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Existence of a True Phosphofructokinase in *Bacillus sphaericus*: Cloning and Sequencing of the *pfk* Gene

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Some strains of *Bacillus sphaericus* are entomopathogenic to mosquito larvae, which transmit diseases, such as filariasis and malaria, affecting millions of people worldwide. This species is unable to use hexoses and pentoses as unique carbon sources, which was proposed to be due to the lack of glycolytic enzymes, such as 6-phosphofructokinase (PFK). In this study, PFK activity was detected and the *pfk* gene was cloned and sequenced. Furthermore, this gene was shown to be present in strains belonging to all the homology groups of this heterogeneous species, in which PFK activity was also detected. A careful sequence analysis revealed the conservation of different catalytic and regulatory residues, as well as the enzyme's phylogenetic affiliation with the family of allosteric ATP-PFK enzymes.

Bacillus sphaericus strains are strictly aerobic and mesophilic bacilli which form spherical endospores (15). On the basis of DNA homology, this species has been divided in five homology groups (11, 17). In turn, group II was divided in two subgroups, IIA and IIB (11). Some strains in group IIA are biotechnologically very interesting, because they can produce insecticidal proteins which are active against mosquito larvae, in particular against various species of the genera *Culex* and *Anopheles*, which are known to be regular transmission vectors of filariasis and malaria (3, 4, 13, 14, 16).

These bacteria can metabolize a wide variety of organic compounds and amino acids (1), but they are unable to use hexoses and pentoses as unique carbon sources (18). In fact, these metabolic limitations hamper likely industrial developments with this species due to the high costs of the proteinaceous media used for toxin production compared to the costs of alternative media based on starch or molasses (5, 18, 26). Consequently, studies disclosing the true metabolic potential of *B. sphaericus* are essential for genetic-improvement programs.

The inability to metabolize carbohydrates in this species has been attributed to the absence of key enzyme activities in both the Embden-Meyerhof-Parnas pathway (phosphoglucoisomerase [EC 5.3.1.9], 6-phosphofructokinase [PFK] [EC 2.7.1.11]) and the Entner-Doudoroff pathway (phosphogluconate dehydratase [EC 4.2.1.12] and phospho-2-keto-3-deoxygluconate [EC 4.1.2.14]) (18). Furthermore, Russell et al. did not detect either glucose or sucrose transport (18). However, the observation that *B. sphaericus* 2362 could use *N*-acetylglucosamine as a carbon source (A. Alice, unpublished results) indicated that some metabolic enzymes had to be present in this microorganism.

PFK activity in *B. sphaericus*. *B. sphaericus* and *Bacillus subtilis* strains were grown aerobically in Luria-Bertani (LB) medium at 30 and 37°C, respectively. At mid-log phase (optical density at 600 nm, 0.6 to 0.8), cells were harvested by centrifugation, washed twice with TDP buffer (20 mM Tris-HCl [pH 7.5], 1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride), and resuspended in the same buffer at 4°C. Crude extracts were prepared by disrupting cells twice by 20-s sonication in the presence of glass beads and maintained refrigerated in an acetone-ice bath. Samples were centrifuged in a microcentrifuge at 17,400 × *g* for 15 min, and the supernatants were used for the enzymatic assays. Reactions were performed in a 500-μl final volume containing 50 mM Tris-HCl (pH 7.5), 5 mM Cl_2Mg, 2 U each of aldolase (Sigma Chemical Co.) and triose phosphate isomerase–glyceraldehyde-3-phosphate dehydrogenase (Sigma Chemical Co.), 1 mM fructose-6-phosphate (F-6-P) (Sigma Chemical Co.), 0.15 mM NADH, 0.5 mM ATP, and 10 to 50 μl of cell extract. NADH oxidation was determined by measuring the absorbance variation at 340 nm. Specific activity is expressed in nanomoles per minute per milligram of total protein. Protein concentrations were determined with a protein assay kit (Bio-Rad) with bovine serum albumin as the standard. PFK activity could be detected in all strains tested, and with the exception of some strains, they showed values similar to those obtained with *B. subtilis* 168 (Table 1). The detected activities were absolutely dependent on the presence of both ATP and F-6-P in the reaction mixture. Moreover, no ATP-PFK activity was detected when crude extracts were omitted from these mixtures, indicating that the commercial enzymes and reagents used in the assays were not contaminated with an ATP-PFK activity. The affinity constants (K_m), calculated from crude extracts, of ATP-PFK for ATP and F-6-P were estimated to be 0.11 and 0.29 mM, respectively, and the maximum rates (V_{max}) of metabolism were 28.5 nmol min^{-1} for ATP and 25.9 nmol min^{-1} for F-6-P.

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TABLE 1. Detection of PFK activity in *B. sphaericus*

Strain	DNA homology group	Sp act (nmol min^{-1} mg of $protein^{-1}$)[a]
B. sphaericus strain		
ATCC 14577	I	19.16 ± 1.14
2362	II A	23.08 ± 1.06
1593	II A	6.61 ± 3.9
2297	II A	12.55 ± 0.63
ATCC 7055	II B	20.05 ± 0.35
NRS592	III	6.82 ± 0.31
NRS400	IV	11.9 ± 1.54
NRS1198	V	25.88 ± 3.79
B. subtilis strain 168		27.7 ± 0.66

[a] The results are the means of results of three distinct experiments ± the standard deviations, except for strains 2362 (for which the results are the means of results of eight experiments) and 168 (for which the results are the means of results of five experiments).

PCR amplification and sequencing of the *pfk* gene of *B. sphaericus* 2362. In order to ratify the previous finding, two degenerated primers, PFK1 (5′-TCCCMNGGNATGAAYG C-3′) and PFK2 (5′-CCNCGYTGDAYRTGNCC-3′), were designed from the alignment of different microbial ATP-PFK enzymes. Total DNA from *B. sphaericus* 2362 prepared as described previously (6) was subjected to PCR amplification. The reactions were performed in a 50-μl final volume containing the following reagents: 20 to 100 ng of DNA template, 1× buffer (Life Technologies), 1 U of *Taq* polymerase, 100 μM concentrations of each deoxynucleoside triphosphate, and a 1 μM concentration of each primer. Control reactions were performed with the DNA of *B. subtilis* 168. The temperature profile was as follows: initial denaturation at 94°C for 5 min followed by 30 cycles of 94°C for 30 s, 51°C for 60 s, and 72°C for 90 s and then an additional extension step of 72°C for 5 min.

A fragment of the expected size (720 bp) was amplified and sequenced with an ABI PRISM dRhodamine Terminator Cycle Sequencing Ready Reaction kit with AmpliTaq DNA polymerase and an automatic ABI 310 DNA sequencer (Applied Biosystems). Analysis of the translated sequence showed great similarity to ATP-dependent PFK (ATP-PFK) enzymes of *B. subtilis*, *Bacillus stearothermophilus*, and *Paenibacillus macquariensis*. A 658-bp fragment upstream of the 5′ end of the amplicon and a 1,542-bp fragment downstream of the 3′ end could also be amplified by reverse PCR. At least two independent clones were sequenced. Sequence analysis of all the fragments was performed with the BLAST server of the National Center for Biotechnology Information at the National Institutes of Health, Bethesda, Md. (http://www.ncbi.nlm.nih.gov). This analysis showed three ORFs (Fig. 1). The central ORF (962 bp) showed high identity to ATP-PFK amino acid sequences of *B. subtilis* (68% identical residues), *B. stearothermophilus* (67% identical residues), and *P. macquariensis* (52% identical residues), and as consequence, it was named *pfk*. The deduced amino acid sequence corresponded to a protein of 319 amino acids with a predicted molecular mass of 33,948 Da. A putative ribosome binding site (AGGAGG) was detected 7 bp upstream of the start codon, a distance that has been reported to be optimal for efficient translation in *B. subtilis* and *Escherichia coli* (24). Also, a putative promoter region was identified upstream of this gene with potential −10 and −35 regions similar to those described for σ^A in *B. subtilis* (9) and a putative −16 region was identified (25).

Upstream from *pfk*, we found an incomplete ORF (*orf1*, 312 bp) with an intergenic region of 311 bp which encodes a protein with similarity to the acetyl coenzyme A carboxylase carboxyltransferase alpha subunit (AccA) protein of *B. subtilis*. Moreover, downstream of the *pfk* gene there was another ORF (*orf3*) possibly encoding a protein with high identity to pyruvate kinase of *Bacillus psychrophilus* and *B. subtilis*. This gene organization (*accA pfk pykA*) is similar to that observed in *B. subtilis* and *Bacillus halodurans* (12, 22).

In order to determine if this cloned gene was transcribed, a reverse transcription-PCR (RT-PCR) assay was performed on

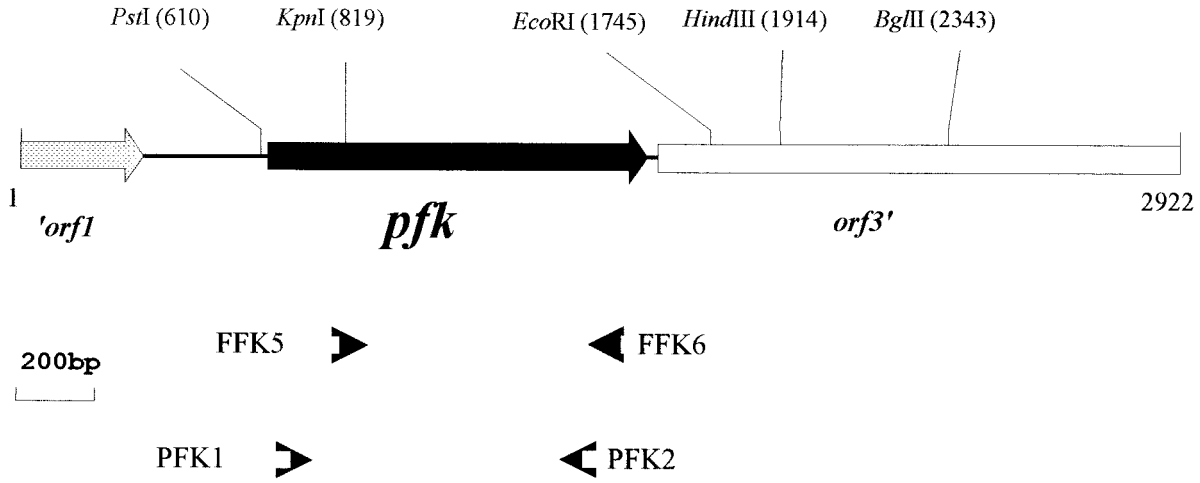

FIG. 1. Organization of the *B. sphaericus* 2362 genomic region containing the *pfk* gene. *orf1* is similar to the *B. subtilis accA* gene, and *orf3* resembles the *B. subtilis pykA* gene (see the text). Black arrows indicate the positions and directions of primers used both in the RT-PCR assays (FFK5 and FFK6) and in the synthesis of the probe used in the Southern blot analysis (PFK1 and PFK2). Relevant restriction sites and their positions in the sequence are also indicated.

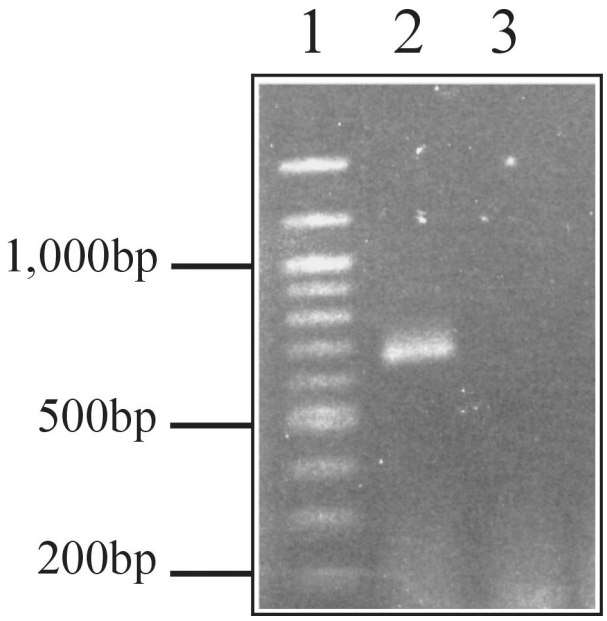

FIG. 2. RT-PCR of the *pfk* gene. RNA was extracted from *B. sphaericus* 2362, and the RT-PCRs were performed as described in the text. Lane 1, 100-bp DNA ladder; lane 2, RT-PCR with RNA from 2362; lane 3, RT-PCR without reverse transcription enzyme in the RT reaction.

B. sphaericus 2362 cells grown in LB medium (optical density at 600 nm, 0.6 to 0.8). Total RNA was isolated by using Trizol (Life Technologies) heated at 70°C and treated with RNase-free DNase (Promega) to eliminate contaminating DNA. For RT reactions, 1 to 5 μg of total RNA was used for the synthesis of the first-strand cDNA with Moloney murine leukemia virus reverse transcriptase enzyme (Ambion) and the primer FFK6 (5′-TAAGTCGACATTAGTAACTCCACTGC-3′). The RT reactions (20-μl mixtures) were carried out according to the manufacturer's recommendations. Then, 5 μl of each RT reaction product mixture was used as the template for PCR amplifications. These amplifications were carried out as described above with an annealing temperature of 60°C and with the primers FFK5 (5′-CACAAGCTTATTCAGCGTGGTGG-3′) and FFK6. A fragment with the expected size (617 bp) was detected with primers FFK5 and FFK6, while PCR products were not found in control reactions when the RT reaction was carried out without reverse transcriptase enzyme (Fig. 2).

Analysis of the deduced protein sequence. The sequence of the PFK from *B. sphaericus* was aligned and compared with sequences of other bacterial species. Multiple alignments were performed with CLUSTAL X software (23) (Fig. 3). Ten out of the 11 amino acids that constitute the substrate-binding domain in the *E. coli* enzyme (21) are conserved in *B. sphaericus* (Fig. 3). Curiously, the R162 residue, which binds F-6-P and is important for the inhibition of the *B. stearothermophilus* ATP-PFK by phosphoenolpyruvate (PEP) (20), is changed to N162 in *B. sphaericus* PFK. The role of this particular site in allosteric inhibition was recently studied, with a finding that an R162A mutation decreased by 30-fold ATP-PFK's affinity for F-6-P but that it diminished the effectiveness of PEP inhibition by only one-third (10). Further biochemical work is required to determine the incidence of the R162N change in *B. sphaericus* PFK and its effects on both ATP-PFK's affinity for F-6-P and PEP inhibition.

Another 10 residues are implicated in the ATP binding of *E. coli* ATP-PFK (21). In the deduced sequence of *B. sphaericus* ATP-PFK, this domain is also highly conserved, with eight identical amino acids, a conservative substitution (R77 to K77), and a nonconservative substitution (F73 to C73); however, these two differences were also present in the very well-characterized ATP-PFK from *B. stearothermophilus*. Also, residue D187, important for PEP inhibition and ADP or GDP activation of the enzyme (2), was conserved in *B. sphaericus*.

Phylogenetic analysis of the evolutionary relationships between these proteins was realized with the programs supplied in the PHYLIP 3.5c package (8). This method showed that this enzyme clustered convincingly with other enzymes of the ATP-PFK family (data not shown).

The presence of these conserved residues and the similarity to other ATP-PFK enzymes suggests that PFK of *B. sphaericus* 2362 is also an allosteric enzyme.

Complementation of the *E. coli pfk* mutant. In order to confirm that the cloned gene encodes a functional ATP-PFK enzyme, a DNA fragment amplified with the primers PFKN (5′-TCGGGATCCAAAAAAATTGCTG-3′ containing a *Bam*HI site [underlined]) and PFKC (5′-CATAAGCTT TTATATTGATAGTTC-3′, which contains a *Hin*dIII site [underlined]) and carrying the complete *pfk* gene from *B. sphaericus* 2362 was cloned into the expression vector pQE30 (QIAGEN), which had been digested with *Bam*HI-*Hin*dIII. The recombinant plasmid, called pALE-232, was used to transform *E. coli* DF1020 [*pro-82 glnV44*(AS) λ-Δ*pfkB201 recA56 endA1* Δ(*rhaD-pfkA*)*200 thi-1 hsdR17*; *E. coli* Genetic Stock Center], which has no ATP-PFK activity (7). This *E. coli* strain does not grow on mannitol as the carbon source. This deficiency was restored only when the pALE-232 plasmid was introduced and not with the pQE30 control vector. When ATP-PFK activities from these same strains cultivated in the presence of IPTG (isopropyl-β-D-thiogalactopyranoside) were compared, *E. coli* DF1020 cells carrying the *B. sphaericus* 2362 *pkf* gene showed an ATP-PFK activity of 27 nmol min^{-1} mg of $protein^{-1}$ while *E. coli* cells harboring pQE30 showed less than 1 nmol min^{-1} mg of $protein^{-1}$. These results proved that the cloned gene indeed encodes an ATP-PFK enzyme from *B. sphaericus*.

***pfk* genes in other strains of *B. sphaericus*.** In order to test whether the presence of the *pfk* gene in strain 2362 was a particular feature of this strain or strains in the IIA homology group, we performed a Southern blot experiment using an internal fragment of the *pfk* gene as the probe against DNA from different strains representing each of the homology groups described in the *B. sphaericus* taxon. DNA of *B. sphaericus* strains was digested with *Eco*RI and analyzed by electrophoresis on 0.8% horizontal agarose slab gels. Southern blot analysis was performed by standard procedures (19), and after being blotted, membranes were hybridized with a fragment of the *pfk* gene, amplified by PCR with the primers PFK1 and PFK2. This probe was labeled with a Bioprime DNA labeling system (Life Technologies). The detection was carried out with a chemiluminescence Phototope-Star CDP-STAR (New England BioLabs) system as described by the manufacturer.

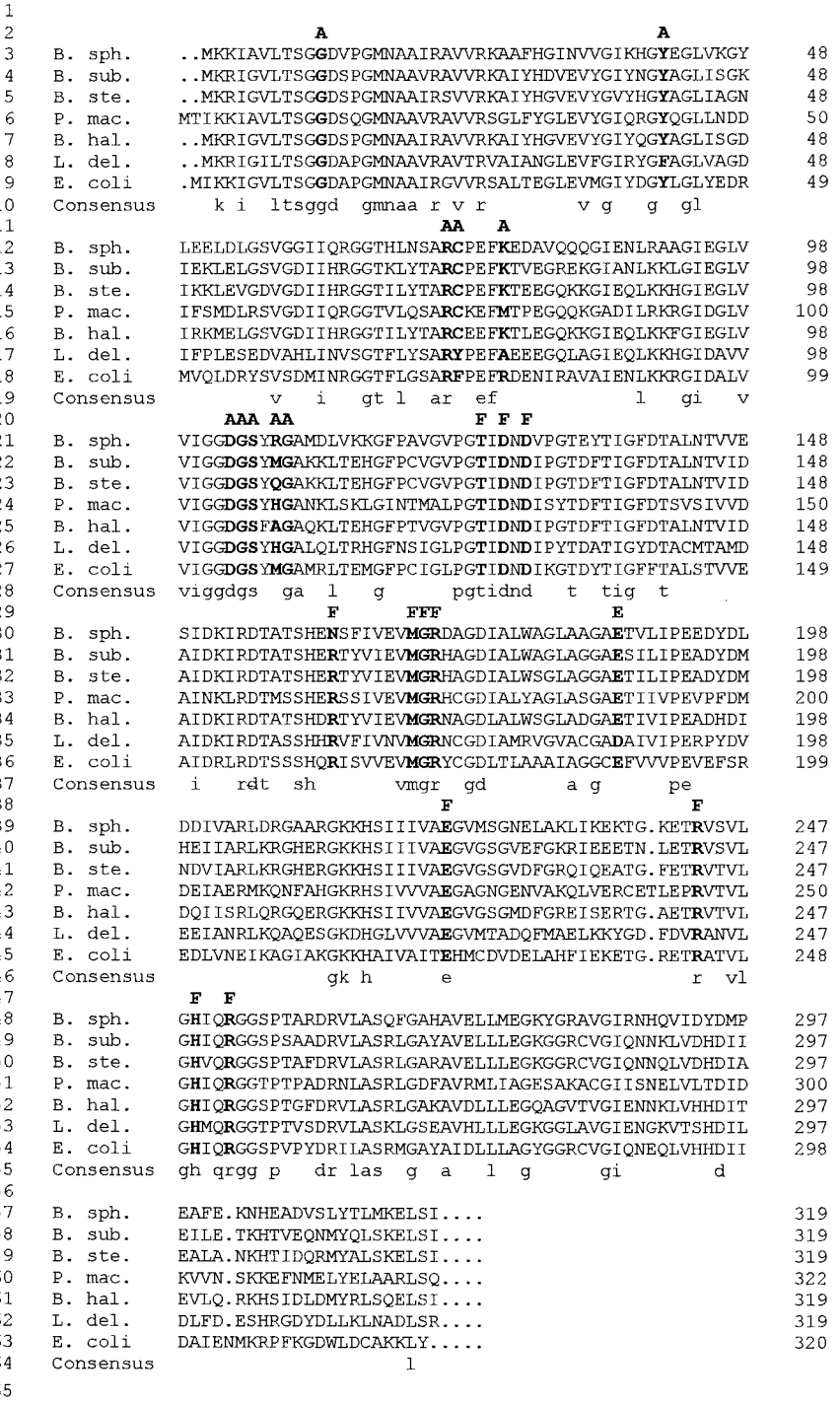

FIG. 3. Alignment of the PFK amino acid sequences from different microorganisms. The consensus sequence is given. The numbers on the right indicate the residue positions in the sequences. A, F, and E refer to residues implicated in the binding of ATP, F-6-P, and PEP, respectively, for *E. coli* ATP-PFK and are indicated in bold. B. sph., *B. sphaericus* (accession number AY034597); B. sub., *B. subtilis* (O34529); B. ste., *B. stearothermophilus* (P00512); P. mac., *P. macquariensis* (Q59214); B. hal., *B. halodurans* (BAB06883); L. del., *Lactobacillus delbrueckii* subsp. *bulgaricus* (P80019); E. coli, *E. coli* (AAC76898).

Figure 4 shows that a clear signal was detected in all strains, indicating that the *pfk* gene (or a very similar one) is present in all DNA homology groups.

Conclusions. Although *B. sphaericus* had a well-known inability to use sugars as unique carbon sources, results obtained in this study provide the first evidence that one of the enzymes of the upper half of the glycolytic pathway is present in *B. sphaericus*. Conservation of the catalytic and regulatory domains of the protein strongly supports its functionality in glycolysis or gluconeogenesis. Complementary studies indicated

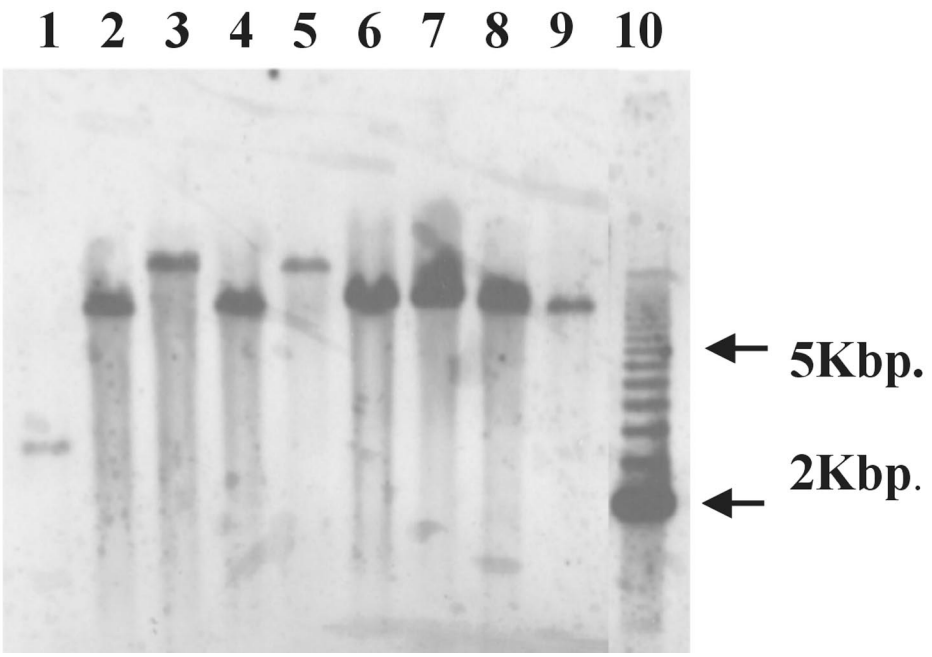

FIG. 4. Southern blot of chromosomal DNAs from different strains of *B. sphaericus* with a *pfk* gene probe. Chromosomal DNAs from different strains were digested with *Eco*RI. Lane 1, ATCC 14577 (I); lane 2, ATCC 7055 (IIB); lane 3, NRS 592 (III); lane 4, NRS 400 (IV); lane 5, NRS 1198 (V); lane 6, 2362 (IIA); lane 7, 1593 (IIA); lane 8, ASB13052 (IIA); lane 9, 2297 (IIA); lane 10, DNA ladder. The positions of certain marker bands are indicated as references.

the presence of a PEP phosphotransferase system in this bacterium (A. F. Alice and C. Sanchez-Rivas, Abstracts of the 9th International Conference on Bacilli, abstr. 107, 1997), and there is also evidence of the existence of an *N*-acetylglucosamine-specific PEP phosphotransferase system in *B. sphaericus* (A. Alice, unpublished results), suggesting that *B. sphaericus* may in fact be able to translocate and catabolize certain sugars like *N*-acetylglucosamine, most probably through glycolysis.

This study may establish the foundations for other studies of the metabolism of this important mosquito pathogen, and this knowledge might eventually be used to find better carbon sources for the industrial production of toxins.

Nucleotide sequence accession number. The nucleotide sequence presented in this work was entered in the GenBank nucleotide database under the accession number AY034597.

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