Cloning and Nucleotide Sequences of the Genes Encoding Triose Phosphate Isomerase, Phosphoglycerate Mutase, and Enolase from Bacillus subtilis

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The Bacillus subtilis genes tpi, pgm, and eno, encoding triose phosphate isomerase, phosphoglycerate mutase (PGM), and enolase, respectively, have been cloned and sequenced. These genes are the last three in a large putative operon coding for glycolytic enzymes; the operon includes pgk (coding for phosphoglycerate kinase) followed by tpi, pgm, and eno. The triose phosphate isomerase and enolase from B. subtilis are extremely similar to those from all other species, both eukaryotic and prokaryotic. However, B. subtilis PGM bears no resemblance to mammalian, fungal, or gram-negative bacterial PGMs, which are dependent on 2,3-diphosphoglycerate (DPG) for activity. Instead, B. subtilis PGM, which is DPG independent, is very similar to a DPG-independent PGM from a plant species but differs from the latter in the absolute requirement of B. subtilis PGM for Mn²⁺. The cloned pgm gene has been used to direct up to 25-fold overexpression of PGM in Escherichia coli; this should facilitate purification of large amounts of this novel Mn²⁺-dependent enzyme. Inactivation of pgm plus eno in B. subtilis resulted in extremely slow growth either on plates or in liquid, but growth of these mutants was enhanced by supplementation of media with malate. However, these mutants were asporogenous with or without malate supplementation.

The process of sporulation in Bacillus and Clostridium species produces a spore which is extremely resistant to a variety of harsh treatments, such as heat, dessication and radiation, and which can survive for long periods in the absence of exogenous nutrients (19). One reason for the survival of spores in such conditions is that they are metabolically dormant and carry out neither macromolecular biosynthesis nor detectable metabolism of exogenous or endogenous compounds (17–19). Not surprisingly, dormant spores lack significant levels of products of catabolism such as ATP and NADH, which are present at high levels in growing cells, although AMP and NAD are present at significant levels in spores (18). While the spore can remain in this dormant state for long periods of time, with the proper stimulus spor germination is initiated, and within minutes ATP and NADH are produced and macromolecular biosynthesis begins (17, 18). The dormant spore contains a number of energy reserves which are mobilized in the first minutes of germination, thus facilitating the rapid resumption of metabolism. One such reserve is the large depot of small, acid-soluble proteins which are degraded to amino acids early in germination, with a significant amount of these amino acids catabolized for energy (17, 18). A second energy reserve is a depot of 3-phosphoglyceric acid (3PGA), which constitutes 0.2 to 0.5% of spore dry weight (18). The 3PGA depot is accumulated late in sporulation within the developing spore, and there is a body of evidence indicating that phosphoglycerate mutase (PGM) is the enzyme which is specifically inhibited to allow 3PGA accumulation (18, 20, 22). In the first minutes of germination, the 3PGA depot is converted to acetate, NADH, and ATP through the action of PGM, enolase (ENO), pyruvate kinase, and pyruvate dehydrogenase (18).

As noted above, PGM appears to be the enzyme whose inhibition allows 3PGA accumulation during sporulation and whose reactivation permits 3PGA utilization during germination (9, 19, 22). PGM has been purified from Bacillus megaterium and Bacillus subtilis and is a member of the 2,3-diphosphoglycerate (DPG)-independent PGM class (5, 23, 30). In addition, PGM from Bacillus species is unique among PGMs in that it has an absolute and specific requirement for Mn²⁺ (5, 9, 11, 23, 30). This Mn²⁺ requirement renders the Bacillus enzyme exquisitely pH sensitive, and a model has recently been proposed in which changes in intrasporal pH during sporulation and germination play a key role in modulating PGM activity and thus 3PGA accumulation and utilization (9, 19). In order to carry out further experiments to prove or disprove this model and in order to generate significant amounts of Bacillus PGM for investigation of its pH and Mn²⁺ dependence in detail, we undertook the cloning of the gene coding for B. subtilis PGM (pgm). In this communication we report the cloning and nucleotide sequence of B. subtilis pgm, as well as those of two adjacent genes coding for glycolytic enzymes. We also report the effects of pgm mutations in B. subtilis and the use of the cloned gene for PGM overexpression.

MATERIALS AND METHODS

Bacteria, plasmids, and sources of DNA. The B. subtilis strain used was a trp⁻ revertant of strain 168, the Escherichia coli strain used for plasmid cloning was JM83, and the E. coli strain used for λEMBL3 propagation was P2-392 (Stratagene). B. subtilis chromosomal DNA was isolated and purified as described previously (4), and DNA fragments were cloned in plasmid pUC19 or EMBL3. The ermC gene, encoding resistance to macrolides-lincosamides-streptogramin (MLS) (erythromycin and lincomycin), and 25 μg/ml, respectively, was originally isolated as a 1.4-kb HpaII fragment and cloned in the Accl site of pUC12 with the direction of ermC transcription from the EcoRI site toward the HindIII site in the polylinker (25). Digestion of this plasmid (pPS936) with XbaI and ClaI
releases a 1.2-kb fragment containing the intact ermC gene with its promoter (25). Chromosomal DNA from Bacillus stearothermophilus ATCC 7953 was a gift from H. Cudny. In experiments analyzing the growth of B. subtilis eno and pgm mutants, the MLS' wild-type strain used was PS1804, in which the ermC gene has been inserted in the pbgE gene of B. subtilis 168 (12).

**Enzymes, antisera, enzyme assays, and Western blots (immunoblots).** PGM and ENO were purified to homogeneity from vegetative cells of B. megaterium as previously described (21, 23), and antisera against each protein were raised in rabbits by use of the regimen described previously (14). Cell extracts of B. subtilis or E. coli strains were prepared by sonication; glass beads (1 g) were added to B. subtilis cell suspensions to facilitate sonication. Cells from ~20 ml of culture were harvested by centrifugation (10 min; 8,000 × g), washed once with 10 ml of 0.15 M NaCl, and suspended in 1.5 ml of 50 mM Tris-HCl (pH 7.5)−2 mM MgSO4−10 μM MnCl2−0.1 mM dithiothreitol. After sonic disruption in the cold for 30 s to 1 min, the extract was centrifuged in a microcentrifuge, and the supernatant fluid was saved for enzyme assays or polyacrylamide gel analysis. Enzymes extracts were dialyzed for 18 h against 50 mM Tris-HCl (pH 7.5)−50 mM KCl−2 mM MgSO4−10 μM MnCl2−0.1 mM dithiothreitol−20% glycerol prior to assays.

Assays of PGM, ENO, and protein were carried out as previously described (21, 23). Analysis of proteins in cell extracts by sodium dodecyl sulfate (SDS)−10% polyacrylamide gel electrophoresis was as described previously (14). In some experiments, proteins resolved by polyacrylamide gel electrophoresis were transferred to polyvinylidene difluoride paper (Immobilon; Millipore Corp.). The paper was then treated with either a 1/500 dilution of anti-PGM or a 1/1,000 dilution of anti-ENO in a Western blot procedure as described previously, using alkaline phosphatase-coupled goat anti-rabbit immunoglobulin G and with LumiPhos as the substrate (14). In other experiments, the paper was lightly stained with Coomassie brilliant blue R and destained, and appropriate bands were subjected to automated protein sequenator analysis as described previously (14).

**Cloning and DNA manipulations.** E. coli strains were routinely grown at 37°C in 2× YT medium (tryptone, 16 g/liter; yeast extract, 10 g/liter; NaCl, 5 g/liter) and transformed, and plasmid DNA was isolated and analyzed by standard procedures. B. subtilis strains were routinely grown in 2× YT medium, and sporulation was assessed either in NS medium (11) supplemented with 50 μM MnCl2 or in the resuspension medium of Sterlini and Mandelstam (24). When noted, these media were supplemented with glucose to 27 mM and 1-malate to 10 mM. Formation of heat-resistant spores was analyzed by heating aliquots of sporulating cultures at 85°C for 15 min, plating appropriate dilutions on 2× YT plates supplemented with malate, and incubating the plates for 24 to 48 h prior to enumeration of survivors. This heat treatment gives only ~20% killing of wild-type spores but more than 8 log units of killing of growing cells.

Generation of the partial Sau3A library of B. subtilis DNA in λEMBL3, as well as plating, screening, and isolation of DNA from recombinant phage, was by standard methods. B. subtilis 168 was made competent and transformed with Scal-linearized pUC19 derivatives as described previously (14). Transformants were selected on 2× YT plates containing 10 mM L-malate plus erythromycin (1 μg/ml) and lincomycin (25 μg/ml). After growth for 36 h at 37°C, transformants giving small colonies (>95% of transformants) were restreaked three times over the next week on 2× YT plus malate plates. Chromosomal DNA from transformants was isolated, digested with appropriate restriction enzymes, and subjected to Southern blot analysis. For hybridization to these blots, as well as all others, we used probes labeled with digoxigenin coupled to dUTP, alkaline phosphatase coupled to antidigoxigenin antiserum, and LumiPhos as the substrate (3, 14).

PCR was carried out essentially as described previously (26) with B. stearothermophilus DNA as the template. The primers used were 5′-GCTGTCAAATTTGCGAGGC-3′ (oligo A) and 3′-GCCGGAAATCGCAGCC-5′ (oligo B). These are homologous to nucleotides 385 to 326 and 947 to 954, respectively, in B. stearothermophilus tpi (GenBank accession number X66129). The 650-bp PCR product was isolated by electrophoresis on low-gelling-temperature agarose and then labeled and used as a hybridization probe with Southern blots of restriction enzyme-digested B. subtilis chromosomal DNA. A 0.45-kb HindIII fragment hybridized well and was cloned in pUC19; DNA sequence analysis revealed that this fragment was within the B. subtilis tpi sequence. This 0.45-kb HindIII fragment was then used to probe the partial Sau3A library of B. subtilis DNA in λEMBL3, and one phage which hybridized well was isolated. This phage, which contained the original 0.45-kb HindIII fragment, was used as a source of DNA to clone a 3-kb hybridizing EcoRI fragment (Fig. 1) in pUC19. A 0.9-kb PstI-EcoRI fragment from the right end of the EcoRI fragment was then used to clone an overlapping 4-kb PstI fragment in pUC19 (Fig. 1). Southern blot analyses using these various fragments as probes, as well as DNA sequencing, showed that the 4-kb PstI fragment represented continuous chromosomal DNA, as did most of the 3-kb EcoRI fragment. However, the leftmost 300 to 450 bp of the 3-kb EcoRI fragment were derived from a second Sau3A fragment.
DNA sequences were determined by the chain termination method with DNA fragments cloned in pUC19. All sequences shown in this communication were determined completely in both directions, and all restriction sites used in cloning were overlapped.

**Nucleotide sequence accession number.** The DNA sequence reported in this work has been assigned GenBank accession number L29475.

**RESULTS**

Cloning and nucleotide sequence of *B. subtilis* pgm. The *B. megaterium* PGM was purified and analyzed by SDS-polyacrylamide gel electrophoresis, the resolved proteins were transferred to poly(vinylidene difluoride) paper and stained lightly, and the amino-terminal sequence of PGM was determined to be SKKPVIALILDGFALDEDKG?AV (? denotes a residue that could not be identified). Comparison of this sequence with those in the GenBank data base revealed a perfect match to the first 13 amino acids of an open reading frame (ORF) beginning just prior to the stop codon of the *B. stearothermophilus* tpi gene (GenBank accession number X66129). The homology continued even further if a sequencing error in the *B. stearothermophilus* sequence was assumed. These findings made it most likely that *B. subtilis* pgm was also located just downstream of tpi. Consequently, the known *B. stearothermophilus* sequence was used to construct two PCR primers which allowed amplification of a 0.65-kb fragment of *B. stearothermophilus* DNA from within tpi. This fragment was then used as a probe to initiate the cloning of *B. subtilis* pgm. Eventually, ~7 kb of contiguous chromosomal DNA was cloned, including the complete pgm gene (Fig. 1; see Materials and Methods).

DNA sequence analysis of the cloned DNA revealed the presence of three complete ORFs, all transcribed in the same direction, as well as the end of a fourth ORF (Fig. 1 and 2). All three complete ORFs were preceded by sequences exhibiting good complementarity to the 3' end of 16S rRNA (underlined residues in Fig. 2), initiated with ATG, and terminated with TAA (Fig. 2). The leftmost partial ORF exhibited 85% identity with the product of the pgk gene (coding for phosphoglycerate kinase) of *B. megaterium* (15) (GenBank accession number M87648); however, the *B. subtilis* pgk locus was not sequenced completely in both directions. The first complete ORF was 81% identical to the products of the *B. megaterium* and *B. stearothermophilus* tpi genes (data not shown). The second complete ORF appeared to code for PGM, as the first 24 residues showed excellent homology with the sequence determined for *B. megaterium* PGM. In addition, this gene product showed good homology (33% identity and an additional 22% similarity) with the DPG-independent PGM of corn (7) (GenBank accession number M80912), and it resulted in PGM synthesis in *E. coli* (see below). The third ORF exhibited excellent homology (an average of 43% identity and an additional 19% similarity) with ENOs of several species (i.e., *Zea mays* [10] [GenBank accession number X55981] and *Zymomonas mobilis* [2] [GenBank accession number M99380]) and also directed synthesis of ENO in *E. coli* (see below). Just downstream of eno there is a stem-loop structure followed by a T-rich region that may well be a transcription terminator (arrows in Fig. 1 and 2). Downstream of this putative terminator there were no obvious other ORFs in the same direction as eno. Translation of this downstream region in all six reading frames gave no sequences homologous to those in currently available data bases.

Previous work with *B. megaterium* has shown a close association of pgk and tpi, with tpi followed by a partial ORF whose product has excellent homology to *B. subtilis* PGM (15) (GenBank accession number M87648). In addition, pgk in *B. megaterium* is preceded by gap, coding for glyceraldehyde 3-phosphate dehydrogenase (15). While the gap gene of *B. subtilis* has been cloned and sequenced (29), we cannot definitively assign gap to the restriction map of the *B. subtilis* pgk-tpi-eno cluster. In *B. megaterium* there is a 312-bp spacing between the end of gap and the beginning of pgk (5), and this spacing is at least 252 bp in *B. subtilis* (29). Given the significant sequence similarity between these spacer regions in the two species (5, 29), it seems very likely that gap also precedes pgk in *B. subtilis*.

Expression of PGM and ENO in *E. coli*. The 4-kb PstI fragment (Fig. 1) carrying both pgm and eno was cloned in PstI-cut pUC19 either (i) such that the direction of pgm transcription was identical to that of the plasmid's lac promoter (pML5) or (ii) in the opposite orientation (pML3). The growth properties of *E. coli* JM83 were unaffected by the presence of this insert in either orientation (data not shown). Analysis of extracts from cells harvested shortly after the optical density at 600 nm of cultures stopped increasing revealed two prominent protein bands in extracts of cells carrying pML5 which were not present in cells with pUC19 or pML3 (Fig. 3). The sizes of these new proteins (65 and 44 kDa) are similar to those found previously for PGM and ENO of *Bacillus* species (21, 23). Their identities were confirmed by Western blot analysis (data not shown), since the 65-kDa protein reacted with anti-PGM and the 44-kDa band reacted with anti-ENO. In addition, automated protein sequenator analysis of the 44-kDa band gave PYIVDV as the first six residues, which are identical to those predicted from the eno gene (Fig. 2) (with the exception of the amino-terminal methionine, which is presumably removed posttranslationally).

Enzyme assays of extracts prepared at various times during growth of these strains showed that pML5 gave PGM and ENO specific activities 10- to 25-fold higher than those in the *E. coli* host (Table 1). The high PGM specific activity in cells carrying pML5 was not increased when growth was in medium supplemented with 25 μM MnCl₂ (Table 1). The specific activities of PGM and ENO in *E. coli* with pML5 are also 10- to 60-fold higher than those in *E. coli* strain pPS2020, indicating that this *E. coli* strain should provide a good source from which to purify large amounts of these enzymes.

**Construction and analysis of pgm and eno mutations in** *B. subtilis*. With the *B. subtilis* pgm and eno genes in hand, it was relatively simple to create deletion mutations in these genes in vitro and then introduce these mutations into *B. subtilis*. This was carried out by replacing an internal fragment of pgm or pgm plus eno (Fig. 1) with an antibiotic resistance gene and then integrating the mutant genes into the *B. subtilis* chromosome by a double crossover event with concomitant replacement of wild-type DNA. The antibiotic resistance gene used was a 1.2-kb ClaI-XbaI fragment containing the emrC gene (see Materials and Methods), which was isolated and had the ends filled in with the large fragment of *E. coli* DNA polymerase. This fragment was cloned into pML5 which had been cut either with Ncol plus AavII or with EagI (Fig. 2), and the ends were filled in as described above. Appropriate recombinant plasmids were isolated in *E. coli* and designated pPS2019 and pPS2020, respectively. These plasmids were then linearized with ScaI, which cuts in the pUC sequence, and used to transform *B. subtilis* 168 to MLS'. Southern blot analysis of a transformant with plasmid pPS2019 (termed PS2028) or plasmid pPS2020 (termed PS2027) confirmed the expected chromosomal structures, since transformation with pPS2019 re-
FIG. 2. Nucleotide sequence in the pgm region of the chromosome. The region in Fig. 1 shown in this sequence is from ~690 bp upstream of the leftmost PstI site in pgm to the rightmost EcoRI site downstream of eno. Amino acids are given in the single-letter code above the first nucleotide of a codon; stop codons are denoted by asterisks. Regions prior to translation start sites which show good complementarity to the 3' end of B. subtilis rRNA are underlined; these are undoubtedly ribosome binding sites. The apposed arrows downstream of eno denote a stem-loop structure which is likely to be a transcription stop signal. The locations of restriction sites used in constructing plasmids to give pgm and eno inactivation in B. subtilis are as follows: AvrII, nucleotide 2288; EagI, nucleotides 1292, 1878, and 2500; and NcoI, nucleotide 1277.
sulted in replacement of ~1 kb from within *pgm* by *ermC* and transformation with pPS2020 resulted in replacement of ~1.2 kb covering the end of *pgm* and the beginning of *eno* by *ermC* (Fig. 1 and 2 and data not shown). Western blot analysis of extracts from late-log-phase cells grown in 2× YT medium plus 10 mM l-malate (see below) showed that strains PS2027 and PS2028 had no detectable PGM (Fig. 4A) and also lacked detectable ENO (Fig. 4B).

Analysis of the growth of strains PS2027 and PS2028 indicated that both strains grew extremely poorly on 2× YT plates with or without glucose, and they never gave more than pinpoint-sized colonies. However, growth on 2× YT plates was significantly stimulated by l-malate, although neither the growth rate nor the colony size was that of the wild-type strain; similar results were obtained when 2× YT medium was supplemented with malate plus glucose. These qualitative results on plates were confirmed in liquid media (Table 2). Notably, glucose actually decreased the growth rate of both mutants, and while malate did not stimulate the growth rate of the mutants tremendously, it greatly increased the maximum cell density achieved. In general, the mutant strain in which a DNA fragment covering both *pgm* and *eno* had been lost grew more slowly on all media tested. Analysis of the sporulation of both of these mutant strains on NS medium (either plates or liquid) with or without malate or glucose resulted in no significant (<0.05%) sporulation after 2 to 5 days as observed in the phase-contrast microscope and resulted in <10⁵ heat-resistant spores per ml. Under these same conditions, the wild-type strain gave >50% sporulation after 36 h and 5 × 10⁸ heat-resistant spores per ml. With the Sterlini-Mandelstam resuspension medium (24) used to induce sporulation, strains PS2027 and PS2028 gave <10⁵ heat-resistant spores per ml after 1 to 5 days, while the wild-type strain gave 10⁸ heat resistant spores per ml after 18 h.

![Figure 3](http://example.com/fig3.png)

**FIG. 3.** Proteins made in *E. coli* with and without cloned *pgm* and *eno* genes. The *E. coli* strains were grown at 37°C in 2× YT medium, and cells were harvested at the beginning of the stationary phase of growth. Extracts were prepared as described in Materials and Methods, and 25 μg of protein from each extract was examined by SDS-polyacrylamide gel electrophoresis. The horizontal arrows denote the positions of molecular mass markers run in an adjacent lane. The samples are from strains with pML5 (lane A), pUC19 (lane B), and pML3 (lane C).

<table>
<thead>
<tr>
<th>Time of harvest</th>
<th>Sp act (μmol/ml · mg of protein) with:</th>
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<tbody>
<tr>
<td></td>
<td>pUC19</td>
</tr>
<tr>
<td></td>
<td>PGM</td>
</tr>
<tr>
<td>Logarithmic phase</td>
<td>0.32</td>
</tr>
<tr>
<td>Early stationary phase</td>
<td>0.41</td>
</tr>
<tr>
<td>Overnight growth</td>
<td>0.38</td>
</tr>
<tr>
<td>Without MnCl₂</td>
<td></td>
</tr>
<tr>
<td>With MnCl₂</td>
<td></td>
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</table>

a Cells were grown in 2× YT medium with or without 25 μM MnCl₂ harvested, disrupted, and assayed for PGM, ENO, and protein as described in Materials and Methods.

b *B. subtilis* *pgm* and *eno* in the orientation opposite to that of the plasmid's *lac* promoter.

c *B. subtilis* *pgm* and *eno* in the same orientation as the plasmid's *lac* promoter.
space between pgk, tpi, pgm, and eno suggests that these genes are likely to be cotranscribed. In *B. megaterium* there is a 312-bp gap between the end of gap and the beginning of pgk (15), and this gap is at least 232 bp in *B. subtilis* (29). Although the presence of gap just upstream of pgk in *B. subtilis* has not yet been shown definitively, this certainly seems likely. Thus, significant transcription of *B. subtilis* pgk may initiate between gap and pgk, although some pgk transcription may initiate upstream of gap. The existence of clusters of glycolytic genes with multiple transcripts has been shown for other organisms (1, 16). Indeed, in *B. stearothermophilus* the genes coding for phosphofructokinase and pyruvate kinase may constitute an operon (13).

The high degree of sequence similarity of PGK, triose phosphate isomerase, and ENO of *B. subtilis* with the corresponding enzymes of other species, either prokaryotic or eukaryotic, is not surprising, since glycolytic enzymes almost always exhibit a high degree of sequence conservation throughout evolution. We note, however, that the sequence we determined for *B. subtilis* ENO is very different from that previously reported in the literature (28). While the latter sequence certainly appears to be that of an ENO, the cloning of the DNA for sequencing by cDNA methodology in that work makes its identification as arising from *B. subtilis* questionable.

Comparison of the *B. subtilis* PGM sequence with those of DPG-dependent PGMs reveals no obvious homology (data not shown). This is not surprising because PGM from *Bacillus* species is DPG independent and is a monomer of 61 to 74 kDa as determined by SDS-polyacrylamide gel electrophoresis (23, 30). DPG-dependent PGMs are monomers, dimers, or tetramers with a subunit molecular mass of 23 to 30 kDa (5). In contrast, plant PGMs are ~60-kDa monomers and are DPG independent (5), and *B. subtilis* PGM exhibits good homology with the one PGM from plant species (Z. mays; GenBank accession number M80912) whose sequence is in current data bases. Previous work has indicated that the Z. mays PGM shows no obvious homology with DPG-dependent PGMs, but sequence similarity was noted between the Z. mays PGM and conserved regions in alkaline phosphatases thought to be involved in catalysis by the latter group of enzymes (7). However, we did not observe such significant homology between *B. subtilis* PGM and these conserved regions of alkaline phosphatases (data not shown). The *B. subtilis* PGM also exhibits good sequence homology (45% identity) with an ORF identified on the plastid genome from the red alga *Antithia-minion* sp. (27) (GenBank accession number X64705). While this algal ORF has not been shown directly to code for a PGM, it is known that plant plastids contain a DPG-independent PGM that probably is distinct from the cytosolic PGM (5).

Thus, we believe that this algal plastid gene codes for a DPG-independent PGM. While most DPG-independent PGMs may have a requirement for divalent cations (5), these enzymes differ from the *B. subtilis* PGM in that the latter enzyme has an absolute and specific requirement for Mn$^{2+}$ (23, 30). The reason for this Mn$^{2+}$ requirement is not clear, but there is evidence that histidine residues are involved in Mn$^{2+}$ binding (9). The involvement of histidine residues in Mn$^{2+}$ binding by *Bacillus* PGM may be at least one of the reasons for the high degree of pH sensitivity of this enzyme (9). Given the overexpression of *B. subtilis* PGM which we have achieved in *E. coli*, it should be feasible to purify large amounts of this enzyme for studies to elucidate the detailed mechanisms of Mn$^{2+}$ binding and pH sensitivity.

The cloning and analysis of the pgm and eno genes reported in this work allowed us to readily construct knockout mutations in these genes via marker replacement. Insertion of ermC into

**DISCUSSION**

Previous work with *B. megaterium* has indicated that the gap, pgk, and tpi genes are clustered on the chromosome with an additional unidentified ORF just upstream of gap and a second ORF, which we have now shown is pgm, just downstream of tpi (15). This same cluster of genes encoding glycolytic enzymes seems likely to be present in *B. subtilis* as well and includes eno just downstream of pgm. Since eno is followed by an apparent transcription terminator and there is no obvious ORF downstream of eno, this may be the last gene in this cluster. Detailed transcript analyses of this region of the chromosome have not yet been carried out. However, the small (or no) intergenic

**TABLE 2. Growth rates of the wild-type strain and pgm and eno mutants**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Doubling time (min)$^a$ with the following addition(s) to the medium:</th>
<th>glucose</th>
<th>malate</th>
<th>glucose plus malate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td></td>
<td>29 (7.5)</td>
<td>21 (9)</td>
<td>28 (10.5)</td>
</tr>
<tr>
<td>Δpgm mutant</td>
<td></td>
<td>115 (0.6)</td>
<td>210 (0.7)</td>
<td>95 (3.8)</td>
</tr>
<tr>
<td>Δeno Δpgm mutant</td>
<td></td>
<td>175 (0.5)</td>
<td>&gt;300 (0.5)</td>
<td>120 (1.8)</td>
</tr>
</tbody>
</table>

$^a$ Strains were grown from colonies freshly streaked the day before on 2x YT plus malate and MLS plates. Growth was in 2x YT medium at 37°C with MLS and supplements of glucose (27 mM) and/or malate (10 mM) as noted.

$^b$ The Δpgm and Δeno Δpgm mutants are strains PS2028 and PS2027, respectively, which were generated by transformation with plasmids pPS2019 and pPS2020, respectively.

$^c$ Values in parentheses are the maximum optical density at 600 nm reached by these cultures (generally after ~18 h of growth).
pgm abolished production of both PGM and ENO. The elimination of ENO production by integration into pgm was not unexpected, since these two genes appear to be in an operon. Replacement of parts of both pgm and eno by ermC also eliminated production of PGM and ENO. While these two mutant strains exhibited very similar growth phenotypes, the strain with both pgm and eno sequences removed grew more slowly; the reason for this difference is not clear. Possibly it might be due to differential expression of the inserted ermC in the two mutant strains.

Since marker replacement of pgm or pgm and eno resulted in complete loss of PGM and ENO, these mutations effectively abolished both the lower part of the glycolytic pathway and the capacity for gluconeogenesis from three carbon compounds. To our knowledge these are the first such knockout mutations in these genes isolated in a bacterium, although mutations causing ENO deficiency have been isolated in E. coli (8). Clearly, the growth of these B. subtilis mutants was severely compromised, as growth rates of the mutants were always 3- to 15-fold lower than those of the wild-type strain, and the mutant strains generally grew to much lower cell densities than did the wild-type strain. While the mutants grew, albeit very slowly, in a rich medium, their growth was stimulated appreciably by supplementation with malate. In this respect, these B. subtilis mutants behave similarly to the eno mutants of E. coli (8). However, even in the best medium the growth rate of the mutants was significantly lower than that of the wild-type strain.

We were most interested in analyzing the sporulation of our pgm and eno mutants, in particular to obtain further information on the role of PGM in regulating 3PGA metabolism during sporulation and germination. Previous work has indicated that growth of wild-type B. subtilis in Mn2+-deficient media results in inhibition of PGM and poor growth on glucose. While malate supplementation stimulates growth on Mn2+-poor media, this supplementation did not restore sporulation (11). In contrast, a pgk mutant of B. subtilis sporulates well if malate is added to an Mn2+-containing medium (6). However, as was found previously by using Mn2+-poor medium to inhibit PGM (11), while malate stimulated the growth of our strains lacking PGM and ENO, there was no significant sporulation, even in a resuspension medium. Consequently, as suggested previously (11), PGM or ENO (or both) may be essential for sporulation, possibly to provide energy for the developing forespore, which lacks most enzymes of the tricarboxylic acid cycle (20). Given the dramatic effect of loss of PGM and ENO on B. subtilis sporulation, we may now be able to restore sporulation of this mutant strain by introduction of genes coding for (i) ENO and (ii) a PGK not subject to the regulation of B. subtilis PGM (for example the DPG- and Mn2+-independent PGM of Z. mays [7]). In addition, analysis of 3PGA metabolism during sporulation and spore germination of such a strain may provide further insight into the role of B. subtilis PGM in 3PGA accumulation during sporulation.

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