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Evolution of a *Saccharomyces cerevisiae* metabolic pathway in *Escherichia coli*

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Abstract

The *Saccharomyces cerevisiae* glycerol pathway (GPD1 and GPP2) was evolved in vivo in *Escherichia coli*. The central metabolism of *E. coli* was engineered to link glucose consumption and glycerol production. The engineered strain was evolved in a chemostat culture and a high glycerol producer was rapidly obtained. The evolution of the strain was associated to a deletion between GPD1 and GPP2, resulting in the production of a fusion protein with both glycerol-3-P dehydrogenase and glycerol-3-P phosphatase activities. The higher efficiency of the fusion protein was due to partial glycerol-3-P channeling between the two active sites. The evolved strain produces glycerol from glucose at high yield, concentration and productivity.

Keywords: Metabolic pathway evolution; Central metabolism engineering; Genes fusion; Protein fusion

1. Introduction

It is now well documented that when a microorganism is growing under a strong pressure of selection (high temperature, strong substrate limitation, etc.), variants are likely to emerge bearing changes in the DNA sequence that bring about an advantageous change in phenotype. If the selection is being applied to cell growing in liquid, the population with the original phenotype will be rapidly displaced by the variants (Atwood et al., 1951, Dykhuizen, 1990).

The genetic adaptation of a microorganism under a strong pressure of selection was later used to change the substrate specificities of enzymes for the directed evolution of new functions. The first demonstration of this approach was done by using an *Escherichia coli* strain with a lacZ deletion and by selecting spontaneous mutants that grow on lactose and other beta-galactoside sugars (Hall and Zuzel, 1980). In all the variants, the target of the evolution was the *ebgA* gene where point mutations in the structural gene alter the enzyme so that it hydrolyzes lactose, or lactulose or galactosylarabinose or lactobionic acid (Hall, 1981).

More recently, experiments have demonstrated that organisms are capable of evolving whole new metabolic pathways. The most common experimental process is to put a microorganism in a novel environment that contains a chemical that the organism has not been exposed to in the past. If that new chemical is the sole source of carbon or nitrogen the organism requires for survival, most of the time, the organism will die. However, if any of the organisms existing enzymes have the slightest ability to enhance reactions with the new resource, selection will strongly favor the evolution of the gene that produces that enzyme, and future mutations will further improve the ability of the enzyme to process the new chemical resource at high rate. This approach was successfully used in *E. coli* to evolve pathways for the use of propylene glycol (Lu et al., 1998) or ethanol (Membrillo-Hernandez et al., 2000) as carbon sources.

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The objective of the present work was to demonstrate, under appropriate "metabolic pressure", the evolutionary adaptation of a heterogeneous pathway in a strain specifically designed for this purpose. To validate this concept, the accelerated evolution of a yeast metabolic pathway (the glycerol pathway from *Saccharomyces cerevisiae*) was examined in a bacterium (*E. coli*).

2. Materials and methods

2.1. Bacterial strains and plasmids and oligonucleotides

All bacterial strains and plasmids used or derived from this study are listed in Table 1.

2.2. Growth conditions

Continuous and fed-batch cultures of *E. coli* were done under aerobic conditions in the mineral medium previously described (Hartlep et al., 2002). The continuous culture was done in a 0.4 L bioreactor at 35 °C and pH 6.5 (by addition of 6 M NH_4OH). The fed-batch fermentations were carried out in a 2 L fermentor (Setric, Toulouse, France) with a working volume of 1 L. The initial glucose concentration was 10 g/L, the substrate concentration was subsequently limited by pumping a glucose concentrated solution at a rate of 5 g/h. The temperature, the pH, the aeration and the agitation were, respectively, regulated at 37 °C, 6.8 (by addition of NH_4OH (6 M)), 0.4 vvm and 500 rpm.

During the construction of plasmid and strains, cultures were done in LB broth (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) or on LB plates (1.5% agar). Antibiotics were included as appropriate (200 mg/L erythromycin; 100 mg/L ampicillin; 25 mg/L chloramphenicol).

2.3. Analysis of fermentation products

The concentrations of substrate and fermentation products were measured as previously described (Raynaud et al., 2003).

2.4. Construction of the E. coli (ΔtpiA::FRTΔmgsA::FRTΔedd::FRT) pS3

The replacement of the *tpiA*, *mgsA* or *edd* genes by a selectable antibiotic resistance gene in the *MG1655 E. coli* strain to produce the Ecdtpi, ECdedd and Ecdmgs strains (Table 1) was facilitated by pKD46 containing an arabinose-inducible Red recombinase (Datsenko and Wanner, 2000).

The Δ*mgsA*::*FRT-cm-FRT* mutation was transduced from ECdmgs into *MG1655*(Δ*tpiA*::*FRT*) to produce the *MG1655*Δ*tpiA*::*FRT*Δ*mgsA*::*FRT-cm-FRT*) strain. After removal, the *cm* gene by using FLP recombinase (Cherepanov and Wackernagel, 1995) the *MG1655* Δ*tpiA*::*FRT*Δ*mgsA*::*FRT*) strain was selected. The Δ*edd*::*FRT-cm-FRT* mutation was transduced from ECedd into *MG1655* (Δ*tpiA*::*FRT*Δ*mgsA*::*FRT*) to produce the *MG1655* Δ*tpiA*::*FRT*Δ*mgsA*::*FRT*Δ*edd*::*FRT-cm-FRT*) strain. After removal the *cm* gene, the resulting strain *MG1655* (Δ*tpiA*::*FRT*Δ*mgsA*::*FRT*Δ*edd*::*FRT*) was electroporated with the pS3 plasmid to produce the *E. coli* (Δ*tpiA*::*FRT*Δ*mgsA*::*FRT*Δ*edd*::*FRT*) pS_3 strain.

2.5. Sampling, quenching, extraction and analysis of DHAP, G3P and GAP

Sampling, quenching, and extraction of intracellular metabolites was done by the method of Buchholz et al. (2001).

Glyceraldehyde 3-phosphate (GAP) was measured in the presence of 0.5 mM TEA/EDTA buffer (0.5 mM TrisEthanolAmine, 5 mM EDTA, pH 7.6) and 0.224 mM NADH by the addition of 0.096 U/ml glycerol-3-phosphate dehydrogenase (GDH) and 5 U/ml triosephosphate isomerase (TIM).

Dihydroxyacetone phosphate (DHAP) was measured in the same assay mixture as the GAP determination by adding 0.096 U/ml GDH.

Glycerol 3-phosphate (G3P) was measured in the presence of 0.5 mM TEA/EDTA buffer (pH 7.6) and 1 mM NAD^+ by the addition of 0.096 U/ml GDH.

Table 1
Sources and characteristics of strains and plasmids

Strain/plasmid	Relevant characteristics[a]	Source/reference
Strains		
MG1655	K12 Wild type (*F*⁻, *LAM rph-1*)	Genetic stock center, New Haven, Conn.
Ecdtpi	MG1655 Δ*tpiA*::*FRT-cm-FRT*	This study
Ecdedd	MG1655 Δ*edd*::*FRT-cm-FRT*	This study
Ecdmgs	MG1655 Δ*mgsA*::*FRT-cm-FRT*	This study
Plasmids		
pS3	Ap^r, MLS^r, Yeast GPD1 and GPP2 genes, ColE1 origin	Hartlep et al. (2002)
pS3*	Ap^r, MLSr,GPD1-GPP2 fusion genes, ColE1 origin	This study

[a]MLSr: macrolide, lincosamide, Streptogramin B resistance; Apr: ampicillin resistance.

2.6. Preparation of cell extract and enzyme assays

Cell free extracts were prepared from *E. coli* (Δ*tpiA*::*FRT*Δ*mgsA*::*FRT*Δ*edd*::*FRT*) pS3 and *E. coli* (Δ*tpiA*::*FRT*Δ*mgsA*::*FRT*Δ*edd*::*FRT*) pS3* cells previously cultured in 1 L LB + 50 μg/ml carbenicillin and stored as a cell paste at −80 °C. Cells (1.9 g wet weight) were washed twice in 100 ml of ice cold potassium phosphate buffer (25 mM, pH 7.0) and resusupended in 8 ml of ice cold MOPS buffer (10 mM, pH 7.5) containing 1 mg/ml of DNaseI and 2 mM $MgSO_4$. Resuspended cells were lysed twice in a French pressure cell at ~18,000 psi. Cell debris were pelleted by spinning 30 min at 24,800*g*, 4 °C. The cell free extract was stored at 4 °C before purification of the enzymes. GDH activity (GPD1) and glycerol-3-phosphate phosphatase (GPP2) activity were measured as previously described (Albertyn et al., 1992, Norbeck et al., 1996).

2.7. Purification of the proteins

Proteins purification was carried out at 4 °C, and whenever possible samples were kept on ice. GPD1 was purified by Anion-Exchange Chromatography and affinity chromatography on Blue Sepharose Cl-6B (Albertyn et al., 1992). GPP2 and the GPD1–GPP2 fusion protein were purified by Anion-Exchange Chromatography and Affinity Chromatography on Dyematrex RedA.

Cell extracts from *E. coli* (Δ*tpiA*::*FRT*Δ*mgsA*::*FRT*-Δ*edd*::*FRT*) pS3 for GPD1 and GPP2 and *E. coli* (Δ*tpiA*::*FRT*Δ*mgsA*::*FRT*Δ*edd*::*FRT*) pS3* for the GPD1–GPP2 fusion protein were loaded onto an anion exchange column (PerSeptive Biosystems, POROS® HQ20) equilibrated with 10 mM MOPS (pH 7.5) containing 2 mM $MgSO_4$ and eluted with a linear NaCl gradient (0–0.5 M). Fractions were collected and GPD1 and GPP2 activities measured.

To purify GPD1, the most active fractions (from the Anion-Exchange Chromatography) for the GPD1 activity were pooled and applied to a Blue Sepharose Cl-6B column equilibrated with 10mM MOPS (pH 7.5), washed until the baseline was reached and eluted with the same buffer containing 5 mM NADH. Fractions with the highest GPD1 activities were pooled and concentrated and dialyzed on an Amicon cell with a 10,000 mol weight cut-off polyethylene sulfone membrane.

To purify GPP2 and the GPD1–GPP2 fusion protein, the most active fractions for GPP2 activity were pooled and applied to a Dyematrex RedA column equilibrated with 10 mM MOPS (pH 7.5) containing 2 mM $MgSO_4$ and eluted with a linear NaCl gradient (0–1 M). Fractions with the highest GPP2 activities were pooled and concentrated on an Amicon cell with a 10,000 mol weight cut-off polyethylene sulfone membrane.

2.8. Glycerol 3-phosphate channeling experiment

The progress curve for the GPD1–GPP2-coupled reaction was obtained by measuring the time-dependent formation of inorganic phosphate in the presence of an NADH recycling system (formate dehydrogenase). The assay mixture consisted of 10 mM dihydroxyacetone-phosphate, 20 mM formate, 0.2 mM NADH and 20 U formate dehydrogenase, 20 mM MES pH 6.5 containing 10 mM $MgSO_4$, 1 mM DTE, in a total volume of 5 ml. Assays were conducted at 30 °C in the presence of 1 nmol of each purified protein. Steady-state rate of phosphate and glycerol production was determined by a least squares fit of the data. The transient time (τ) was obtained by extrapolating the steady-state rate to the time axis.

3. Results

3.1. Engineering of the central metabolism of E. coli for the in vivo evolution of a yeast glycerol pathway

The strategy used was to engineer the central metabolism of *E. coli* to get an absolute link between glucose consumption and glycerol production (Fig. 1) from the *S. cerevisiae* glycerol pathway introduced on a plasmid. Three key modifications were introduced in the central metabolism of *E. coli*. The *tpiA* gene encoding the triose phosphate isomerase an enzyme that interconverts dihydroxyacetone-P (DHAP) and glyceraldehyde-3-P (GAP) was first deleted. Such a mutant is unable to grow on sugars and is very sensitive to the accumulation of dihydroxyacetone-P, due to the conversion of this intermediate (Hopper and Cooper, 1972) to the bactericidal agent methylglyoxal (Cooper and Anderson, 1970). Although *E. coli* possesses a glyoxalase that converts methylglyoxal to D-lactate, and thus a pathway for dihydroxyacetone-P dissimilation, it has low capacity and is insufficient to prevent methylglyoxal toxicity in the *tpiA* mutant (Cooper and Anderson, 1970). To avoid any evolution of the DHAP to D-lactate pathway the *mgs* gene encoding the methyl glyoxal synthase was deleted. A *tpiA mgsA* double mutant could in theory grow on glucose by using the first two steps of the pentose-P pathway to convert glucose-6-P to 6-P-gluconate and then the Entner–Doudoroff pathway to produce GAP and pyruvate. To avoid this possible evolution of the *tpiA mgsA* double mutant, the *edd* gene encoding the 6-P-gluconate dehydratase, the first specific step of the Entner–Doudoroff pathway was also deleted. Such a mutant is unable to grow on glucose but retains growth on LB.

In yeast, glycerol can be produced from DHAP in two steps (1) reduction to glycerol-3-P (G3P) by GPD1 an NADH-dependent G3P dehydrogenase and (2) dephosphorylation of G3P to glycerol by GPP2, a G3P phosphatase. We cloned the GPD1 and GPP2 genes and organized them into a bicistronic artificial operon (see Section 2) in the pSOS95 expression vector (GenBank accession no. AY187686). The resulting plasmid pS3 was then introduced in the *E. coli* (Δ*tpiA*::*FRT*Δ*mgsA*::*FRT*-Δ*edd*::*FRT*) strain.

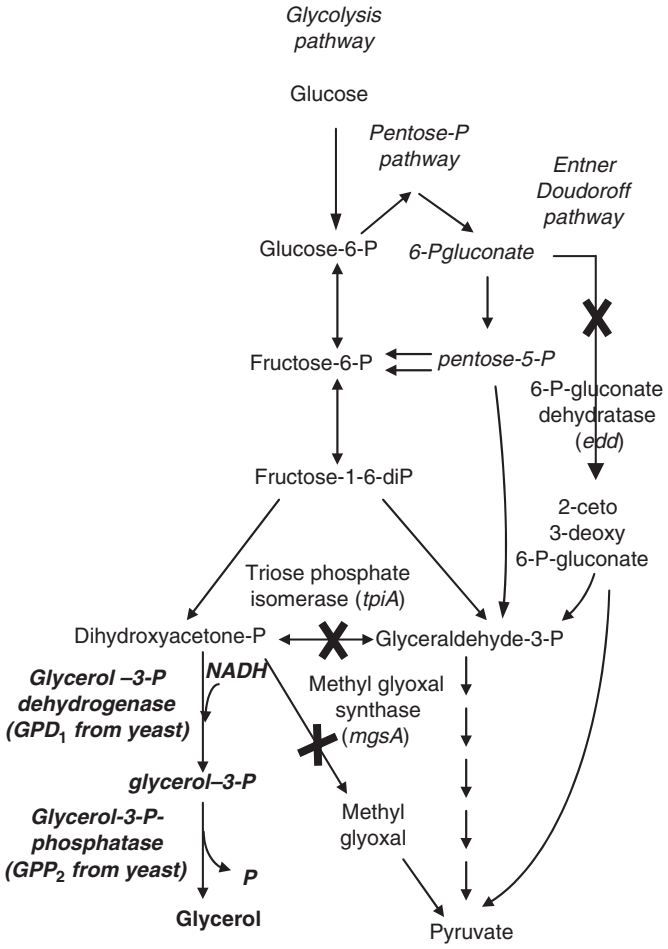

Fig. 1. Schematic representation of the *E. coli* (ΔtpiA::FRTΔmgsA::FRTΔedd::FRT) pS3 metabolism.

As in the engineered strain DHAP can only be converted to glycerol, *E. coli* (Δ*tpiA*::*FRT*Δ*mgsA*::*FRT*Δ*edd*::*FRT*) pS3 has to produce 1 mol of glycerol for each mole of glucose consumed. This implies that if the rate of glycerol formation is low, it will control the rate of glucose metabolism. Thus, any improvement in the rate of glycerol production will lead to higher glucose consumption, higher ATP production and thereby a higher growth rate. This strain should therefore be a good tool for the in vivo evolution of the glycerol pathway.

3.2. In vivo evolution of the glycerol pathway in continuous culture and analysis of the variants

We chose to evolve the glycerol pathway of *E. coli* (Δ*tpiA*::*FRT*Δ*mgsA*::*FRT*Δ*edd*::*FRT*) pS3 in chemostat culture. Based on the maximal specific growth rate of this strain in batch culture on glucose mineral medium ($0.17 + 0.02\,h^{-1}$, data not shown), the chemostat culture was run on the same medium at a dilution rate of $0.15\,h^{-1}$. For 22 generations (~100 h) an apparent steady state was obtained under a large excess of all the components of the medium, suggesting that the rate of glycerol production was limiting glucose consumption and therefore controlling the chemostat culture. Between 170 and 350 h (~40 generations) a rapid evolution was observed that progressively led to a chemostat culture controlled by glucose limitation. From the population obtained at 750 h, 10 individual clones were isolated and their specific growth rates analyzed in batch culture on the glucose mineral medium. The obtained values were not significantly different between all the isolated clones ($0.35 + 0.04\,h^{-1}$) but were more than double compared to the original strain. For 4 of them, the pS3 plasmid was isolated and the artificial glycerol operon sequenced. For all the isolated plasmids the sequences were identical and the same 44 bp deletion and one base insertion between *GPD1* and *GPP2* were observed (Fig. 2). A careful analysis of the sequence revealed that this deletion resulted in a fusion of the *GPD1* and *GPP2* genes. The transcript size of the fusion *GPD1–GPP2* gene was 1908 base pairs encoding a *GPD1–GPP2* fusion protein of a 70 kDa theoretical size. The pS3 plasmid with the 44 bp deletion and one base insertion between *GPD1* and *GPP2* was named pS3*. No additional point mutations were observed in the sequence of the artificial glycerol operon.

To evaluate the respective effect of these 44 bp deletion and one base insertion in pS3* and other possible

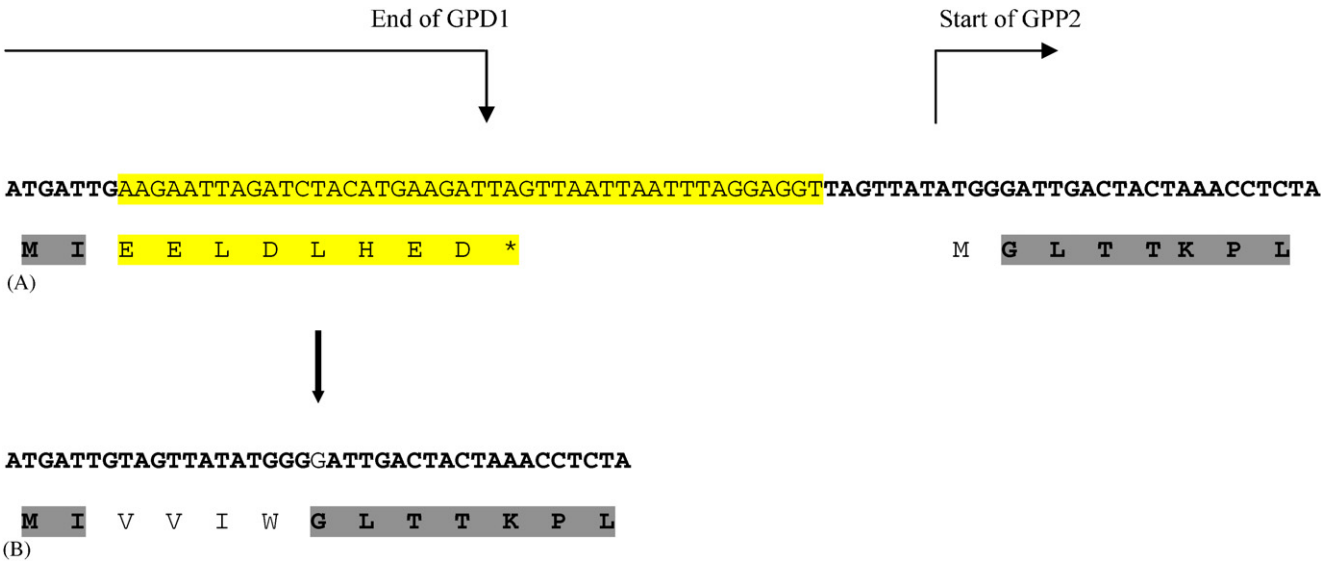

Fig. 2. Comparison of nucleotide and amino acid sequences between GPD1 and GPP2 proteins and the GPD1–GPP2 fusion protein. A-sequence of the GPD1–GPP2 intergenic region of the pS3 plasmid, the shaded sequence is the deleted section. B-corresponding region in the pS3* plasmid. Identical nucleotides and amino acids are in bold letters. The inserted base is indicated with an arrow.

Table 2
Kinetic properties of the purified proteins

	GPD1 activity			GPP2 activity		
	K_m (mM)	K_{cat} (s^{-1})	K_{cat}/K_m (/s/M)	K_m (mM)	K_{cat} (s^{-1})	K_{cat}/K_m (/s/M)
GPD1–GPP2 fusion	0.17 ± 0.04	$0.61 \pm 0.15 \times 10^2$	3.6×10^5	6.4 ± 1.4	$2.1 \pm 0.3 \times 10^2$	3.3×10^4
GPD1	0.36 ± 0.09	$0.56 \pm 0.16 \times 10^2$	1.6×10^5			
GPP2				2.9 ± 0.6	$1.6 \pm 0.2 \times 10^2$	5.6×10^4

mutations on the chromosome of the evolved strain, we transformed the original *E. coli* (Δ*tpiA*::*FRT*Δ*mgsA*::*FRT*-Δ*edd*::*FRT*) with pS3* and measured its specific growth rate in batch culture on the glucose mineral medium. The value obtained was $0.31 \pm 0.05\,h^{-1}$ indicating that the improvement in specific growth rate in the evolved strain was due mainly to the fusion between GPD1 and GPP2.

3.3. Biochemical characterization of the evolved glycerol pathway

The GPD1–GPP2 fusion protein was purified to homogeneity from a cell free extract of *E. coli* (Δ*tpiA*::*FRT*Δ*mgsA*::*FRT*Δ*edd*::*FRT*) pS3* by Anion Exchange Chromatography followed by affinity chromatography on Dyematrex RedA column. The estimated molecular weight measured by SDS-PAGE was 62 kDa in agreement with its theoretical size of 70 kDa. The size of the native protein was determined by size exclusion chromatography on a Superdex 200 column. The protein was present both as a monomer and a homodimer in a 55/45 proportion (data not shown). To compare the kinetic properties of the fusion protein with the kinetic properties of individual enzymes, we also expressed GPD1 and GPP2 in *E. coli* and purified (see material and method for detail) the two enzymes.

The fusion protein and GPD1 have similar kcat but the fusion protein has higher catalytic efficiency and lower K_m for DHAP (Table 2). The fusion protein and GPP2 also have similar kcat but in this case the fusion protein has lower catalytic efficiency and higher K_m for G3P (Table 2).

3.4. Partial substrate channeling in the GPD1–GPP2 fusion protein

One possible explanation to the higher flux in the glycerol pathway in *E. coli* (Δ*tpiA*::*FRT*Δ*mgsA*::*FRT*-Δ*edd*::*FRT*) pS3* might be partial G3P channeling in the fusion protein compared to the mixture of individual enzymes. To evaluate this hypothesis, we performed an *in vitro* experiment with equimolar amount of GPD1 and GPP2 or the GPD1–GPP2 fusion protein under excess of DHAP and in the presence of an NADH recycling system (for details see Section 2). The reaction was followed by the liberation of free phosphate in the reaction mixture and the steady state concentration of G3P was measured (Fig. 3).

Using the GPD1–GPP2 fusion protein, the progress curve for G3P formation in the presence of saturating substrates reaches steady state after a short transient time (τ) and remains constant for at least 10 min. Analysis of this data gave a τ of 10 s and a steady-state G3P concentration of 0.3 mM. In contrast, when an equimolar mixture of GPD1 and GPP2 was used, values of 75 s and 1.6 mM were obtained for τ and G3P steady-state concentration, respectively. Moreover, the phosphate production rate was doubled using the GPD1–GPP2 fusion protein (0.011 mM/s) compared to that measured using the natives GPD1 and GPP2 proteins (0.0058 mM/s). The shorter transient time and lower steady-state G3P concentration is consistent with the idea that the intermediate is partially channeled between the active sites of the GPD1–GPP2 fusion protein.

To verify if partial substrate channeling also occurred *in vivo*, we grew *E. coli* (Δ*tpiA*::*FRT*Δ*mgsA*::*FRT*Δ*edd*::*FRT*) pS3 and *E. coli* (Δ*tpiA*::*FRT*Δ*mgsA*::*FRT*Δ*edd*::*FRT*) pS3* in chemostat culture at a dilution rate of 0.1 h^{-1} and analyzed the intracellular concentrations of DHAP, G3P and GAP and the activities of GPD1 and GPP2 (Table 3). Although DHAP and GAP concentrations changed only slightly, the G3P concentration was much lower for the culture with the GPD1–GPP2 fusion protein. This result can not be explained by a change in GPD1 and GPP2 level or GPP2/GPD1 ratio as all those values were lower for the culture with the fusion protein (Table 3).

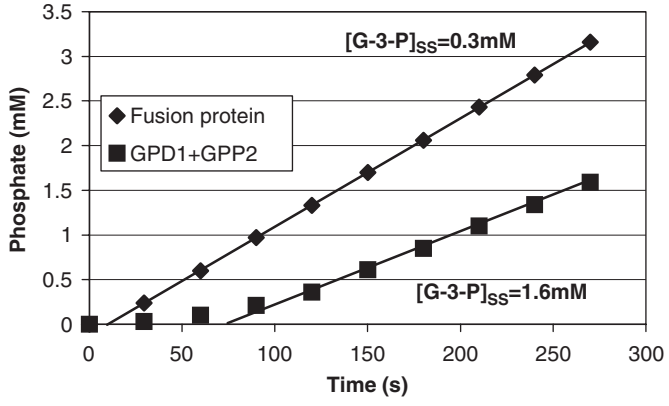

Fig. 3. Progress curves for the formation of inorganic phosphate in the GPD1–GPP2-coupled reactions. The time-dependent formation of inorganic phosphate in the GPD1–GPP2-coupled reaction was measured as described under materials and methods.

3.5. Production of glycerol by the evolved *E. coli* strain

Glycerol production by *E. coli* (Δ*tpiA*::*FRT*Δ*mgsA*::*FRT*Δ*edd*::*FRT*) pS3* and by *E. coli* (Δ*tpiA*::*FRT*Δ*mgsA*::*FRT*Δ*edd*::*FRT*) pS3 was investigated in aerated Fed-batch cultures with glucose as carbon source. Growth of *E. coli* (Δ*tpiA*::*FRT*Δ*mgsA*::*FRT*Δ*edd*::*FRT*) pS3* was accompanied by the accumulation of glycerol to a final concentration of over 130 g/L (Fig. 4A). Glycerol was the major fermentation product. Concentrations of acetate remained below 1 g/L. Throughout the fermentation process, the glycerol yield on glucose was 1.0 mol/mol. Due to the low initial biomass concentration, the overall glycerol production rate was 0.68 g glycerol/L/h. Glycerol consumption did not occur, even after glucose depletion. Most wild-type *E. coli* strains can consume glycerol (Irani

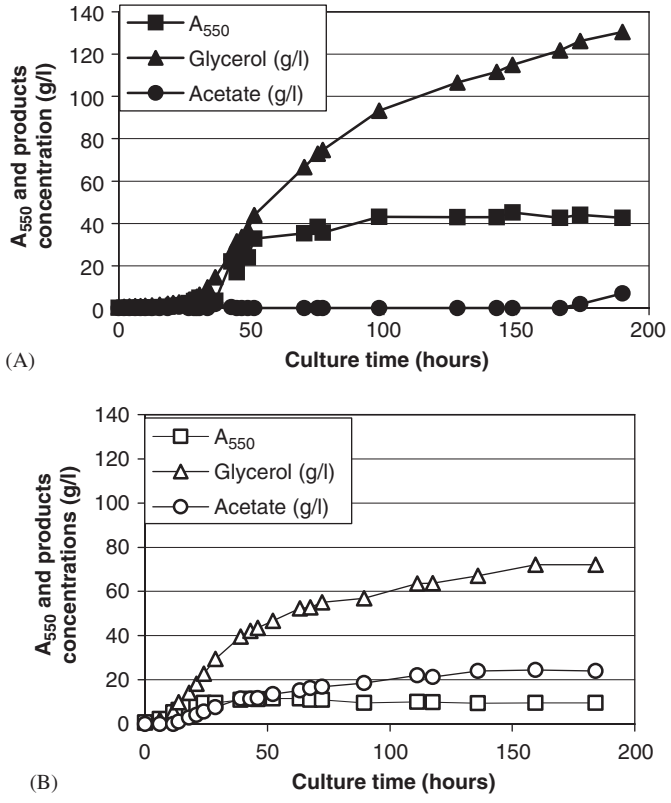

Fig. 4. Comparison of fed-batch cultures of *E. coli* (Δ*tpiA*::*FRT*Δ*mgsA*::*FRT*Δ*edd*::*FRT*) pS3* (A) and *E. coli* (Δ*tpiA*::*FRT*Δ*mgsA*::*FRT*Δ*edd*::*FRT*) pS3 (B).

Table 3
Intracellular concentration of DHAP, GAP, G3P and activities of GPD1 and GPP2 in chemostat culture of *E. coli* (Δ*tpiA*::*FRT*Δ*mgsA*::ΔFRTΔ*edd*::*FRT*) pS3 and *E. coli* (Δ*tpiA*::*FRT*Δ*mgsA*::Δ*FRT*Δ*edd*::*FRT*) pS3*

	DHAP (mM)	GAP (mM)	G3P (mM)	GPD1 (U/mg)	GPP2 (U/mg)
E. coli (Δ*tpiA*::*FRT*Δ*mgsA*::*FRT*Δ*edd*::*FRT*) pS3	2±0.4	0.8±0.3	1.8±0.4	2.1±0.9	9.2±2.3
E. coli (Δ*tpiA*::*FRT*Δ*mgsA*::*FRT*Δ*edd*::*FRT*) pS3*	1.5±0.3	1.2±0.5	0.5±0.2	1.7±0.7	7.4±2.5

Conditions: $D = 0.1\ h^{-1}$, temperature = 35 °C and pH = 6.5.

and Maitra, 1977). However, in the triple mutant, glycerol dissimilation is not possible as the absence of triose phosphate isomerase blocks the oxidation pathway (Anderson and Cooper, 1969). In similar culture conditions, growth of *E. coli* (Δ*tpiA*::*FRT*Δ*mgsA*::*FRT*Δ*edd*::*FRT*) pS3 was associated to the production of both glycerol and acetate at final concentrations of 70 and 24 g/L, respectively (Fig. 4B).

4. Discussion

In this study, we describe the successful *in vivo* evolution of a yeast glycerol pathway in *E. coli* and the molecular and biochemical characterizations of the evolved pathway. The keys for the success of the evolution was (i) to engineer the metabolism of *E. coli* so that glucose could be metabolized only if glycerol was produced (ii) to introduce the yeast glycerol pathway in *E. coli* as an artificial bicistronic operon. The engineering of *E. coli* was achieved (i) by deleting the *tpiA* gene to force half of the carbon from glucose to be catabolized to glycerol and (ii) by deleting the genes coding for the first enzymes specific of the methylglyoxal and the Entner–Doudoroff pathways, two alternative pathways that could allow glucose metabolism without glycerol production. Deletion of the *tpi* gene has previously been used in yeast (Overkamp et al., 2002) to increase the yield of glycerol production from glucose but never with the purpose of evolving GPD1 or GPP2 which encoding genes are located on different chromosomes. The selection procedure used in our study was based on the evolution of in a chemostat culture that was not controlled by substrate limitation but by the flux in the glycerol pathway. The fact that the microorganism has no other possibility to increase the glucose flux than increasing the flux in the glycerol pathway and thereby reach glucose limitation allowed a rapid evolution of the culture. Interestingly, we found that the main reason for the evolution of the strain was due to 44 bp deletion and one base insertion on the pS3 plasmid, between GPD1 and GPP2, which resulted in the production of a fusion protein with both G3P dehydrogenase and G3P phosphatase activities. The biochemical characterization of this fusion protein showed that its higher catalytic efficiency than GPD1 and its lower catalytic efficiency than GPP2 were mainly due to change in the K_m values while the kcat values, for both activities, were fairly similar to those of the individual enzymes. By comparing only the individual kinetic properties of these enzymes, it is difficult to explain why the fusion protein is more efficient *in vivo* and *in vitro* than the mixture of the individual enzymes. GPD1 is a reversible G3P dehydrogenase with high affinities for DHAP and G3P while GPP2 is a unidirectional G3P phosphatase with relatively low affinity for G3P. In agreement with these enzyme properties, we observed, both *in vitro* and *in vivo*, a relatively high steady state concentration of G3P. The net flux of DHAP to G3P conversion is the difference between the rate of G3P production minus the rate of G3P conversion back to DHAP. Thus, the net flux of G3P production will be inversely dependent upon the G3P concentration. Partial channeling of G3P between GPD1 and GPP2 would effectively diminish the G3P concentration, thereby increasing both the net flux of G3P synthesis and the overall rate of DHAP conversion to glycerol. Two lines of evidence support the contention that G3P is partially channeled in the GPD1–GPP2 fusion protein: (1) the transient time for the steady-state rate of glycerol formation is significantly lower for the fusion protein than the individual enzymes. (2) Steady-state kinetics satisfy the criteria devised by Ovadi et al. (1989) for partial channeling of G3P.

There are a number of well-characterized examples of substrate channeling within bifunctional enzymes. In aromatic amino acid biosynthesis the α-subunit of tryptophan synthase catalyzes the production of indole, which is then channeled to the α-subunit to condense with serine for the synthesis of tryptophan (Dunn et al., 1990). Two different bifunctional enzymes catalyze the conversion of chorismate, directing the intermediate prephenate to the synthesis of either tyrosine (Christopherson et al., 1983) or phenylalanine (Zhang et al., 1998). The bifunctional enzyme glutamate formimidoyl transferase-cyclodeaminase has been proposed to channel N5-formiminotetrahydrofolate in consecutive reactions in the histidine degradative pathway (MacKenzie and Baugh, 1980). A recent structure of the transferase domain shows the presence of an electrostatic tunnel through the domain that is postulated to be responsible for channeling the folate intermediate (Kohls et al., 2000).

However, to our knowledge there is no example of individual consecutive enzymes in a pathway where partial substrate channeling was artificially created by a fusion of the two proteins. In that respect, the creation by *in vivo* evolution of a fusion protein with a higher efficiency due to partial substrate channeling is unique. As in *S. cerevisiae* the two genes are located on different chromosomes such an evolution would be almost impossible in yeast.

At the end of the evolution process in chemostat culture not only was an improved catalyst for the conversion of DHAP to glycerol obtained but also a very efficient microorganism for the conversion of glucose to glycerol. Without any optimization of the fermentation conditions high yield (1 mol/mol), titer (130 g/L) and productivity (16 g/L day) were obtained in glucose limited Fed-batch cultures. The performances of this strain compared favorably with the best *S. cerevisiae* strain obtained by a metabolic engineering approach (Overkamp et al., 2002).

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