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Analysis of the Function of a Putative 2,3-Diphosphoglyceric Acid-Dependent Phosphoglycerate Mutase from *Bacillus subtilis*

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A *Bacillus subtilis* gene termed *yhfR* encodes the only *B. subtilis* protein with significant sequence similarity to 2,3-diphosphoglycerate-dependent phosphoglycerate mutases (dPGM). This gene is expressed at a low level during growth and sporulation, but deletion of *yhfR* had no effect on growth, sporulation, or spore germination and outgrowth. YhfR was expressed in and partially purified from *Escherichia coli* but had little if any PGM activity and gave no detectable PGM activity in *B. subtilis*. These data indicate that *B. subtilis* does not require YhfR and most likely does not require a dPGM.

Phosphoglycerate mutase (PGM) catalyzes the interconversion of 3-phosphoglyceric acid (3PGA) and 2PGA in both glycolysis and gluconeogenesis. Two types of PGM have been identified; one is dependent on 2,3-diphosphoglycerate (DPG) for activity (dPGM), and the other is not (iPGM) (5, 6). These two types of PGM differ strikingly in their structures, mechanisms, and amino acid sequences (3, 5, 6, 8, 9, 10). Some organisms appear to contain only a single type of PGM, while others contain both types of PGM (2, 5, 6, 7). Among the latter is *Escherichia coli*, which contains genes for both an iPGM and a dPGM; both genes are expressed at somewhat similar levels, and both give functional enzymes (7).

In *Bacillus subtilis* the great majority (≥90%) of PGM activity is due to an iPGM (20, 26), and mutation of the coding gene (termed *pgm*) has very severe effects on cell growth, especially in the presence of glucose (13). Although this iPGM appears to be the major PGM in *B. subtilis*, determination of the complete sequence of the *B. subtilis* genome revealed only a single gene, termed *yhfR*, that codes for a protein with significant sequence similarity to dPGMs (11), (see below). This raised the possibility that, like *E. coli*, *B. subtilis* also might contain two types of PGM under some conditions.

In order to probe the possible function of *yhfR* in *B. subtilis*, we first determined if this gene was expressed at any significant level by construction and analysis of the expression of translational *yhfR-lacZ* fusions. A fragment from 191 bp upstream of to 28 bp into the *yhfR* coding sequence was amplified by PCR; the primers used contained extra residues with either *Bam*HI or *Eco*RI sites at their 5′ ends. The 226-bp PCR product was cut with *Bam*HI and *Eco*RI and cloned between these sites in plasmid pJF751, a vector for construction of translational *lacZ* fusions (4), giving plasmid pPS3083. This plasmid was sequenced to confirm the expected DNA sequence in the *yhfR-lacZ* region and then used to transform our wild-type *B. subtilis* 168 strain (PS832) to chloramphenicol resistance (Cm^r) by integration at the *yhfR* locus through a single-crossover event. Southern blot analysis confirmed that the resultant strain (PS3113) contained a single copy of the *yhfR-lacZ* fusion at *yhfR*. To insert the translational *yhfR-lacZ* fusion at the *amyE* locus, plasmid pPS3083 was digested with *Eco*RI and *Cla*I and the resulting fragment carrying the *lacZ* fusion was cloned between the *Eco*RI and *Cla*I sites of plasmid pDG268 (22) giving plasmid pPS3150. This plasmid was linearized with *Pst*I and used to transform PS832 to Cm^r by integration at the *amyE* locus through a double-crossover event. Southern blot analysis again confirmed the expected chromosome structure of the resultant strain, PS3169. Further details of these strain constructions are available upon request.

Analysis of β-galactosidase expression in strain PS3113 during growth and sporulation in 2× SG medium (16) showed that *yhfR* was expressed during the log phase of growth; expression then increased slightly, but the level of β-galactosidase decreased as sporulation progressed (Fig. 1). These assays used the fluorescent substrate methylumbelliferyl-β-D-galactoside (MUG), with samples assayed as described previously (16). Analysis with orthonitrophenyl-β-D-galactoside as a substrate gave a maximal β-galactosidase-specific activity of only ~15 Miller units (data not shown), which is a rather low level of expression. The kinetics and level of *yhfR-lacZ* expression were essentially identical when the *lacZ* fusion was either at the *yhfR* locus (strain PS3113) (Fig. 1) or at *amyE* (strain PS3169; data not shown), indicating that the *yhfR* promoter is within the 191 bp upstream of *yhfR* in the original PCR fragment.

The data noted above indicated that *yhfR* was expressed in *B. subtilis*, albeit at a low level, and suggested that it could be worthwhile to examine the function of *yhfR*. In order to make a *yhfR* deletion strain, PCR was used to amplify a fragment encompassing 285 bp upstream of *yhfR* to 355 bp downstream. The primers used had extra nucleotides at their 5′ ends containing *Hin*dIII or *Xba*I sites, and, after the 1,235-bp PCR product was digested with *Hin*dIII and *Xba*I, it was cloned between these sites in plasmid pUC19 in *E. coli* TG1 giving plasmid pPS3111. The 440-bp *Pst*I/*Hin*cII fragment from within the *yhfR* coding sequence was then removed from plasmid pPS3111 and replaced with the spectinomycin resistance (Sp^r) cassette from plasmid pJL74 (12) giving plasmid pPS3114. This plasmid was linearized with *Sca*I and used to transform *B. subtilis* PS832 to Sp^r, giving strain PS3168. Southern blot analysis confirmed that strain PS3168 contained the expected deletion of

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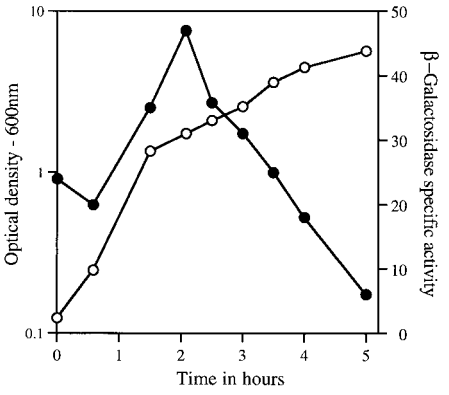

FIG. 1. Expression of *yhfR-lacZ* during growth and sporulation. Strain PS3113 (*yhfR-lacZ* at *yhfR*) was grown and sporulated at 37°C in 2× SG medium, and aliquots were taken and assayed for β-galactosidase with MUG as described in the text. β-Galactosidase-specific activity is expressed as nanomoles of MUG hydrolyzed per minute per milliliter of cell culture per OD_{600} unit of the culture. Symbols: ○, OD_{600}; ●, β-galactosidase-specific activity. The wild-type strain without a *lacZ* fusion (PS832) had a β-galactosidase-specific activity of less than 2 nmol/min/ml/OD_{600} unit throughout growth and sporulation.

>60% of the *yhfR* coding sequence; details of the construction of this strain are available on request. Comparison of strains PS832 (wild type) and PS3168 (Δ*yhfR*) indicated that both strains had identical growth rates and extents of growth at 37°C in 2× YT medium (16 g of tryptone, 10 g of yeast extract, and 5 g of NaCl per liter) or Spizizen's minimal medium (21) containing 1% glucose (data not shown). Both strains also exhibited identical sporulation at 37°C in 2× SG medium (16), and the purified spores from both strains had identical levels of heat resistance as well as germination and outgrowth at 37°C in 2× YT medium plus 4 mM L-alanine (data not shown). Thus analysis of this *yhfR* deletion mutant did not reveal any function for YhfR.

One reason that YhfR might have no function in *B. subtilis* could be that this protein is not active as a dPGM even though YhfR does exhibit significant amino acid sequence homology to dPGMs, with YhfR and *Saccharomyces cerevisiae* dPGM having 27% identical residues in a 190-amino-acid overlap, including the two histidine residues known to be essential for dPGM catalysis. In order to determine if *yhfR* does encode a functional dPGM, we decided to overexpress, purify, and assay YhfR. PCR was used to amplify *yhfR* including the coding region and stop codon, using chromosomal DNA from PS832 as a template; the primers used also contained extra nucleotides at their 5′ ends including *Nde*I or *Bam*HI sites. The 728-bp PCR product was cut with *Nde*I and *Bam*HI and cloned between these sites in plasmid pET11c (23) in *E. coli* TG1 giving plasmid pPS3112. DNA sequence analysis confirmed that the sequence of the insert in pPS3112 was as expected. This plasmid was used to transform *E. coli* BL21 carrying the gene for T7 RNA polymerase under control of the *lac* promoter, giving strain PS3112. Strain PS3112 was grown at 37°C in 1 liter of 2× YT medium containing 50 μg of ampicillin/ml; at an optical density at 600 nm (OD_{600}) of 0.8 the culture was made 0.1 mM in isopropyl-β-D-thiogalactopyranoside (IPTG), growth was continued for 2 1/2 h, the culture was harvested by centrifugation, and the cells were stored frozen. Analysis of aliquots of IPTG-induced *E. coli* cells with or without plasmid pPS3112 showed that cells carrying pPS3112 expressed a large amount of a soluble protein of 23 kDa, the approximate molecular mass expected for the YhfR polypeptide (22 kDa) (Fig. 2, lanes 1 and 2). However, crude extracts prepared by sonication of cells in cold 50 mM HEPES (pH 7.4)–2 mM $MgCl_2$–0.2 mM dithiothreitol (buffer A) followed by centrifugation and dialysis of the supernatant fluid overnight against buffer A plus 50 mM KCl had dPGM-specific activities of 0.12 μmol/min · mg of protein in the conversion of 3PGA to 2PGA in the presence of DPG (7) for both IPTG-induced strain PS3112 and cells without the plasmid (data not shown). We also purified YhfR 5- to 10-fold (Fig. 2, lane 3) by preparation of a crude extract as described above and isolation of the protein precipitating between 50 and 85% ammonium sulfate, followed by chromatography on DEAE-Sephadex A-50 in buffer A plus 50 mM KCl and elution of the protein with a gradient of 50 to 400 mM KCl in buffer A. The amino-terminal sequence of the purified YhfR was determined, as described previously (14), to be TAVCLVR as predicted from the *yhfR* coding sequence, with the exception of the amino-terminal methionine, which is presumably removed posttranslationally. The molecular mass of the purified YhfR was determined by matrix-assisted laser desorption–time of flight mass spectrometry (17) to be 21,847 Da, very close to the predicted value of 21,733 Da. Assays of fractions containing the highest percentage (>75%) of total protein as YhfR (Fig. 2, lane 3) gave a specific activity of only ~1.3 μmol/min · mg of protein, in contrast to values of >500 μmol/min · mg protein for purified dPGMs (6, 7); even the low activity of the most-purified YhfR fractions could be due to the endogenous *E. coli* dPGM, which would likely purify with YhfR (7). We also measured the PGM-specific activities for both wild-type (PS832) and *pgm* (PS2028) (13) *B. subtilis* strains using the one-step PGM assay (2) with dialyzed crude extracts prepared as described previously (2) from cells grown at 37°C to an OD_{600} of ~1.5 in 2× YT medium. The PGM-specific activity (with or without DPG) in extracts of strain PS832 was 320 nmol/min/mg of protein, similar to values found previously (26), while the PGM-specific activity in the *pgm* strain was <0.5 nmol/min/mg of protein, even with DPG (data not shown). Thus, while we cannot be sure that YhfR has no dPGM activity, it clearly has at most extremely low activity

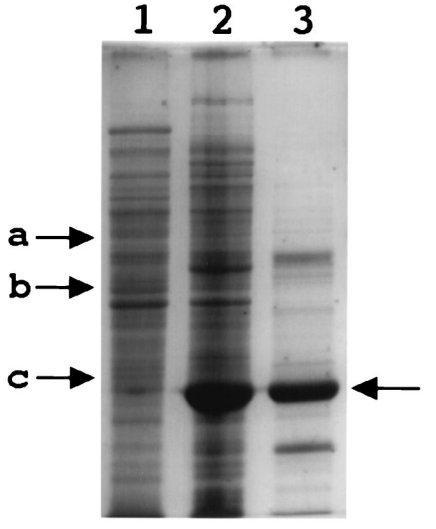

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of dPGM. Samples of soluble protein (lane 1, 24 μg; lane 2, 34 μg; lane 3, 10 μg; as determined by the Lowry procedure [15]) from lane 1, the host *E. coli* cells without plasmid, lane 2, IPTG-induced cells of strain PS3112 (overexpressing YhfR), or lane 3, YhfR purified 5- to 10-fold as described in the text, were subjected to SDS-PAGE, and the gel was stained with Coomassie blue. Arrows a, b, and c, migration positions of molecular mass markers of 50, 37, and 25 kDa, respectively; arrow on the right, position of YhfR.

compared with other dPGMs under the conditions normally used for assays of dPGMs.

Previous work has shown that the great majority of PGM activity in *B. subtilis* is due to an iPGM with a specific requirement for Mn^{2+} for activity (20, 26). In addition, deletion of the gene coding for this enzyme had a huge effect on cell growth (13). These data strongly suggest that, under the conditions that have been tested, *B. subtilis* has and needs only one PGM, an iPGM. This is consistent with our new data, which indicate no effects on cell growth and differentiation due to deletion of the *yhfR* gene, which has been suggested to encode a dPGM. We cannot exclude the possibility that *B. subtilis* has a protein other than YhfR that is a dPGM, but YhfR is the only *B. subtilis* protein with significant sequence homology to known dPGMs (11). Although *yhfR* is expressed, albeit at a very low level, the encoded protein appears to be either a poorly functional or a nonfunctional dPGM, again consistent with the lack of effect of deletion of *yhfR*. The lack of or relatively low PGM activity of YhfR is somewhat surprising, given the sequence homology of this protein with dPGMs including the enzyme from *S. cerevisiae* noted above. However, the yeast enzyme has 36 internal residues not present in YhfR, and the sequences in the carboxy-terminal regions of the two proteins are very different; there are also a variety of data indicating that the carboxy-terminal regions of dPGMs are important for enzyme function (18, 24, 25, 27). In addition, comparison of putative dPGM sequences from *Bacillus stearothermophilus*, *B. subtilis*, *Clostridium acetobutylicum*, and *Clostridium difficile*, all of which are spore formers, showed a surprising lack of sequence conservation, with only ~13% identical residues in the proteins from these four species (data not shown). In contrast, the iPGMs from these same four species have 44% identical residues (3). Thus it is possible that, while *yhfR* may have once coded for a dPGM, the need for this enzyme in these species has been lost. As a consequence, the coding gene may have evolved with very little selection such that the protein has become poorly functional or nonfunctional. An alternative possibility is that, while the protein encoded by *yhfR* may not catalyze PGM activity, it may catalyze some similar reaction; for example, it appears possible that, with only minor changes, a dPGM could evolve into a phosphatase (1, 19). Indeed, the structure of the active sites of iPGMs is almost identical to that of *E. coli* alkaline phosphatase (9). While further work will be required to definitively assess possible enzymatic reactions catalyzed by YhfR, this enzyme is clearly not needed for normal growth and differentiation of *B. subtilis*.

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