JOURNAL OF BACTERIOLOGY, Oct. 1995, p. 5899–5905 Vol. 177, No. 20
0021-9193/95/$04.00+0
Copyright © 1995, American Society for Microbiology

Synthesis of *sn*-Glycerol 3-Phosphate, a Key Precursor of Membrane Lipids, in *Bacillus subtilis*

HÉCTOR RICARDO MORBIDONI,[1] DIEGO DE MENDOZA,[2] AND JOHN E. CRONAN, JR.[1,3]*

Departments of Microbiology[1] and Biochemistry,[3] University of Illinois, Urbana, Illinois 61801, and Departmento de Microbiología, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, 2000 Rosario, Argentina[2]

Received 22 May 1995/Accepted 6 August 1995

The *Bacillus subtilis gpsA* gene was cloned by complementation of an *Escherichia coli gpsA* strain auxotrophic for *sn*-glycerol 3-phosphate. The gene was sequenced and found to encode an NAD(P)H-dependent dihydroxyacetone phosphate reductase with a deduced molecular mass of 39.5 kDa. The deduced amino acid sequence showed strong conservation with that of the *E. coli* homolog and to other procaryotic and eucaryotic dihydroxyacetone phosphate reductases. The physical location of *gpsA* on the *B. subtilis* chromosome was at about 200°. Disruption of the chromosomal *gpsA* gene yielded *B. subtilis* strains auxotrophic for glycerol, indicating that the *gpsA* gene product is responsible for synthesis of the *sn*-glycerol 3-phosphate required for phospholipid synthesis. We also found that transformation of the classical *B. subtilis* glycerol auxotrophs with a *gpsA*-containing genomic fragment yielded transformants that grew in the absence of glycerol. In agreement with prior work, our attempts to determine the reductase activity in *B. subtilis* extracts were unsuccessful. However, expression of the *B. subtilis gpsA* gene in *E. coli* gave reductase activity that was only slightly inhibited by *sn*-glycerol 3-phosphate. Since the *E. coli* GpsA dihydroxyacetone phosphate reductase is very sensitive to allosteric inhibition by *sn*-glycerol 3-phosphate, these results indicate that the *B. subtilis gpsA*-encoded reductase differs from that of *E. coli*. It seems that *B. subtilis* regulates *sn*-glycerol 3-phosphate synthesis at the level of gene expression rather than through the *E. coli* mechanism of strong allosteric inhibition of an enzyme produced in excess.

Our knowledge of lipid metabolism in *Bacillus subtilis* is scant. Although this bacterium is often considered the paradigm gram-positive organism, the mechanisms involved in lipid biosynthesis have been little studied and much is argued by analogy with *Escherichia coli* (11). A possible exception to agreement with the *E. coli* paradigm was the synthesis of the key phospholipid precursor, *sn*-glycerol 3-phosphate (G3P). The lipid composition of *B. subtilis* suggested that G3P is a key intermediate in the synthetic pathway, and in 1970 this premise was confirmed by Mindich (25), who reported the isolation and characterization of a mutant (Gly$^-$) that required supplementation with glycerol for growth. Starvation of Gly$^-$ strains for glycerol blocked phospholipid synthesis (26) in a manner similar to that seen upon starvation of a class of *E. coli* G3P auxotrophs called *gpsA* (5, 6, 10, 13). These *E. coli* mutants lack the enzyme that catalyzes the NAD(P)H-dependent reduction of dihydroxyacetone phosphate (DHAP) to G3P [NAD(P)H-dependent DHAP reductase has also been called the biosynthetic G3P dehydrogenase and G3P synthase]. Although the *B. subtilis* Gly mutant had the phenotype expected of a *gpsA* mutant, no conversion of DHAP to G3P could be detected in extracts of either wild-type or mutant cells, thus raising the possibility that G3P synthesis proceeded by a different mechanism in this organism. A possible alternative mechanism would be the reduction of a glycolytic intermediate other than DHAP, such as glyceraldehyde-3-phosphate or 3-phosphoglycerate. Although the enzymatic defect of the Gly$^-$ mutant was unknown, this strain was used by Mindich (26) in pioneering studies of coupling between phospholipid biosynthesis and the synthesis of fatty acids and membrane proteins. Later, Freese and coworkers (15, 16, 28) used derivatives of the Gly$^-$ strain (renamed Gol$^-$) to study effects of the balance between G3P synthesis and catabolism on cell membrane integrity and sporulation proficiency. These observations showed that a delicate balance between synthesis and catabolism of G3P was necessary for growth and sporulation of *B. subtilis.*

In this paper we report that *B. subtilis* synthesizes G3P by a pathway and enzyme analogous to those of *E. coli*, although the enzyme activity is not regulated by the same mechanism and is synthesized at levels below those detectable by the standard enzyme assay.

MATERIALS AND METHODS

Materials. Dihydroxyacetone phosphate, G3P, NADPH, ampicillin, chloramphenicol, and erythromycin were obtained from Sigma Chemical Co. Restriction enzymes, DNA modifying enzymes and molecular weight markers (protein and DNA) were purchased from Gibco BRL and used according to the supplier's recommendations. Amersham was the source of [α-^{32}P]dCTP (specific activity, 3,000 Ci/mmol) and ^{35}S-α-dATP (specific activity, 1,000 Ci/mmol). ICN was the supplier of [^{35}S]methionine (specific activity, 1,139 Ci/mmol).

Bacterial strains, plasmids, and phages. The bacterial strains, plasmids and phages used are listed in Table 1. All *E. coli* strains were derivatives of strain K-12, except for the *E. coli* B strain BL21(λDE3) used for overexpression studies. Strains DH5α, JM109, and LE392 (30) were used as standard host strains for transformation. All *B. subtilis* strains were derivatives of strain JH642, except strain B42.

Media and bacterial growth. *E. coli* and *B. subtilis* strains were routinely grown in Luria-Bertani (LB) broth (30). LB agar was used as the solid medium for *E. coli*, and TBAB (Difco) containing 33 g of tryptose blood agar base (Difco) per liter was used for the propagation of *B. subtilis*. The minimal medium used for *E. coli* was M9 (24) supplemented with 0.4% glucose, 0.2% casein hydrolysate, 10 mM $MgSO_4$, and 0.01% thiamine. Spizizen salts (1) with the appropriate supplements (0.4% glucose, 0.2% casein hydrolysate, or 25 μg of the required L-amino acids per ml, when needed) was used as the minimal medium for *B. subtilis*. Glycerol and racemic G3P were used at final concentrations of 25 μg/ml and 0.1%, respectively. Antibiotics were added to media as follows (in micrograms per milliliter): ampicillin, 100; chloramphenicol, 30 (for *E. coli*) and 5 or

* Corresponding author. Mailing address: Department of Microbiology, Burrill Hall, University of Illinois at Urbana-Champaign, S. Goodwin Ave., Urbana, IL 61801. Phone: (217) 333-0425. Fax: (217) 244-6697.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)[a]	Source or reference[b]
E. coli		
BB20-14	*gpsA20 glpD phoA8* λ	Laboratory stock
JA200	F^+ *recA* Δ*trpE5*	CGSC
B. subtilis		
168	*trpC2*	Laboratory stock
JH642	*trpC2 pheA1*	BGSC
B42	*gly glpD ind*	25
61106	*gol metC trpC2*	BGSC
1A476	*met recA*::ery	BGSC
MBU1	JH642 *gpsA*::Cm	This work
MBU2	61006 *recA*::Ery	This work
Plasmids		
pHSG575	Cm^r	34
pHB201	*E. coli-B. subtilis* shuttle vector Cm^r Ery^r	BGSC
pJM103	Integrative cloning vector Ap^r Cm^r	J. Hoch
pBAD18	*E. coli* expression vector Ap^r	J. Beckwith via S. Maloy
pRMU15	pHGS575 carrying a 6.7-kbp *Eco*RI fragment from λpRM2, Cm^r *gpsA*$^+$	This work
pRMU45	Tn*1000* insertion in pRMU15, *gpsA*$^+$	This work
pRMU62	Tn*1000* insertion in pRMU15, *gpsA* mutant	This work
pRMU108	637-bp *Hin*dIII-*Acc*I fragment from pRMU15 cloned into pJM103, Ap^r Cm^r	This work
pRMU117	1.44-kbp *Eco*RI-*Bam*HI fragment cloned in pGEM7z(+), Ap^r *gpsA*$^+$	This work
pRMU121	1.56-kbp *Bam*HI-*Hin*cII fragment from pRMU15 cloned into pHB201	This work
pRMU127	1.5-kbp *Eco*RI-*Xba*I fragment cloned into pBAD18, Ap^r *gpsA* expression under arabinose control	This work
pRMU132	Filled *Acc*I site in pRMU117, Ap^r	This work
pRMU134	1.67-kbp *Hin*cII fragment from pRMU15 cloned in pBluescript KSII(−) T7 promoter	This work
pRMU135	1.67-kbp *Hin*cII fragment from pRMU15 cloned in pBluescript KSII *lacZ* promoter	This work
pRMU136	1.44-kbp *Eco*RI-*Bam*HI fragment from pRMU132 cloned into pBluescript KSII(−)	This work

[a] Ap^r, Cm^r, and Ery^r denote resistance to ampicillin, chloramphenicol, and erythromycin, respectively

[b] CGSC and BGSC denote strains obtained from the *E. coli* Genetic Stock Center and the *Bacillus* Genetic Stock Center, respectively.

10 (for *B. subtilis*) (chromosomal *cat* insertion or plasmid, respectively); and erythromycin, 1 (for *B. subtilis*). Bacterial growth was monitored by measuring turbidity with a Klett-Summerson colorimeter (green filter) calibrated by determining the number of CFU per milliliter as a function of colorimeter units.

DNA methods. The *B. subtilis* 168 chromosomal DNA library in vector λ charon 4A was previously described (14). Transducing lysates were prepared (2), and lambda-mediated transductions were done as described previously (32). λ DNA was purified by Qiagen columns (Qiagen Inc., Chatsworth, Calif.) according to the instructions of the supplier. Extraction of high-molecular-weight *B. subtilis* DNA was done according to the method of Hoch (19). *B. subtilis* total RNA was obtained by a whole-cell lysate method (4) from log-phase cultures grown in 2× yeast extract-tryptone medium supplemented with chloramphenicol (5 μg/ml) and glycerol (25 μg/ml) when necessary. For manipulations of DNA molecules standard methods were used (30). Large-scale plasmid preparations were made by using Qiagen columns, and DNA fragment elution from agarose gels was done with the Qiaex gel extraction kit. Southern and Northern (RNA) blots were carried out according to the method of Sambrook et al. (30). DNA probes were synthesized by using a random primer DNA labeling kit (Amersham) and [α-^{32}P]dCTP (3,000 Ci/mmol), purified by column chromatography (Clontech, Palo Alto, Calif.), and detected by exposing the nylon membranes to X-ray film or a PhosphoImager screen (Molecular Dynamics, Sunnyvale, Calif.).

Physical mapping of the *B. subtilis gpsA* gene was done by Southern hybridization on a membrane containing an ordered array of YAC clones carrying *B. subtilis* DNA (the generous gift of V. Azevedo and S. D. Ehrlich) according to standard protocols (3). Plasmids in which the insertion of Tn*1000* abolished the *gpsA* complementing activity were mapped by restriction digestion. Two such plasmids were modified for DNA sequencing by digestion with *Sal*I followed by religation at low DNA concentrations. This resulted in deletion of one of the ends of Tn*1000* together with part of the insert in the plasmid and allowed the use of a sequencing primer complementary to the Tn*1000* terminal repeat (9). DNA sequencing was done by the dideoxy chain termination method by using a Sequenase version 2.0 kit (United States Biochemical Corp.). Custom-made primers (synthesized at the Genetic Engineering Facility, University of Illinois at Urbana-Champaign) were used for further sequencing of both strands of the *gpsA* gene with *Taq* DNA polymerase cycle sequencing of plasmid DNA and an Applied Biosystems 373 DNA sequencer and DNASTAR software.

Plasmid pRMU15 was constructed by ligation of a 6.7-kbp *Eco*RI fragment from λpRM2 into *Eco*RI-digested pHGS575. A 1.67-kbp *Hin*cII fragment from pRMU15 was cloned in both orientations into pBluescript KSII(−) digested with *Hin*cII to give plasmids pRMU134 and pRMU135. Plasmid pRMU121 was constructed by ligation of a 1.56-kbp *Bam*HI-*Hin*cII fragment from pRMU15 into the *E. coli-B. subtilis* shuttle vector pHB201 (29) digested with *Bam*HI and *Eco*RV. Plasmid pRMU117 was constructed by ligation of a 1.44-kbp fragment containing *gpsA* and adjacent sequences obtained by PCR amplification from the chromosome of *B. subtilis* into pGEM7z(+). The primers used were 5′-TAGCG GAATTCGGGGACATACTCTG-3′ and 5′-CATTGGATCCCAAGACTTGG GTG-3′ (*Eco*RI and *Bam*HI restriction sites were introduced at the positions underlined). The fragment obtained was gel purified, digested with *Eco*RI and *Bam*HI, and ligated into *Eco*RI-*Bam*HI-digested pGEM7z(+) to give plasmid pRMU117.

Genetic methods. The chromosomal copy of *gpsA* was disrupted by first ligating a 637-bp *Acc*I-*Hin*dIII fragment of pRMU15 into the integrative vector pJM103 (12). The resulting plasmid, pRMU108, was transformed into *E. coli* LE392 to obtain multimeric plasmids which were used to transform *B. subtilis* JH642. Transformants resistant to chloramphenicol were selected on TBAB-glycerol-chloramphenicol plates and subsequently scored for glycerol auxotrophy by replica plating onto chloramphenicol-minimal salts medium with or without glycerol. Competent *B. subtilis* cultures were obtained by the two-step method of Dubnau and Davidoff-Abelson (12). Insertions of Tn*1000* into plasmids were isolated by bacterial conjugation as described by Guyer (17). *E. coli* JA200 was transformed with pRMU15 and then mated with strain BB20-14. Colonies resistant to both chloramphenicol and streptomycin were selected on LB plates supplemented with 0.1% G3P, and their *gpsA* phenotypes were scored on minimal medium with or without G3P.

Expression analysis. Plasmid pRMU117 containing the *B. subtilis gpsA* gene under T7 promoter control was transformed into *E. coli* BL21(λDE3). Induction of the gene expression was done according to the method of Studier et al. (33). Briefly, 2 ml of mid-log-phase cultures carrying the relevant plasmids were induced by adding 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). After 10 min, half of the culture was labeled by adding 5 μl of [^{35}S]methionine (specific activity, 1,000 Ci/mmol; ICN) followed by an additional 10-min incubation. The cells were harvested by centrifugation, washed twice with 10 mM Tris-HCl buffer (pH 7.4), and stored frozen until analysis. The remaining half of each culture received rifampin (200 μg/ml) to inhibit mRNA synthesis from the *E. coli* chromosome, was incubated at 37°C with shaking for 1 h, and then was labeled with [^{35}S]methionine as described above. The labeled proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and detected by autoradiography or exposed to a PhosphoImager screen (Molecular Dynamics).

NAD(P)H-dependent DHAP reductase assay. NAD(P)H-dependent DHAP reductase (biosynthetic G3P dehydrogenase) activity was assayed at 25°C with a Beckman DU64 spectrophotometer as previously described (13, 20). The assay was performed in a total volume of 1 ml, which contained 0.1 M Tris-HCl buffer (pH 7.4), 1 mM dithiothreitol, 1 mM DHAP, 0.1 mM NADPH, and up to 100 μg of cell extract protein. The assay was based on the DHAP-dependent oxidation of NADPH and consisted of two stages. The background of NADPH oxidation was first determined in reaction mixtures lacking DHAP. DHAP was then added, and the reductase activity was measured. The assay was linear with protein concentration and time. Inhibition by the G3P addition was determined by adding increasing amounts of G3P to the assay and monitoring the rate of NADPH oxidation. Cell extracts for enzymatic activity assays were grown in LB medium to mid-log phase, collected by centrifugation, washed once with 20 mM Tris-HCl buffer (pH 7.4), and lysed by passage through a French pressure cell.

The lysate was clarified by centrifugation and stored frozen until assay. In other experiments, membrane-bound NADPH oxidase activity was removed by ultracentifugation of cell extracts. The protein contents were determined by the method of Bradford (7).

RESULTS

Cloning and mapping of a *B. subtilis* gene encoding NAD(P)H-dependent DHAP reductase. To explore the mechanism of G3P synthesis in *B. subtilis*, we attempted to complement the defect of an *E. coli gpsA* strain. Our rationale was that, given a single gene (or linked genes) that encoded the G3P synthetic enzyme of this organism, expression of the gene(s) in *E. coli* should bypass the *gpsA* mutation and allow growth regardless of the glycolytic intermediate utilized by the *B. subtilis* enzyme. A *B. subtilis* 168 chromosomal library in λ Charon 4A (the generous gift of J. Hoch) was used to infect *E. coli* BB20-14, a strain unable to synthesize G3P because of a mutant *gpsA* gene encoding an inactive NAD(P)H-dependent DHAP reductase (5, 6). Strain BB20-14 is a λ lysogen, and thus complementation would involve homologous recombination between a library phage and the resident λ prophage. Several colonies that gained the ability to grow on minimal medium lacking G3P were isolated, purified, and then induced with mitomycin C to obtain transducing lysates. The resulting lysates transduced strain BB20-14 to protrotrophy at high frequency. Clear plaque phages (λ charon 4A lacks the λ*c*I repressor gene) from one of these lysates were plaque purified and again tested for complementation. A phage clone isolated in this way was denoted λpRM2. *Eco*RI digestion of λpRM2 gave two DNA fragments with sizes of 1.5 and 6.7 kbp. Cloning of the smaller *Eco*RI fragment into pBluescript KSII gave recombinant plasmids that failed to complement strain BB20-14. Several attempts to ligate the larger fragment into vectors of high or medium copy number gave only unstable complementing clones. After further growth, these clones accumulated plasmids having large deletions of the insert DNA and lost the ability to complement strain BB20-14. Finally, we cloned the larger *Eco*RI fragment into pHSG575, a low-copy-number plasmid derived from pSC101 (34), to give plasmid pRMU15. This plasmid readily transformed BB20-14 to G3P protrotrophy, showing that it contained an intact and functional gene. The *B. subtilis* chromosomal DNA insert of pRMU15 was stable in both strains BB20-14 and DH5α. The physical location of the complementing gene within the insert was then located by restriction mapping and Tn*1000* mutagenesis (17). Several Tn*1000* insertions that failed to complement BB20-14 were mapped by restriction digests. A derivative of one of these insertion plasmids, pRMU45, was used as template for sequencing with a primer complementary to the Tn*1000* terminal inverted repeat (9). The sequence obtained gave a deduced amino acid sequence similar to that of the C-terminal end of the *gpsA* encoded NAD(P)H-dependent DHAP reductase (biosynthetic G3P dehydrogenase) of *E. coli*. Further DNA sequencing by primer walking resulted in a 2.2-kbp DNA sequence which contained only two open reading frames (ORFs) (Fig. 1) of significant length. Partial sequence of the upstream ORF gave a deduced amino acid sequence similar to that of GTP-binding protein from *Mycobacterium leprae* (8; GenBank accession number U00021) and was not further studied.

The downstream ORF (ORF2) was completely sequenced on both strands and contained two possible translation initiation sites at positions 778 and 970, the latter being that consistent with sequence conservation with the *E. coli* GpsA protein. Preceding this ATG by 12 nucleotides we found a potential weak *B. subtilis* ribosome binding site. ORF2 was 1,037 nucleotides in length, ending at position 1815 with a TAA codon. No obvious *B. subtilis* sigma A-type promoter sequence was found upstream of the proposed start codon for the ORF (Fig. 2). This finding and the close proximity between

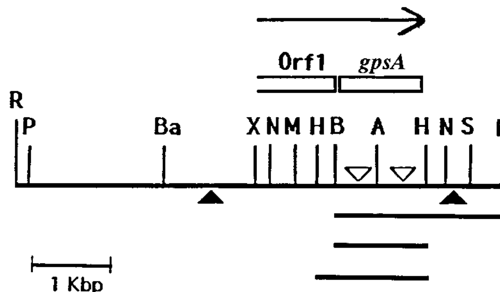

FIG. 1. Physical map of *B. subtilis* chromosomal region encoding *gpsA*. The longest solid line represents the 6.7-kbp *Eco*RI-*Eco*RI fragment cloned during this study. The restriction map shows the most relevant restriction sites. R, *Eco*RI; S, *Sal*I. N, *Nco*I; H, *Hinc*II; A, *Acc*I; B, *Bam*HI; M, *Mlu*I; X, *Xma*III; Ba, *Bal*I; P, *Pst*I. The location of the ORFs is indicated by open rectangles; ORF1 (partially sequenced) encodes a putative GTP-binding protein and *gpsA*. The direction of transcription is shown by arrow above the ORFs. Tn*1000* insertions that lack or retain complementation activity are displayed as open inverted and solid triangles, respectively, and the DNA fragments that conferred Gly$^-$ (Gol$^-$) mutant strains with the ability to grow in the absence of glycerol (because of recombinational rescue) are shown by the bars beneath the map (the full-length *Eco*RI-*Eco*RI fragment also rescued the mutant).

```
   1 CCAATTGTCGGAAATCTTCACTTGTGAATGCGATGCTCGGCGAAGAACGCGTTATTGTCAGCAACGTGGCTGAACGACAA   80
  81 GAGATGCTGTTGATACGTCATTTACTTACAACCAGCAGGAGTTTGTCATTGTCGATACTGCAGGTATGCGAAAAAAAGGG  160
 161 AAAGTCTATGAAACGACTGAGAAGTATAGTGTACTGCGGGCGCTAAAAGCGATTGACCGCTCAGAAGTCGTGGCGGTTGT  240
 241 GCTGGATGGCGAAGAAGGCATTATTGAACAGGACAAGCGTATCGCCGGTTATGCACACGAAGCGGGCAAGGCCGTCGTCA  320
 321 TCGTCGTAAACAAATGGGATGCTGTTGACAAAGATGAGAGCACGATGAAAGAATTTGAAGAAAATATTCGCGATCATTTT  400
 401 CAATTTCTGGATTATGCGCCAATCCTATTTATGTCTGCCTTAACGAAAAAACGGATCCATACTCTGATGCCTGCGATTAT  480
 481 CAAAGCTAGTGAAAATCATTCACTTCGAGTTCAAACAAACGTCTTAAATGATGTCATCATGGACGCTGTGGCAATGAATC  560
 561 CGACACCGACTCATAACGGTTCTCGTTTGAAAATTTACTATGCGACTCAAGTGTCGGTAAAGCCGCCAAGCTTCGTTGTG  640
 641 TTTGTAAACGATCCGGAACTGATGCATTTTTCATACGAACGGTTTTTAGAAAACCGAATCAGAGACGCGTTCGGTTTTGA  720
 721 GGGGACACCAATCAAAATATTTGCAAGAGCTAGAAAATAAAAAGGTGTGAATCAAACATGAAAAAAGTCACAATGCTTGG  800
                                                                  M  K  K  V  T  M  L  G
 801 AGCGGGGAGTTGGGGAACAGCACTGGCTTTAGTTCTAACTGATAATGGAAATGAAGTGTGTGTGTGGGCTCACCGTGCAG  880
      A  G  S  W  G  T  A  L  A  L  V  L  T  D  N  G  N  E  V  C  V  W  A  H  R  A  D
 881 ATTTAATTCATCAAATTAATGAGTTGCATGAAAACAAAGATTATTTGCCGAATGTTAAGCTGTCTACATCCATTAAAGGA  960
       L  I  H  Q  I  N  E  L  H  E  N  K  D  Y  L  P  N  V  K  L  S  T  S  I  K  G
 961 ACAACAGATATGAAAGAGGCTGTTTCAGACGCAGATGTCATTATCGTTGCGGTCCCGACAAAAGCAATTCGGGAAGTGCT 1040
     T  T  D  M  K  E  A  V  S  D  A  D  V  I  I  V  A  V  P  T  K  A  I  R  E  V  L
1041 GAGACAGGCTGTCCCTTTTATAACGAAAAAGGCAGTCTTTGTTCATGTCAGCAAGGGTATTGAGCCAGATTCACTTCTGC 1120
      R  Q  A  V  P  F  I  T  K  K  A  V  F  V  H  V  S  K  G  I  E  P  D  S  L  L  R
1121 GCATTTCTGAAATTATGGAAATTGAGCTCCCGAGTGATGTCAGAAGAGATATCGTTGTCCTTTCCGGCCCGAGTCATGCG 1200
       I  S  E  I  M  E  I  E  L  P  S  D  V  R  R  D  I  V  V  L  S  G  P  S  H  A
1201 GAAGAAGTAGGGCTGCGGCACGCCACAACTGTTACTGCATCTTCAAAGAGCATGAGGGCAGCAGAAGAGGTGCAGGATCT 1280
     E  E  V  G  L  R  H  A  T  T  V  T  A  S  S  K  S  M  R  A  A  E  E  V  Q  D  L
1281 ATTTATTAATCACAATTTTCGGGTGTACACAAATCCTGACATTATCGGAGTTGAAATCGGAGGGGCTTTAAAAAATATTA 1360
      F  I  N  H  N  F  R  V  Y  T  N  P  D  I  I  G  V  E  I  G  G  A  L  K  N  I  I
1361 TTGCCCTTGCTGCAGGAATTACAGATGGTTTAGGGTACGGTGACAATGCCAAAGCTGCTTTGATTACACGCGGACTTGCC 1440
       A  L  A  A  G  I  T  D  G  L  G  Y  G  D  N  A  K  A  A  L  I  T  R  G  L  A
1441 GAAATCGCGAGACTCGGAACGAAAATGGGCGGAAATCCCTTGACGTTCTCTGGATTGACAGGAGTAGGCGATCTGATTGT 1520
     E  I  A  R  L  G  T  K  M  G  G  N  P  L  T  F  S  G  L  T  G  V  G  D  L  I  V
1521 GACGTGCACAAGTGTTCATTCCAGAAACTGGCGTGCGGGCAATTTGCTCGGAAAAGGGTACAAGCTTGAAGATGTTCTTG 1600
      T  C  T  S  V  H  S  R  N  W  R  A  G  N  L  L  G  K  G  Y  K  L  E  D  V  L  E
1601 AAGAGATGGGAATGGTAGTCGAAGGCGTCCGCACGACGAAAGCGGCTTATCAGCTTTCGAAGAAATATGATGTTAAAATG 1680
       E  M  G  M  V  V  E  G  V  R  T  T  K  A  A  Y  Q  L  S  K  K  Y  D  V  K  M
1681 CCGATTACAGAAGCTCTCCATCAGGTCCTATTCAACGGACAAAAAGTGGAAACCGCTGTTGAATCTTTAATGGCGAGAGG 1760
     P  I  T  E  A  L  H  Q  V  L  F  N  G  Q  K  V  E  T  A  V  E  S  L  M  A  R  G
1761 GAAAACCCACGAGATGGAGGATTTGGTAAATACGTTTGAAAATCAAGTGAAGTAAAAGTGTCAATCAAATGGTGAATCGC 1840
      K  T  H  E  M  E  D  L  V  N  T  F  E  N  Q  V  K  *
1841 ATATCTTAATTGAACGAAAGCCCGAAAAACAGAAGAACACCCAAGTCTTGGGATCTCCTGAAACATTTTGCCGATTTAGG 1920
1921 CAATAGAGGATGCATCTGTATGCCAAGTCGCATACTATAGCATCGAAGTCCAAGAGAGTGTTTCTTAAACGTGCAGGTGA 2000
2001 ATATAGTCAAGTTGACTGAAGCGAAGGTCCCCCTCTTTGCTTCAGTTTTTTTGTTCTATCAATGGATGGAAAATCTGGTT 2080
2081 GATCTTTTCCGGCGCTGTATATTAAAATATAATATAACGCTTATATCTTACATATCGGGGAGGCGCACGTCATTGAATCT 2160
2161 GGCATTGATGAAAATGTCGGTTTGCTCTAGGGTCCATGGGTCCTGATGTTTCTGGCCTGTAGCTTCCATCTATTTAAAGC 2240
2241 CGCTTT  2246
```

FIG. 2. Nucleotide and deduced amino acid sequence of *B. subtilis gpsA*. The predicted amino acid sequence is shown below the DNA sequence. A putative ribosomal binding site for *gpsA* is underlined and a putative translational start site is shown in boldface type. The GenBank accession number is U32164.

```
B subt    M K K V - - - - T M L G A G S W G T A L A L V L T - - - D N G N E V C V W A H R A D - L I H Q I N E  42
E coli    M N Q R N A S M T V I G A G S Y G T A L A I T L A - - - R N G H E V V L W G H D P E - H I A T L E R  46
Dros      - - - - - N W G S A I A K I V G A N A A A L P E - F E K R V T M F V Y E E M I D G K K L T E I I N E  57
S. cerev  V I G S G N W G T T I A K V V A E N C K G Y P E V F A P I V Q M W V F E E E I N G E K L T E I I N T  50

B subt    L H E N K D Y L P N V K L S T S I K G T T D M K E A V S D A D V I I V A V P T K A I R E V L R Q A V  92
E coli    D R C N A A F L P D V P F P D T L H L E S D L A T A L A A S R N I L V V V P S H V F G E V L R Q I K  96
Dros      T H E N V K Y L K G H K L P T N V V A V P D L V E A A K N A D I L I F V V P H Q F I P N F C K Q L L  107
S. cerev  R H Q N V K Y L P G I T L P D N L V A N P D L I D S V K D V D I I V F N I P H Q F L P R I C S Q L K  100

B subt    P F I T K K A V F V H V S K G I E P D S L L R I S E I M E I E L P S D V R R D I V V L S G P S H A E  142
E coli    P L M R P D A R L V W A T K G L E A E T G - R L L Q D V A R E A L G D Q I P L A V I - S G P T F A K  144
Dros      G K I K P N A I A I S L I K G F D K A E G G G I D L I S H I I T R H L K I P C A V - L M G A N L A N  156
S. cerev  G H V D S H V R A I S C L K G F E V G A K G V Q - L L S S Y I T E E L G I Q C G A - L S G A N I A T  148

B subt    E V - - G L R H A T T V T A S S K S M R A A E E V Q - - - - - - - - D L F I N H N F R V Y T N P D I I  183
E coli    E L A A G L P T A I S L - A S T D Q T F A D D L Q Q - - - - - - - - L L H C G K S F R V Y S N P D F I  186
Dros      E V A E G N F C E T T I G C T D K K Y - - - - G K - - - - - - V L R D L F Q A N H F R V V V V E D A D  197
S. cerev  E V A Q E H W S E T T V A Y H I P K D F R G E G K D V D H K V L K A L F H R P Y F H V S V I E D V A  198

B subt    G V E I G G A L K N I I A L A A G I T D G L G Y G D N A K A A L I T R G L A E I A R L G T K M - - G  231
E coli    G V Q L G G A V K N V I A I G A G M S D G I G F G A N A R T A L I T R G L A E M S R L G A A L - - G  234
Dros      A V E V C G A L K N I V A C G A G F V D G L K L G D N T K A A V I R L G L M E M I R F V D V F Y P G  247
S. cerev  G I S I C G A L K N V V A L G C G F V E G L G W G N N A S A A I Q R V G L G E I I R F G Q M F F P E  248

B subt    G N P L T F S G L - T G V G D L I V T C T S V H S R N W - R A G N L L G K G Y K L E D V L E E M G M  279
E coli    A D P A T F M G M - A G L G D L V L T C T D N Q S R N R - R F G M M L G Q G M D V Q S A Q E K I G Q  282
Dros      S K L S T F F - E S C G V A D L I T T C Y G G R N R R V S E A F V T S G K T I E D L E K E M L N G Q  296
S. cerev  S R E E T Y Y Q E S A G V A D L I T T C A G G R N V K V A R L M A T S G K D A W E C E K E L L N G Q  298

B subt    V V E G V R T T K A A Y Q L S K K Y D V K M P I T E A L H Q V L F N G Q K V E T A V E S L M A R G K  329
E coli    V V E G Y R N T K E V R E L A H R F G V E M P I T E E I Y Q V L Y C G K N A R E A A L T L L G R A R  332
Dros      K L Q G P P T A E E V N Y M L K N K G L E D K F P L F T A I H K I C T N Q L K P K D L I D C I R N H  346
S. cerev  S A Q G L I T C K E V H E W L E T C G S V E D F P L F E A V Y Q I V Y N N Y P M K N L P D M I E E L  348

B subt    T H E M E D L V N T F E N Q V K .                                                                  346
E coli    K D E R S S - - - - - - - - - - H                                                                  339
Dros      P E H M Q T L                                                                                      353
S. cerev  D L H E D                                                                                          353
```

FIG. 3. Sequence alignment of *B. subtilis* GpsA and homologous proteins. The deduced amino acid sequences were aligned with the multiple alignment (Clustal method) feature of the DNASTAR software package. The numbers indicate positions in the amino acid sequences. Dashes within the sequences indicate gaps introduced to give optimal alignments. Residues identical to *B. subtilis* GpsA are boxed. Designations (GenBank accession numbers in parentheses) are B. subt, *B. subtilis*, (U32164); E. coli, *E. coli* GpsA (U00039); S. cerv, *S. cerevisiae* (Q00055); and Dros, *Drosophila virilis* (S31790).

the stop codon of the upstream ORF and the putative ribosome binding site of ORF2 suggested that the two ORFs are cotranscribed. Subcloning of ORF2 (plus flanking sequences) in both orientations into several *E. coli* and *B. subtilis* vectors gave plasmids that complemented the *E. coli gpsA* mutant, suggesting that expression in *E. coli* is due to a *B. subtilis* sequence fortuitously acting as an *E. coli* promoter.

Sequence analysis of ORF2 predicted an encoded protein of 346 amino acid residues with a molecular mass of 36.5 kDa. A GenBank search showed that the predicted amino acid sequence was similar to those of several (NAD(P)H-dependent DHAP reductases (G3P dehydrogenases) of both prokaryotic and eukaryotic origin (Fig. 3). The ORF2-encoded protein was virtually identical in size to the *E. coli* GpsA protein, and the two protein sequences could be readily aligned throughout their lengths, with 41% identical residues (Fig. 3). It should be noted that no GXGXXG ADP-binding fold is conserved among the four reductases.

Gene disruption of ORF2 and recombinational repair of classical glycerol auxotrophs. Gene disruption of the chromosomal copy of ORF2 was done by cloning a 637-bp *Acc*I-*Hin*dIII fragment from pRMU15 into the *B. subtilis* integrative vector pJM103 (29). This plasmid (pRMU108) was transformed into *B. subtilis* JH642, and chloramphenicol-resistant colonies were selected on media containing glycerol. All transformant colonies failed to grow in the absence of glycerol. Growth of these auxotrophs was only weakly rescued by the addition of high concentrations of G3P in place of glycerol. This finding is consistent with prior reports by Freese and Oh (15) and Lindgren and Rutberg (22, 23), who found that (unlike *E. coli*) *B. subtilis* transports G3P poorly.

Transformation of a *B. subtilis* Gly$^-$ (Gol$^-$) mutant with several DNA fragments (Fig. 1) excised from pRMU15 resulted in strains that grew in medium lacking glycerol. A small *Bam*HI-*Hin*cII fragment, which contained only ORF2 plus 521 bp of the flanking sequence, gave recombinational rescue of the mutation (Fig. 1). Therefore, we conclude that the mutation present in Mindich's strain B42 (and in the Freese strain 61106) is an allele of the gene that we cloned by complementation of the *E. coli gpsA* mutant. These findings, together with the ability of ORF2 to complement the *E. coli gpsA* mutant and the sequence similarities, indicated that we had cloned the NAD(P)H-dependent DHAP reductase (biosynthetic G3P dehydrogenase) of *B. subtilis* and that the encoded protein was responsible for the production of the G3P required for phospholipid synthesis. We propose that given the marked similarity of the ORF2-encoded protein to the product of the *E. coli gpsA* gene and the analogous role in metabolism, the *gpsA* designation should replace the Gly$^-$ (Gol$^-$) nomenclature. The location of the *gpsA* gene on the physical map of *B. subtilis* was determined by Southern blotting with a mapping membrane containing an ordered array of *B. subtilis* overlapping DNA fragments cloned in a YAC vector (3). Two different fragments from pRMU15 were used as probes, and both hybridized to clones 15-6B and 11-501 of the ordered array, thus giving a map position at about 200° on the *B. subtilis* chromosome (data not shown). This map position is consistent with a prior transductional mapping of glycerol requiring mutants (23).

Expression of the *B. subtilis gpsA* gene in *E. coli*. Analysis of the product of the *B. subtilis gpsA* gene was performed by using the T7 promoter expression system of Studier et al. (33). SDS-PAGE of the [^{35}S]methionine-labeled cell extracts showed expression of two proteins (Fig. 4). The larger of the two proteins

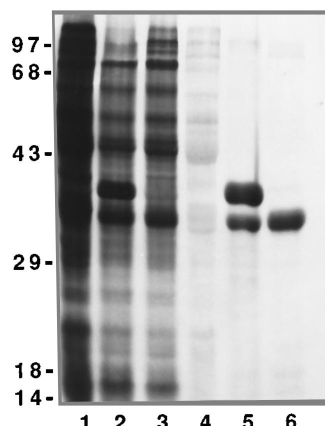

FIG. 4. Expression of *B. subtilis gpsA* gene in *E. coli* BL21(λDE3). Cultures harboring the following plasmids were induced with IPTG and pulse-labeled with [^{35}S]methionine in the absence (lanes 1 to 3) or presence (lanes 4 to 6) of rifampin. Lanes: 1 and 4, pGEM7 vector plasmid; 2 and 5, plasmid pRMU117 carrying the intact *gpsA* gene; 3 and 6, plasmid pRMU132 carrying the frame-shifted *gpsA*. Samples were analyzed by SDS-PAGE. The positions of molecular mass markers (in kilodaltons) are indicated at the left.

had an apparent molecular mass of 39.8 kDa, a value slightly larger than that predicted from the DNA sequence. However, the presence of the smaller protein (apparent molecular mass of 36.3 kDa) suggested that a methionine codon internal to the ORF might function as a second initiation codon. Since a methionine codon with a plausible *E. coli* ribosomal binding site was present at position 970 of the ORF (Fig. 2), the shorter translation product seemed likely to be the product of an aberrant initiation at this site. To test this possibility, a frame-shift was introduced at position 944 by filling in of the unique *Acc*I site in the *gpsA* nucleotide sequence (Fig. 1 and 2). This +2 frameshift should disrupt translation starting at position 778 and thus result in loss of the larger protein, and this was the result observed (Fig. 4). The frame-shifted *gpsA* gene was moved into pBluescript SKII(−) under the transcriptional control of the *lacZ* promoter to test if the shorter protein was functional in complementation of the *E. coli gpsA* mutant. Transformation of this construct (pRMU136) into BB20-14 failed to complement the G3P auxotrophy, indicating that the shorter protein was nonfunctional, a result consistent with the *gpsA* nucleotide sequence data and the protein sequence similarities to other G3P dehydrogenases (Fig. 3).

NAD(P)H-dependent DHAP phosphate reductase activity in *B. subtilis* and *E. coli*. The sequence similarities to known NAD(P)H-dependent DHAP reductases strongly indicated that the *B. subtilis gpsA* gene encoded an enzyme with the same catalytic activity. However, no direct data supported this conclusion. We postulated that the failure to detect reductase activity in *B. subtilis* could be due to very low levels of expression of the gene and/or to the presence of inhibitors of the reaction in the cell extracts. To test the latter possibility we assayed NADH-dependent DHAP reductase in cell extracts from various *E. coli* strains including strains that expressed the *B. subtilis gpsA* gene (Table 2). We also assayed *B. subtilis* 168. In agreement with the findings of Mindich (25), we were unable to detect NADH-dependent DHAP reductase in extracts of wild-type *B. subtilis* strains whereas activity was readily detected in extracts of a wild-type *E. coli* strain in good quantitative agreement with results reported previously. Mixtures of the *B. subtilis* and *E. coli* cell extracts failed to inhibit the *E. coli* activity, indicating that the presence of a reductase inhibitor in

TABLE 2. DHAP reductase activities

E. coli strain[a]	Addition[b]	Sp Act (U/mg of protein)[c]
JM109		10.8
JM109	0.15 mM G3P	4.5
JM109	0.5 mM G3P	3.7
JM109	B. subtilis 168	9.4
BL21(λDE3) pGEM7		11.6
BL21(λDE3) pRMU117		14.7
BL21(λDE3) pRMU117	0.5 mM G3P	8.8
BL21(λDE3) pGEM7 (+ rifampin)		2.8
BL21(λDE3) pRMU117 (+ rifampin)		113.0
BL21(λDE3) pRMU117 (+ rifampin)	0.5 mM G3P	102.8
BL21(λDE3) pRMU117 (+ rifampin)	*B. subtilis* 168	107.3
None	*B. subtilis* 168	<0.1
BB20-14		<0.1
BB20-14 pRMU127 (+ fucose)		<0.1
BB20-14 pRMU127 (+ arabinose)		12.8
BB20-14 pRMU127 (+ arabinose)	0.5 mM G3P	9.3
BB20-14 pRMU127 (+ arabinose)	1.2 mM G3P	8.9
BB20-14 pRMU15		6.0
BB20-14 pRMU15	0.5 mM G3P	4.7

[a] The compounds indicated in parentheses were added to the growth media (see Materials and Methods).
[b] Either G3P at the concentrations indicated or 0.06 mg of *B. subtilis* cell extract protein was added to the assay.
[c] One unit equals 1 nmol of NADPH oxidized per min at 25°C.

B. subtilis cell extracts could be ruled out, unless the putative inhibitor was somehow specific for the *B. subtilis* enzyme. To test this possibility, we used expression in *E. coli* as a source of the *B. subtilis* reductase activity. Expression of the *B. subtilis gpsA* gene from a phage T7 promoter in *E. coli* BL21(λDE3) gave a small increase in the levels reductase activity compared with those in wild-type *E. coli* extracts (Table 2). However, if the induced cells were treated with the *E. coli* RNA polymerase inhibitor rifampin following T7 polymerase synthesis, we found specific activities 5- to sixfold higher than those in wild-type *E. coli* extracts. (We attribute the effect of rifampin to inhibition of *E. coli* chromosomal mRNA synthesis [Fig. 4] which thus increased the number of ribosomes available to translate the mRNA produced by the rifampin-insensitive T7 polymerase). The addition of 1 mM G3P produced only a small decrease in the specific activity of these extracts whereas the addition of 0.5 mM G3P to extracts of a wild-type *E. coli* strain (JM109) produced a large inhibition of reductase activity (Table 2). This latter decrease is explained by the known strong allosteric inhibition of the endogenous *E. coli* enzyme (6, 13, 20) and demonstrated that the enzyme produced by the *B. subtilis gpsA* gene was fundamentally different from the host enzyme (Table 2). Since most of the activity in these extracts was encoded by the *B. subtilis gpsA* gene, we mixed these extracts with extracts of *B. subtilis* to test for the presence of a specific inhibitor. No inhibition was observed (Table 2). We also failed to detect any activity in *B. subtilis* cell extracts following ultracentrifugation (although this treatment substantially reduced the background activity) or ammonium sulfate fractionation.

The *B. subtilis gpsA* gene was also expressed in *E. coli* from the powerful *araBAD* promoter (18). Introduction of a plasmid carrying this construct readily complemented the *E. coli gpsA* mutation in the presence of arabinose but complemented weakly (if at all) in the presence of the anti-inducer fucose. Arabinose induction of the *B. subtilis gpsA* gene in the *E. coli gpsA* strain gave reductase activity slightly higher than that in

wild-type *E. coli* strains, whereas extracts of the strain lacking the plasmid or grown with fucose (rather than arabinose) had no detectable activity (Table 2). The induced activity was almost completely resistant to inhibition by G3P, as expected from the results obtained upon expression from the T7 promoter. Finally, transformation of *B. subtilis* MBU2, a *recA* derivative of the *gpsA* (Gly$^-$) strain 61106, with plasmid pRMU121 (a pHB201 derivative in which *gpsA* was part of a transcriptional fusion with *lacZ*) restored growth in the absence of glycerol, but no enzyme activity could be detected in cell extracts of these transformants.

DISCUSSION

In contrast to the current detailed information on the anabolic and catabolic pathways of G3P metabolism in *E. coli* (10, 21), only the catabolic pathway has received significant study in *B. subtilis* (22, 23, 27). Indeed the route of G3P synthesis in *B. subtilis* was unclear, since no NAD(P)H-dependent DHAP reductase activity could be detected in wild-type cells (25). Thus, the enzymatic defect of mutant strains auxotrophic for glycerol was unknown. Mindich (25) attempted numerous combinations of cell extract preparation methods and assay conditions to detect the enzyme but was unable to detect activity. It therefore seemed possible that the immediate precursor of G3P was a glycolytic intermediate other than DHAP or that the glycerol auxotrophy of these strains was due to a defect in G3P utilization rather than G3P synthesis. An example of the latter possibility is a class of *plsB* mutants of *E. coli* (5, 6, 10) which have a defective G3P acyltransferase with a Michaelis constant 10-fold greater than that of wild-type strains. This decreased G3P affinity engenders a requirement for an elevated G3P pool (supplied by exogenous supplementation plus blockage of G3P catabolism). We have now demonstrated that the enzyme missing in the Mindich (25) glycerol auxotroph is an NAD(P)H-dependent DHAP reductase and have renamed the gene *gpsA* in concordance with *E. coli*. We have also failed to detect the enzyme in *B. subtilis* cell extracts, and (since enzyme inhibition can be ruled out) this situation seems likely to be due to extremely low levels of the enzyme. It should be noted that studies of various strains of *E. coli* suggest that a reductase activity of only 1 to 2 U/mg of protein may allow growth of this organism. Therefore, given the slower growth and lower lipid content of *B. subtilis*, an activity of a few tenths of a unit per milligram of protein (a value approaching the signal-to-noise ratio of the assay) could support lipid biosynthesis in *B. subtilis*. We assume that the low reductase levels result from inefficient gene expression. The *B. subtilis gpsA* gene seems very poorly transcribed since we have been unable to detect a *gpsA* mRNA, using highly radioactive probes. Translation of the *gpsA* mRNA may also be inefficient. The codon usage of *gpsA* is characteristic of genes with low levels of expression (31) and expression of the gene in *E. coli* is strongly stimulated by inhibition of host mRNA synthesis (Table 2), suggesting that the *B. subtilis gpsA* transcript may compete poorly for ribosomes.

G3P inhibits the *E. coli* reductase by two mechanisms: (i) binding to an allosteric site distinct from the active site and (ii) by the simple product inhibition characteristic of enzymatic reactions (13). The allosteric inhibition is much more efficient than the product inhibition and is known to regulate the intracellular G3P concentration. A mutant *E. coli* reductase refractory to allosteric inhibition remains sensitive to product inhibition (13), and since this mutant enzyme has properties similar to those of the *B. subtilis* reductase, we attribute the weak G3P inhibition of the latter enzyme to simple product inhibition. A plausible rationale for low levels of *gpsA* expression in *B. subtilis* is that because of the lack of allosteric inhibition, the NAD(P)H-dependent DHAP reductase levels must be closely regulated.

High-level production of the *B. subtilis* reductase would produce G3P and induce G3P catabolism, resulting in a wasteful cycle of oxidation-reduction. A block in G3P catabolism would avoid this cycle, but accumulation of large G3P pools is known to result in abnormal septation and inhibition of sporulation (15, 16, 28). The decreased sporulation efficiency suggests that this process requires strict coordination between cell membrane phospholipid biosynthesis and cell wall synthesis. Moreover, accumulation of G3P in a mutant defective in G3P catabolism led to membrane collapse, abnormal septation, and inhibition of sporulation. Thus, the *Bacillus* organism's life cycle seems to require close regulation of G3P levels.

E. coli avoids a futile cycle of G3P synthesis-degradation by maintaining a low intracellular G3P concentration through efficient allosteric inhibition of the reductase by its product, G3P (6, 13, 20). Thus, the pool is kept sufficiently low that the degradative enzymes are not induced, while consumption of G3P by phospholipid synthesis partially relieves inhibition of the enzyme, thereby restoring the G3P pool. The validity of this model is strongly supported by the isolation of *gpsA* mutants insensitive to allosteric inhibition (6, 13) which accumulate elevated intracellular G3P pools (6). In contrast, *B. subtilis* must regulate the G3P pool by another means. This organism seems to have chosen the option of expressing only minimal levels of the G3P synthetic enzyme. Given the importance of G3P pools in *B. subtilis* metabolism, it seems surprising that allosteric inhibition does not occur. Perhaps G3P acts as a regulatory ligand of *gpsA* transcription, a possibility that seems worthy of experimental testing.

ACKNOWLEDGMENTS

This work was supported by NIH grant AI15650 and the Rockefeller Foundation. D.d.M. is a Career Investigator from Consejo Nacional de Investigaciones Científicas y Téchicas (CONICET).

REFERENCES

1. **Anagnostopoulos, C., and J. Spizizen.** 1961. Requirements for transformation in *Bacillus subtilis*. J. Bacteriol. **18:**741–746.
2. **Arber, W., L. Enquist, B. Hohn, N. Murray, and K. Murray.** 1983. Experimental methods for use with lambda, p. 433–466. *In* R. W. Hendrix, J. E. Roberts, F. W. Stahl, and R. A. Weisberg (ed.), Lambda II. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
3. **Azevedo, V., E. Alvarez, E. Zumstein, G. Damiani, V. Sgaramella, S. D. Ehrlich, and P. Serror.** 1993. An ordered collection of *Bacillus subtilis* DNA segments cloned in yeast artificial chromosomes. Proc. Natl. Acad. Sci USA **90:**6047–6051.
4. **Barry, T., S. Geary, S. Hannify, C. MacGearailt, M. Shalloo, D. Heery, F. Gannon, and R. Powell.** 1992. Rapid mini-preparations of total RNA from bacteria. Nucleic Acids Res. **20:**4940.
5. **Bell, R. M.** 1973. Mutants of *Escherichia coli* defective in membrane phospholipid synthesis: macromolecular synthesis in an *sn*-glycerol 3-phosphate acyltransferase Km mutant. J. Bacteriol. **117:**1065–1076.
6. **Bell, R. M., and J. E. Cronan, Jr.** 1975. Mutants of *Escherichia coli* defective in membrane phospholipid synthesis: phenotypic suppression of *sn*-glycerol 3-phosphate acyltransferase Km mutants by loss of feedback inhibition of the biosynthetic *sn*-glycerol 3-phosphate dehydrogenase. J. Biol. Chem. **250:** 7153–7158.
7. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
8. **Bron, S.** Personal communication
9. **Cronan, J. E., Jr., W.-B. Li, R. Coleman, M. Narasimhan, D. de Mendoza, and J. M. Schwab.** 1988. Derived amino acid sequence and identification of active site residues of *Escherichia coli* β-hydroxydecanoyl thioester dehydrase. J. Biol. Chem. **263:**4641–4646.
10. **Cronan, J. E., Jr., and C. O. Rock.** 1987. Biosynthesis of membrane lipids, p. 474–497. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*

typhimurium: cellular and molecular biology. American Society for Microbiology, Washington, D.C.

11. **de Mendoza, D., R. Grau, and J. E. Cronan, Jr.** 1993. Biosynthesis and function of membrane lipids, p. 411–421. *In* A. L. Sonenshein, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis* and other gram-positive bacteria: physiology, biochemistry, and molecular genetics. American Society for Microbiology, Washington, DC.
12. **Dubnau, D., and R. Davidoff-Abelson.** 1971. Fate of transforming DNA following uptake by competent *Bacillus subtilis*. 1. Formation and properties of the donor-recipient complex. J. Mol. Biol. **56:**209–221.
13. **Edgar, J. R., and R. M. Bell.** 1978. Biosynthesis in *Escherichia coli* of *sn*-glycerol 3-phosphate, a precursor of phospholipid: kinetic characterization of wild type and feedback-resistant forms of the biosynthetic *sn*-glycerol 3-phosphate dehydrogenase. J. Biol. Chem. **253:**6354–6361.
14. **Ferrari, E., D. J. Henner, and J. A. Hoch.** 1981. Isolation of *Bacillus subtilis* genes from a charon 4A library. J. Bacteriol. **146:**430–432.
15. **Freese, E. B., and Y. K. Oh.** 1974. Adenosine 5′-triphosphate release and membrane collapse in glycerol-requiring mutants of *Bacillus subtilis*. J. Bacteriol. **120:**507–515.
16. **Freese, E., Y. K. Oh, E. B. Freese, M. D. Diesterhaft, and C. Prasad.** 1972. Supression of sporulation of *Bacillus subtilis*, p. 212–221. *In* H. O. Halvorson, R. Hanson, and L. L. Campbell (ed.), Spores V. American Society for Microbiology, Washington, D.C.
17. **Guyer, M. S.** 1983. Uses of transposon γ-δ in the analysis of cloned genes. Methods Enzymol. **101:**362–369.
18. **Guzman, L.-M., D. Belin, M. J. Carson, and J. Beckwith.** 1992. Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. J. Bacteriol. **177:**4121–4130.
19. **Hoch, J. A.** 1991. Genetic analysis in *Bacillus subtilis*. Methods Enzymol. **204:**305–320.
20. **Kito, M., and L. I Pizer.** 1969. Purification and regulatory properties of the biosynthetic *L* glycerol 3-phosphate dehydrogenase of *Escherichia coli*. J. Biol. Chem. **244:**4381–4385.
21. **Lin, E. C. C.** 1987. Dissimilatory pathways for sugars, polyols and carboxylates, p. 244–284. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
22. **Lindgren, V.** 1978. Mapping of a genetic locus that affects glycerol 3-phosphate transport in *Bacillus subtilis*. J. Bacteriol. **133:**667–670.
23. **Lindgren, V., and L. Rutberg.** 1974. Glycerol metabolism in *Bacillus subtilis*: gene-enzyme relationships. J. Bacteriol. **119:**431–442.
24. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
25. **Mindich, L.** 1970. Membrane synthesis in *Bacillus subtilis*. I. Isolation and properties of strains bearing mutations in glycerol metabolism. J. Mol. Biol. **49:**415–432.
26. **Mindich, L.** 1970. Membrane synthesis in *Bacillus subtilis*. II. Integration of membrane proteins in the absence of lipid synthesis. J. Mol. Biol. **49:**433–439.
27. **Nilsson, R. P., L. Beijer, and B. Rutberg.** 1994. The *glpT* and *glpQ* genes of the glycerol regulon in *Bacillus subtilis*. Microbiology **140:**723–730.
28. **Oh, Y. K., E. B. Freese, and E. Freese.** 1973. Abnormal septation and inhibition of sporulation by accumulation of L-α-glycerophosphate in *Bacillus subtilis* mutants. J. Bacteriol. **113:**1034–1045.
29. **Perego, M.** 1993. Integrational vectors for genetic manipulation in Bacillus subtilis, p. 617–624. *In* A. L. Sonenshein, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis* and other gram-positive bacteria: biochemistry, physiology, and molecular genetics. American Society for Microbiology, Washington, D.C.
30. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
31. **Sharp, P. M., D. G. Higgins, D. C. Shields and K. M. Devine.** 1990. Protein-coding genes: DNA sequence database and codon usage, p. 557–569. *In* C. R. Hardwood and S. M. Cutting (ed.), Molecular methods for *Bacillus*. John Wiley & Sons, New York.
32. **Silhavy, T. J., M. L. Berman, and L. W. Enquist.** 1984. Experiments with gene fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
33. **Studier, F. W., A. H. Rosenberg, J. J. Dunn, and J. W. Dubendorff.** 1990. Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol. **185:**60–89.
34. **Takeshita, S., M. Sato, M. Toba, W. Masahashi, and T. Hashimoto-Gotoh.** 1987. High-copy-number and low-copy-number plasmid vectors for *lacZ* α-complementation and chloramphenicol or kanamycin-resistance selection. Gene **61:**63–74.