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Response of the central metabolism of *Escherichia coli* to modified expression of the gene encoding the glucose-6-phosphate dehydrogenase

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Abstract The deletion of the *zwf* gene encoding G6PDH activity led to restructuring of the carbon flux through central metabolism in *Escherichia coli*, though over-expression of this gene had only minor consequences for overall carbon flux. The modified carbon flux seen in the *zwf* deletion mutant enabled alternative routes of anabolic precursor formation and an adequate supply of NADPH synthesis via a modified TCA cycle to be generated so as to sustain growth rates comparable to the WT.

Keywords: Metabolic network; Fluxomics; ^{13}C-labelling experiments; Mass isotopomer analysis; *Escherichia coli*

1. Introduction

Microorganisms can efficiently adapt their metabolism in response to genetic or environmental changes, and understanding the extent of this metabolic robustness has become an emergent issue in both basic and applied research. Part of the robustness is directly related to the network organization of metabolic systems, where the interplay between all available biochemical reactions provides alternative mechanisms for compensating the perturbations [1,2]. Recently, ^{13}C-metabolic flux analysis (^{13}C-MFA) has been applied to *Escherichia coli* knock-out mutants lacking key enzymes of central metabolism to determine the phenotypic effects of structural changes in the metabolic network, providing direct evidence for the nature and extent of the mechanisms that compensate the effects of such perturbations [3–6]. Compensatory mechanisms involve enzyme redundancy and alternative pathways.

Glucose-6-phosphate dehydrogenase (G6PDH) is a key enzyme in central metabolism, involved in the partition of carbon between glycolysis and the pentose phosphate pathway (PPP) which provides a large proportion of the NADPH needed for anabolism. The consequences of the lack of G6PDH have been already reported [7–9], but there is currently no report on the effects of increased G6PDH activity on the metabolic network. A strain expressing *zwf* to high levels has been constructed and compared to both its parent strain and an isogenic strain in which *zwf* has been deleted as regards to the growth characteristics and metabolic flux distribution. To define clearly the consequences of specific genetic modifications, isotopic flux distribution techniques were used to access real in vivo metabolic fluxes within central metabolism.

2. Materials and methods

2.1. Bacterial strains

Escherichia coli MG1655 was used as the parent strain for the construction of both the deletion and overexpression mutants. The deletion mutant (Δzwf) was obtained by a one-step disruption protocol [10] and the overexpression mutant (Pzwf) was obtained by a one-step method to modulate expression of chromosomal genes [11] based on sequence substitution within the promoter region. In order to confirm the mutations, polymerase chain reaction (PCR) was used to amplify fragments containing the modified sequences. Lengths of amplified fragments were tested by agarose gel electrophoresis and compared with those of the wild type strain (WT). PCR products were also sequenced to confirm *zwf* removal and to determine the exact sequence of the artificial promoter.

2.2. Media and growth conditions

All *E. coli* strains were grown on minimal synthetic medium containing 48 mM Na_2HPO_4, 22 mM KH_2PO_4, 9 mM NaCl, 19 mM NH_4Cl, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.1 g/l of thiamine and 2 g/l of glucose. Magnesium sulfate and calcium chloride were autoclaved separately. Glucose and thiamine were sterilized by filtration. Exponentially growing cells pre-cultured in the same medium were harvested by centrifugation, washed in the same volume of fresh medium (lacking glucose and thiamine) and used for inoculation with 1% (v/v). Cultivation

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Abbreviations: G6PDH, glucose-6-phosphate dehydrogenase; ^{13}C-MFA, ^{13}C-metabolic flux analysis; GC–MS, gas chromatography–mass spectrometry; PPP, pentose phosphate pathway; TCA, tricarboxylic acid cycle; ED, Entner Doudoroff

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was at 37 °C in a 2 l stirred reactor with a dissolved oxygen content maintained above 20% saturation and with pH regulated at 6.9 by addition of 2 M NaOH.

2.3. Analytical procedures and physiological parameters

Cell growth was monitored by measuring optical density at 600 nm (OD_{600}). Glucose and acetate concentrations were determined by HPLC as described previously [12]. Physiological parameters (maximum growth, specific glucose consumption and specific acetate production rates) were determined during exponential growth phase using a previously validated correlation factor of 0.38 g cellular dry weight per OD_{600} U.

The specific activity of G6PDH was measured in crude cell extracts. After two washings, the cells were resuspended in buffer containing Tris–HCl (275 mM) and tricarballylate (10 mM) at pH 7.8, glycerol (10% v/v), $MgCl_2$ (11 mM) and DTT (1 mM). Cells were disrupted by sonication using an ultrasonic disruptor (Bioblock Vibracell 72412). Cell debris was removed by centrifugation (20 000 × *g* for 10 min at 4 °C), and the crude cell extracts were used for enzyme assays. Enzyme activity was measured spectrophotometrically at 340 nm under thermostatically controlled conditions (37 °C). All compounds of the reaction mixture, Tris–HCl buffer pH 7.6 (100 mM), $MgCl_2$ (12.5 mM), $NADP^+$ (0.6 mM), 350 µl extract, diluted as necessary, were pipetted into a micro-cuvette with a 1 cm light path. The reaction was initiated by adding glucose 6-phosphate (2 mM) to a final volume of 1 ml. Protein concentrations were determined using the Lowry method with bovine serum albumin as a standard [13].

2.4. ^{13}C-labelling experiments

Cultures for ^{13}C-labelling experiments employed a mixture of 20% (mol/mol) [U-^{13}C] glucose and 80% (mol/mol) [1-^{13}C] glucose (99% of ^{13}C atom, Eurisotop, France). Samples were collected at the mid-exponential phase to ensure isotopic and metabolic steady state. Mass isotopomer fractions of amino acids were determined in triplicate by gas chromatography–mass spectroscopy (GC–MS) as described previously [14]. Data acquisition were performed in the selected ion monitoring (SIM) mode, monitoring mainly fragments at $[M-57]^+$ except for isoleucine. The mean experimental error for mass isotopomer fractions was 0.38%. The GC–MS derived mass isotope distributions were corrected for naturally occurring isotopes [15]. The measurement of positional isotopomer and carbon enrichments was performed by NMR spectroscopy, from 2D-HSQC [16] and 2D-TOCSY [17] experiments, respectively. Samples for NMR analysis were prepared as described previously [17] from the same cultures as the samples used for GC–MS analysis.

2.5. Metabolic flux analysis

Metabolic fluxes were calculated from the ^{13}C-labeling patterns of metabolites using both metabolite and isotope balancing equations. The metabolic network considered for flux calculations contained the main pathways of *E. coli* central metabolism: glycolysis, PPP, tricarboxylic acid cycle (TCA), the glyoxylate shunt and anaplerotic reactions. It included additionally the Entner Doudoroff (ED) pathway and reactions of amino acid biosynthesis. The reaction between phosphoenolpyruvate and oxaloacetate was defined as the net flux through a bidirectional conversion involving phosphoenolpyruvate carboxylase and phosphoenolpyruvate carboxykinase.

Flux calculations were performed using the 13C-Flux software developed by Wiechert [18], in which both mass balances and carbon atom transitions describing the bioreaction network were used. Measurable extracellular fluxes (glucose uptake, acetate production and specific precursor requirements for biomass [19]) were constrained as well as the labelling pattern of glucose, thereby obtaining direct evidence for possible modifications in in vivo fluxes.

3. Results and discussion

3.1. Construction and characterization of the mutants

The behaviour of the strain over-expressing *zwf* (Pzwf strain) (in which the promoter sequence was modified as shown in Supplementary data S1) was compared to its parent strain (*E. coli* MG1655) but also to a knock-out mutant lacking G6PDH activity (Δzwf strain), generated by removal of the entire sequence of the *zwf* gene.

The expression of *zwf* in these strains was assessed by measuring G6PDH activity in cell-free extracts. The $NADP^+$-dependent G6PDH activity of the WT strain was found to be 190 ± 4 nmol/min/$mg_{protein}$. This value was similar to the values reported in the literature for comparable cultivation conditions [4,5]. As expected, no G6PDH activity was detectable in the deletion mutant while high activity was measured in the over-expression mutant (2870 ± 20 nmol/min/$mg_{protein}$). This value was more than 15 times higher than in the parent strain, indicating that the *zwf* gene was efficiently over-expressed when placed under the control of the artificial promoter.

The growth behaviour of the strain over-expressing *zwf*was compared to the growth of the two other strains (Table 1). The growth of the parent strain in the medium considered in this work was similar to that seen in previous reports using synthetic media, and was accompanied by the production of acetate. As described previously [9], the deletion of the *zwf* gene had little effect on cell growth (slight decrease in growth rate). In the same way, the strain over-expressing *zwf* showed growth characteristics close to those observed for the WT strain, indicating that the increased G6PDH activity had no significant consequence on the overall phenotypic behaviour. Obviously, the metabolic network of *E. coli* can compensate for such changes in *zwf* expression without major deleterious effects on the growth characteristics.

3.2. Metabolic flux response to modified expression of zwf

The distribution of carbon flux throughout the central metabolic pathways was measured in the three strains, using ^{13}C-labelling experiments. Flux analysis was based on the GC–MS mass isotopomer analysis of proteinogenic amino acids following proteolysis of biomass collected under both metabolic and isotopic steady-state conditions [20]. For this purpose, cells were grown in minimal medium containing a mixture of 80% [1-^{13}C] glucose and 20% [U-^{13}C] glucose. The specifically labelled [1-^{13}C] glucose was used, in addition to the uniformly labelled glucose, to obtain direct data concerning flux through the oxidative PPP since CO_2 loss within this pathway involves specific loss of [1-^{13}C] glucose [21]. Thus, the M0 abundances will reflect the extent to which glucose is catabolized via PPP. The M0 abundances, corresponding to unlabelled molecules, for all amino acids were lower in the *zwf* deletion mutant than in the two other strains (see Supplementary Table S2). The higher content in ^{13}C-atom in this strain thus reflects a diminished contribution of the oxidative PPP in this strain. The relative importance of M0 abundances was quite similar in the WT and the strain over-expressing *zwf*, indicating that flux distribution was not significantly altered in this background.

Table 1
Growth characteristics of the different strains: μ, specific growth rate; q_{Glc}, glucose uptake rate; v_{Ac}m acetate production rate

Strains	μ (h^{-1})	q_{Glc} (mmol/g/h)	v_{Ac} (mmol/g/h)
Δzwf	0.60 ± 0.04	7.71 ± 0.43	3.40 ± 0.19
WT	0.65 ± 0.01	7.15 ± 0.61	3.44 ± 0.42
Pzwf	0.66 ± 0.02	7.32 ± 0.65	3.23 ± 0.29