Engineering a central metabolic pathway: glycolysis with no net phosphorylation in an *Escherichia coli gap* mutant complemented with a plant *GapN* gene

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Abstract A cDNA fragment containing the *Pisum sativum GapN* gene, which encodes the non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase, was cloned in a prokaryote expression vector. This construct enabled *Escherichia coli* strain W3CG, a mutant which lacks the glycolytic phosphorylating G3P dehydrogenase, to grow aerobically on sugars. The functionally complemented mutant exhibited high levels of the catalytically active plant enzyme, which renders 3-phosphoglycerate and NADPH, thus bypassing the first substrate level phosphorylation step of the glycolysis. As expected if such a glycolytic bypass would be operative in vivo, this clone failed to grow anaerobically on sugars in contrast to W3CG clones complemented with phosphorylating glyceraldehyde-3-phosphate dehydrogenases. According to the irreversible catabolic character of the non-phosphorylating reaction, the *GapN*-complemented clone was unable to grow on gluconeogenic substrates. This metabolic engineering approach demonstrates that a pure catabolic Embden-Meyerhof pathway with no net energy yield is feasible.

Key words: Non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase; Functional complementation; Glycolysis; Gluconeogenesis; Metabolic engineering

1. Introduction

Two phosphorylating D-glyceraldehyde-3-phosphate dehydrogenases (GAPDH) that perform key roles in the carbon metabolism are present in photosynthetic eukaryotic organisms: the cytosolic NAD-dependent GAPDH (EC 1.2.1.12) of the glycolysis/gluconeogenesis and the chloroplastic NADP-dependent GAPDH (EC 1.2.1.13) of the photosynthetic Calvin cycle [1]. Both enzymes catalyze the same reversible reaction, the oxidation of D-glyceraldehyde-3-phosphate (G3P) to 1,3-bisphosphoglycerate (1,3-BPGA) with the concomitant addition of a molecule of Pi, but they are coded by distinct, although related, nuclear genes (*GapC* and *GapA*/*B*, respectively), differ in the coenzyme specificity and are located in different cellular compartments [2,3]. All photosynthetic eukaryotes investigated so far, both plants and microalgae, contain in addition a third, distinctive, cytosolic non-phosphorylating NADP-dependent G3P dehydrogenase, or G3P:NADP oxidoreductase (GAPDHN, EC 1.2.1.9), encoded by the nuclear gene *GapN* that irreversibly oxidizes G3P to 3-phosphoglycerate (3-PGA) with the concomitant addition of a molecule of H_2O [4–7]. As a member of the aldehyde dehydrogenase superfamily, the GAPDHN differs from GAPDHs both in primary structure and molecular mass [4,5,8]. On the other hand, the ubiquitous NAD-dependent GAPDH involved in the glycolysis/gluconeogenesis is encoded in eubacteria by a heterogeneous family of *GapC*-like genes [1], but photosynthetic cyanobacteria possess in addition a phosphorylating NAD(P)-dependent enzyme (GAPDH2, EC 1.2.1.59), encoded by the *GapA*-like gene *gap2* [9].

The cytosolic GAPDH is responsible for the first substrate level phosphorylation step of the glycolysis, rendering 1,3-BPGA, the substrate of 3-PGA kinase which eventually produces 3-PGA and ATP [1]. The GAPDHN, in contrast, does not use Pi as a substrate and directly produces 3-PGA, thus bypassing the ATP-generating reaction [5,7]. It has been proposed [4,7] that the cytosolic GAPDHN could participate, with the cooperation of the chloroplastic GAPDH and the triose-phosphate/Pi translocator of the chloroplast inner membrane [10], in the indirect shuttling of reducing power (H^-) and protons from the chloroplast stroma to the cytosol, thus contributing to the maintenance of the pH gradient between these two cellular compartments.

Functional complementation of the *Escherichia coli gap* mutant W3CG, which has a Tn10 transposon inserted in the *GapC*-like gene *gap-2* and lacks GAPDH [11], has been used as a cloning strategy for eubacterial *gap* genes [9,12,13]. We have cloned the pea *GapN* gene under a strong *lac*-derived promoter in the prokaryote expression vector p*Trc* 99A, so that a functional plant GAPDHN could be expressed in *E. coli*. This construct partially complemented the W3CG *E. coli* strain, which recovered the ability to grow aerobically on sugars but failed to perform anaerobic sugar fermentation or aerobic growth on gluconeogenic substrates. This metabolic engineering approach illustrates the operation of a strict catabolic glycolytic pathway with no net substrate level phosphorylation. To our knowledge, this is the first time that such a route has been demonstrated in vivo.

2. Materials and methods

All chemicals were of analytical grade and were purchased from

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Abbreviations: GAPDH, D-glyceraldehyde-3-phosphate dehydrogenase; G3P, D-glyceraldehyde-3-phosphate; 3-PGA, D-3-phosphoglycerate; DHAP, dihydroxyacetone-phosphate; 1,3-BPGA, D-1,3-bisphosphoglycerate; IPTG, isopropyl-β-D-thiogalactopyranoside; NPA, *p*-nitrophenyl acetate

PII: S0014-5793(99)00430-5

Sigma-Aldrich Chemical or Merck. Restriction and modification enzymes were from Pharmacia Biotech.

2.1. Organisms and growth conditions

Wild-type *E. coli* strain K12 and strain W3110, its derived strain W3CG (*E. coli* W3110 *gap-2*::Tn10 [11]) and complemented W3CG clones were cultured at 37°C in Luria-broth (LB) or minimal M63 media supplemented with the following organic carbon sources: (a) 20 mM glucose, (b) 20 mM Na-succinate plus 5 mM glycerol or (c) 20 mM Na-succinate plus 20 mM Na-acetate. Other sugars used (at 20 mM) instead of glucose were fructose, galactose, maltose, mannose and sucrose. Either solid (plus 1.5%, w/v, Difco-Bactoagar) or liquid cultures with continuous stirring were used. When necessary, ampicillin (Ap) or tetracycline (Tet) were added at a concentration of 100 μg/ml or 25 μg/ml, respectively. Growth rates of the different cultures were determined in 96 well (250 μl each) microtiter plates using an Applied Biosystem microtiter lector with 10 agitation cycles per minute and measurements of A_{690} every hour for a 48 h cycle. Anaerobic growth conditions were achieved by bubbling the media for 1 h with N_2 into 100 ml screw-capped bottles that were closed inmediately afterwards. When required, KNO_3 was added to a 25 mM final concentration.

2.2. Enzyme assays and protein techniques

The phosphorylating G3P dehydrogenase oxidizing and reductive activities were measured spectrophotometrically by monitoring changes in the A_{340} as described in [6]. The GADPHN activity was measured using the same procedure but without Pi or arsenate in the reaction medium [4,6]. The esterase activity of GAPDHN was determined spectrophotometrically with *p*-nitrophenyl acetate (NPA) as described in [14] for *E. coli* GAPDH except that the reaction pH was 7.5. Its optimal pH was estimated in the range from 6 to 11 using different mixtures of Tris and glycine (50 mM total concentration) as buffers. One unit was defined as the amount of enzyme able to reduce/oxidize 1 μmol of NAD(P)/NAD(P)H (dehydrogenase activity) or hydrolyze 1 μmol of NPA (esterase activity) per min. Polyacrylamide gel electrophoresis of proteins in the presence of sodium dodecyl sulfate (SDS-PAGE) was carried out by the Laemmli method in 12% (w/v) acrylamide slab gels using a Miniprotean-II (Bio-Rad) cuvette. Other protein techniques were as described in [15].

The pea recombinant GAPDHN was purified to electrophoretic homogeneity from cultures of the *E. coli* W3CG mutant transformed with the pFVNP1 plasmid (see below) by a procedure modified from that described for the GAPDH2 from the cyanobacterium *Synechocystis* sp. PCC 6803 [9]. 100 ml of LB medium supplemented with Ap and Tet were cultured until an A_{700} of 0.5 was reached. The expression was then induced for 3–6 h by 0.5 mM IPTG and the cells were eventually harvested by centrifugation (15 000 × *g*, 10 min). Cell pellets were washed twice in 50 mM Tris-HCl buffer, pH 7.5, and resuspended in the same buffer supplemented with 2 mM dithiothreitol, 2 mM phenylmethylsulfonyl fluoride and 10% (v/v) glycerol. After cell breakage by ultrasonic treatment, a cell-free extract (soluble protein fraction) was obtained by centrifugation (40 000 × *g*, 20 min) to remove cell debries and membranes. The purification protocol consisted of a 40–85% ammonium sulfate fractionation of the cell-free extract followed by three column chromatography steps, an ionic exchange chromatography on DEAE-cellulose (DE-52 Whatman) and two consecutive hydrophobic chromatographies on Phenyl-Sepharose HP (Pharmacia Biotech), the second one replacing the final affinity cromatography step on Blue-Sepharose used in [9]. The recombinant GAPDHs (GAPDH2 from *Synechocystis* sp. PCC 6803, GAPDH1 from *Anabaena* sp. PCC 7120 and GAPDH from *E. coli*) were purified by the procedure described in [9]. Western blots were carried out after SDS-PAGE of cell-free extracts as described in [9] by using monospecific polyclonal antibodies raised in rabbits against the G3P dehydrogenases purified from the *E. coli* W3CG mutant transformed with plasmids pFV8 (GAPDH2 of *Synechocystis* sp. PCC 6803) [9], pFVA1 (GAPDH1 of *Anabaena* sp. PCC 7120) and pFVNP1 (*Pisum sativum* GAPDHN) (see below).

2.3. Cloning strategy and DNA manipulation

Plasmid pFVNP1 was constructed from plasmid pPsGAPN1 [8], a pBS SK$^+$ (Stratagene) derivative with a cDNA fragment of 1.63 kb containing the *GapN* gene from pea (*P. sativum*) and the prokaryote

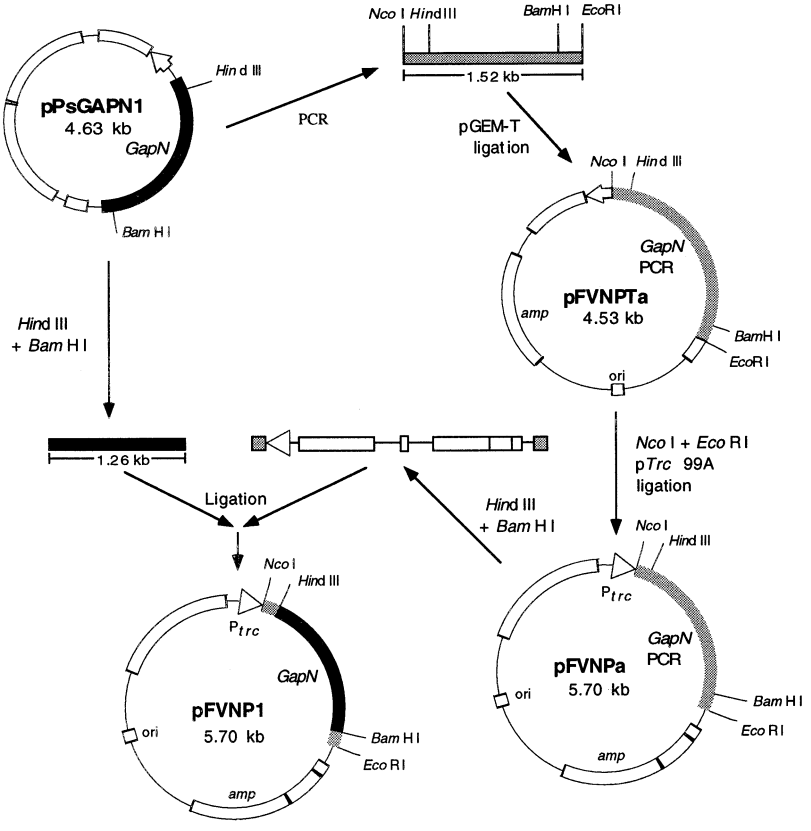

Fig. 1. Subcloning of the pea *GapN* gene from the original pPsGAPN1 plasmid into the expression vector p*Trc* 99A. *amp*, ampicillin resistant gene; ori, origin of replication; P_{trc}, *lac*-derived *trc* promoter; *GapN*, gene for the GAPDHN from *P. sativum*.

expression vector p*Trc* 99A (Pharmacia Biotech) (Fig. 1). A PCR amplification was carried out at 50°C with two strict oligonucleotides (forward, 5′-ATTTCCTA**CCATGG**CTGC-3′; reverse, 5′-AATATG-CTACCGATTATT **CTTAAG**AT-3′) using plasmid pPsGAPN1 as a template. In this way, a new *Nco* I restriction site (in bolds in the forward primer) was created at the 5′-end of the gene incorporating the start codon ATG (underlined) at the beginning of the translated sequence and another *Nco* I site present in the natural sequence was deleted at the 3′-end. This strategy also created an *Eco*R I site (in bolds in the reverse primer) after two stop codons (underlined) originally present in the *GapN* gene. The 1.52 kb DNA fragment thus obtained was ligated into the pGEM-T vector (Promega) and transformed into *E. coli* DH5α strain, three independent clones (named pFVNPTa, b and c) being selected and tested by restriction analysis. These plasmids were restricted with *Nco* I-*Eco*R I endonucleases and the resulting 1.49 kb DNA fragment was ligated into plasmid p*Trc* 99A restricted with the same enzymes, so that the *GapN* gene was directionally cloned with the ATG of the *Nco* I site immediately after the *trc* promoter. A *Bam*H I-*Hin*d III fragment of 1.26 bp was extracted from the original pPsGAPN1 vector by partial restriction and ligated into the *Bam*H I-*Hin*d III-restricted pFVNPa, b and c plasmids. These plasmids were transformed into *E. coli* DH5α and cell-free extracts were obtained as above and tested for GAPDH and GAPDHN activities. Plasmid pFVNP1 was obtained from one of these constructs (pFVNTa) and 300 bp starting from both ends of the *Nco* I-*Eco*R I insert were sequenced in both strands to confirm that no mutation either due to PCR or ligation processes occurred. Plasmid pFVA1 was obtained by functional complementation in LB-Ap (50 μg/μl) medium of *E. coli* W3CG transformed with a DNA library from the cyanobacterium *Anabaena* sp. PCC 7120 in pBS SK$^+$ [9] and subcloning until no other complete open reading frame than the one corresponding to the *gapC*-like *gap1* gene was present in the vector insert (approximately 3 kb). Plasmid pF61A is a pBR322 derivative containing the *gapC* gene of *E. coli* K12 [16].

3. Results and Discussion

Fig. 1 shows the construction of plasmid pFVNP1, which contains the pea *GapN* gene (1488 bp corresponding to a polypeptide of 496 amno acids (aa)) under the control of a strong *trc* promoter, from the cDNA insert of plasmid pPsGAPN1 [8]. Since the cloning strategy involved a PCR step, most of the original translated region (1257 bp) was re-introduced in the final construct and the rest was checked by sequencing. In this way, a high transcription level of the *GapN* gene and, consequently, a high production of the 52 kDa GAPDHN protein were expected in *E. coli* cells in the presence of the IPTG inducer. To avoid any contamination with the host GAPDH, we used the *E. coli gap* mutant W3CG, which has the *gapC* gene disrupted by insertional mutagenesis [11]. This strain, that fails to metabolize sugars, showed an aerobic growth in minimal medium with glucose as the sole carbon source when transformed with pFVNP1, so the *gap*$^+$ phenotype was regained, although lower growth rates than the wild-type K-12 strain were observed (5 h versus 1.5 h, doubling times). However, in contrast with the wild-type, no growth was observed in M63 medium supplemented with gluconeogenic substrates (acetate plus succinate) (see below). Cell-free extracts from this complemented clone exhibited in the presence of G3P an irreversible and Pi (or arsenate)-independent generation of NADPH that was markedly inhibited by Pi (Fig. 2A, first kinetic). This distinctive feature of the non-phosphorylating G3P dehydrogenase reaction [4,5] indicated that the functional plant GAPDHN was present in the bacterial extracts. Moreover, the specific activity achieved (0.7–1.0 U/mg of protein) was two orders of magnitude higher than that found in photosynthetic cells [4,6]. Since the natural

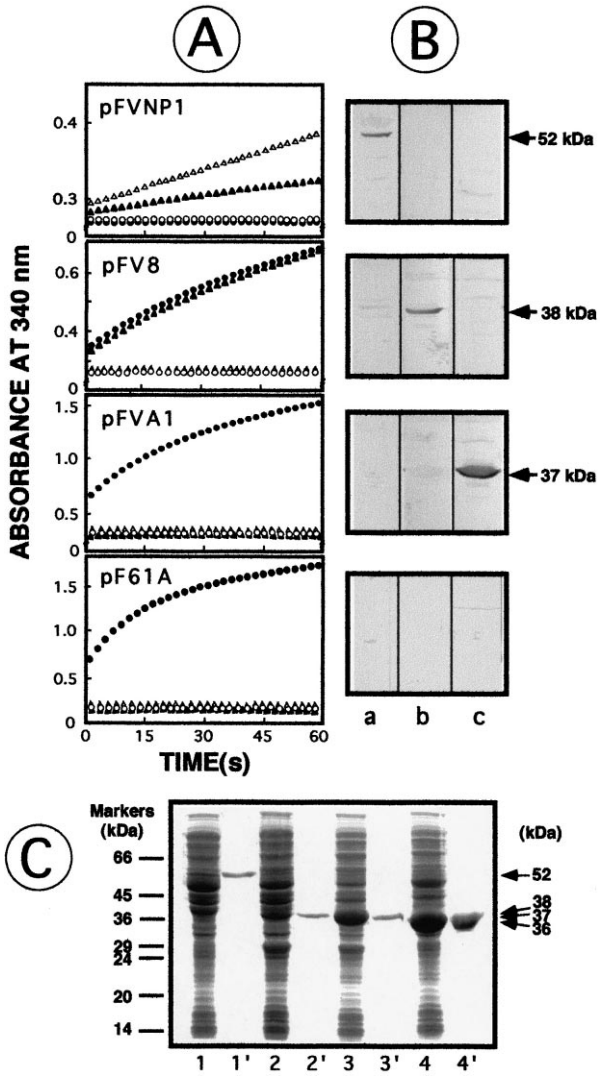

Fig. 2. (A) G3P dehydrogenase reductive activities determined spectrophotometrically at 340 nm in the presence or absence of Pi in cell-free extracts from W3CG clones transformed with plasmids containing different *gap* genes. From top to bottom: W3CG/pFVNP1, producing the GAPDHN from *P. sativum*; W3CG/pFV8, producing the NAD(P)-dependent GAPDH2 from *Synechocystis* sp. PCC 6803; W3CG/pFVA1, producing the NAD-dependent GAPDH1 from *Anabaena* sp. PCC 7120 and W3CG/pF61A, producing the NAD-dependent GAPDH from *E. coli* K-12. (▲/△) NADP-dependent activity with/without Pi; (●/○) NAD-dependent activity with/without Pi. (B) Western blots of cell-free extracts corresponding to the W3CG clones showed in A using monospecific antibodies against: pea GAPDHN (lane a), *Synechocystis* GAPDH2 (lane b) and *Anabaena* GAPDH1 (lane c). About 50 μg of protein was loaded per lane, fractionated by SDS-PAGE and transferred to nitrocellulose membranes. The apparent molecular masses of the immunodetected protein bands are indicated. (C) Coomassie blue-stained SDS-PAGE electropherogram of cell-free extracts (50 μg of protein per lane) from the W3CG clones described above, showing the level of overproduction achieved and the corresponding purified recombinant proteins (5–10 μg per lane) on their right. 1, W3CG/pFVNP1; 1′, purified pea GAPDHN (52 kDa subunit); 2, W3CG/pFV8; 2′, purified *Synechocystis* GAPDH2 (38 kDa subunit); 3, W3CG/pFVA1; 3′, purified *Anabaena* GAPDH1 (37 kDa subunit); 4, W3CG/pF61A and 4′, purified *E. coli* GAPDH (36 kDa subunit). The apparent molecular masses of the recombinant proteins are indicated on the right margin.

E. coli GAPDH is not present in the complemented W3CG clone (no NAD-dependent GAPDH activity was detected), the only possible pathway of sugar catabolism must be, therefore, a modified Embden-Meyerhof route with an irreversible and non-phosphorylating bypass involving the GAPDHN. Failure to grow with acetate plus succinate is in agreement with this proposal. Although functional complementation of *E. coli gap* mutants has been achieved with different GAPDHs [9,12,13], to our knowledge, this is the first time that a non-phosphorylating G3P dehydrogenase has been used for this purpose.

For comparison, the *E. coli* W3CG strain was also transformed with plasmids harboring genes which encode three different bacterial GAPDHs: pFV8, containing the *gap2* gene from the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 [9], pFVA1, having the *gap1* gene from the filamentous cyanobacterium *Anabaena* sp. PCC 7120 ([9] and this work) and pF61A, with the *gapC* gene from *E. coli* [16]. All these plasmids, although derived from different vectors, have genomic DNA inserts of similar sizes (2–3 kb) containing both the bacterial *gap* genes and its corresponding natural promoters. Consequently, these constructs functionally complemented the W3CG *gap* mutant and cell-free extracts of the complemented clones exhibited high levels of reversible phosphorylating G3P dehydrogenase activity, in the absence of IPTG. Noteworthy, the pFV8-transformed clone showed comparable levels (4–6 U/mg of protein) of both NAD- and NADP-dependent activities, as it was expected since the cyanobacterial GAPDH2 is able to reduce both cofactors with a similar efficiency [9] (Fig. 2A, second kinetic). In contrast, the cyanobacterial GAPDH1 (6–10 U/mg of protein) and the *E. coli* GAPDH (12–15 U/mg of protein), produced by the W3CG/pFVA1 and W3CG/pF61A clones, respectively, are NAD-dependent glycolytic enzymes (Fig. 2A). The diverse specific activity values obtained could result from differences in the expression levels achieved by each particular plasmid. It should be noted that, in contrast to the *GapN*-complemented W3CG clone, the three clones harboring recombinant GAPDHs are able, as the wild-type is, to fermentate sugars and grow on gluconeogenic substrates (acetate plus succinate) as the sole carbon sources (see below).

The presence of only one G3P dehydrogenase protein in the different W3CG-transformed clones was clearly demonstrated by Western analysis of cell-free extracts using three different monospecific polyclonal antibodies raised against pea GAPDHN and the cyanobacterial enzymes GAPDH2 and GAPDH1. As can be seen in Fig. 2B, each antibody immunodetected only its respective antigenic protein and neither of the three reacted with the *E. coli* GAPDH [16]. The overexpression levels achieved in the recombinant clones allowed us to visualize the G3P dehydrogenase proteins as major bands in the corresponding SDS-PAGE analysis of cell-free extracts (Fig. 2C). The apparent molecular masses of the purified recombinant proteins (52, 38, 37 and 36 kDa for GAPDHN, GAPDH2, GAPDH1 and *E. coli* GAPDH, respectively) correlate well with those predicted from gene sequences and match with the immunodetected protein bands. The overexpression level of *E. coli* GAPDH is evidently higher than all the other three recombinant enzymes and can be explained by the fact that the transcription is performed from a multicopy plasmid containing both the promoter and the encoding region of the bacterial host enzyme. Overall, these results confirm the identity of the recombinant G3P dehydrogenases and the corresponding natural enzymes.

Further kinetic studies also confirmed the identity of natural and recombinant GAPDHNs. The purified recombinant plant GAPDHN from the transformed W3CG clone (purification factor approximately 40-fold, yield 70%) presented a specific activity of about 20 U/mg protein. Calculated K_m values were 17 ± 2 and 30 ± 3 μM (means of three independent determinations) for NADP and D-G3P, respectively, the activity being strictly dependent on D-G3P and strongly inhibited (K_i, 50 ± 3 μM) by the L-isomer of the triose. Although the optimal pH of the recombinant GAPDHN was somewhat lower than those of natural enzymes (7.5 versus 8.5), the overall results are in good agreement with those reported for the dehydrogenase reaction of native plant and algal GAPDHNs [4,5]. On the other hand, GAPDHs as well as the members of the aldehyde dehydrogenase family exhibit an esterase activity with NPA which involves the same aa residues than the dehydrogenase reactions [14]. We have now found that both natural and recombinant pea GAPDHNs exhibit a esterase activity, the K_m value for NPA being 0.3 ± 0.02 mM in both cases. This value is clearly lower than those calculated for GAPDHs (i.e. $K_m(\text{NPA}) = 1.2 \pm 0.05$ mM for GAPDH2). To our knowledge, this is the first report on the esterase activity of a GAPDHN.

As mentioned above, the pFVNP1-transformed W3CG clone exhibited growth characteristics different from those of the recombinant clones producing GAPDHs or the wild-type K-12 strain (used as a control) when tested in various culture media (LB or M63 supplemented with different carbon sources). Similar aerobic growth rates (4–6 h, doubling times) were obtained in M63 medium with glucose for pF61A-, pFV8- and pFVNP1-transformed clones, indicating that GAPDHN is efficiently working in the sugar catabolism under these conditions, but all transformant clones showed lower growth rates than the wild-type strain (doubling time, 1.5 h). The GAPDHN-producing W3CG clone exhibited reduced growth rates in M63 media supplemented with other sugars, in accordance with the preference order for these substrates previously reported for the wild-type *E. coli* strain [17,18]. Surprisingly, the pFVA1-transformed mutant producing the cyanobacterial GAPDH1 has a much higher doubling time (approximately 9 h), indicating that this enzyme does not complement the *gap* mutation as efficiently as the others (cf. [9]). Noteworthy, the GAPDHN-producing clone failed to grow on M63 medium supplemented with the gluconeogenic substrates succinate plus acetate, showing in this medium the same pattern as the parental W3CG strain, while all three GAPDH-producing clones exhibited growth rates similar to the wild-type strain (7–9 h, doubling times). All transformant clones presented aerobic growth rates (5–7 h, doubling times) comparable to the parental W3CG strain in the non-selective succinate plus glycerol M63 medium.

Dramatic differences were also found in the anaerobic growth patterns of the GAPDHN and the GAPDH-producing W3CG clones in glucose-supplemented media, no growth being observed with the *GapN*-expressing clone (Fig. 3). Since under these conditions glycolysis is the only energy-supplying route for the enterobacterium, the lack of growth of the pFVNP1-transformed clone is the result of the in vivo operation of a non-phosphorylating bypass involving the GAPDHN, i.e. a modified glycolytic pathway with no energy

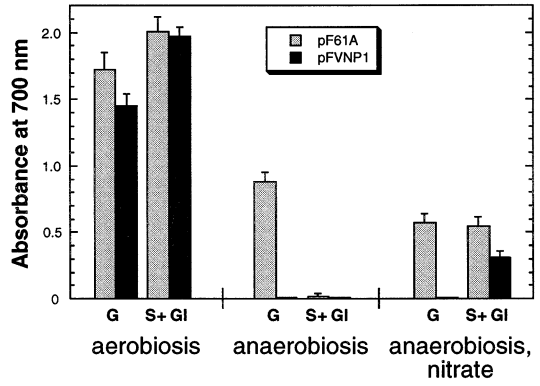

Fig. 3. Comparison of growth (absorbance at 700 nm) under different metabolic conditions of 48 h old cultures of *E. coli* W3CG clones transformed with either plasmid pF61A, producing the *E. coli* K-12 GAPDH, or plasmid pFVNP1, producing the pea GAPDHN. Cultures were grown at 37°C in M63 liquid media supplemented either with glucose (G) or succinate plus glycerol (S+Gl) under aerobiosis, anaerobiosis and anaerobiosis in the presence of potassium nitrate. Results are mean values (±S.D.) of three independent determinations.

yield. Nitrate, that can replace oxygen as the terminal electron acceptor in the respiratory chain, does not revert this growth failure (Fig. 3).

Another remarkable growth characteristic of the pFVNP1-transformed W3CG clone is the marked acidification of the medium, a decrease of 1–1.5 U of pH in the weak-buffered culture media being observed for the GAPDHN-producing clone (pH circa 6.0–6.5) when compared to clones producing GAPDHs (pH circa 7.5). In connection with this, it should be noted that the purified GAPDHN produces an intense acidification of the assay medium as a consequence of the irreversible Pi-independent G3P dehydrogenase reaction that it catalyzes [7]:

$$G3P + NADP + H_2O \rightarrow PGA + NADPH + 2H^+$$

This reaction has both kinetic and thermodinamic characteristics very different from the reversible Pi-dependent overall reaction catalyzed by GAPDH and PGA kinase:

$$G3P + NAD + ADP + Pi \Leftrightarrow PGA + NADH + ATP + H^+$$

Thus, although no ATP is produced by the GAPDHN reaction, two protons are generated as a result of water ionization [7].

The fact that the GAPDHN is able to functionally complement the *gap* mutation, producing a modified glycolytic pathway, suggests that it could have an equivalent function in its natural environment (the plant or algal cytosol). Experiments with the green alga *Chlorella fusca* showing the occurrence of a differential regulation by trophic conditions of the three algal G3P dehydrogenases [19] also indicate that, in the presence of metabolizable sugars, the GAPDHN can compete with advantage over the cytosolic NAD-dependent GAPDH due to its lower K_m values for G3P and the pyridine nucleotide. Thus, the substrate availability and enzyme kinetic characteristics could explain why the pFVNP1-transformed clone exhibited in minimal media with sugars growth rates comparable to the GAPDH-producing W3CG transformants, despite having about 10-fold less of both recombinant protein and G3P dehydrogenase activity. The presumable function of GAPDHN in a non-phosphorylating bypass of the glycolysis in plants and algae, together with the presence of a pyrophosphate(PPi)-dependent phosphofructokinase in these organisms, could be adaptations of photosynthetic eukaryotes to phosphate deficiency, a trophic stress condition often found in their natural environments that drastically reduces the cellular pools of both Pi and nucleotides, but not the PPi cellular pool [20]. These photosynthetic cells could overcome this situation by using the Pi-independent or PPi-dependent glycolytic reactions, adenine nucleotides being likely reserved for photosynthetic phosphorylation.

It was reported that GAPDHN activity was neither detected in non-photosynthetic eukaryotes, nor in eubacteria and archaea [4,6]. However, this enzyme was later found in some prokaryotic microorganisms, a GAPDHN similar to the plant enzyme having been discovered in certain cariogenic *Streptococcus* strains that lack the NADPH-generating enzymes of the oxidative pentose phosphate pathway [21]. These strains also contain the NAD-dependent phosphorylating GAPDH and a regulation of sugar degradation similar to that of photosynthetic eukaryotes has been proposed, either NADPH or ATP being generated depending of the metabolic demands of the cell. Moreover, it has recently been found [22] that the hyperthermophilic archaeum *Thermoproteus tenax*

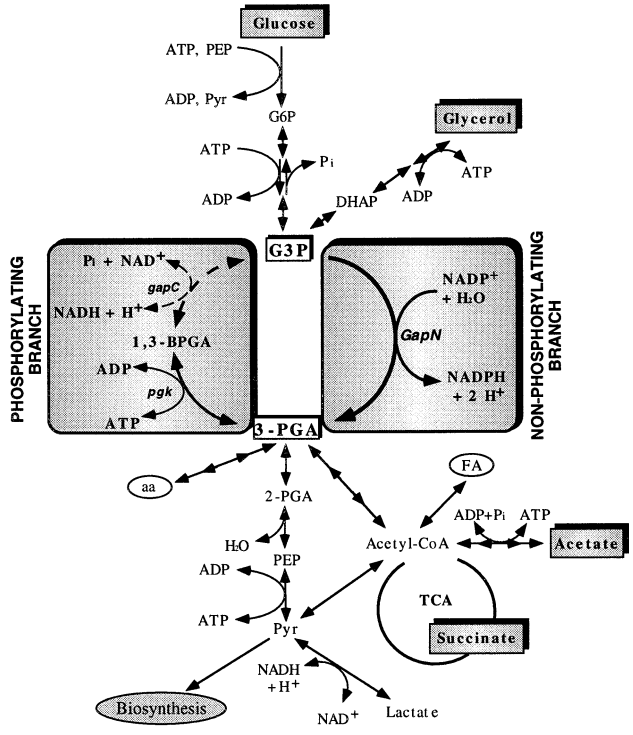

Fig. 4. The phosphorylating and non-phosphorylating branches of the glycolysis, that should be functional in the recombinant *E. coli* W3CG clones containing, respectively, either the bacterial NAD-dependent GAPDHs or the plant GAPDHN, are boxed in this metabolic scheme showing the trunk glycolytic route and its connections with other central and peripheral pathways. The reaction in which the W3CG strain is defective is shown in dashed arrows. The substrates used as carbon sources in this work have also been highlighted. TCA, tricarboxylic acid cycle; FA, fatty acids pool; aa, amino acids pool.

posseses, in addition to the GAPDH, a NAD-dependent GAPDHN that, although related to the plant enzyme, seems to be strongly regulated by the energy charge of the cell. This enzyme could have a catabolic role without any energy yield, deriving a quick pool of metabolites for other reactions. This assumption is further assesed by our recombinant pFVNP1-transformed W3CG clone, in which the strict catabolic role of the GAPDHN is established since no other GAPDH is present and the reaction is irrerversible.

The phosphorylating and non-phosphorylating branches of the glycolysis presumably operative in the recombinant *E. coli* W3CG clones described in this work and in the organisms cited above are diagramatically represented in Fig. 4, together with other central and peripheral metabolic pathways.

Summarizing, we have shown that the replacement of a single key enzyme in a central metabolic pathway can produce dramatic modifications in the physiology and growth characteristics of the bacterial host. This metabolic engineering approach has demonstrated the in vivo operation of a non-phosphorylating bypass involving a glycolytic route with no net energy yield that may be functional in photosynthetic eukaryotes and some bacteria. This work also illustrates the possible application of this strategy in the biotechnology of fermentative processes.

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