similar. Identical growths were also observed with *B. licheniformis*. In contrast, the growth rate of *E. coli* DH5α, harboring the *Bli*-TagP on the pDG TAG plasmid (Tag⁺ phenotype) in the presence of D-tagatose was rather similar to that observed in the presence of the non-PTS sugar

D-galactose [Van der Heiden et al., 2013]. Our previous results suggested the possibility that the phosphate transfer between the endogenous protein HPr from *E. coli* to the exogenous EIIA^{Tag,Bli} was not efficient. Support for this hypothesis is provided in this study by the results of transformation of *E. coli* DH5 α (pDGTag) with a second plasmid (pPtsHI) encoding the PTS general cytoplasmic components HPr and EI of *B. subtilis*. The constitutive *ptsHI* operon of *B. subtilis* [Gonzy-Treboul et al., 1989] was chosen for the construction of this second plasmid because HPr proteins from *B. subtilis* and from *B. licheniformis* present 98% identity. *E. coli* harboring *Bli*-TagP and exogenous HPr from *B. subtilis* is now able to catabolize tagatose at the same rate as glucose (fig. 6b). This result demonstrates that HPr is sufficient for the phosphate transfer to EIIA^{Tag,Bli} in the *Bli*-TagP, and is consistent with the fact that a TPr domain is lacking in the EIIA^{Tag,Bli} component. Interestingly, our results contrast with functional heterologous interactions reported between HPr of *E. coli* and glucose-specific enzyme EIIA^{Glc} of *B. subtilis* [Reizer et al., 1992]. Nevertheless, it is well known that precise electrostatic interactions assure correct amino acid orientation for in-line phosphotransfer reactions. Further investigations should be undertaken to study the residues implicated in the interactions between both the EIIA^{Tag,Bli} and HPr proteins in *B. licheniformis* in order to compare the data to the previously reported interfaces required for interactions between HPr and EIIA^{Glc} of *E. coli*, and between HPr and EIIA^{Glc} of *B. subtilis* [Herzberg, 1992; Wang et al., 2000].

Studies by Koh et al. [2013] demonstrated that probiotic strains such as *L. rhamnosus* GG and *L. casei* catabolize tagatose via tagatose-specific PTS. This study highlights the potential of tagatose as a supplement (symbiotic partner) for *L. casei* and/or *L. rhamnosus* strain GG in dairy foods and therapeutic dietary regimens. The study reports differential expression of genes of *L. rhamnosus* GG (ATCC 53103) in the presence of tagatose compared those present in glucose-grown (control) cells. For example, *tagT* and *fruK* gene expressions were dramatically increased 31- and 56-fold, respectively. The *tagT* gene (LGG_02647) codes for the PTS system fructose-specific transporter subunit IIABC, and the *fruK* gene (LGG_02648) encodes a 1-phosphofructokinase [Koh et al., 2013]. The IIABC protein comprises domains B, C and A. Whereas we found 54% identity between IIBC^{Tag,Bli} and IIBC^{Tag,Kox}, we found that the IIABC exhibits 53% identity with IIBC^{Tag,Bli} and 47% identity with IIBC^{Tag,Kox}. In addition, FruK protein from *L. rhamnosus* exhibits 39% amino acid sequence identity with both TagK of

K. oxytoca and *B. licheniformis*. Presumably, a PTS-mediated D-tagatose pathway including a Tag-1P intermediate is also extant in other Gram-positive strains, including nutritionally important probiotic organisms, such as *L. casei* and *L. rhamnosus*.

Materials and Methods

Materials

D-Tagatose and D-tagatofuranose-6-phosphate, lithium salt (Tag-6P) were purchased from Carbosynth US LLC (San Diego, Calif., USA). PEP monosodium salt, barium acetate (99%), NADH, Triose phosphate isomerase from baker's yeast (*Saccharomyces cerevisiae*), glycerophosphate dehydrogenase type I (rabbit muscle) and D-fructose 6-phosphate, disodium salt hydrate (Fru-6P) were obtained from Sigma-Aldrich. ATP disodium trihydrate was from Amresco and D-fructose 1-phosphate, sodium salt (Fru-1P) was from Santa Cruz Biotechnology.

Bacterial Strain and Culture Medium

S. aureus subsp. *aureus* ATCC 25923 and *B. licheniformis* ATCC 14580 were obtained from the American Type Culture Collection (Manassas, Va., USA). *Bacillus subtilis* 168 was from the Bacillus Genetic Stock Center (BGSC accession number 1A1). The *E. coli* BL21(DE3) strain (Stratagen, La Jolla, Calif., USA) was used to overexpress proteins.

K. pneumoniae subsp. *pneumoniae* ATCC 23357 was obtained from the American Type Culture Collection. This strain was used for the enzymatic synthesis of Tag-1P. The organism was grown in a DM containing (per liter): Na₂HPO₄, 7.1 g; KH₂PO₄, 1.5 g; (NH₄)₂SO₄, 3 g; MgSO₄ · 7H₂O, 0.1 g, and FeSO₄ · 7H₂O, 5 mg. Filter-sterilized tagatose was added to autoclaved medium to a final concentration of 0.4% (w/v).

Growth and Preparation of *K. pneumoniae* ATCC 23357

The organism was grown (without aeration) at 37°C in 3 × 1-liter bottles, each containing 800 ml of medium. After growth to the stationary phase (18 h), the cells were harvested by centrifugation (13,000 g for 10 min at 5°C) and washed twice in 25 mM of Tris-HCl buffer (pH 7.5) containing 1 mM of MgCl₂ · 6H₂O. The yield was ~2 g wet weight of cells/liter.

Preparation of Tag-1P

The enzymatic synthesis of this novel hexose phosphate was catalyzed via the multicomponent PEP-PTS present in tagatose-grown cells of *K. pneumoniae* (see fig. 1b). The procedure, with slight modification, is essentially that described previously for the biosynthesis of a variety of 6'-O-phosphorylated disaccharides [Thompson et al., 2001a, b]. Tagatose-grown cells were added to 5 ml of 25 mM Tris-HCl buffer (pH 8) containing 1 mM of MgCl₂ to a density of 10 mg dry weight/ml. After chilling on ice, the cells were permeabilized by the addition of 50 μ l of a mixture of acetone/toluene (9:1 v/v), and the suspension was agitated vigorously for 30 s on a Vortex mixer. This procedure was performed three times, and the permeabilized cell suspension was then placed in ice. For preparative purposes, 15 such suspensions were prepared. Thereafter, PEP (330 mg) and tagatose (1 g) were dissolved in 12 ml of

25 mM Tris-HCl buffer (pH 8) and, after adjustment to pH 8 with ~0.5 ml of 5 N NaOH, water was added to a final volume of 15 ml. Subsequently, 1 ml of PEP/tagatose solution was added to each of the permeabilized cell suspensions to provide approximately 100 μmol of PEP and 350 μmol of tagatose per reaction mixture. The 15 suspensions were transferred to a 37°C water bath and, after 1.5 h of incubation (with occasional inversion), the preparations were chilled to 0°C. During incubation there was usually a decrease of 0.5 units in the pH of the reaction mixture. The suspensions were pooled and cells were removed by centrifugation. The supernatant fluid was adjusted to pH 8.2 with NaOH, and 8 ml of aqueous barium acetate (25% w/v) was added slowly with continuous stirring. The mixture was chilled on ice for 30 min and the heavy white precipitate of water-insoluble Ba^{2+} compounds was removed by centrifugation. The clarified supernatant was filtered through a 0.45- μm pore-size membrane, and 4 volumes of ice-cold absolute ethanol were added. The solution, containing a white flocculent precipitate of the ethanol-insoluble Ba^{2+} salt of Tag-1P was placed overnight in a cold room.

Purification of Tag-1P

The barium salt of Tag-1P was collected by centrifugation and upon drying (at 37°C) the ethanolic white pellet yielded a yellow/white crystalline residue. Ba^{2+} ions were exchanged for H^+ by addition of 2–3 ml of an aqueous suspension of Bio-Rad AG 50W X2 (H^+ -form) resin. Exchange resin was removed by filtration and, after adjustment to pH 7.2 (by addition of NaOH or NH_4OH), the solution was frozen and lyophilized. Further purification of Tag-1P and removal of trace contaminants (e.g. nucleotides, P_i and residual PEP) was effected by descending paper chromatography (Whatman 3 MM, 20 h) in a solvent containing n-butanol/glacial acetic acid/ H_2O (5:2:3 v/v/v). The location of Tag-1P was visualized as a vivid white zone against a pink background by Wade-Morgan stain [Wade and Morgan, 1953]. The appropriate strips containing Tag-1P were excised from the chromatogram and the hexose monophosphate was collected by centrifugal elution with distilled water. The pH of the eluate was adjusted to 7.2 with NaOH or NH_4OH , and the solution was frozen and lyophilized. As outlined, the procedure usually yielded 20–30 mg of white crystalline Tag-1P salt.

Physicochemical Properties of Tag-1P

Thin-layer chromatographic analysis provided evidence for the purity of the compound. When 20–100 nmol of Tag-1P were applied to 0.1-mm layers of Polygram Cellulose MN 300 and eluted using the previously described solvent, only a single white spot was revealed by Wade-Morgan stain. Under these conditions, the migration positions of Tag-1P and Tag-6P were virtually identical. High-resolution negative mode electrospray mass spectra data for Tag-1P were acquired on a Thermo Q-Exactive Orbitrap, calculated for $\text{C}_6\text{H}_{12}\text{O}_9\text{P}$ [$\text{M}-\text{H}$] $^{-1}$, measuring 259.0224. ^1H , ^{13}C and ^{31}P NMR spectral data for Tag-1P are presented in Results.

NMR Experiments

All NMR data were collected in a Bruker Avance II, 700 MHz NMR spectrometer, equipped with a QXI xyz-gradient, room temperature probe. For tagatose chemical shift assignment, two samples of tagatose were prepared: (1) a 200-mM tagatose solution in 90:10 $\text{H}_2\text{O}:\text{D}_2\text{O}$ and (2) a 200-mM tagatose solution in 50:50 $\text{H}_2\text{O}:\text{D}_2\text{O}$ solution. The pH was measured at 25°C and adjusted to 6.5 with NaOH. The data were collected at –10°C. Low tempera-

tures were utilized to ensure sample preservation and the observation of hydroxyl proton signals [Battistel et al., 2014]. Chemical shift assignment was carried out utilizing a series of 2D HSQC-TOCSY experiments (10, 30 and 60 ms of DIPSI-2 isotropic mixing) [Rucker and Shaka, 1989]. These experiments also afforded hydroxyl proton assignments, as previously reported [Battistel et al., 2013].

For the enzymatically synthesized tagatose phosphate, a 50-mM aqueous solution, with 10% D_2O , was prepared. The pH was adjusted to 7.5 with NaOH, at 25°C. Chemical shift assignments were carried out as for tagatose at –10°C and 25°C (table 1). A pH of 7.5 was required to decrease the OH exchange rate with the solvent and allow observation of the isotope shift in ^{13}C signals. ^1H - ^1H coupling constants were estimated from HSQC traces or from phase-sensitive COSY experiments.

For control experiments with Tag-6P, a 3.8-mM aqueous solution, 10% D_2O , pH 7.5, was prepared. Chemical shift assignments were obtained as for tagatose and Tag-1P.

Phosphorylation Position

The position of phosphate substitution was determined using three methods: (1) we measured and assigned the OH groups in tagatose and compared them to phosphorylated tagatose; (2) we measured the isotope effect on the ^{13}C chemical shifts of the carbohydrate ring in a 50:50 $\text{H}_2\text{O}:\text{D}_2\text{O}$ mixture and in 90:10 $\text{H}_2\text{O}:\text{D}_2\text{O}$, and (3) we measured two- and three-bond ^{31}P - ^{13}C coupling constants ($^2J_{^{13}\text{C}-^{31}\text{P}}$, $^3J_{^{13}\text{C}-^{31}\text{P}}$, respectively; table 1). It has been shown [Eyrisch et al., 1993] that $^3J_{^{13}\text{C}-^{31}\text{P}}$ are often larger than $^2J_{^{13}\text{C}-^{31}\text{P}}$; thus, these NMR observables can help in establishing which carbon atoms are two or three covalent bonds removed from the phosphate group.

When OH signals cannot be directly observed, deuterium isotope effects can be useful in establishing which OH groups are substituted by other functional groups. Deuterium two-bond isotope effects on ^{13}C can be observed if the hydroxyl proton exchange rate is slow enough (exchange rate constant, $k_{\text{ex}} < |\sim 2 \cdot \Delta\delta^{13}\text{C}|$, where $\Delta\delta^{13}\text{C}$ is the frequency difference, in Hz, between ^{13}C measured in H_2O with respect to that measured in D_2O). If the exchange is fast ($k_{\text{ex}} \gg |\sim 2 \cdot \Delta\delta^{13}\text{C}|$), then a single peak will be observed; however, its chemical shift will change depending on the $\text{D}_2\text{O}:\text{H}_2\text{O}$ ratio used.

As OH groups could not be directly observed at the sample concentration available, the H/D isotope effect was monitored as follows: the 90:10 $\text{H}_2\text{O}:\text{D}_2\text{O}$, tagatose phosphate sample was used to conduct ^{13}C 1D NMR experiments. Thereafter, the sample was lyophilized and then dissolved in 50:50 $\text{H}_2\text{O}:\text{D}_2\text{O}$, pH 7.5, at 25°C, and ^{13}C 1D data were again collected. Carbon atoms with OH groups in slow exchange with the solvent will display two ^{13}C signals because of isotope shifts originating from two different kinds of ^{13}C atoms: one signal for ^{13}C attached to OH and the other for ^{13}C attached to OD. Several factors may account for the absence of signal duplication for a given ^{13}C , including: (1) OH is in fast exchange with the solvent; (2) the OH is phosphorylated, alkylated or substituted by some other functional group without an exchangeable hydrogen atom, or (3) the exchangeable hydrogen atom was lost during formation of a sugar ring or a glycosidic linkage. In the first case, the existence of an OH group can be verified by varying the D_2O percentage. The presence of an OH group will lead to a change in the apparent ^{13}C chemical shift, whereas its absence will not. In the second case, other NMR observables can be

measured to determine which type of substituent is present. In this report, we have measured ^{13}C - ^{31}P coupling constants (table 1) and collected 1D ^{31}P and 2D ^1H , ^{31}P NMR experiments (online suppl. fig. S1B). In the third case, one can rely on available data, such as chemical shifts, the coupling constant of reference compounds and molecular formulae.

Modeling of TagK

The structure of fructose 1-phosphate kinase from *B. halodurans* (PDB ID: 2ABQ) was used to generate figure 7. Unfortunately, the structure of 2ABQ has not yet been published, nor has the Fru-1P binding site been established. TagK has not yet been crystallized, and axiomatically the Tag-1P binding site is also unknown. However, because fructose differs from tagatose only in the orientation of the OH moiety at C4, we proceeded to use 2ABQ as a potential model, since it may bear structural resemblance with TagK. The chemical structures for Tag-1P, Tag-6P and Tag 1,6-BP were created in the HyperChem software package (Gainesville, Fla., USA). The model depicted in figure 7 was created in PyMOL (the PyMOL Molecular Graphics System, v.1.7.0.3; Schrödinger LLC, New York, N.Y., USA), the magnesium ion and the glycans chemical structure coordinates were modified with the translate command, so as to appear approaching and leaving the protein surface.

Plasmids, Enzymes and Oligonucleotide Primers

The expression plasmids pET28a(+) and pColdTF were purchased from Novagen and Takara, respectively. The plasmid pLysS was purchased from Novagen. Restriction endonucleases, Phusion High-Fidelity DNA Polymerase kit and In-Fusion HD Cloning kit were purchased from Promega, Fisher Scientific and Clontech, respectively. Oligonucleotides primers were from Eurogentec (Seraing, Belgium).

The Complementation Test: Construction of the pPtsHI Plasmid and Growth Experiments

The constitutive *B. subtilis* ptsHI operon was amplified by PCR with oligonucleotides primers ptsHI forward 5'-CCCGTCCTGTGGATCCGGGTGTTAGTACGCCGTGC-3' and ptsHI reverse 5'-CCGGCGTAGAGGATCCCTTACTTATGAAAAAACCAGACAG-3' using the genomic DNA of *B. subtilis* 168 as the template DNA. The PCR product was subcloned between the two BamHI sites of pLysS plasmid replacing the T7 lysozyme coding sequence yielding pPtsHI. Construct was verified by DNA sequencing (GIGA, Seraing, Belgium). The pLysS plasmid confers resistance to chloramphenicol (Cam^R) and contains the p15A origin compatible with that of the pDGTag plasmid (pBR322-derived plasmid) previously introduced in the *E. coli* DH5α strain [Van der Heiden et al., 2013]. This strain, harboring the pDGTag plasmid expressing the Bli-TagP (fig. 1a), was transformed with pPtsHI or the pLysS plasmid (negative control).

E. coli DH5α strain harboring pDGTag and either pPtsHI or pLysS plasmid was grown in a DM previously described [Van der Heiden et al., 2013] supplemented with either 20 mM of tagatose or 20 mM of glucose, or deionized H₂O (sugar negative control). Spectinomycin and chloramphenicol antibiotics were added in the DM at 50 and 12.5 µg/ml, respectively. Cultures were performed in 30 ml of the supplemented DM with 600 µl of preculture under agitation (250 rpm) at 28°C. Growth of the cultures was monitored by the increase in absorbance at 600 nm during 40 h.

Cloning and Overexpression of TagK-TF^{His6}, GatY^{His6} and LacD^{His6}

The *fruK2* gene was amplified by PCR with oligonucleotide primers FruK2-UP 5'-TCGAAGGTAGGCATATGATCTATACATGCACGATGAACACCGCAG-3' and FruK2-RP 5'-ATTACCTATCTAGACTGCAGTTATAAATGAGTGATTTGAATTTGTGAAGCTGTTTGATC-3' using the genomic DNA from *B. licheniformis* ATCC 14580 as the template DNA. The PCR product was cloned into the NdeI and PstI sites of pColdTF yielding pCold-TagK-TF^{His6}. The *gatY* gene was amplified by PCR with oligonucleotide primers ALD-UP 5'-AGGAGATATACCATGGCCCTGACAAATACGAAAAATGCTGCTGGACC-3' and ALD-RP 5'-GGTGGTGGTGCTCGAGGTATCTTGTCATTGCTCATGCACATC-3' using the genomic DNA from *B. licheniformis* ATCC 14580 as the template DNA. The PCR product was cloned into the NcoI and XhoI sites of pET28a yielding pET28a-GatY^{His6}. The *lacD* gene was amplified by PCR with oligonucleotide primers LacD-UP 5'-AGGAGATATACCATGGCGAAATCGAATCAAAAAATCGCATC-3' and LacD-RP 5'-GGTGGTGGTGCTCGAGTTTTCTTTGTTTCCAAGATGTCGC-3' using the genomic DNA from *S. aureus* subsp. *aureus* ATCC 25923 as the template DNA. The PCR product was cloned into the NcoI and XhoI sites of pET28a yielding pET28a-LacD^{His6}. The three resulting constructs were verified by DNA sequencing (GIGA).

Overexpression and Purification of TagK-TF^{His6}, GatY^{His6} and LacD^{His6}

E. coli BL21(DE3) cells were transformed with each construct for protein overexpression. Cultures were grown at 37°C under agitation (250 rpm) in liquid 2XYT medium (10 g of tryptone, 10 g of yeast extract, 5 g of NaCl for 1 liter of medium) supplemented with ampicillin (100 µg/ml) or kanamycin (50 µg/ml) for the pColdTF and pET28a constructs, respectively. Medium for the overexpression of GatY^{His6} was supplemented with 0.5 mM of ZnSO₄. When the absorbance (600 nm) of the cultures reached 0.6–0.8, the overexpression of TagK-TF^{His6} was induced by the addition of IPTG 0.5 mM and the culture was incubated overnight at 15°C under agitation (250 rpm). Similarly, the overexpression of GatY^{His6} and LacD^{His6} were induced by the addition of 1 mM of IPTG and cultures were incubated for 3 h at 28°C.

Cell pellets of the culture were resuspended in Tris-HCl 50 mM buffer (pH 8.0) and lysed with a high-pressure homogenizer (Emulsiflex C3, Avestin Europe GmbH, Mannheim, Germany). The supernatants were recovered after 30 min of centrifugation at 25,000 g. For protein purification, we used an automated two-step Profinia system composed of a Bio-Scale Mini Profinity IMAC cartridge followed by a desalting cartridge (Bio-Rad). The internal programmed method 'Native IMAC and Desalting' was applied.

Elution from the desalting cartridge was performed in buffer Tris-HCl 50 mM (pH 8.0), KCl 2.7 mM and NaCl 137 mM for TagK-TF^{His6}, in Tris-HCl 20 mM (pH 8.0) for GatY^{His6} and in Tris-HCl 20 mM (pH 8.0), NaCl 200 mM for LacD^{His6}. Concentrations of the purified proteins were determined spectrophotometrically by measuring the absorbance at 280 nm and were 0.7 mg/ml for GatY^{His6}, 2.5 mg/ml for LacD^{His6} and 2.1 mg/ml for TagK-TF^{His6}. The purity of His-tagged proteins was estimated to be no less than 90%. GatY^{His6} and LacD^{His6} can be conserved at -20°C for at least 6 months without the need for additive, while TagK-TF^{His6} requires the addition of 5% glycerol before freezing.

Enzyme Assays

Phosphotagatokinase and phosphofructokinase activities of TagK-TF^{His6} were measured by coupling the formation of Tag 1,6-BP or Fru 1,6-BP to NADH oxidation via class I ketohexose 1,6-BP aldolase (LacD), triose phosphate isomerase (TPI) and glycerophosphate dehydrogenase type I (G3PDH; fig. 8). The assay contained: 50 mM of Tris-HCl (pH 8.0), 10 mM of MgCl₂, 0.2 mM of NADH, 2 mM of ATP and variable concentrations of substrate. Non-rate-limiting amounts of LacD (13 µM), of TPI (49.5 U/ml) and of G3PDH (9 U/ml) were added to the assay mixture. The concentration of TagK-TF^{His6} was rate limiting (2.4 µM) in the assay. Enzyme activities were monitored spectrophotometrically at 340 nm for 12 min with a microplate reader (Tecan Infinite M200Pro) in a total volume of 200 µl at 25°C.

TagK-TF^{His6} Enzyme Specificity and Inhibition Assays

Fru-1P, Fru-6P, Tag-1P or Tag-6P was added at a final concentration of 2 or 10 mM to test the specificity of TagK-TF^{His6}. For the inhibition assays, the Tag-1P concentration was fixed at 2 mM and Fru-1P, Fru-6P or Tag-6P were then each added to the reaction, at 2 or 10 mM. The rate of the reaction with the Tag-1P at 2 mM was designated as 100% maximum activity. Subsequent assays were expressed as a relative activity according to the formula: relative activity (%) = [slope_(assay)/slope_(Tag-1P 2 mM)] × 100. All assays were performed in triplicate.

Kinetic Parameters of TagK-TF^{His6}

For the determination of the kinetic parameters, Tag-1P was added at 8, 4, 2, 1, 0.5 or 0.25 mM in the mixture assay. Fru-1P was added at 80, 40, 20, 10, 5, 2.5 and 1.25 mM. The respective *k*_{cat} and *K*_m values were calculated by nonlinear fit of the Michaelis-Menten curve with the help of the GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, Calif., USA). Kinetic parameters were determined from three independent experiments.

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