Research Paper

Effect of *pfkA* chromosomal interruption on growth, sporulation, and production of organic acids in *Bacillus subtilis*

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Phosphofructokinase (Pfk) plays a key role in the regulation of carbohydrate metabolism. Its activity can be used as an indicator of glycolytic flux in a microorganism. We have cloned and characterized the *pfkA* gene from *Bacillus subtilis,* which encodes the enzyme phosphofructokinase. This gene was insertionally inactivated at the chromosomal level in a wild type strain and in strains lacking the PEP:sugar phosphotranferase system (PTS). Although the *pykA* gene is immediately downstream of the *pfkA* gene, forming a constitutive operon in *B. subtilis,* the pyruvate kinase activity was not altered in the *pfkA* mutant. The inactivation of the *pfkA* gene had a strong impact on the growth of the *B. subtilis* wild type strain and PTS mutants in Spizizen's minimal media and Schaeffer's sporulation media. Pfk inactivation was also reflected by the timing and percentage of sporulation of the wild type and PTS mutants in sporulation media as well as in the production of organic by-products (pyruvate, lactate, and acetate).

Keywords: Phosphofructokinase mutant/ PTS/ Carbon metabolism/ HPLC/ Carboxylic acid

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Introduction

6-Phosphofructokinase (ATP:D-fructose-6-phosphate 1-phosphotransferase, Pfk, EC 2.7.1.11) catalyzes the phosphorylation of fructose-6-phosphate to fructose-1,6-bisphosphate (FBP) (Fig. 1). Pfk, together with the pyruvate kinase (Pyk), plays a major role in controlling carbon flux through the glycolytic pathway [1]. The *pfkA* and *pykA* genes form an operon in *B. subtilis* and *B. stearothermophilus* [2, 3].

B. subtilis has been used commercially for the production of several industrial vitamins and other products including enzymes such as amylases, proteases and lipases. The preferred source of carbon and energy in this microorganism is glucose [4]. This sugar is taken up and concomitantly phosphorylated by the glucose permease of the phosphoenolpyruvate:sugar phosphotransferase system (PTS) and further catabolism by glycolysis and the pentose phosphate pathway [5].

The pyruvate generated by PTS and by the oxidation of triose phosphates in glycolysis is finally oxidized in the tricarboxylic acid (TCA cycle), and can also be reduced to lactate and other compounds such as acetate, acetyl-CoA, ethanol, acetolactate, diacetyl, acetoin, and 2, 3-butanediol [6, 7].

The PTS elements are also involved in other important functions, such as the general regulatory process of carbon catabolite repression, metabolic and transcriptional regulation, and chemotaxis [8]. HPr-kinase/phosphorylase is a sensor for catabolite repression, phosphorylates HPr, a phosphocarrier protein of PTS, in the presence of ATP and FBP but dephosphorylates P-Ser-HPr when phosphate prevails over ATP and fructose-1,6-bisphosphate. This mode of regulation links

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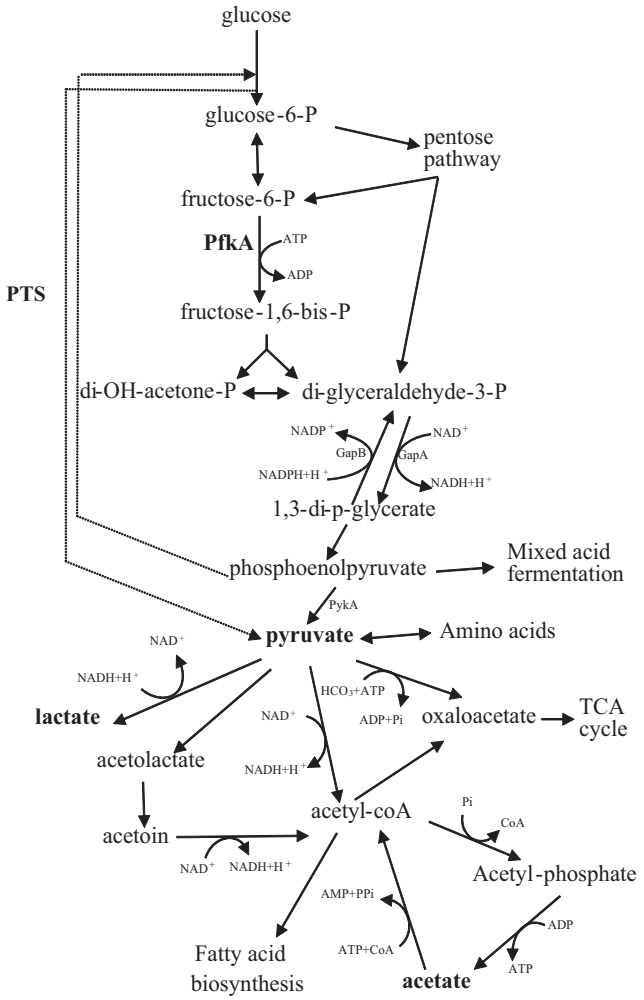

Figure 1. Principal metabolic pathways in *Bacillus subtilis.*

the HPr to the metabolic and energetic state of the cell [4, 9].

Pfk has been studied extensively both structurally and functionally in *Escherichia coli*, *Lactococus lactis,* and *Bacillus stearothermophilus* [10–12], but little attention has been paid to this enzyme in *B. subtilis*. *B. subtilis* PfkA mutants have been obtained by ethyl methane sulfonate mutagenesis [13], by phage transduction experiments, and by selection for auxotrophy [14, 15]. Even though chemical procedures have opened the door to study PfkA in *B. subtilis,* a good PfkA knockout *B. subtilis* strain has not been previously developed. The new *pfkA* mutant insertionally obtained in this study provides a relatively clean genetic background for metabolic studies. PfkA mutants and wild type strains were evaluated for growth in rich and minimal media supplemented with glucose. Also, because PTS is an important source of pyruvate and serves both transport and transduction purposes we isolated a deletion mutant on *ptsXHI* genes. Results showed that PfkA and PTS are essential for a normal growth in both culture media, and also to maintain the precise balance of metabolites required for differentiation and organic acid production.

Materials and methods

Bacterial strains, plasmids and antibiotics

All bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* JM101 was transformed by standard procedures and used for plasmid construction and amplification [16]. The *B. subtilis* strain lacking PTS was obtained by transferring the Δ(*pts'XHI'*)::*erm* from strain MM3 [17] into the Δ*(nprE)* strain MM2 (used as a wild type) [18], according to standard procedures [19]. Finally, the insertionally inactivated *pfkA* gene was incorporated into the Δ*pts* strain (see below). Antibiotics were used in plates at the indicated concentration (mg l^{-1}): ampicillin, 50; erythromycin, 15; spectinomycine 100.

Growth conditions

Luria-Bertani medium was used for routine liquid growth maintenance of *E. coli* and *B. subtilis* [16]. The strains were grown aerobically for 48 h on flasks of 2.8 l capacity with 500 ml of media in orbital agitation at 300 rpm and 37 °C. The Spizizen minimal medium (SMM), used for growth evaluation of the mutants,

Table 1. Bacterial strains and plasmids.

Strain or plasmid		Relevant Genotype	Reference
B. subtilis	MM2 (WT)	*Δ(nprE) hisA glyB* derived of 168	[18]
	MM3	*trpC2 sacR::lacZ Δ(pts'XHI')::erm*	[17]
	EMP06	MM2 but *pfkA::sp*	This work
	MM4	MM2 but *sacR::lacZ (pts'XHI')::erm*	This work
	EMP07	MM4 but *pfkA::sp*	This work
E. coli	JM101	*supE thi* (*lac⁻proAB*) (F′ *traD36 proAB lac* l^qZ *M15*)	[39]
Plasmids	pE512	pUC19 and 512 pb *pfk-pyk* fragment	[3]
	pDG1726	*sp* gene of *Staphylococcus aureus*	[20]
	pEH5	pE512 but *pfkA::sp*	This work

contained per liter: 2 g $(NH_4)_2SO4$, 6 g KH_2PO_4, 14 g K_2HPO_4, 1 g Na_3 citrate · 2 H_2O, pH 7.4 and was supplemented with 1 M $MgSO_4$, 0.1 M $CaCl_2$, 2.5 mM $FeCl_3 \cdot 6\ H_2O$ and glucose 2 g l^{-1} as the only carbon source [19]. Additionally, Schaeffer's sporulation medium (SSM) was used to evaluate the growth and the organic acids production at OD_{600} 0.95, at the transition time between the end of exponential growth and the onset of stationary phase (T0), and 2 and 4 h after T0 (T2 and T4). One liter of SSM media contained 8 g Nutrient Broth (DIFCO), 1 g KCl, 0.13 g $MgSO_4 \cdot 7\ H_2O$ and was suplemented with 1 mM $FeSO_4$, 1 M Na_2SO_4 and 10 mM $MnCl_2$, pH 7.4. Amino acids used at limited concentrations were also added to the media: glycine (2 μg ml^{-1}), histidine (2 μg ml^{-1}) and tryptophan (5 μg ml^{-1}). The percentages of heat-resistant spores present in cell cultures at 4, 8 and 15 h after T0 (T4, T8 and T15) were evaluated using a standard protocol described by Harwood and Cutting [19] using a spore buffer solution (10 mM K_2HPO_4, 50 mM KCl and 1 mM $MgSO_4$). Data are presented as average of at least three independent biological replicates.

Construction of a *pfkA* insertional inactivation mutant

Two plasmids were constructed to interrupt the *pfkA* gene in the *B. subtilis* chromosome. The plasmid pE512 carries a 512 bp fragment, including 222 bp of the *pfkA* gene and 255 bp of the *pykA* gene (Fig. 2). This DNA segment was corroborated by PCR, mapped by enzyme digestion and sequenced. Plasmid pE512 possesses a unique *Hind*III site in *pfkA* fragment, where a 1,100 bp DNA segment carrying the spectinomycin resistance gene from plasmid pDG1726 [20] was inserted. This derivative, named pEH5 (see Table 1) was used for insertional inactivation of the chromosomal *pfkA* gene on wild type and Δ*pts* strains using standard procedures based in a double cross-over event [19]. Disruption of the *pfkA* gene was confirmed by antibiotic resistance and PCR using chromosomal DNA as template and a *Taq* polymerase kit (Perking-Elmer/Cetus, Norwalk, CT).

Enzymatic activity assays

The PfkA and PykA activities of the wild type and PfkA mutant *B. subtilis* strains were measured in cell extracts obtained by sonication. Cells were grown in Schaeffer's medium and harvested at an OD_{600} of approximately 0.8. The activity was determined from the rate of NADH oxidation at A_{340} at 25 °C using a Beckman spectrophotometer (GMI, MN, USA). Proteins were determined according to Lowry *et al.* [21]. All the enzymes used for the enzymatic activity assays were from Sigma (Sigma-Aldrich Corp. St. Louis, MO, USA). The enzymatic activity was reported as international units of enzyme per milligram of protein (U mg^{-1}). One unit of activity was defined as the production of 1 μmol of fructose-1, 6-bisphosphate per minute. Data are presented as average at least of three independent experiments.

For Pyk activity, harvested cells were washed with buffer solution (50 mM Tris-acetate, pH 7.5, 0.1 mM EDTA, 10 mM $MgCl_2$, 100 mM KCl, 10 mM β-mercaptoetanol) and then resuspended in ice-cold sonication buffer (same as wash buffer plus 2 mM PEP) as reported by Diesterhaft and Freese [22]. After sonication (five cycles of 15 s interspaced by 30 s cooling periods) the cell debris was removed by centrifugation for 20 min at 37,000 g and 4 °C. As a measure of the degree of cell disruption, the optical density at 280 nm was used. Pyk activity was determined as described by Malcovati and Valentini [23], except that the final concentrations of the components of the reaction mixture were: 50 μM Tris-acetate, pH 7.0, 250 μM KCl, 10 μM $MgCl_2$, 5 μM ADP, 0.3 μM NADH, 4 μM PEP, 1 μM AMP, 1 μM FBP, and 2.75 U of lactate dehydrogenase.

For PfkA activity, the cells were washed with buffer solution (50 mM Tris-acetate pH 7.5, 0.1 mM EDTA, 50% glycerol, 1 mM dithiothreitol), resuspended and sonicated three times for 40 s with intervals of 30 s, as reported by Solem and Jensen [24], followed by centrifugation at 20,000 *g* for 20 min at 4 °C. As a measure of the degree of cell disruption, optical density at 280 nm was used. PfkA activity was performed using the coupled enzyme assay in which the production of FBP leads to the oxidation of NADH as described by Fordyce *et al.* [25], except that the final concentrations in the assay mixture were: 1 mM ATP, 1 mM fructose 6-phosphate, 0.2 mM NADH, 10 mM $MgCl_2$, 10 mM NH_4Cl, 0.3 U of triose phosphate isomerase per ml, 1 U of glycerol 3-phosphate dehydrogenase per ml, and 0.3 U of aldolase.

Theoretical motif of transcription

In order to establish a theoretical motif of transcription for the two genes in the *pfkA* and *pykA* operon, we used the database of transcriptional regulation in *B. subtilis* (DBTBST) [26] with p values of 0.01 and 0.5. The genome sequence template was *B. subtilis* subsp *subtilis* 168, the total number of genes analyzed was 4106 and the number of genes grouped was 711 (17.3% of the total). The motif matrices available from DBTBS are the result of clustering of regions conserved to different extents between genus-based upstream intergenic regions (UPIR) of homologous genes from Gram-positive bacteria. The UPIR of each of the subgroup containing more than 2 members were then aligned with ClustalW, and the

degree of conservation of the last 300 positions of the alignment, representing the nucleotides directly upstream of the gene start sequence, was calculated based on information content [27].

Organic acids determination by HPLC

The cells samples of each culture were removed by centrifugation and the supernatants were passed through filter of 0.45 µm pore size. High performance liquid chromatography (HPLC) was carried out using an isocratic HPLC pump, Agilent 1100 series. The ultraviolet wavelength detection was set at 210/230 nm and a ZORBAX 300 SB-C18 column was used for the separation of analytes (Agilent Technologies, Waldbronn, Germany). A sample stock solution and further dilutions of each carboxylic acid (lactate, pyruvate and acetate) standards were prepared in ultrapure water. A solution containing 25 mM potassium phosphate pH 3 and 0.3% acetonitrile was used as running buffer. All reagents were HPLC grade from Sigma (St. Louis, MO, USA). Reversed phase HPLC using aliquots of 20 µl from the filtrates were then injected and the mobile phase was pumped at 0.5 ml min^{-1} in the isocratic mode, with the column temperature maintained at 25 °C. The organic acids were identified based on their retention times with known standards of each acid. Concentrations of pyruvate, acetate and lactate were determined from standard curves made with solutions of the appropriate acid using linear regression analysis and after substracting the media culture values. The values of pyruvate and acetate were obtained at 230 nm and lactate values at 210 nm and all were expressed in g l^{-1}. All the measurements were performed in triplicate and the standard deviations calculated were not higher than 10%.

Results and discussion

The aim of this study was to investigate the metabolic and physiological effects of the *pfkA* inactivation in both wild type and Δ*pts* backgrounds of *B. subtilis*. The cloning and chromosomal interruption of the *pfkA* gene in *B. subtilis* MM2 was accomplished using the strategy shown in Fig. 2. Interruption of the gene was analyzed by selection by spectinomycin as well as by PCR amplification. As can be observed in Fig. 3, a 2.242 Kb PCR product was obtained for the *pfkA* mutant (EMP06), in contrast with the 1.142 Kb fragment observed for the wild type *pfkA* gene. The interruption of the *pfkA* gene was corroborated by the measurement of the PfkA enzymatic activity. The specific activity of Pfk in the wild type strain was

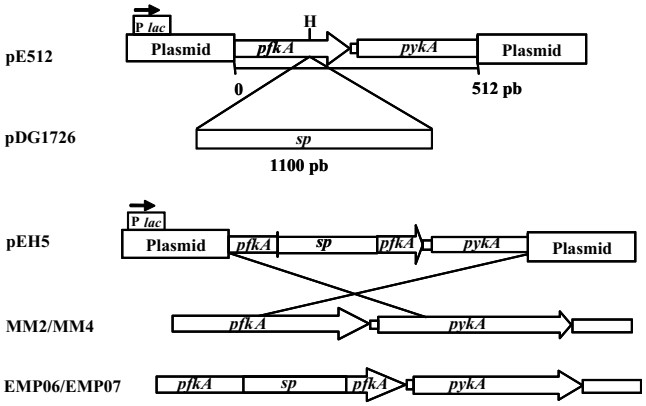

Figure 2. Construction of a *pfkA* insertional inactivation mutants.

0.4 ± 0.007 U mg^{-1} of protein, and in the *pfkA* mutant was 0.02 ± 0.007 U mg^{-1} of protein.

In *B. subtilis* pyruvate kinase gene (*pykA*) and *pfkA* gene are in the same operon, and *pykA* is immediately down stream of the *pfkA* gene. Because of that positioning, it was necessary to determine that the chromosomal interruption of *pfkA* gene would not affect the function of the *pykA* gene. Our results showed that the specific activity of the pyruvate kinase enzyme was similar in both wild type (0.6 ± 0.008 U mg^{-1} of protein) and *pfkA* mutant (0.7 ± 0.009 U mg^{-1} of protein).

The absence of polar effect may be explained by transcriptional read through from the *pfkA* or the *spc* promoter. Interesting results were obtained by Andersen *et al.* [10] when the promoter of the *las* operon of *Lactococcus lactis*, harboring *pfk*, *pyk,* and *ldh*, was replaced by a synthetic promoter. In that study phosphofructokinase activity showed a twofold decrease, whereas the activities of pyruvate kinase and lactate dehydrogenase remained closer to those of the wild type.

In order to locate possible transcriptional regions (motifs) in the *pfkA pykA* operon we used the DBTBS program. The analysis showed two hexameric motifs: one at −54 from the start point for *pykA* (aatgta), and second motif at −163 from the start point from *pfkA* (ttaatt) that may have regulatory functions (Fig. 4). Additionally, we found binding sites of transcription factors in the UPIR of *pfkA*–*accA*, but none in the UPIR *pykA*–*pfkA*. The Shine Dalgarno (SD) site located in the UPIR of the *pykA* gene, reported previously by Muñoz *et al.* [3], was also observed. Interestingly, we found some possible binding sites of transcription factors in the sequence of the *pfkA* gene (Fig. 4). On this regard, conserved regions potentially indicate binding sites of minor or specialized transcription factors, which typically bind at only a few places in a genome. Alternatively, they may indicate target-binding sites of global

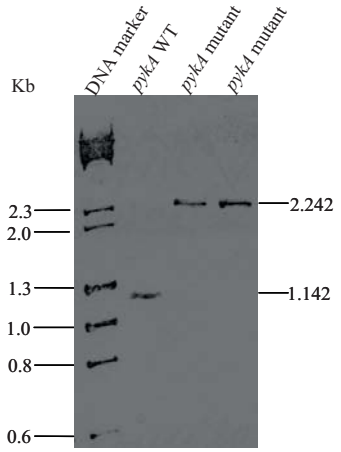

Figure 3. Chromosomal PCR products. Lane 1, DNA marker; Lane 2, wild type *pfkA* gene; Lane 3, *pfkA* mutant (EMP06).

regulators, which occur much more frequently. These factors can be induced in response to different environmental conditions such as heat shock, pleiotropic regulators, glucose and oxygen limitation or oxidative stress [27]. *B. subtilis* makes extensive use of secondary σ factors to coordinately activate groups of genes under specific cellular conditions. The genomic sequence analysis of *B. subtilis* has indicated that it encodes for at least 17 σ factors, including 7 extracytoplasmic functions family members which had not been identified prior to genomic sequencing [28]. The co-occurrence of motifs found in our analysis provides interesting information about co-regulation by several transcription factors. Additional studies would be helpful in understanding the relationship between these factors and gene activation.

The effect of the *pfkA* gene inactivation in the wild type strain and in strains in which the $EIII^{Glc}$, HPr and EI enzymes of the PTS were inactivated (Δ*ptsXHI*) was evaluated by culturing them in two different media: Schaeffer's sporulation medium and Spizizen minimal medium with glucose (2 g l^{-1}) as the only carbon source. As can be seen in Fig. 5 and Table 2, the absence of PfkA activity produced a strong impact on *B. subtilis* physiology. Growth was higher in Schaeffer's rich medium than in minimal medium in all the strains examined in this work. The generation time of the *pfkA* mutant, in rich medium, was 43.5% higher than the wild type (Fig. 5A). These results are in agreement with the growth reduction in complex medium observed in *L. lactis* mutants with a reduced phosphofructokinase activity when compared with the wild type strain [10]. Interestingly, *pts* and *pts pfkA* mutations caused a discrete increment in the generation time (19%) compared with the wild type strain. Despite mutations in *pfkA* and *pts*, all the strains were able to reach the same optical final density in Schaeffer's medium. These results suggest that all strains are ultimately able to obtain enough building blocks from the culture medium.

The effect of the *pfkA* inactivation was more evident in minimal media with glucose (2 g l^{-1}) than in the rich medium (Fig. 5). As can be seen in Table 2, the generation time of the *pfkA* mutant increased 45% in comparison with the wild type strain. However, at the end of the culture, the *pfkA* mutant reached an optical density very similar to that of the wild type strain. These results suggest that PfkA is needed for reaching maximal growth rates. It is possible that the absence of PfkA activity could result in the accumulation of upstream

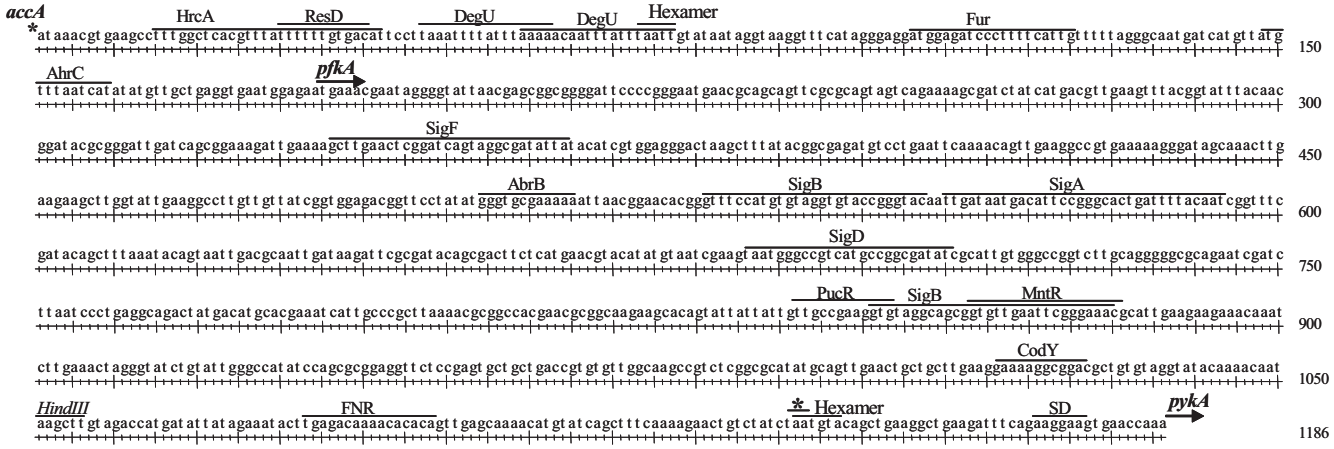

Figure 4. Location of theoretical transcription sites using DBTBS program. In intergenic region *AccA–pfkA*: HrcA, ResD, DegU, hexamer motif, Fur, and AhrC. In *pfkA* gene sequence: SigF, AbrB, SigB, SigA, SigD, PucR, SigB, MntR, CodY, FNR, hexamer motif, and cut site *HindIII*. In intergenic region *pfkA–pykA:* Shine Dalgarno (SD). *AccA*, acetyl CoA carboxylase; *pfkA,* phosphofructokinase; *pykA*, pyruvate kinase. The asterix indicates the end of the ORF sequence.

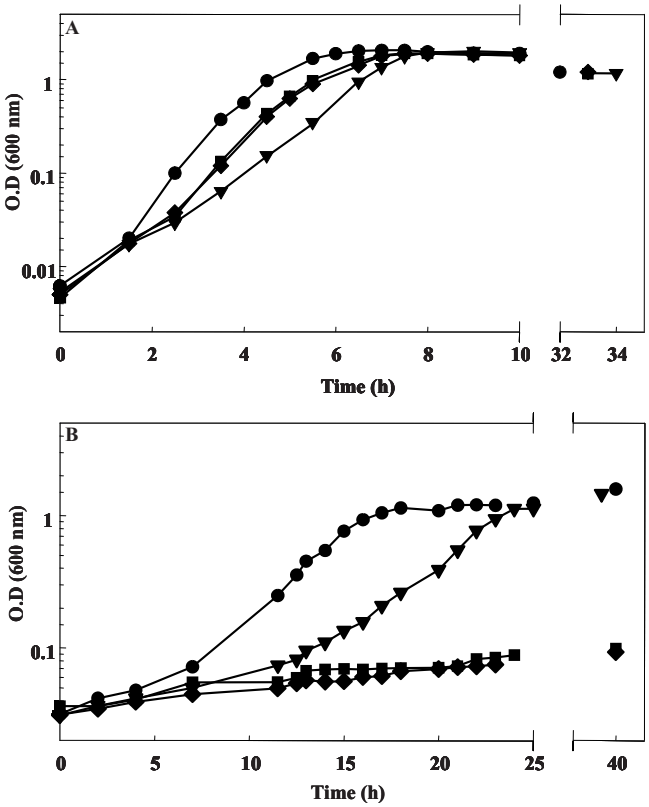

Figure 5. Growth kinetic behavior of the wild-type strain, *pfk*A and *pts* mutants. (A) In Schaeffer's sporulation medium and (B) Supplemented Spizizen's minimal medium (see text). $A_{600\,nm}$: ●, WT; ▼, *pfkA::sp;* ■, Δ*pts*; ♦, *pfkA::sp* Δ*pts.*

sugar-phosphates and redirection of glucose-6P to the pentose pathway to again enter the glycolytic pathway as glyceraldehyde-3-P (Fig. 1). Glycolytic intermediates, at least FBP, ATP and phosphate, control the activity and phosphorylation state of HPr in a complex manner. The HPr protein phosphorylates not only the EII for sugar transport but also catabolic enzymes and transcriptional regulators for modulation of their activities [9]. The ability of *pfkA* mutant to grow on glucose could also be explained by the existence of a particular isoenzyme or by a reaction bypassing the phosphofructokinase step. This behavior was observed in *Sacharomyces cerevisiae* mutants in one of the two *pfk* genes since they did not show any detectable Pfk activity *in vitro.* However, they still fermented glucose as the sole carbon source [29].

Contrary to the results obtained in this study for the PfkA knockout strain grown in minimal medium with glucose at 2 g l^{-1}, *pfkA* mutants obtained by mutagenesis with methanesulfonate or by transduction mediatedby bacteriophage PSB1 have been reported to be unable to grow in minimal media with glucose at 5 g l^{-1} [14–15]. Those results suggest that not only the glucose concentration, but also additional mutations that are reported to be present in some of those *pfkA* mutants, like *purB, sacA, metC* and possibly in other genes, could interfere with the metabolic and physiological effects related directly with PfkA in *B. subtilis*.

The *pts* and *pts pfkA* mutants showed residual growth on minimal media with glucose, which is in agreement with the observation that the *ptsH* mutants grow very slowly with glucose [4]. Our results support the possibility of an additional uptake system for glucose as was previously suggested by Gonzy-Tréboul *et al*. [30]. GlcP and GlcU glucose transporters are possible candidates for PTS-independent glucose uptake [31]. Additionally, it has been suggested that the intracellular glucose originating from non-PTS glucose transport or degradation of disaccharides is phosphorylated by glucose kinase [4].

In order to study the impact of the inactivation of *pfkA* and *pts* on sporulation, the percentage of sporulation was quantified in Schaeffer's media at different time points after the cultures reached the stationary phase. The process of sporulation is very complex, integrating several intra- and extracellular signals related to: nutrients supply, the metabolic state of the cell, cell density, and the bacterium's position in the cell cycle [32].

No spores were observed when the strains were grown on minimal media with glucose. These results were expected since it is already known that a rapidly metabolizable carbon source such as glucose suppresses

Table 2. Kinetic t_Ds and sporulation percentage.

Strain	t_D (min)[a]		% of sporulation[b]		
	SSM[b]	SMM[c]	T4	T8	T15
WT	37.2 (±1.62)	120 (±3.80)	0.07 (±0.08)	13.4 (±0.80)	32.4 (±0.78)
pfkA	53.4 (±1.68)	174 (±3.32)	0.19 (±0.01)	27.3 (±0.60)	30.1 (±0.57)
pts	43.1 (±1.40)	ND[d]	0.01 (±0.003)	19.0 (±0.66)	19.0 (±0.53)
pts pfkA	44.3 (±1.90)	ND[d]	1.60 (±0.06)	22.2 (±0.57)	51.3 (±0.45)

[a] Data presented as average of at least 3 independent experiments.
[b] SSM = Schaeffer's sporulation medium.
[c] SMM = Spizizen's minimal medium.
[d] ND = No determined.

the onset of sporulation until the carbon source is depleted [14]. However, interesting results were obtained on Schaeffer's sporulation medium. As shown in Table 2, at early time points, the *pfkA* mutant produced more spores than the wild type strain. However at T15, both strains reached the same sporulation percentage. Except for the wild type at T8 (13.4%), the *pts* mutant produced fewer spores at all the time points in comparison with the other strains. Interestingly, at T4, the *pts pfkA* double mutant produced around ten-fold more spores than the *pfkA* mutant, and almost 23-fold more spores than the wild type strain. However, at T15, the *pts pfkA* double mutant produced only around 1.7 fold more spores than the *pfkA* mutant and the wild type strain. These results suggest the importance of PfkA for normal sporulation of *B. subtilis* and that a deficiency of this enzyme signals the cells to start spore formation earlier, without changing the final percentage of sporulation. These results differ from those obtained with the *pfkA* mutant obtained by chemical mutagenesis since the mutant sporulated normally in a phosphate-buffered nutrient sporulation medium (NSMP) supplemented with methionine and tryptophan, and its sporulation was suppressed by glucose and other carbon sources [14]. In contrast, mutant cells lacking the Krebs cycle enzymes citrate synthase, aconitase and isocitrate dehydrogenase have been shown to be defective in the initiation of sporulation and in the expression of genes controlled by Spo0A~P [33].

Interestingly, the concentration of spores in the *pts pfkA* double mutant was even higher than the observed in the *pfkA* mutant, especially at T4. Since PTS plays a role in many different aspects of bacterial cellular physiology, the affectation of some of these functions in strains lacking PTS could explain the generation time increment in the *pts* mutants in Schaeffer's medium as well as the high concentration of spores observed in the *pts pfkA* mutant. These results are also in agreement with the decrease of acetate, observed in *pts pykA* mutant at T2 and T4 (Fig. 6d), since the decrease of acetate has been associated with the induction of TCA cycle enzymes that are needed for sporulation [34]. In fact, the ability of many microorganisms to produce a high concentration of spores in a short cultivation time has attracted commercial interest for the development of products such as a bio-insecticide [35].

Since pyruvate, lactate and acetate are some of the most important organic acids produced by *B. subtilis,*

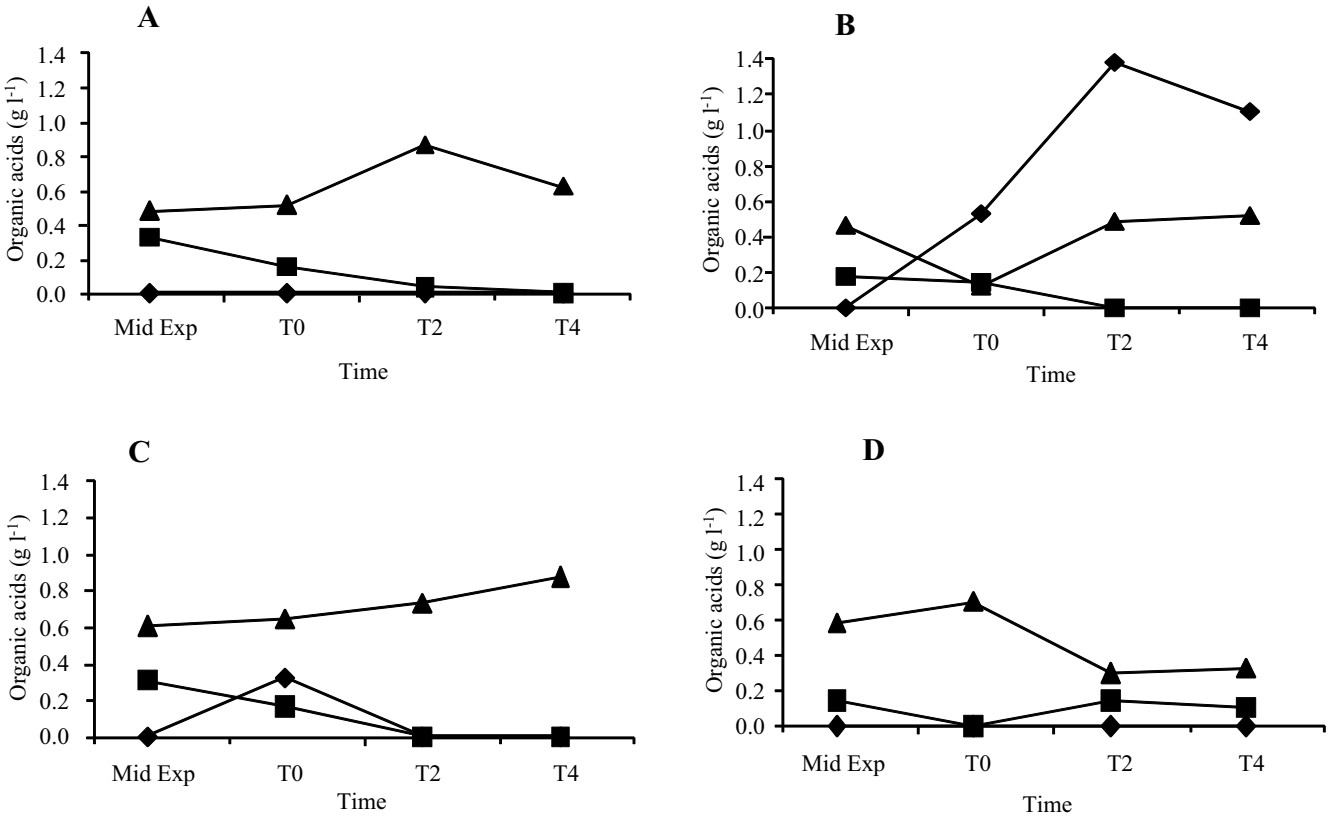

Figure 6. Growth kinetic behavior of the wild-type strain, *pfk*A and *pts* mutants. (A) In Schaeffer's sporulation medium and (B) Supplemented Spizizen's minimal medium (see text). $A_{600\,nm}$: ●, WT; ▼, *pfkA::sp;* ■, Δ*pts*; ♦, *pfkA::sp* Δ*pts.*

HPLC was used to determined the profile of formation of these carboxylic acids in PTS and PfkA mutants in Schaeffer's medium, at different times points of the growth curve (Fig. 6). A rich medium such as Schaeffer contains intermediates and precursors for the biosynthesis of amino acids and nucleotides that can enter central points of the glycolytic pathway and TCA cycle, like oxaloacetate, PEP or pyruvate (Fig. 1).

Interestingly, the concentration of pyruvate for wild type strain, and *pfkA* and *pts* mutants decreased throughout the time and was completely exhausted at T2–T4 (Fig. 6). However, *pts pfkA* double mutant showed similar values at all the times, except at T0. Pyruvate is a key compound for the TCA cycle, and can also be reduced to lactate and other compounds such as acetate, acetyl-CoA, ethanol, acetolactate, diacetyl, acetoin, and 2, 3-butanediol [6]. Lactate dehydrogenase catalyses the reduction of pyruvate to lactate, with the simultaneous oxidation of one molecule of NADH per molecule of pyruvate reduced [36, 37]. In this way, glycolysis continues, but lactate accumulates. HPLC analysis detected only a small amount of lactate in *pts* mutant at T0, whereas larger amounts were detected in *pfkA* mutant from T0 to T4. These results suggest an important participation of lactate dehydrogenase in *pfkA* mutants growing in Schaeffer's rich media. Lactate dehydrogenase is used to regenerate NAD+, a coenzyme of glyceraldehyde 3-P dehydrogenase (Fig. 1). Glyceraldehyde-3-P and fructose 6-P are the carbohydrates that participate in the convergence of the pentose pathway with glycolysis.

Acetate was the major fermentation product of the wild type strain and mutants, except for *pfkA* mutant that produced lactate as the principal organic acid (Fig. 6B). These results are in agreement with the literature that describes to acetate as one of the major by-products of carbon metabolism detectable during the growth of *B. subtilis* in rich media [38]. Contrary to the pyruvate profile, acetate showed similar values between times sampled except for the *pts* mutant, which showed an apparently progressive accumulation. These results suggest that in rich media, even in the absence of the PTS, acetate is produced as the principal by-product. Acetate can be produced by conversion of pyruvate to acetyl-CoA, and in a two-step reaction catalyzed by phosphotransacetylase (Pta) and acetate kinase (Ack). Because one ATP molecule is produced per molecule of acetate, this is the fermentation pathway most energetically efficient. Acetate synthesis allows an additional substrate-level phosphorylation without regenerating NADH; however, the pathways leading to lactate cannot be used for energy conservation. This observation is in agreement with the high levels of acetate produced by all the mutants in rich media and with the high lactate level produced by *pfkA* mutant, the strain that showed the highest generation time in this media.

Concluding remarks

The new *pfkA* mutant insertionally obtained in this study provided a relatively clean genetic background for physiological and metabolic studies in *B. subtilis*. Results showed that PfkA as well as PTS are essential to maintain the precise balance of metabolites required for differentiation and to reach maximal growth rates. The organic acids analysis provides insight into the energetic requirements of the cell when *pfkA* and *pts* mutants are grown in a rich medium. Since PEP is the phosphate donor for sugar phosphorylation in PTS, but also the allosteric inhibitor of key glycolytic enzymes like Pyk and Pfk, one interesting aspect to consider is that the absence of Pfk and/or PTS could change the levels of phosphorylated compounds in the cell, thereby affecting the PEP/PYR pool and the enzymes regulated by these metabolic intermediates. Studies presently in progress concerning the phenotype microarray and transcriptome analysis could help to clarify the role of PfkA in carbon metabolism and cell physiology.

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