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 Mn^{2+} . 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 Mn^{2+} -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 spore 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 Mn^{2+} (5, 9, 11, 23, 30). This Mn^{2+} requirement renders the *Bacillus* enzyme exquisitely pH sensitive, and a model has recently been proposed in which changes in intraspore 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 Mn^{2+} 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 λ EMBL3 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 λ EMBL3. The *ermC* gene, encoding resistance to macrolides-lincosamides-streptogramin (MLS) (erythromycin and lincomycin, 1 and 25 μ g/ml, respectively), was originally isolated as a 1.4-kb *Hpa*II fragment and cloned in the *Acc*I site of pUC12 with the direction of *ermC* transcription from the *Eco*RI site toward the *Hind*III site in the polylinker (25). Digestion of this plasmid (pPS936) with *Xba*I and *Cla*I

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FIG. 1. Restriction map and location of ORFs in the *pgm* region of the *B. subtilis* chromosome. The ORFs in this region are denoted by the various shaded boxes and code for phosphoglycerate kinase (*pgk*), triosephosphate isomerase (*tpl*), PGM (*pgm*), and ENO (*eno*). Note that *pgk* was not fully sequenced; the size of the gene was estimated from that for *B. megaterium pgk* (5). The apposed arrowheads downstream of *eno* represent a putative transcription stop site. The horizontal double-headed arrows below *pgm* or *pgm* and *eno* denote the regions deleted in generating Δpgm or $\Delta pgm \Delta eno$ mutants by marker replacement. Restriction enzyme cleavage sites are denoted by vertical arrowheads and are as follows: E, *EcoRI*; P, *PstI*; and V, *PvuII*. The horizontal arrow below the map denotes the direction of transcription.

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^r wild-type strain used was PS1804, in which the *ermC* gene has been inserted in the *pbpE* 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. *B. subtilis* 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 L-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 *ScaI*-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'-GCTGTCCAATTTGTCGAGGACG-3' (oligo A) and 3'-GCCCGGAATCAGCCGCC-5' (oligo B). These are homologous to nucleotides 305 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 polyvinylidene difluoride paper and stained lightly, and the amino-terminal sequence of PGM was determined to be SKKPVALIILDGFAL?DEDKG?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. stearotheophilus tpi* gene (GenBank accession number X66129). The homology continued even further if a sequencing error in the *B. stearotheophilus* sequence was assumed. These findings made it most likely that *B. subtilis* *pgm* was also located just downstream of *tpi*. Consequently, the known *B. stearotheophilus* sequence was used to construct two PCR primers which allowed amplification of a 0.65-kb fragment of *B. stearotheophilus* 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. stearotheophilus 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 (apposed 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 associ-

ation 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-pgm-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 232 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 *Pst*I fragment (Fig. 1) carrying both *pgm* and *eno* was cloned in *Pst*I-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 μ M 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 extracts of *Bacillus* spp. (21, 23, 30), 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 *Cla*I-*Xba*I fragment containing the *ermC* 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 *Nco*I plus *Avr*II or with *Eag*I (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 *Sca*I, which cuts in the pUC sequence, and used to transform *B. subtilis* 168 to MLS^r. 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-

pgk →
 L E F M E G K E L P G A A A L N R ***
 1 CTTGAGTTTATGCAAGGCAAGAGCTTCCAGGGCTGCTGACCTGAACCGATTAATATCTCAAACTGCTATAGGAGTGGAAACAGATGAGAAACCA
 I I A G N W K M N K T L G E A V S F V E E V K S S I P A A D K A E A
 101 ATTATCGCGCGTAACTGGAAATGAACAAACACTCGGCGAGCTGTCAGCTTCGTTGAGAGAGTGAATCTTCCTTCCAGCAGCAGCAAGCTGAAG
 V V S P A L F L E K L A S A V K G T D L K V G A Q N M H F E E S G
 201 CTGTTGTTTGGCCAGCGCTTTTCTTAGAAAAGCTTCTCTGCTGTGAAGGCACTGACCTAAAGTGGCGGCTCAAAACATGCACTTGAAGCAAGCGG
 A F T G E I S P V A L K D L G V D Y C V I G H S E R R E M F A E T
 301 CGCGTTCCAGCGCGAATCAGCGCGCTTCTCTGAAGAGCTTGGCGTTGACTACTCGGTCATCGGCGCACTCTGAGCGCGCTGAATGTTGGCTGAACCT
 D E T V N K K A H A A F K H G I V P I I C V G E T L E E R E A G K T
 401 GATGAACAGTTAAACAAAAGCAACCGAGCTTTCAACACCGCATTTGTGCGGATCTCTCTGTGTAGGTGAACCGCTTGAAGAGCTTGAAGCGCGCAAAA
 N D L V A D Q V K K G L A G L S E E Q V A A S V I A Y E P I W A I
 501 CAATGATCTTCTGTGCTGCAAGCACTGAAGAAAGCGCTTCTGCTCTCTGAGAACAGTTGCTGCTCTCTGTTATTGCGTATGAGCGCAATCTGGCGAAT
 G T G K S S T A K D A N D V C A H I R K T V A E S F S Q E A A D K
 601 CGGACAGCGCAATCTCTTACTGCGAAGATCGGATGAGCTGTGCGCGCATATCCGTAACCGTGGCTGAAGCTTCAAGCAAGCTTCAAGCAAGCTTCAAGCAAG
 L R I Q Y G G S V K P A N I K E Y M A E S D I D G A L V G G A S L E
 701 CTTGCGATCAATATGCGCGAGCGTAAAGCGCTCAACACTTAAAGATATATGCGAGCTCGGATATTGACGGTCTTTGCTGCGCGCGCGGCGCTTGC
 P Q S F V Q L L E E G Q Y E ***
pgm → M S K K P A A L I I L D G F G L R N E T V
 801 AGCCACAGTCATTCGTTCAATATTGCGAGGAGGTCATATGAGTAAACACAGCTGCACTCTCTCTGATGGCTTGGCATTACGTAAACGAAACAG
 G N A V A L A K K P N F D R Y W N Q Y P H Q T L T A S G E A V G L
 901 TAGGCAACCGAGTCCGCTTAGCGAAAAACCGAATTTTGACCGCTATTGGAACCGCATCTCTCTCAACCTTGAAGCTTCTGAGCGGCTGAGGCTCT
 P E G H S E V G H L N I G A G R I V Y Q S L T R V N V A I R
 1001 TCTGAGGGCAATGGGGAATCCGAGTAGGTCACTTAAATATCGGTGCGCGAGCTATTGTGTACCAAGCTTAAACAGCGTAAATGTTGCCATTGCT
 E G E F E R N Q T F L D A I S N A K E N N K A L H L F G L L S D G G
 1101 GAAGGAGAGTTGCAACCAATCAACATTCCTTGAAGCGCTCAGCAACCGCGAAGCAACCAACCAAGCGCTTGAAGCTTGGCTTTTATCTGACCGAG
 V H S H I N H L F A L L K L A K E G L T K F V L Y I H G F L D G R D
 1201 CGCTGCAAGCCATTAATTTATTCGCACTGTTAAAGCTTGGCAAAAAGAGCGCTGACAAAGCTTATATCATGCTTCTGACCGCGCTGA
 V G P Q T A K T Y I N Q L N D Q I K E I G V G E I A S I S G R Y Y
 1301 TGTAGGTCGCGCAGCAGCGCAACCGTACATCAACAGTTCGAAGATCAATCAAGCAATCGGCTGAGGTGAATGCGCAGATTTCGCGACGTTACTAC
 S M D R D K R W D R V E K A Y R A M A Y G E G P S Y R S A L D V V D
 1401 TCTTACGCGCGCAACCGCTGGGACGCTGTAGAAAACCTTACCGCGCATGCGCGCAAGCGCGATCTTACCGCGCGCGCGCGCTGATGTTG
 D S Y A N G I Y D E F V I P S V I T K E N G E P V A K I Q D G D S
 1501 ATGATTCAATGCAATGGTATCTACGATGAATTCGTGATTCATCTGTCAACCAACCAACCGGTGAGCGCTGTTGCGAATCCAGACCGCGGATTC
 V I F Y N F R P D R A I Q I S N T F T N K D F R D F D R G E E Y P
 1601 TGTGATTTTCTATAATTTCAAGCGCGACCGCGCGCTACGATTTCCACAGCTTCAACCAACCAAGCTTCCGTCAGCTTGAACCGCGCGCGAATTATCCA
 K N F L T H F L S E T V D G Y V A F K P I N L D N T V G E V L
 1701 AAGAACCTGTATTTGCTGCTGACTCTCTCTGTAAGACAGTTGACCGGCTATGTAGCGTTTAAACCGTAAATCTTGATTAACACAGTCGGAGAGTAT
 S Q H G L K Q L R I A E T E K Y P H V T F F M S G G R E A E F P G
 1801 TATCTCAGCAGCGGTTAAACAGCTTCCGATTCGCAAGCTGAAAGTATCCGACGTCACGCTTCTTATGAGCGCGCGCGCTGAAGCGTAAATCCCGGG
 E E R I L I N S P K V A T Y D L K P E M S A Y E V K D A L V K E I
 1901 TGAGGAGCTTCTCATCTACTCGCTTAAAGTGTCAACGATGACCTGAAGCTGAGTGAAGTGAAGCGCGCTTGTCAAGAAAT
 E A D K E D A I I L N F A N P D M V G H S G M V E P T I K A I E A V
 2001 GAAGCTGACAGCATGACCGGATCATTCTTAACCTGCAACCGCTGATATGGTGGCGCACTCGGATGCTTGAACCAACANTTAAAGCAATGAAGCAG
 D E C L G E V V D A I L A K G G H A I I T A D H G N A D I L I T E
 2101 TGGCGAATGCTTGGCGAAGTCTTGAAGCGGATCTTCTGTAAGCGGACAGCTATCATTACCGCTGNTCAGCGGATGCTGACATCTCTGATTACAGA
 S G E P H T A H T N P V P V I V T K E G I T L R E G G I L D L
 2201 ATCAGCGCAACCGCACACTGCGGATACCAACAAACCGAGTCTGCTGATTGTAAAGCAAGCAAGCAATACCGTGGTGAAGCGGCAATCTAGGCGAAGCTT
 A P T L L D L L G V E K P K E M T G T S L I Q K ***
 2301 GCACCAACGTTATTAACCTTCTTCTGCTGTGAACACCGGAAAGAAATGACAGGACATCTTTATTCAAAATAATTTGAAATCAAAAGGAGAGACAAA
eno → M P Y I V D V Y A R E V L D S R G N P T V E V E V Y T E T G A F
 2401 ACTCATGCCATACATTGTTGATGTTTATGCAAGCGAGTATTAGACTCCCGCGGCAACCGCAACAGTTGAAGTTGAAGTATATACAGAACAGGCGCTTTC
 G R A L V P S G A S T G E Y E A V E L R D G D K D R Y L G K G V L T
 2501 GCGCGCGCATAGTGCAAGCGGAGCTTCTACAGGTGAATACGAGCGGTTGAGCTTGTGACCGCGCAAGAGCGTTACCTTGGAAAGCGGCTGTAA
 A V N N V N E I I A P E L L G F D V T E Q N A I D Q L L I E L D G
 2601 CAGCTGTTAAACACGTAACGAAATCATTGCTCCAGAGCTTCTTGGCTTGTATGTAACGAACAAACCGCAATCGATCAGCTTTTAAATCGAGCTTGAACCG
 T E N K G K L G A N A I L G V S M A C A R A A A D F L Q I P L Y Q
 2701 TACTGAAACAAAGGCAACTTGGTGGCAACGCAATCCTTGGCGTATCTATGGCTTGTGCGCGTGTCTGCTGCTGATTCTTACAGATTCTCTTACCAA
 Y L G G F N S K T L P V P M M N I V N G G E H A D N N V D I Q E F M
 2801 TACCTTGGAGGATTCACTCAAAACCGCTTCTGTACCGATGATGACATCGTAAACCGCGGAGAGCATGCTGATACCAACGTTGATATTCAAGATTCA
 I M P V G A P N F R E A L R M G A Q I F H S L K S V L S A K G L N
 2901 TGATCATGCTGTAGGTGCTGCTCAACTTCCGTCAGACACTTCCGCTGGCGGCTCAAACTCTCCACAGCTGAAATCAGTATTAAGCGCAAAAGCGCTTAA

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 *Pst*I site in *tpi* to the rightmost *Eco*RI 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: *Avr*II, nucleotide 2288; *Eag*I, nucleotides 1292, 1878, and 2500; and *Nco*I, nucleotide 1277.

T A V G D E G G F A P N L G S N E E A L Q T I V E A I E K A G F K
 3001 CACTGCTGTAGGTGACGAGGCGGATTCGCTCCAAACCTTGGTTCGAACGAGAGCGCTTCMAACAATCGTTGAGCAATCGAAAGACCGCGCTTCAAA
 P G C E E V K L A M D A A S S E F T Y N K E D G K Y H L S G E G V V T
 3101 CCTGGCGAGCACTTAAACTTCTCTATGCTAGCTCTCTCTGAGTCTACAAAGAGAGCGCAAAATCCATCTCTCTGGCGAGCGGCTTGTGAAT
 S A E M V D W Y E E L V S K Y P I I S I E D G L D E N D W E G H K
 3201 CATCTGCTGAATGGTGTGACTGGTATGAGAGCGTTGTCTTCAAATACCCAACTCATCTGGATCGAGAGCGGACTTGCAGAAACGAGCTGGGAGGCCACAA
 L L T E R T L G K K V Q L V G D D L F V T N T K K L S E G I K N G V
 3301 GCTCTTACTGAGCGCTTGGCAAAAAGTTCAGCTTGTGTGGTGAGCAGCTCTTTCGTACAAACGAGAAAACCTTCTGAGGATTAATAAAGCGCGGA
 G N S I L I K V N Q I G T L T E T F D A I E M A K R A G A Y T A A V I S
 3401 GGCAACTCTATCTCTGATCAAGGTAAACCAATCGGTACATTGACTGAACCTTCGATCGCATCGAAATGGCGAAGCGCGCGGCTACACAGCTGTATCT
 H R S G E T E D S T I A D I A V A T N A G Q I K T G A P S R T D R
 3501 CTCACCGTCTCTGGTGAACCTGAGACAGGCACATCGCGTGACATCGCTGTGGCAACAAACGAGGACAACTCAAAACAGGTGCTCGGCTCTGTCGGGACCG
 V A K Y N Q L L R I E D Q L A E T A Q Y H G I N S F Y N L N K ***
 3601 TGTTCGAAATACAAACAGCTCTCTTCGATCGAGATCAGTTCGGCTGAAACCTCTCAATACCGGCTTCACTCTTCTCAAACTTAACAGTAATAA

 3701 AAGACCGCGCGGATTTTCTCTCGCGCGCTTTTCTTATCGCTTCACAGATCCAAATAMAGCTTACCATTCGCGGCATTGAATCCCGCTTTTCMAAATGT

 3801 CCTCATCATATGCTCTGAGAAAGAAATCGTGGTGGATCGCTGAATCGGAATCCGCTTTTCTGATAGAGGGAAGCTGAATGAATGCTCGAATTC

FIG. 2—Continued.

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 PS207 and PS208 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 $2\times$ 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 $2\times$ 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 $<10^3$ heat-resistant spores per ml. Under these same conditions, the wild-type strain gave $>50\%$ sporulation after 36 h and 5×10^8 heat-resistant spores per ml. With the Sterlini-Mandelstam resuspension medium (24) used to induce sporulation, strains PS2027 and PS2028 gave $<10^5$ heat-resistant spores per ml after 1 to 5 days, while the wild-type strain gave 10^9 heat resistant spores per ml after 18 h.

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 1. Specific activities of PGM and ENO in *E. coli* carrying various plasmids^a

Time of harvest	Sp act (μmol/ml · mg of protein) with:					
	pUC19		pML3 ^b		pML5 ^c	
	PGM	ENO	PGM	ENO	PGM	ENO
Logarithmic phase	0.32	0.54	0.27		4.1	24
Early stationary phase	0.41	0.58	0.35	0.85	6.7	
Overnight growth						
Without MnCl ₂	0.38	0.67	0.65	2.9	3	18
With MnCl ₂					4	

^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.

FIG. 4. Western blot analysis of wild-type and *pgm* and *eno* mutants of *B. subtilis*. Strains PS1804 (wild type), PS2027 (Δ *eno* Δ *pgm*), and PS2028 (Δ *pgm*) were grown to late log phase (optical density at 600 nm of 0.8 to 1.9) in 2 $\times$ YT medium plus L-malate. Cells were harvested by centrifugation, and extracts were prepared as described in Materials and Methods. Aliquots of 2 μ g of protein were examined by SDS-polyacrylamide gel electrophoresis, proteins were transferred to polyvinylidene fluoride paper, and PGM or ENO was detected immunologically as described in Materials and Methods. (A) Samples reacted with anti-PGM; (B) samples reacted with anti-ENO. Lanes: 1, wild type; 2, Δ *eno* Δ *pgm* mutant; 3, Δ *pgm* mutant. The horizontal arrows in panels A and B denote the migration positions of purified PGM and ENO, respectively.

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

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 exhibits 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 *Antithamion* 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

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

Strain ^b	Doubling time (min) ^c with the following addition(s) to the medium:			
	None	Glucose	Malate	Glucose plus malate
Wild type	29 (7.5)	21 (9)	28 (10.5)	21 (12)
Δ <i>pgm</i> mutant	115 (0.6)	210 (0.7)	95 (3.8)	105 (3.9)
Δ <i>eno</i> Δ <i>pgm</i> mutant	175 (0.5)	>300 (0.5)	120 (1.8)	125 (2.4)

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

^b The Δ *pgm* and Δ *eno* Δ *pgm* 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 Mn^{2+} -deficient media results in inhibition of PGM and poor growth on glucose. While malate supplementation stimulates growth on Mn^{2+} -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 Mn^{2+} -containing medium (6). However, as was found previously by using Mn^{2+} -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 PGM not subject to the regulation of *B. subtilis* PGM (for example the DPG- and Mn^{2+} -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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