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Two Glyceraldehyde-3-phosphate Dehydrogenases with Opposite Physiological Roles in a Nonphotosynthetic Bacterium*

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Bacillus subtilis possesses two similar putative phosphorylating glyceraldehyde-3-phosphate dehydrogenase (GAPDH) encoding genes, *gap* (renamed *gapA*) and *gapB*. A *gapA* mutant was unable to grow on glycolytic carbon sources, although it developed as well as the wild-type strain on gluconeogenic carbon sources. A *gapB* mutant showed the opposite phenotype. Purified GapB showed a 50-fold higher GAPDHase activity with NADP⁺ than with NAD⁺, with K_m values of 0.86 and 5.7 mM, respectively. *lacZ* reporter gene fusions revealed that the *gapB* gene is transcribed during gluconeogenesis and repressed during glycolysis. Conversely, *gapA* transcription is 5-fold higher under glycolytic conditions than during gluconeogenesis. GAPDH activity assays in crude extracts of wild-type and mutant strains confirmed this differential expression pattern at the enzymatic level. Genetic analyses demonstrated that *gapA* transcription is repressed by the *yvbQ* (renamed *cggR*) gene product and indirectly stimulated by CcpA. Thus, the same enzymatic step is catalyzed in *B. subtilis* by two enzymes specialized, through the regulation of their synthesis and their enzymatic characteristics, either in catabolism (GapA) or in anabolism (GapB). Such a dual enzymatic system for this step of the central carbon metabolism is described for the first time in a nonphotosynthetic eubacterium, but genomic analyses suggest that it could be a widespread feature.

Glycolysis is the main pathway for degradation of carbohydrates and is found in nearly all groups of organisms. The formation of the final product of glycolysis, pyruvate, from glucose is achieved by nine enzymatic steps, most of which function in the reverse direction during gluconeogenesis. The phosphorylating NAD-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPDH)¹ occupies a pivotal role in the Embden-Meyerhoff pathway not only in glycolysis but also in gluconeogenesis because of the reversibility of the oxidation of glyceraldehyde 3-phosphate (G3P) into 1,3-diphosphoglycerate

(1,3dPG). In plants, two distinct types of phosphorylating GAPDH co-exist: (i) a strictly NAD-dependent cytoplasmic GAPDH involved in glycolysis and gluconeogenesis; and (ii) a chloroplastic GAPDH, which is involved in photosynthetic CO₂ assimilation and exhibits a dual coenzyme specificity with a preference for NADP (1, 2).

Recently, two *gap* genes, named *gap1* and *gap2*, have been characterized in the cyanobacterium *Synechocystis* sp. PPC 6803. The NAD-dependent enzyme Gap1 was reported to be essential for glycolytic glucose breakdown, whereas the enzyme Gap2, which exhibits dual coenzyme specificity, was shown to be operative in the photosynthetic Calvin cycle and in nonphotosynthetic gluconeogenesis (3). Thus, at least in some photoautotrophic bacterial species (in which the photosynthetic Calvin cycle and glycolysis/gluconeogenesis function in the same cellular compartment, in contrast to what happens in land plants and algae) two distinct GAPDHs, a strictly NAD-dependent one and the photosynthetic one, catalyze the two opposing directions of the reaction.

The primary structure of GAPDH is highly conserved in bacteria and eukarya. Their active form is tetrameric. Different GAPDH functions can be correlated to differences in GAPDH structure, which favor either NAD or NADP binding and then a catabolic rather than an anabolic carbon flow, and *vice versa*. Indeed, it has been shown from studies combining structural, enzymatic, and protein engineering approaches that both the presence of Asp at position 32 and Leu-Pro at positions 187–188, the latter being located at the subunit interfaces of the tetramer, excluded any activity with NADP⁺, whereas the presence of Gly or Ala at position 32 and Ala-Ser at position 187–188 favored an NADP-dependent activity (4, 5). Removing steric hindrance and/or electrostatic interactions from position 32 or introducing side chains of amino acids at positions 187–188 that stabilize the 2'-ribose phosphate binding can confer specificity for NADP⁺ and thus discriminate against NAD⁺. Thus, the presence of such signatures is a good indicator of efficient NADP⁺ binding and therefore of the involvement of the GAPDH in anabolic carbon flux.

The analysis of the genome sequence of *Bacillus subtilis* (6) revealed the presence of two putative *gap* genes, the previously described *gap* (renamed *gapA*) and *gapB*. The deduced amino acid sequences indicate different signatures at positions 32 and 187 (see Fig. 2), which suggested different physiological roles of both GAPDHs. To verify this hypothesis, both genes have been inactivated and the resulting mutant strains were tested for the utilization of various carbon sources. The enzymatic properties of GapB were also studied and compared with those of a mutant GapA from *Bacillus stearothermophilus* in which the amino acids present in GapB from *B. subtilis* at positions 32 and 187 were introduced. Taken together, our data reveal that

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¹ The abbreviations used are: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; G3P, glyceraldehyde 3-phosphate; 1,3dPG, 1,3-diphosphoglycerate; IPTG, isopropyl-1-thio-β-D-galactopyranoside; ORF, open reading frame; PCR, polymerase chain reaction; PIPES, 1,4-piperazine diethanesulfonic acid.

TABLE I
B. subtilis strains used

Strain	Genotype or relevant genotype	
168CA	<i>trpC2</i>	Laboratory stock
BFA1080	<i>yvbQ</i> :: pMUTIN2	pSC11 → 168CA
GM1500	<i>ΔgapB</i> :: pMUTIN2	pMUTgapB → 168CA
GM1501	<i>gapA</i> :: pMUTIN2	pMUTgapA → 168CA
GM1511	<i>amyE</i> :: pSF111(<i>PyvbQ</i> :: <i>lacZ-cat</i>)	pSF111 → 168CA
GM1512	<i>amyE</i> :: pSF112(<i>PyvbQgapA</i> :: <i>lacZ-cat</i>)	pSF112 → 168CA
GM1513	<i>amyE</i> :: pSF113(<i>yvbQgapA</i> :: <i>lacZ-cat</i>)	pSF113 → 168CA
GM1514	<i>amyE</i> :: pSF114(<i>PgapB</i> :: <i>lacZ-cat</i>)	pSF114 → 168CA
GM1520	<i>amyE</i> :: <i>yvbQ-cat yvbQ</i> :: pMUTIN2	pSF120 → BFA1080
GM1521	<i>amyE</i> :: <i>cat yvbQ</i> :: pMUTIN2	pDG1662 → BFA1080
GM1530	<i>amyE</i> :: <i>yvbQ-cat yvbQ</i> :: pMUTIN2 <i>ΔccpA</i> :: <i>spc</i>	QB5407 → GM1520
GM1531	<i>amyE</i> :: <i>cat yvbQ</i> :: pMUTIN2 <i>ΔccpA</i> :: <i>spc</i>	QB5407 → GM1521
QB5407	<i>ΔccpA</i> :: <i>spc</i>	Ref. 21

the reversible oxidative phosphorylation of G3P into 1,3dPG is catalyzed by two different proteins in *B. subtilis*.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Culture Conditions—The *B. subtilis* strains used are listed in Table I.

Growth tests were performed at 37 °C with vigorous shaking in MM (7) mineral medium (the strain 168CA is not able to use the citrate present in the MM as carbon source for growth) supplemented with tryptophan (0.005%) and carbon sources (25 mM). Cultures for β -galactosidase assays were performed in CQT medium (C mineral medium (8) supplemented with tryptophan 0.005% and glutamine 0.15%) supplemented with 1% glucose or glycerol (v/v) or in CQTHC medium (CQT medium plus casein hydrolysate 0.05%) supplemented with 1% succinate plus 1% glutamate or 1% proline. For GAPDH activity assays in crude extracts, strains were cultivated in LB medium (DIFCO, Detroit, MI) supplemented with proline (25 mM) and IPTG (1 mM) until the middle of the exponential phase of growth. These precultures were diluted 50-fold into LB medium supplemented with IPTG (1 mM) and glucose (25 mM) or proline (25 mM) and incubated at 37 °C until the A_{595} reached 0.5–1.0.

Assay of GAPDH Activity in Cell-free Extracts—Bacterial cells were harvested by centrifugation at 4 °C and washed twice in 0.2% ice-cold KCl. Cells were then concentrated 50-fold in extraction buffer (45 mM Tris, 15 mM tricarballic acid, pH 8.2, 20% (v/v) glycerol, 50 mM MgCl₂, 1 mM dithiothreitol) and stored at –20 °C. After the addition of 1 mM phenylmethylsulfonyl fluoride and lysozyme at 100 μ g/ml, cell lysis was achieved by incubation at 37 °C during 10 min and then sonication at 4 °C. Cell-free extracts were obtained by centrifugation at 12,000 \times g, 4 °C during 10 min. The supernatant was used for the enzymatic assay in reaction buffer at 20 °C (125 mM triethanolamine, 5 mM L-cysteine, 20 mM potassium arsenate, 50 mM K₂HPO₄, pH 9.2) with G3P (4 mM) and NAD⁺ (2 mM) or NADP⁺ (5 mM). Reduction of NAD(P)⁺ was monitored spectrophotometrically at 340 nm. Protein concentration of the extracts were estimated relatively by absorption at 280 nm. Activities were expressed as nmol of NAD(P)⁺ reduced/min and per A_{280} .

Plasmid Constructions—Plasmids pMUT2gapA and pSC11 designed for *gapA* and *yvbQ* disruption, respectively, were obtained by cloning an internal fragment of the corresponding ORF synthesized by PCR (oligonucleotides listed in Table II) into the plasmid pMUTIN2mcs (9) between the *Hind*III and the *Bam*HI sites. The plasmid pMUT2gapB, designed for *gapB* deletion, was derived from pMUTIN2mcs by inserting two PCR-generated fragments (Table II) corresponding to the DNA regions upstream and downstream from *gapB* between the *Hind*III and *Bam*HI sites. Plasmids pSF111 to pSF114 were constructed by insertion of PCR-generated (Table II) *Eco*RI-*Hind*III-digested fragments between the *Eco*RI and *Hind*III sites of the plasmid pDG1661 (10). Plasmid pSF120, used for complementation by *yvbQ*, was generated by cloning a *yvbQ*-containing *Eco*RI-*Hind*III PCR fragment (Table II) into pDG1662 (10) linearized by *Eco*RI and *Hind*III. For *gapB* overexpression in *Escherichia coli*, the *gapB* coding sequence was amplified by PCR using 5'- and 3'-primers (Table II) that contain a *Nco*I or a *Hind*III site, respectively, at their extremity and cloned between the *Nco*I and *Hind*III sites of the plasmid pT7–7 (Life Technologies, Inc.) resulting in plasmid pT7GapB1.

β -Galactosidase Assay— β -Galactosidase activities were measured using the method of Miller (11) on extracts prepared by lysozyme treatment and centrifugation. Protein concentration was determined using the Bio-Rad protein assay reagent (Bio-Rad). One unit of β -ga-

TABLE II
Oligonucleotides used

Purpose	Primer (5' → 3')
Interruption	
<i>gapA</i> (pMUT2gapA)	GCCAAAGCTTCTGTACACGGAAAATTA CGGGATCCACAGTTGTCCATACACCG
<i>yvbQ</i> (pSC11)	GCCAAAGCTTCTGTGTTTTGGAAAGTAC CGGGATCCGCCTAAACCCTCTTTCG
Deletion	
<i>gapB</i> (pMUT2gapB)	GGAATTCGCTTGGGGAGCGAATC CGGGATCCAGCGTTTCTGC GGAATTCGCTTACGCCTTGC GCCAAAGCTTGAACATCCGTCTGCTG
Fusion	
pSF111	GGAATTCGCTTTAATGAAACAGCCC GCCAAAGCTTCTGGTTCATGACTCAAACGTTCC
pSF112	GGAATTCGCTTTAATGAAACAGCCC GCCAAAGCTTTACTGCCATGATTTGTTTC
pSF113	GGAATTCGCTTCGTTATGCAAAAG GCCAAAGCTTTACTGCCATGATTTGTTTC
pSF114	GGAATTCGCTTGGGGAGCGAATC GCCAAAGCTTTCATGTTGGACACCCCTT
Gene cloning	
<i>yvbQ</i> (pSF120)	GGAATTCGCTTTAATGAAACAGCCC GCCAAAGCTTTACTGCCATGATTTGTTTC
<i>gapB</i> (pT7GapB1)	GGAATTCATAGAAAGTAAAGTAGCCG CGGGATCCTTATACAGCAGACGGATG

lactosidase activity is defined as the amount of enzyme that produces 1 nmol of *O*-nitrophenol/min at 20 °C/mg of protein.

GAPDH Assays and Kinetics—Initial rate measurements were carried out at 25 °C on a Kontron Uvikon 933 spectrophotometer by following the absorbance of NAD(P)H at 340 nm. The experimental conditions for the enzymatic assays were 40 mM triethanolamine, pH 9.2, and 10 mM PIPES, pH 7, for the forward and the reverse reactions, respectively. The turnover number (k_{cat}) was calculated using a molar extinction coefficient at 280 nm of 1.53×10^5 M⁻¹cm⁻¹ and $1.17 \cdot 10^5$ M⁻¹ cm for the apoenzymes of *B. subtilis* and *B. stearothermophilus*, respectively. The k_{cat} is expressed per site (N). The initial rate data of the forward reaction were fitted to the Michaelis-Menten relationship using least squares analysis to determine k_{cat} and K_m . All cofactor K_m values were determined at saturating concentration of G3P and P_i.

Production and Purification of the Mutant GAPDHs of *B. stearothermophilus*—Site-directed mutageneses (12) were performed on a pBlue-scriptII-derived plasmid containing the *gapA* gene of *B. stearothermophilus*. Purification of mutant GAPDHs was performed as described previously for other *B. stearothermophilus* mutants (13). All the mutants were isolated as apo form, as judged by the ratio A_{280}/A_{260} of 2.

Production and Purification of GapB of *B. subtilis*—The enzyme was produced in *E. coli* strain BL21(DE3)pLysS transformed with plasmid pT7GapB1. The overexpression of GapB was performed by the addition of 0.4 mM IPTG in the culture medium at 0.8 A_{600} . After 3 h of induction, cultures were harvested by centrifugation and resuspended in buffer A (50 mM phosphate, pH 8.2) containing 5 mM β -mercaptoethanol. After sonication, *B. subtilis* GapB was purified by ammonium sulfate fractionation (50–70%) and exclusion size chromatography on ACA 34 resin at pH 8.2 (buffer A). Purified fractions were then pooled and applied to a Q-Sepharose column equilibrated with buffer A followed by a linear gradient of KCl (0–1 M) using a fast protein liquid chromatography system (Amersham Pharmacia Biotech). The *B. subtilis* GapB was

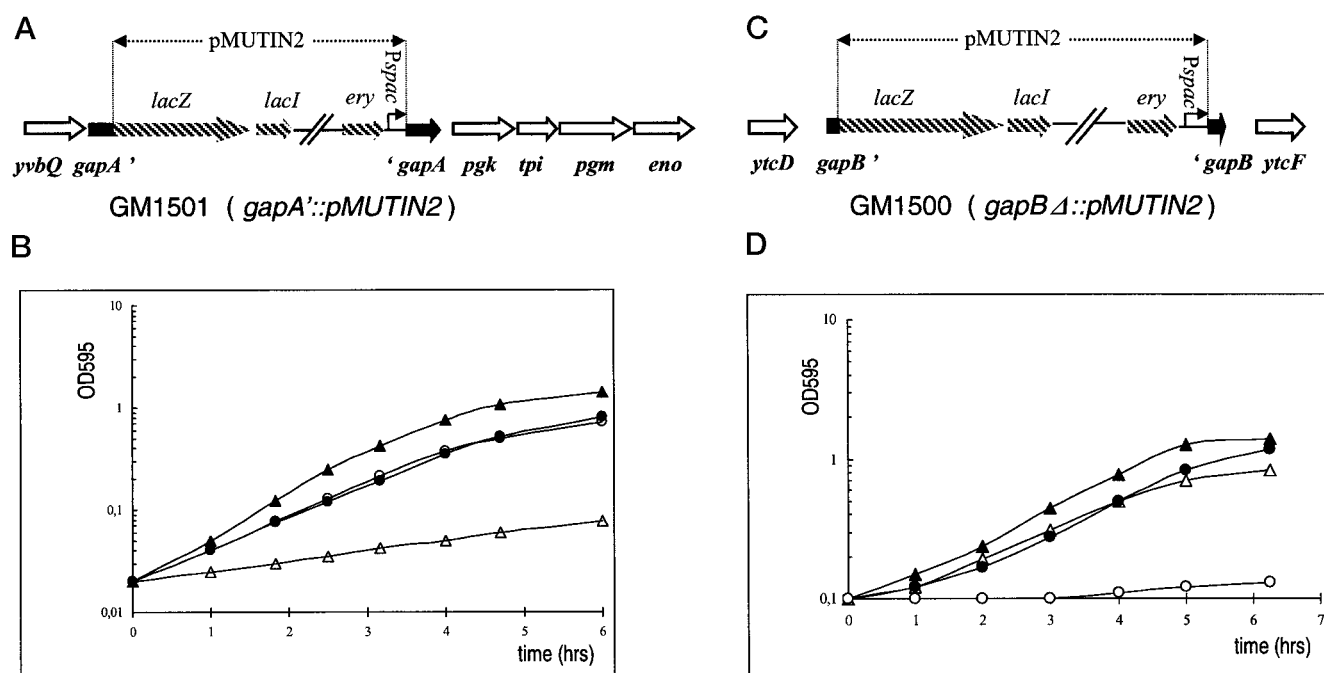


FIG. 1. A, schematic representation of the *gapA* disruption in the strain GM1501. B, growth curves of the GM1501 strain in MM glucose (open triangles) and in MM asparagine (open circles) compared with those of the wild-type strain in the same media (closed triangles and circles, respectively). C, schematic representation of the *gapB* deletion in the strain GM1500. D, growth curves of the GM1500 strain in MM glucose (open triangles) and in MM asparagine (open circles) compared with those of the wild-type strain in the same media (closed triangles and circles, respectively).

eluted at 300 mM KCl. At this stage, the protein was pure as revealed by electrophoresis on 10% SDS-polyacrylamide gel followed by Coomassie Blue staining. The subunit molecular weight of GapB was verified by mass spectrometry. The protein was isolated as apo form, as judged by the ratio A_{280}/A_{260} of 1.8. GapB concentration was estimated spectrophotometrically using an extinction coefficient of $1.53 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$, as deduced from the Scopes method (14). Purified enzyme was stored at 4 °C in the presence of 1 mM β -mercaptoethanol and of 70% ammonium sulfate.

RESULTS

gapA and *gapB* Mutants Have Opposite Phenotypes—To investigate the physiological roles of the two *B. subtilis* GAPDH-encoding genes, we constructed the corresponding *gapA* and *gapB* mutant strains, GM1501 and GM1500, respectively. The pMUTIN2mcs-derived plasmids (9) used for these constructions allowed, simultaneously with the disruption or the deletion of the target gene, (i) the creation of a transcriptional fusion of the 5'-part of the target gene to the *lacZ* gene; and (ii) the control of the expression of downstream gene(s) putatively belonging to the same operon via the IPTG-inducible *Pspac* promoter, to reduce possible polar effects (Fig. 1, A and C). The GM1501 *gapA* insertional mutant grew as well as the parental strain on LB only when IPTG was added. This suggested that the downstream glycolytic genes, *pgk*, *tpi*, *pgm*, and *eno*, are significantly transcribed from a promoter located upstream of *gapA*. Conversely, the growth rate of the GM1500 *gapB* mutant strain on LB was not affected by the presence of IPTG.

Growth tests of both mutant strains, GM1500 and GM1501, revealed opposite phenotypes as shown in Fig. 1, B and D. GM1500 could not grow in MM medium containing asparagine as a sole carbon source but presented the same growth rate as the 168CA parent in glucose-containing MM medium. The phenotype of GM1500 was identical when IPTG was added to the growth medium (data not shown), indicating that only the *gapB* inactivation was responsible for the growth defect. The opposite phenotype was observed for the *gapA* mutant strain, GM1501 (strongly reduced growth in glucose medium and nor-

mal growth in asparagine medium (both evaluated in the presence of IPTG)). Similar results were obtained when glycerol or glucitol were used instead of glucose, and when proline or succinate plus glutamate was used instead of asparagine (data not shown). Thus *gapA* is required for glycolysis, whereas *gapB* is required for gluconeogenesis.

GAPDH Activity in gapA and gapB Mutant Strains—Both mutant and wild-type strains were cultivated in LB medium supplemented with glucose, representing glycolytic physiological conditions, and LB medium supplemented with proline, representing gluconeogenic conditions, to ensure significant growth of the three strains in both media. GAPDH activity was measured by the oxydation of G3P to 1,3dPG using either NAD^+ or NADP^+ as coenzyme. Extracts from the 168CA strain showed roughly 5 times higher NAD-dependent activity under glycolytic conditions than under gluconeogenic conditions (Table III). NADP-dependent activity could be detected only under gluconeogenic growth conditions. This latter activity completely disappeared in the GM1500 (*gapB*⁻) strain, and no NAD-dependent GAPDH-activity could be detected in the GM1501 (*gapA*⁻) strain. These results suggest that GapA and GapB proteins have different cofactor specificities, NAD^+ for GapA and NADP^+ for GapB. In glucose-containing medium the *gapA* encoded NAD-dependent activity is higher than in proline medium, whereas NADP-dependent GAPDH activity probably encoded by *gapB* can only be detected under gluconeogenic growth conditions.

GapA and GapB Exhibit Different Cofactor Specificity Sequence Signatures—Amino acid sequences of bacterial GAPDHs were aligned (Fig. 2). Based on the known cofactor and substrate signatures, three types of phosphorylating GAPDHs can be distinguished. The first two types, named GapA and erythrose-4-phosphate dehydrogenase, possess a typical NAD^+ signature with Asp or Glu at position 32 and amino acids at position 187–188, which are known to prevent an efficient binding of NADP^+ (4, 5). But they differ in substrate specific-

TABLE III
 NAD⁺- and NADP⁺-dependent GAPDH activity in *B. subtilis* wild-type (168CA), *gapA* (GM1501), and *gapB* (GM1500) strains

Culture medium ^a	Strain					
	168CA (wild-type)		GM1501 (<i>gapA</i> ')		GM1500 (Δ <i>gapB</i>)	
	Glc	Pro	Glc	Pro	Glc	Pro
NAD ⁺ -dependent GAPDH activity ^b	450 (+/-150)	80 (+/-25)	ND ^c	ND	95 (+/-20)	25 (+/-5)
NADP ⁺ -dependent GAPDH activity ^b	ND	25 (+/-5)	ND	15 (+/-5)	ND	ND

^a LB supplemented by glucose (Glc) or proline (Pro).

^b One unit corresponds to 1 nmol of NADH (or NADPH) produced/min and per A₂₈₀. The assays were repeated at least three times with independent clones; the average value is indicated with standard deviation.

^c ND, not detectable, i.e. lower than 2 units.

	32	//	179	187	//	208
Gap A						
<i>B. stearothermophilus</i>	NDLT		HSYTNDQRILDLPH			TTTGA
<i>B. subtilis</i> (<i>gapA</i>)	NDLT		HSYTNDQQILDLPH			TSTGA
<i>E. coli</i> (<i>gapA</i>)	NDLL		HATTATQKTVDGSPS			SSTGA
<i>H. pylori</i> (<i>gap1</i>)	NDPA		HSYTSQKLLIDLAH			TTTKA
<i>N. gonorrhoeae</i> (<i>gap1</i>)	NDLT		HAYTGDQNTLDAPH			NSTGA
<i>N. meningitidis</i> (<i>gap1</i>)	NDLT		HAYTGDQNTLDAPH			NSTGA
<i>P. aeruginosa</i> (<i>gap1</i>)	NDLG		HAYTADQRLQDAPH			TTTGA
<i>S. putrefaciens</i> (<i>gap1</i>)	NDPA		HDITNTQTILDAPH			TTTGS
<i>S. typhimurium</i> (<i>gap1</i>)	NDLL		HATTATQKTVDGSPS			SSTGA
<i>V. cholerae</i> (<i>gap1</i>)	NDVA		HNLTNTQTILDAPH			TTTGS
Cyanobacteria :						
<i>A. variabilis</i> (<i>gap1</i>)	NDLV		HAMTATQPTVDGSPS			SSTGA
<i>G. violaceus</i> (<i>gap1</i>)	NDLV		HAMTATQPTVDGSPS			SSTGA
<i>Synechococcus</i> (<i>gap1</i>)	NDLV		HAVTATQPTVDGSPS			ASTGA
<i>Synechocystis</i> (<i>gap1</i>)	NDLV		HAMTATQPTVDGSPS			SSTGA
E4PDH						
<i>E. coli</i> (<i>gapB</i>)	NELA		HSAMHDQQVIDAYH			VDTKL
<i>P. aeruginosa</i> (<i>gap2</i>)	NDLA		HSAMNDQPVIDAYH			VSTGL
<i>S. putrefaciens</i> (<i>gap2</i>)	NELA		HSAMNDQQVIDAYH			VDTKL
<i>S. typhimurium</i> (<i>gap2</i>)	NELA		HSAMNDQQVIDAYH			VDTKL
<i>V. cholerae</i> (<i>gap2</i>)	NELA		HSSMNDQQVIDAYH			VDTKL
<i>Y. pestis</i> (<i>gap2</i>)	NELA		HSSMNDQPVIDAYH			VDTKL
GapB						
<i>B. subtilis</i> (<i>gapB</i>)	NASY		HAYTNDQKNIDNPH			TTTGA
<i>H. pylori</i> (<i>gap2</i>)	NSTA		HSYTNDQNLDDTKH			TSTGV
<i>N. gonorrhoeae</i> (<i>gap2</i>)	NASG		HALTNDQTVTDVVRH			TKTGA
<i>N. meningitidis</i> (<i>gap2</i>)	NASG		HALTNDQTVTDVVRH			TKTGA
Cyanobacteria :						
<i>A. variabilis</i> (<i>gap2</i>)	NDTS		HSYTGDQRLLDASH			SSTGA
<i>G. violaceus</i> (<i>gap2</i>)	NDTS		HSYTGDQRLLDASH			TSTGA
<i>Synechococcus</i> (<i>gap2</i>)	NNTS		HSYTLDQRILLDASH			TTTGA
<i>Synechocystis</i> (<i>gap2</i>)	NDLS		HSYTGDQRILLDASH			TSTGA
Plants :						
<i>Chloroplastic GAPDH</i>	ND		HSYTG QRLLDAS			T TGA

FIG. 2. Alignment of the sequence signatures of GAPDH deduced from complete or unfinished genome sequences relevant for cofactor and substrate specificity predictions. Only bacterial species that possess at least two GAPDHs were considered (the *GapA* sequence of *Yersinia pestis* being not completed is not indicated), except for the *GapA* reference sequence of *B. stearothermophilus*. The consensus sequence of chloroplastic GAPDHs is also indicated. Source of sequences: GAPDH amino acid sequences of *B. stearothermophilus*, *E. coli*, *H. pylori*, *Salmonella typhimurium*, *Anabaena variabilis*, and *Synechocystis* were obtained from Swissprot; those of *Synechococcus* and *Gloeobacter violaceus* were from GenBank™/EBI; those of *B. subtilis*, *N. gonorrhoeae*, *N. meningitidis*, *Pseudomonas aeruginosa*, *Vibrio cholerae*, and *Y. pestis* were from PEDANT directly; and that of *Shewanella putrefaciens* was from TIGR. *E4PDH*, erythrose 4-phosphate dehydrogenase.

ity, with typical signatures at position 179 and around position 208, which favor the binding of either G3P for *GapA* or erythrose 4-phosphate for erythrose-4-phosphate dehydrogenase (15). The third type, named *GapB*, which is G3P-specific (3),

contains a cofactor signature that supports a dual coenzyme specificity with a preference for NADP⁺ (5). Indeed, this signature either resembles that of chloroplastic GAPDH, with Asp at position 32 but with amino acids at position 187–188 that stabilize NADP⁺ binding, or presents no Asp at position 32 but amino acids at positions 32 and/or 187–188, which could stabilize NADP⁺ binding.

In this context, *GapA* from *B. subtilis* has the typical signature of an NAD-dependent GAPDH with Asp-32, Leu-187, and Pro-188. This signature and its overall 85% sequence identity with the *B. stearothermophilus* *GapA* suggest that *GapA* from *B. subtilis* has enzymatic characteristics very similar to those of the well studied *GapA* from *B. stearothermophilus* (5, 16). In contrast, the *B. subtilis* *GapB*, with Ala and Asn at position 32 and 187, respectively, probably presents a dual cofactor signature.

Purification and Enzymatic Characterization of *GapB*—The *B. subtilis* *GapB* protein was overproduced in *E. coli*, purified, and separated from the *E. coli* *GapA* protein by taking advantage of its higher hydrophobicity and its lower isoelectric point. *GapB* showed activity with G3P in the forward direction but only in the presence of P_i, whereas 1,3dPG was the substrate in the reverse direction (data not shown). This demonstrated that the GAPDH activity was of the phosphorylating type. The kinetic parameters determined for *GapB*, in the forward direction (*k*_{cat} and *K*_m for cofactors), are summarized in Table IV. The apparent affinity is about 7-fold higher for NADP⁺ than for NAD⁺ with a 50-fold higher catalytic efficiency. These results demonstrated that *B. subtilis* *GapB* has a strong preference for NADP⁺ as a cofactor. Mutations at the two signature positions, 32 and 187, in the *B. stearothermophilus* *GapA* protein were shown to increase NADP-dependent GAPDH activity and to decrease NAD-dependent GAPDH activity (Table IV). Whereas the wild-type *B. stearothermophilus* *GapA* protein had no detectable activity with NADP⁺ as cofactor, the D32A/L187N double mutant *GapA* protein clearly showed a catalytic efficiency with NADP⁺ similar to that observed with the *B. subtilis* *GapB* protein and higher than that measured with NAD⁺.

The *gapA* and *gapB* Genes Are Transcriptionally Regulated—As the levels of GAPDH activities encoded by *gapA* and *gapB* are modulated in response to the growth conditions, we looked for a transcriptional regulation of the expression of these genes. A series of transcriptional fusions to the *lacZ* gene were constructed. As *gapA* is predicted to be the second gene of an operon, three different reporter constructs have been designed (Fig. 3A) to analyze its transcription pattern and to test whether its expression is driven from the region located immediately upstream of the *gapA* ORF or from the region located between the *araE* terminator and the initiation codon of the *yubQ* ORF, the first gene of the putative *gapA* operon. These fusions, carried by plasmids pSF111 to pSF113, as well as the fusion of the *gapB* promoter region with *lacZ* carried by the plasmid pSF114 (Fig. 3), were integrated as single copy at the *amyE* locus of *B. subtilis* 168CA strain, and the β -galactosidase activities of the resulting strains, GM1511 to GM1514, respectively, were measured during growth in glucose or succinate

TABLE IV

Kinetic parameters of purified GapB GAPDH from *B. subtilis* and of purified wild-type and mutant GapA GAPDH from *B. stearothermophilus*

Coenzyme kinetic parameters ^a	NAD			NADP		
	K_M	k_{cat}	k_{cat}/K_M	K_M	k_{cat}	k_{cat}/K_M
	<i>mM</i>	<i>s</i> ⁻¹	<i>s</i> ⁻¹ · <i>mM</i> ⁻¹	<i>mM</i>	<i>s</i> ⁻¹	<i>s</i> ⁻¹ · <i>mM</i> ⁻¹
<i>B. subtilis</i>						
GapB	5.7	1.0	0.18	0.86	8.0	9.3
<i>B. stearothermophilus</i>						
GapA ^b	0.1	70	700	ND ^c	ND ^c	ND ^c
GapAD32A ^b	0.5	70	140	7.1	2.0	0.3
GapAL187N	1.0	112	112	7.1	8.0	1.1
GapAD32AL187N	0.75	2.5	3.3	1.7	17.7	10.4

^a GAPDH activity was determined at saturating concentrations of G3P and P_i; each value is the average of at least three independent determinations.

^b From Ref. 5.

^c Not detectable ($k_{cat} < 10^{-3} \text{ s}^{-1}$).

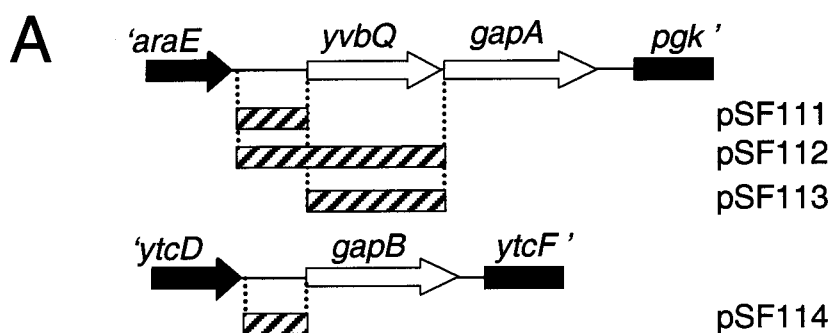
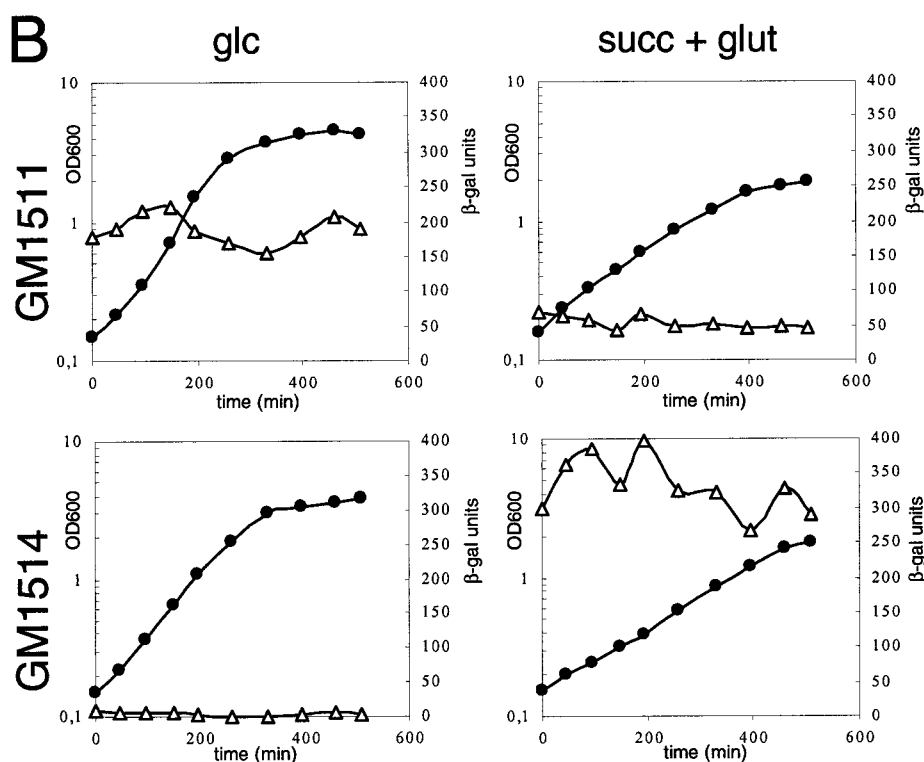


FIG. 3. A, schematic representation of the DNA fragments (striped bars) transcriptionally fused to *lacZ* in the pSF111 to pSF114 series of plasmids used to generate the GM1511 to GM1514 series of reporter strains. B, growth curve (closed circles) and β -galactosidase synthesis (open triangles) of GM1511 and GM1514 strains in minimal CQT glucose medium or in minimal CQTHC succinate plus glutamate medium. β -Galactosidase activities are measured as described under "Experimental Procedures."



plus glutamate minimal medium.

In GM1511, *lacZ* expression was found to be roughly constant during the exponential and the beginning of the stationary phases of growth, but 4-fold higher in glucose than in succinate plus glutamate medium (Fig. 3B). Similar patterns of *lacZ* expression were found in GM1512 with a 4–5-fold stimulation of the expression in glucose medium (data not shown). On the other hand, no expression was detected in GM1513 (data not shown). These results indicated that *yvbQ* and *gapA* form an operon and that *gapA* transcription is driven from a

promoter located between *araE* and *yvbQ*. The β -galactosidase synthesis in GM1514, reflecting *gapB* expression, remained also roughly constant through the exponential and the beginning of the stationary phase of growth but was at least 50-fold higher in succinate plus glutamate than in glucose medium. Very similar patterns of expression were found when glycerol and proline were used instead of glucose and succinate plus glutamate, respectively (data not shown). Thus, we concluded that *gapA* and *gapB* expressions are transcriptionally regulated in an opposite manner; under glycolytic conditions, *gapA*

TABLE V
Effect of *yvbQ* and *ccpA* mutations on transcription of the *yvbQ-gapA* operon

Strain	β -galactosidase activity ^a					
	Glc	Succ + Glut	Glc + Succ + Glut	Gly	Pro	Gly + Pro
GM1520 (<i>yvbQ</i> ':: <i>lacZ</i> , <i>yvbQ</i> ⁺)	339	52	402	715	52	648
GM1521 (<i>yvbQ</i> ':: <i>lacZ</i>)	1127	1558	1101	1162	1229	1458
GM1530 (<i>yvbQ</i> ':: <i>lacZ</i> , <i>yvbQ</i> ⁺ , Δ <i>ccpA</i>)	38	29	30	620	30	593
GM1531 (<i>yvbQ</i> ':: <i>lacZ</i> , Δ <i>ccpA</i>)	934	1252	1438	1236	1182	1173

^a Activity was assayed at least three times from independent clones; the average value is indicated (standard deviations were less than 15% of the mean). Strains were cultivated in glucose (Glc) or glycerol (Gly) CQT media or in succinate plus glutamate (Succ + Glut), glucose plus succinate plus glutamate (Glc + Succ + Glut), proline (Pro), or glycerol plus proline (Gly + Pro) CQTHC media.

expression is stimulated, whereas that of *gapB* is nearly completely repressed. Conversely, during gluconeogenesis, *gapB* transcription is strongly activated, whereas *gapA* is expressed at a basal level.

yvbQ Represses *gapA* Expression during Gluconeogenesis—Searches for similarities revealed that the deduced product of *yvbQ*, located upstream of *gapA*, belongs to the SorC/DeoR family of transcriptional regulators (data not shown). Its similarity is particularly strong with three ORFs, Urf1 (17), YgaP (18), and ORF1' (19), linked to *gap* genes in *Bacillus megaterium*, *Lactobacillus delbrueckii*, and *Clostridium acetobutylicum*, respectively. The functions of these ORFs have not yet been identified. To test a possible role of *yvbQ* in the regulation of *gapA* transcription, we constructed the strain BFS1080, which carries a transcriptional fusion between the promoter region of the *yvbQ-gapA* operon and the *lacZ* gene, and is disrupted for *yvbQ*. Then, either the pSF120 plasmid, a pDG1662-derived vector expressing the wild-type *yvbQ* gene, or the parental pDG1662 plasmid was integrated into BFS1080 at the *amyE* locus creating the GM1520 or the GM1521 strains, respectively.

Although *lacZ* was expressed constitutively at a high level in GM1521 (*yvbQ*⁻), it was expressed at a low level in the presence of succinate plus glutamate and 6-fold stimulated in the presence of glucose in GM1520 (*yvbQ*⁺) (Table V). A similar stimulation of *lacZ* expression was observed when the strain GM1520 was grown in minimal medium containing both glucose and succinate plus glutamate. The *lacZ* expression pattern in GM1520 and GM1521 remained the same when glycerol was used in place of glucose or proline in place of succinate plus glutamate (Table V). These results demonstrated that the *yvbQ* gene product is a repressor of the *yvbQ-gapA* operon and that its activity is inhibited by the presence of glycolytic carbon sources.

An Indirect Role of CcpA in Regulation of gapA Expression—CcpA is a key regulator of carbon flow in *B. subtilis*, acting both as a negative regulator of numerous carbon utilization genes and as a positive regulator of genes involved in the excretion of byproducts when rapidly metabolizable carbon sources are in excess (20, 21). To investigate a possible role of *ccpA* in the regulation of the *gapA* operon, a *ccpA* deletion was introduced in the GM1520 (*yvbQ*⁺) and GM1521 (*yvbQ*⁻) strains, respectively, and β -galactosidase activity in the resulting strains, GM1530 and GM1531 was measured. The stimulation of *lacZ* expression in the presence of glucose observed in GM1520 was not found in its Δ *ccpA* derivative, GM1530 (Table V). However, using glycerol as sole carbon source, no significant difference could be detected between both strains. The expression of the *lacZ* reporter gene was found to be high and constitutive in all the media tested in GM1531 (*yvbQ*⁻, Δ *ccpA*) as it is in GM1521 (*yvbQ*⁻), demonstrating an epistatic effect of the *yvbQ* mutation over the deletion of the *ccpA* gene. Therefore, *ccpA* is required for stimulating the transcription of the *gapA* operon by glucose but not by glycerol. Moreover, this role of *ccpA* is indirect and probably mediated by YvbQ.

DISCUSSION

The complete genome sequence of *B. subtilis* revealed the co-existence of two putative GAPDH encoding genes, *gapA* (formerly *gap*, (22)) and *gapB* (6). To study the physiological role of these genes, we inactivated both of them independently. The phenotypical analysis of the mutants revealed their opposite physiological functions; *gapA* is required for glycolysis, whereas *gapB* is necessary for gluconeogenesis (Fig. 1). Enzymatic assays on crude extracts of the *gapA* and *gapB* mutants (Table III) demonstrated that *gapA* encodes a NAD-dependent GAPDH, whereas *gapB* encodes a NADP-dependent GAPDH. Moreover these experiments suggested that their synthesis and/or their activity are modulated in response to the physiological conditions.

Indeed, we were able to show that transcription of the two *gap* genes is inversely regulated. Under glycolytic growth conditions, *gapA* expression is stimulated 4–5-fold, although that of *gapB* is nearly completely switched off (Fig. 3). By contrast, growth on Krebs cycle intermediates or amino acids strongly induces *gapB* expression and maintains *gapA* transcription at a basal level. Transcriptional regulation of both genes estimated through the expression of *lacZ* reporter gene fusions at an ectopic locus accounts for the differences of GAPDH activity measured in extracts from cultures grown under glycolytic or gluconeogenic conditions (Table III), suggesting that the regulation mainly acts at the transcriptional level. Our data indicate that *gapA* and *yvbQ* are cotranscribed from a promoter located upstream of *yvbQ* (Fig. 3). The *pgk-eno* operon starts 320 base pairs downstream of *gapA*. The *gapA* gene is frequently clustered with other glycolytic genes in bacteria (17, 18, 23), but it is also encountered as an individual gene or transcription unit, e.g. in *E. coli* (24), in *Synechocystis* (25), and in *Streptomyces aureofaciens* (26). In *B. subtilis*, the polar effect of the *gapA* disruption (Fig. 1) suggests that significant transcription of the *pgk-eno* genes may initiate upstream of *yvbQ* despite the presence of a predicted transcription terminator just downstream of *gapA*. Indeed, our preliminary transcription analysis² of this region suggested the existence of a transcript corresponding to the *pgk-eno* operon and of a longer one extending from *yvbQ* to *eno*. This cotranscription would ensure the coordinated expression of all central glycolytic enzymes under glycolytic growth conditions.

Our genetic analyses demonstrated that the first gene of the operon, *yvbQ*, regulates glucose stimulation of *gapA* (and possibly of the downstream genes). The *yvbQ* gene product acts as a repressor, the activity of which would be inhibited under glycolytic growth conditions (Table V). We propose to rename this gene *cggR* for central glycolytic gene Regulator. CggR belongs to the SorC/DeoR family of transcriptional regulators, and homologues of unknown function of this protein are found upstream of *gap* genes in *B. megaterium*, *C. acetobutylicum*, or *L. delbrueckii*. It is therefore probable that also in these latter

² S. Fillinger and S. Aymerich, unpublished observation.

organisms *gapA* expression is regulated *via* the *cggR*-like genes. We also found that the general carbon catabolite repressor protein CcpA is necessary for the stimulation of *gapA* transcription by glucose (Table V), in agreement with the results of Tobisch *et al.* (27). However, our genetic analysis suggested that CcpA indirectly regulates *gapA* transcription; the signal recognized by CggR would be altered in the *ccpA* mutant context when glucose, but not glycerol, is the sole carbon source utilized for growth.

We are currently investigating the mechanisms involved in the regulation of *gapB* expression. A *ccpA* deletion only partially abolished the repression of *gapB* transcription in the presence of glucose.³ Thus an additional CcpA-independent mechanism for carbon catabolite repression must be considered for *gapB* regulation.

Our results clearly showed that purified GapB presents a strong preference and a better catalytic efficiency for NADP⁺ compared with NAD⁺. Introducing the predicted signature responsible of the NADP⁺ binding preference into a strictly NAD⁺-dependent GAPDH converts the latter into a dual cofactor enzyme with a marked preference for NADP⁺ (Table IV). Interestingly, the protein signature involved in the NADP⁺ preference differs from that of the chloroplastic GAPDHs (which also present dual coenzyme specificity with a preference for NADP⁺) with Ala instead of Asp at position 32 and Asn instead of Leu at position 187 (Fig. 2).

Catabolic reactions lead to the formation of reduced coenzymes, which are reoxidized by the oxidative phosphorylation in combination with ATP-production. Only NADH enters the oxidative phosphorylation pathway. The oxidized form of NAD⁺ appears to be predominant over NADH by 50–1000-fold in the bacterial cell depending on the environmental redox state (28–30), whereas NADPH appears to be more abundant than NADP⁺ (31). Redox potentials of both co-enzymes are similar. As expected, NADPH is indeed the preferred cofactor of those enzymes catalyzing anabolic reductions. From this point of view, our discovery of a NADP-dependent GAPDH specialized for the gluconeogenesis in *B. subtilis* is not surprising, especially when one considers that the catalysis of the reduction of 1,3dPG into G3P by the canonical NAD-dependent GAPDH is thermodynamically not favorable because of the *in vivo* NADH/NAD ratio (29).

In many eukaryotic organisms, more than a single GAPDH has been identified, *e.g.* TDH1-TDH3 in *Saccharomyces cerevisiae*. In plants, two types of GAPDHs coexist with distinct physiological roles and distinct cellular localizations (see Introduction). Among bacteria, four cyanobacterial species, which are photosynthetic bacteria, possess at least two putative GAPDHs with different cofactor specificity (Fig. 2). In one of these cyanobacterium species, *Synechocystis*, distinct roles have indeed been demonstrated for these GAPDH. The NAD⁺-dependent GAPDH is required for glycolysis, whereas the dual NADP⁺/NAD⁺-dependent GAPDH, operative in the anabolic Calvin cycle, is also active during gluconeogenesis (3).

Different situations have been described in Archaea; in the hyperthermophilic *Pyrococcus furiosus* and *Thermotoga tenax*, the GAPDH (NADPH-dependent) enzyme appears to be active mainly during gluconeogenesis, whereas either glyceraldehyde-3-phosphate ferredoxin oxidoreductase or nonphosphorylating glyceraldehyde-3-phosphate dehydrogenase enzymes catalyze in one step the unidirectional glycolytic conversion of G3P into 3-phosphoglycerate (32–34). With the current knowledge, a thermodynamic advantage of such a variation of the Embden-Meyerhoff pathway is not obvious (34). Apparently, Archaea possess

an energetically less efficient glycolysis variant but in which the interconversion of G3P and 3-phosphoglycerate developed into a novel regulatory site of the glucose metabolism.

Up to now, GAPDHs of nonphotosynthetic eubacteria were supposed to have dual functions in catalyzing the reaction in both directions. The situation in *B. subtilis* that we describe in this report is the first example among nonphotosynthetic bacteria of the coexistence of a NAD-dependent and of a NADPH-dependent GAPDH specialized in glycolysis and in gluconeogenesis, respectively. This situation preserves the high energetic efficiency of the Embden-Meyerhoff glycolysis but also gives rise to a fourth regulatory checkpoint for the central carbon metabolism.

Presently, three other nonphotosynthetic bacterial species, *Nesseiria meningitidis*, *Nesseiria gonorrhoeae*, and *Helicobacter pylori*, can be predicted on the basis of genome sequence analyses (Fig. 2) to possess like *B. subtilis* a NADP-dependent GAPDH in addition to a canonical NAD-dependent GAPDH; they probably share the GAPDH specialization identified in *B. subtilis*.

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³ M. Arnaud and G. Rapoport, personal communication.

Two Glyceraldehyde-3-phosphate Dehydrogenases with Opposite Physiological Roles in a Nonphotosynthetic Bacterium
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