The *Bacillus subtilis* ywjI (glpX) Gene Encodes a Class II Fructose-1,6-Bisphosphatase, Functionally Equivalent to the Class III Fbp Enzyme\(^7\)

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We present here experimental evidence that the *Bacillus subtilis* ywjI gene encodes a class II fructose-1,6-bisphosphatase, functionally equivalent to the *fbp*-encoded class III enzyme, and constitutes with the upstream gene, *murAB*, an operon transcribed at the same level under glycolytic or gluconeogenic conditions.

Under glycolytic growth conditions, unidirectional phosphor-ylation of fructose-6-phosphate to fructose-1,6-bisphosphate is catalyzed by the 6-phosphofructokinase (EC 2.7.1.11). Under gluconeogenic growth conditions, the opposite reaction is catalyzed by the fructose-1,6-bisphosphatase (FBPase) (EC 3.1.3.11) and is required for the synthesis of fructose-6-phosphate and derived metabolites, such as cell wall precursors. *Escherichia coli* possesses two FBPases: the class I FBPase, encoded by *fbp*, is highly similar to eukaryotic enzymes, and the class II FBPase (GlpX) (3) has homologues in nearly all prokaryotic genera but in only a few eukaryotes (a green alga, an amoeba, and a moss) and a few archaeal species (of the *Methanosarcina* genus). Biochemical, physiological, and genetic studies allowed the characterization of a *Bacillus subtilis* enzyme which defined a new class of bacterial FBPases (class III) not structurally related to those previously described and found mainly in *Firmicutes* (5–7). The gene encoding this activity was identified and, although structurally unrelated to the *E. coli* class I FBPase gene, was also named *fbp* (8). In *E. coli*, the major FBPase is the class I Fbp, whereas the class II GlpX seems to play a minor role (3). In other organisms, the major or even the only FBPase belongs to the class II GlpX family: *Bacillus cereus* possesses two *fbp*-like genes and no class I or class III FBPase-encoding gene (26); in *Mycobacterium tuberculosis*, FBPase activity is encoded only by a *glpX*-like gene, which has been shown to complement an *E. coli* mutant lacking such activity (18); in *Corynebacterium glutamicum*, the only FBPase, essential for growth on gluconeogenic carbon sources, belongs to class II (19). It has been shown that a *B. subtilis* *fbp* mutant was still able to grow on substrates such as d-fructose, glycerol, or l-malate as the sole carbon source, which indicated that this mutant could bypass the FBPase reaction during gluconeogenesis (6). Random mutagenesis (ethyl methanesulfonate treatment) performed with this *fbp* mutant enabled the definition of a *B. subtilis* locus (fbf) whose additional mutation prevented growth on gluconeogenic carbon sources, but this locus had not been characterized further (7). Determination of the nucleotide sequence of the whole *B. subtilis* chromosome (16) led to the identification of a putative gene, *ywjI*, encoding a protein displaying strong homologies with GlpX family members (e.g., 54% identity and 74% similarity with GlpX from *C. glutamicum*). This gene has therefore been annotated *glpX*, encoding a class II FBPase, but such annotation has never been validated by genetic or biochemical experimental evidence. In this work, we present experimental evidence that *ywjI* indeed encodes a class II FBPase.

Growth phenotype of *fbp* and/or *ywjI* mutant strains. *B. subtilis* strains GM2771 (*ΔywjI::spc*) and GM2772 (*Δfbp::cat*) have *ywjI* and *fbp* deleted, respectively. They were obtained by transformation of BSB168, a *trp*<sup>−</sup> derivative of the reference strain 168 Marburg (*trpC2*, with PCR products corresponding to the upstream and downstream regions of either gene to be deleted framing an antibiotic resistance cassette. The upstream and downstream regions were generated by PCR using BSB168 chromosomal DNA as the template and primers *ywjIUPST1* (5′-CGGACGTCTTGGTGGTAGCCGGACTG-3′) and *ywjIDOWN1* (5′-GGCATGAAAGGCCATAGTTGGTAC-3′) or *ywjIDOWN3* (5′-GGCGATGCAAAGTGTCCGATGGAAG-3′) and *ywjIDOWN4* (5′-CGGAATTCCTCTCTGTATGC GTAGAAAATGG-3′) for the *ywjI* deletion or primers *fbpUPST1* (5′-ACCGAATGAAAAGGCCATAGTTGG-3′) and *fbpUPST2* (5′-CGGAATTCCTCTATCTGTAATGACACCGC-3′) or *fbpDOWN3* (5′-TACACGCTGTATACAACTCTA-3′) and *fbpDOWN4* (5′-TCAGCTAGCGAGGATATATAGAATGGA-3′) for the *fbp* deletion. The upstream and downstream fragments for each gene were cut with suitable restriction enzymes and ligated either to an XbaI-SphI spectinomycin resistance cassette from plasmid pIC156 (22), for the *ywjI* deletion, or to an EcoRI-SphI chloramphenicol resistance cassette from plasmid pDG166 (11), for the *fbp* deletion. A second PCR was performed on the ligation mixtures with either the *ywjIUPST1/ywjIDOWN4* or the *fbpUPST1/fbpDOWN4* pair of primers to amplify the mutagenic fragments. The purified fragments were then used to directly transform BSB168, with selection on solid LB medium (12) for transformants resistant to the relevant antibiotic, spectinomycin (100 mg/liter) or chloramphenicol (5 mg/liter). Transformation of BSB168 by a mixture of chromosomal DNA from both GM2771 and GM2772 allowed selection of the double mutant GM2773 (*ΔywjI::spc Δfbp::cat*), resistant to both anti-
The correct structures of the ywjI and fbp chromosomal regions of each mutant were verified by PCR with the ywjI UPST1/ywjIDOWN4 and the fbpUPST1/fbpDOWN4 pairs of primers.

Growth phenotypes of these strains were tested in liquid M9 minimal medium (12) supplemented with different carbon sources: glucose, glucitol, and gluconate as carbon sources that do not require FBPase activity for the synthesis of fructose-6-phosphate and glycerol, malate, and a mixture of succinate and glutamate as carbon sources requiring FBPase activity. We also tested fructose, which is mainly transported and phosphorylated in fructose-1-phosphate via the FruA phosphotransferase system fructose-specific enzyme IIABC to enter glycolysis, after phosphorylation by the FruK fructose-1-phosphate kinase, as fructose-1,6-bisphosphate (9). Therefore, with respect to the synthesis of fructose-6-phosphate, most of the fructose represents a “gluconeogenic” substrate. However, B. subtilis possesses a second minor fructose phosphotransferase system, encoded by the levDEFG operon (17), by which fructose is transported and phosphorylated in fructose-6-phosphate. Thus, a minor fraction of the fructose can feed directly into the upper part of glycolysis and can therefore be used for anabolic reactions, without the requirement of FBPase activity. The growth of either GM2771 or GM2772 (ywjI or fbp single mutant, respectively) was as efficient as that of the BSB168 wild-type strain under all conditions tested, whereas the GM2773 ywjI fbp double mutant was unable to grow with carbon sources demanding FBPase activity (Fig. 1). Thus, ywjI appears to be required for fbp bypass during gluconeogenesis, which strongly suggests that it might correspond to the previously identified bflD locus. This hypothesis was strengthened by determining the nucleotide sequence of the ywjI region of the bflD-I mutant strain YF062 (bflD-I fdp-74 glp hisA1 leuA8 metB5 trpC2) (7), which revealed a C-to-T transition of the first base of codon 296 of ywjI, leading in the corresponding protein to replacement by a cysteine residue of an arginine residue extremely conserved in all GlpX family members.

GM2773 still grew with fructose as the sole carbon source, although at a much lower growth rate, which could be explained by the low level of fructose-6-phosphate generated by the minor LevDEFG transport system (Fig. 1). Indeed, transferring both ΔywjI:spc and Δfbp:cat deletions into the nonsense levE mutant OB166 [trpC2 sacL5 (levE)] (17) led to a strain unable to grow with fructose as a unique carbon source (data not shown).

These results and the homology of ywjI with glpX family genes made us consider that ywjI encodes a class II FBPase, functionally equivalent to the fbp-encoded class III FBPase. The role of YwjI in B. subtilis appears rather different than that of the corresponding enzyme (GlpX) in E. coli, as deletion of the class I FBPase-encoding gene fbp was sufficient to prevent growth of this bacterium on a gluconeogenic carbon source. However, in E. coli, glpX could compensate for inactivation of fbp when overexpressed from a multicopy plasmid (3). Thus, GlpX, whose expression in E. coli is induced by glycerol or glycerol-3-phosphate (25), would play a specific role under particular conditions rather than being a general FBPase active under every gluconeogenic condition.

**FBPase activity of YwjI.** Assays to reveal the FBPase activity of YwjI were performed with crude extracts from the fbp and/or ywjI mutant strain cultivated in liquid LB medium. Extracts were prepared from cultures in mid-log growth phase (optical density at 600 nm of ~1.5) by cell breakage using glass microbeads (diameter of 0.4 to 0.6 mm; Braun Biotech Inter-
nation) in a FastPrep FP120 instrument (Bio101), followed by centrifugation to remove beads and cell debris. FBPase activity in the supernatant was assayed by measuring the production of fructose-6-phosphate in a coupled spectrophotometric assay, using conditions described previously for other members of the class II FBPase family (3, 10): the fructose-6-phosphate produced was converted to gluconate-6-phosphate by sequential reactions of phosphoglucoisomerase and glucose-6-phosphate dehydrogenase added to the reaction mixture, and stoichiometric NADPH formation in the latter reaction was followed by measuring the increase of the absorbance at 340 nm. We could detect weak FBPase activities for both GM2771 (ywJI mutant) and GM2772 (fbp mutant) but not for the ywJI fbp double mutant GM2773 (Table 1), which led us to conclude that YwjI indeed possesses FBPase activity. In addition, by use of GM2772 extract, a $K_m$ value for YwjI of about 20 $\mu$M for fructose-1,6-bisphosphate was determined. This value falls within the range of $K_m$ values estimated for other class II FBPases: 14 $\mu$M, 12 to 17 $\mu$M, and 35 $\mu$M for GlpX from C. glutamicum, M. tuberculosis, and E. coli, respectively (3, 18, 19).

Fujita and Freese determined that the B. subtilis Fbp FBPase was fully activated by phosphoenolpyruvate (PEP) at concentrations above 0.1 mM (5). Regarding GlpX FBPase family members, PEP (1 mM) stimulated the activity of the E. coli enzyme by 1.7-fold (3), whereas it neither activated nor inhibited that of the M. tuberculosis enzyme (18). On the contrary, the C. glutamicum GlpX enzyme was inhibited by PEP (50% inhibition with 0.36 mM PEP) (19). This prompted us to test the effect of PEP on the B. subtilis enzyme. We used the same conditions described above, except that both the crude extract buffer and the assay buffer contained in addition 1 mM PEP. As expected, we observed activation (about 30-fold) of FBPase activity in the GM2771 (ywJI) extract due to the activation of Fbp. Conversely, the presence of 1 mM PEP completely abolished any detectable activity in the GM2772 (Δfbp) extract (Table 1), revealing an inhibitory effect of PEP on the YwjI activity under the conditions of the assay. Still, the FBPase specific activity in the GM2772 extract assayed in the absence of PEP was low compared to that of the fully PEP-stimulated Fbp. Nevertheless, there was no difference in growth between this strain and the ywJI mutant GM2771 or the wild-type BSB168 under any condition tested. This could signify either that such a low activity was sufficient for efficient growth under these conditions or that the assay conditions we used, suitable for other members of the GlpX family, are not optimal for the

Table 1. FBPase specific activities$^a$ in single or double mutant crude extracts

<table>
<thead>
<tr>
<th>Strain (genotype)</th>
<th>Sp act (Without PEP)</th>
<th>Sp act (With PEP (1 mM))</th>
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<tbody>
<tr>
<td>GM2771 (ΔywJI:spc)</td>
<td>4.2 ± 0.5</td>
<td>140.3 ± 25.5</td>
</tr>
<tr>
<td>GM2772 (Δfbp:cat)</td>
<td>3.1 ± 0.7</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>GM2773 (ΔywJI:spc Δfbp:cat)</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

$^a$ Specific activities are expressed as nanomoles of NADPH formed per minute and per milligram of protein. Initial rates of NADPH formation were determined at 25°C from the increase in absorbance at 340 nm, using the molar extinction coefficient of 6,220 M$^{-1}$ cm$^{-1}$. Protein concentration in extracts was determined by the method of Bradford (1), using bovine serum albumin as a standard.

In conclusion, we have established that the B. subtilis ywjI enzyme. The latter possibility would also explain the paradox between the efficient growth of GM2772 (Δfbp) under gluconeogenic conditions and the complete inhibition of YwjI FBPase activity by 1 mM PEP, a concentration measured in B. subtilis cells grown on malate (5). Thus, the PEP concentration threshold for the full inhibition of YwjI would be higher in vivo.

ywJI constitutes an operon with the upstream gene murAB.

The B. cereus ywjI homologue has been considered a part of a putative murAB-ywjI operon (26). In B. subtilis, ywjI is down-stream of murAB, a murAB equivalent, which codes for a UDP-N-acetylglucosamine-1-carboxyvinyltransferase (EC 2.5.1.17) involved in cell wall formation. Contrary to its murAB parologue, murAB is not essential in B. subtilis (14, 15), and alleles of this gene have been shown to confer a thermostressive lysis phenotype, to suppress some of the diverse effects of spo0 mutations, or to suppress the sporulation defect caused by certain ribosomal mutations conferring erythromycin resistance (21, 24). The very short (30-bp) murAB-ywjI intergenic region does not contain any obvious transcription terminator or promoter sequences. In addition, the transcription of the fbaA-ywjIH operon has been shown to terminate upstream of murAB (24), and the rho gene, downstream of ywjI, is not cotranscribed with ywjI (13). We examined the possibility that murAB and ywjI constitute an operon by performing a reverse transcriptase PCR experiment as follows. RNA was extracted from strain BSB168 grown in liquid LB medium as previously described (4), and after a DNase treatment (Turbo DNA-free kit; Ambion), a reverse transcription followed by a PCR was performed (Illuma Ready-To-Go RT-PCR bead kit; GE Healthcare) using primers ywjIUPST1 (forward primer, inside murAB) and ywjIUPST2 (reverse primer, inside ywjI). This generated a 409-bp fragment (but no amplification when the reverse transcriptase had been heat inactivated prior to the reverse-transcription step), which clearly revealed the existence of a cotranscript of both genes.

Regulation of expression of the murAB-ywjI operon.

We investigated the potential transcriptional regulation of the murAB-ywjI operon. For this, two strains carrying reporter transcriptional fusions were constructed by single-crossover integration of plasmid pBaSysBio1murmurAB or pBaSysBioIywJl, which placed the gfpmut3 gene (2) directly under the control of the murAB-ywjI promoter or downstream from ywjI, respectively. These strains were cultivated as described above, with glucose, fructose, glycerol, or malate as a unique carbon source, and transcription was estimated by monitoring the fluorescence along the growth (excitation wavelength of 485 nm, emission recorded at 528 nm). For both strains, similar levels of expression were observed with glucose or malate as a carbon source, whereas a somewhat higher level of expression (1.2- to 1.4-fold increase) was observed with fructose or glycerol (Table 2), suggesting that no murAB-ywjI transcriptional regulation occurred due to glycolytic or gluconeogenic conditions. This confirmed a tiling-array analysis performed with strains grown on glucose or malate as a unique carbon source, which revealed only bicistronic murAB-ywjI transcripts and the same level of transcription under both conditions (BaSysBio consortium, unpublished data).

In conclusion, we have established that the B. subtilis ywjI gene encodes a class II FBPase (GlpX family). Although we
observed inhibition of activity of this FBPase by PEP, this enzyme can functionally substitute for the Fbp class III FBPase. We have shown that ywjI constitutes with the upstream gene murAB an operon whose transcription does not depend on the glycolytic or neoglycocogenic condition. Thus, like fbp, and contrary to the two other genes essential for gluconeogenesis, gapB and pckA, ywjI (glpX) is not regulated by CcpN (20, 23).

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Table 2. Expression of murAB- and ywjI-gfpmut3 fusions under different growth conditions

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Reporter activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>murAB-gfpmut3</td>
</tr>
<tr>
<td>Glucose</td>
<td>100 ± 22</td>
</tr>
<tr>
<td>Malate</td>
<td>158 ± 23</td>
</tr>
<tr>
<td>Fructose</td>
<td>135 ± 15</td>
</tr>
<tr>
<td>Glycerol</td>
<td>111 ± 9</td>
</tr>
</tbody>
</table>

* Expressed as relative fluorescence arbitrary units/optical density at 600 nm of culture, corrected for background (strain BSBI68 without gfpmut3) and normalized to expression in M9 glucose.

REFERENCES


