Metabolic flux analysis of wild-type *Escherichia coli* and mutants deficient in pyruvate-dissimilating enzymes during the fermentative metabolism of glucuronate

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The fermentative metabolism of D-glucuronic acid (glucuronate) in *Escherichia coli* was investigated with emphasis on the dissimilation of pyruvate via pyruvate formate-lyase (PFL) and pyruvate dehydrogenase (PDH). *In silico* and *in vivo* metabolic flux analysis (MFA) revealed that PFL and PDH share the dissimilation of pyruvate in wild-type MG1655. Surprisingly, it was found that PDH supports fermentative growth on glucuronate in the absence of PFL. The PDH-deficient strain (Pdh[−]) exhibited a slower transition into the exponential phase and a decrease in specific rates of growth and glucuronate utilization. Moreover, a significant redistribution of metabolic fluxes was found in PDH- and PFL-deficient strains. Since no role had been proposed for PDH in the fermentative metabolism of *E. coli*, the metabolic differences between MG1655 and Pdh[−] were further investigated. An increase in the oxidative pentose phosphate pathway (ox-PPP) flux was observed in response to PDH deficiency. A comparison of the ox-PPP and PDH pathways led to the hypothesis that the role of PDH is the supply of reducing equivalents. The finding that a PDH deficiency lowers the NADH : NAD⁺ ratio supported the proposed role of PDH. Moreover, the NADH : NAD⁺ ratio in a strain deficient in both PDH and the ox-PPP (Pdh[−]Zwf[−]) was even lower than that observed for Pdh[−]. Strain Pdh[−]Zwf[−] also exhibited a slower transition into the exponential phase and a lower growth rate than Pdh[−]. Finally, a transhydrogenase-deficient strain grew more slowly than wild-type but did not show the slower transition into the exponential phase characteristic of Pdh[−] mutants. It is proposed that PDH fulfils two metabolic functions. First, by creating the appropriate internal redox state (i.e. appropriate NADH : NAD⁺ ratio), PDH ensures the functioning of the glucuronate utilization pathway. Secondly, the NADH generated by PDH can be converted to NADPH by the action of transhydrogenases, thus serving as biosynthetic reducing power in the synthesis of building blocks and macromolecules.

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Abbreviations: AcCoA, acetyl-CoA; ACK, acetate kinase; MFA, metabolic flux analysis; non-ox-PPP, non-oxidative pentose phosphate pathway; ox-PPP, oxidative pentose phosphate pathway; PDH, pyruvate dehydrogenase; PFL, pyruvate formate-lyase; PP, pentose phosphate; PPP, pentose phosphate pathway; PTA, phosphotransacetylase; TCA cycle, tricarboxylic acid cycle.

A reaction network model for glucuronate fermentation by *Escherichia coli*, list of metabolites and results of the *in vivo* metabolic flux analysis of wild-type *E. coli* MG1655 and mutants deficient in the pyruvate-dissimilating enzymes pyruvate dehydrogenase and pyruvate formate-lyase, and *in silico* metabolic flux analysis of wild-type *E. coli* and mutants deficient in pyruvate dehydrogenase, pyruvate-formate lyase, glucose-6-phosphate dehydrogenase and phosphotransacetylase enzymes, are available as supplementary information with the online version of this paper.

INTRODUCTION

D-Glucuronic acid (glucuronate) is a naturally occurring six-carbon sugar acid found in both plants and animals, the fermentative metabolism of which is of importance for fundamental and applied research. For example, glucuronic and 4-O-methyl-glucuronic acids are major side chains of xylans present in most plants considered as feedstocks for the production of biofuels (Lawford & Rousseau, 1997), an application in which *Escherichia coli* is one of the preferred organisms (Atsumi & Liao, 2008). Glucuronate is also found in the intestine of mammals, where *E. coli* is the predominant facultative anaerobe (Finegold *et al.*, 1983), and its metabolism plays an important role in the colonization of the large intestine by this bacterium (Peekhaus & Conway, 1998).

E. coli utilizes glucuronate as the sole carbon and energy source under fermentative conditions using the pathways summarized in Fig. 1 (Mandrand-Berthelot *et al.*, 2004). Once inside the cells, glucuronate is isomerized to D-fructuronate by D-glucuronate isomerase (*uxaC*). D-Fructuronate then undergoes an NADH-dependent reduction to D-mannonate by D-mannonate oxidoreductase (*uxuB*). D-mannonate dehydratase (*uxuA*) subsequently catalyses dehydration to yield 2-dehydro-3-deoxy-D-glucuronate (KDG). At this point, 2-keto-3-deoxygluconokinase (*kdgK*) phosphorylates KDG to yield 2-dehydro-3-deoxy-D-glucuronate-6-phosphate (KDPG), which is an intermediate in the Entner–Doudoroff pathway. KDPG is further cleaved by 2-keto-3-deoxyglucuronate-6-phosphate aldolase (*eda*) to produce the glycolytic intermediates pyruvate and glyceraldehyde 3-phosphate (Mandrand-Berthelot *et al.*, 2004). These two intermediates are processed through common glycolytic, gluconeogenic and fermentative pathways, and reductive and oxidative branches of the tricarboxylic acid (TCA) cycle, to generate precursor metabolites, ATP, and reducing power for biomass synthesis along with several fermentation products (Fig. 1). Pyruvate also serves as the main switching point between respiratory and fermentative metabolism. In this context, pyruvate formate-lyase (PFL) and pyruvate dehydrogenase (PDH) are considered the major routes for pyruvate dissimilation in *E. coli* (Sawers & Clark, 2004). During aerobic growth, pyruvate is converted to acetyl-CoA (AcCoA), carbon dioxide and NADH by PDH. For anaerobic growth, on the other hand, PFL replaces PDH in the formation of AcCoA, and generates formate as co-product.

The work reported here studied the fermentative metabolism of glucuronate in *E. coli* with emphasis on pyruvate dissimilation. It was found that PDH can support fermentative growth on glucuronate in the absence of PFL. *In silico* and *in vivo* metabolic flux analyses (MFA) were used to characterize glucuronate fermentation in the wild-type and in recombinant strains lacking PDH and PFL. Changes in metabolic fluxes brought about by the PDH and PFL deficiencies provided the basis to hypothesize that the role of PDH during glucuronate fermentation is to provide reducing equivalents for glucuronate utilization and biosynthesis.

METHODS

Strains, plasmids and genetic methods. Strains, plasmids and primers used in this study are listed in Table 1. K12 strain MG1655, used as the wild-type strain, and mutants Pdh[−] (PDH-deficient) and Pfl[−] (PFL-deficient) were obtained from the University of Wisconsin *E. coli* Genome Project (<http://www.genome.wisc.edu>; Kang *et al.*, 2004). Single-gene deletion mutants JW1841 (Δzwf -FRT-Km-FRT), JW2294 (Δpta -FRT-Km-FRT), JW1228 ($\Delta adhE$ -FRT-Km-FRT) and JW1375 ($\Delta ldhA$ -FRT-Km-FRT) were obtained from the National BioResource Project, National Institute of Genetics, Japan (Baba *et al.*, 2006). Double mutant SthA–PntA[−] (SthA[−], soluble pyridine nucleotide transhydrogenase-deficient; PntA[−], membrane-bound pyridine nucleotide transhydrogenase-deficient) was obtained from

the laboratory of Dr U. Sauer, Institute of Molecular Systems Biology, Zurich, Switzerland (Sauer *et al.*, 2004). Single mutants Zwf[−] (glucose-6-phosphate dehydrogenase-deficient), Pta[−] (phosphotransacetylase-deficient), Ldh[−] (lactate dehydrogenase-deficient) and Adh[−] (alcohol/acetaldehyde dehydrogenase-deficient) were constructed by introducing the *zwf*, *pta*, *ldhA* and *adhE* deletions from JW1841, JW2294, JW1228 and JW1375, respectively, into MG1655 using P1 phage transduction. Double mutant Pdh–Zwf[−] was constructed by transferring the *aceF* mutation from strain Pdh[−] into strain Zwf[−]. Double mutant Pfl–Ldh[−] was obtained by transferring the *pflB* mutation from strain Pfl[−] into strain Ldh[−]. Finally, double mutant Pta–Adh[−] was constructed by transferring the *adhE* mutation from strain JW1228 into strain Pta[−]. Details of the specific protocol used in phage transduction are available elsewhere (Yazdani & Gonzalez, 2008; Miller, 1972).

Manufacturers' protocols and standard methods (Miller, 1972; Sambrook *et al.*, 1989) were followed for DNA purification (Qiagen), restriction endonuclease digestion (New England Biolabs) and DNA amplification (Stratagene and Invitrogen). The strains were kept in 32.5 % v/v, glycerol stocks at -80°C . Plates were prepared using Luria–Bertani (LB) medium containing 1.5 % (w/v) agar, and chloramphenicol and kanamycin were included (when required) at final concentrations of 34 and 50 $\mu\text{g ml}^{-1}$, respectively.

Culture medium and cultivation conditions. The minimal medium designed by Neidhardt *et al.* (1974) supplemented with 5 g glucuronate l^{-1} was used unless otherwise specified. A 10 $\times$ concentrate of this medium was prepared and filter-sterilized before storing at -20°C . Chloramphenicol and kanamycin were included when required at final concentrations of 34 and 50 $\mu\text{g ml}^{-1}$, respectively. Chemicals were obtained from Fisher Scientific and Sigma-Aldrich.

Fermentations were conducted in a SixFors multi-fermentation system (Infors) with six 400 ml working volume vessels and independent control of temperature (37°C), pH (7.4) and stirrer speed (300 r.p.m.) (Dharmadi *et al.*, 2006). The system was fully equipped and computer controlled using the manufacturer's IRIS NT software. Each vessel was fitted with a condenser operated with a 4°C cooling methanol–water supply to minimize evaporation. Anaerobic conditions were maintained as follows. The medium was autoclaved and sparged with ultra-high-purity argon (Matheson, Tri-Gas), which was passed through an oxygen trap (Alltech Associates) to eliminate traces of oxygen. The vessels were then closed to prevent any exchange of gases. NaOH and H_2SO_4 (both 3 M) were used to control the pH.

Pre-cultures used to inoculate the above fermenters were prepared as follows. A single colony was used to inoculate 17 ml Hungate tubes (Bellco Glass) completely filled with MOPS media (pH 7.4) supplemented with 5 g glucuronate l^{-1} , 10 g tryptone l^{-1} and 5 g yeast extract l^{-1} . The tubes were placed in a rotator and kept at 37°C . Upon reaching a cell density between OD₅₅₀ 0.3 and 0.4 (measured using a BioMate 5 spectrophotometer, Thermo Scientific), the cultures were centrifuged and the pellet washed and resuspended in minimal medium (pH 7.4) to OD₅₅₀ 0.02. This inoculated minimal medium was incubated in completely filled Hungate tubes as described above until the cultures reached an OD₅₅₀ between 0.3 and 0.4. An appropriate volume of the culture was harvested, the pellet resuspended in 10 ml minimal medium, and the resulting suspension used to inoculate the fermenters with a target starting OD₅₅₀ of 0.05. Some experiments were conducted in partially filled tubes (10 ml medium) with an argon atmosphere. For this purpose, the medium was dispensed to the tubes and sparged with ultra-high-purity argon as described above for the fermentation vessels. These tubes were then inoculated with a preculture prepared as described above using 17 ml Hungate tubes (Bellco Glass) completely filled with

Fig. 1. Key metabolic pathways involved in the fermentative metabolism of glucuronate by *E. coli*. Glucuronate utilization: [1], D-glucuronate isomerase (*uxaC*); [2], D-mannonate oxidoreductase (*uxuB*); [3], D-mannonate dehydratase (*uxuA*); [4], 2-keto-3-deoxygluconokinase (*kdgK*); [5], 2-keto-3-deoxygluconate-6-phosphate aldolase (*eda*). Embden-Meyerhof-Parnas: [6], glyceraldehyde-3-phosphate dehydrogenase (*gapA*); [7], phosphoglycerate kinase (*pgk*); [8], phosphoglycerate mutase (*gpmA*) and enolase (*eno*); [9], pyruvate kinase I/II (*pykF/pykA*). Gluconeogenesis: [10], triose phosphate isomerase (*tpiA*); [11], fructose bisphosphate aldolase class II (*fbaA*); [12], fructose-1,6-bisphosphatase I (*fbp*); [13], phosphoglucose isomerase (*pgi*). ox-PPP: [14], glucose-6-phosphate dehydrogenase (*zwf*); [15], 6-phosphogluconolactonase (*pgl*); [16], 6-phosphogluconate dehydrogenase (*gnd*). Non-ox-PPP: [17], ribulose phosphate 3-epimerase (*rpe*); [18], ribose-5-phosphate isomerases A (*rpiA*) and B (*rpiB*); [19], transketolases I (*tktA*) and II (*tktB*); [20], transaldolases A (*talA*) and B (*talB*). Oxidative and reductive branches of the TCA cycle: [21], citrate synthase (*gltA*) and aconitases (*acnA* and *acnB*); [22], isocitrate dehydrogenase (*icd*); [23], malate dehydrogenase (*mdh*) and fumarate isozymes A (*fumA*) and B (*fumB*); [24], fumarate reductase (*frdABCD*). Anaplerotic reaction: [25], phosphoenolpyruvate carboxylase (*ppc*). Pyruvate dissimilation: [26], pyruvate formate-lyase (*pflB*); [27], pyruvate dehydrogenase complex (*aceEF-lpdA*). Fermentation: [28], lactate dehydrogenase (*ldhA*); [29], formate hydrogen lyase (*fdhF*, *hycB-F*); [30], phosphotransacetylase (*pta*); [31], acetate kinase (*ackA*); [32], alcohol/acetaldehyde dehydrogenase (*adhE*). Transhydrogenases: [33], soluble (*sthA*) and membrane-bound (*pntAB*) pyridine nucleotide transhydrogenases. Cell growth: [34], synthesis of cell mass from precursor metabolites (★), ATP and reducing equivalents. Broken lines indicate multiple steps. Abbreviations: Acetyl-P, acetyl phosphate; AKG, α -ketoglutarate; DHAP, dihydroxyacetone phosphate; E4P, erythrose 4-phosphate; FRU16P, fructose 1,6-bisphosphate; FRU6P, fructose 6-phosphate; FUM, fumarate; GALP, glyceraldehyde 3-phosphate; GLC6P, glucose 6-phosphate; ICIT, isocitrate; KDG, 2-dehydro-3-deoxy-D-gluconate; KDPG, 2-dehydro-3-deoxy-D-gluconate-6-phosphate; OAA, oxaloacetate; PEP, phosphoenolpyruvate; PGLU, 6-phospho-D-gluconate; PGLUL, D-glucono- δ -lactone-6-phosphate; R5P, ribose 5-phosphate; RL5P, ribulose 5-phosphate; S7P, sedoheptulose 7-phosphate; 13PG, 1,3-diphosphoglycerate; 3PG, 3-phosphoglycerate; X5P, xylose 5-phosphate.

Table 1. Strains, plasmids and primers used in this study

Strain, plasmid or primer	Genotype, structure or description	Reference or source	
Strains			
MG1655	F [−] λ [−] <i>ilvG[−] rfb-50 rph-1</i>	Kang <i>et al.</i> (2004)	
Pdh [−]	MG1655, <i>aceF::Tn5KAN-I-SceI</i> (pyruvate dehydrogenase-deficient)	Kang <i>et al.</i> (2004)	
Pfl [−]	MG1655, <i>pflB::Tn5KAN-I-SceI</i> (pyruvate formate-lyase-deficient)	Kang <i>et al.</i> (2004)	
JW1841	BW25113, Δ <i>zwf-FRT-Km-FRT</i>	Baba <i>et al.</i> (2006)	
JW2294	BW25113, Δ <i>pta-FRT-Km-FRT</i>	Baba <i>et al.</i> (2006)	
JW1228	BW25113, Δ <i>adhE-FRT-Km-FRT</i>	Baba <i>et al.</i> (2006)	
JW1375	BW25113, Δ <i>ldhA-FRT-Km-FRT</i>	Baba <i>et al.</i> (2006)	
Ldh [−]	MG1655, Δ <i>ldhA::FRT</i> (lactate dehydrogenase-deficient)	This study	
Pfl-Ldh [−]	MG1655, Δ <i>ldhA::FRT pflB::Tn5KAN-I-SceI</i>	This study	
Pta [−]	MG1655, Δ <i>pta::FRT</i> (phosphotransacetylase-deficient)	This study	
Adh [−]	MG1655, Δ <i>adhE::FRT</i> (alcohol/acetalddehyde dehydrogenase-deficient)	This study	
Pta-Adh [−]	MG1655, Δ <i>pta::FRT ΔadhE::FRT</i>	This study	
Zwf [−]	MG1655, Δ <i>zwf::FRT</i> (Zwf [−] glucose-6-phosphate dehydrogenase-deficient)	This study	
Pdh-Zwf [−]	MG1655, Δ <i>zwf::FRT aceF::Tn5KAN-I-SceI</i>	This study	
SthA-PntA [−]	MG1655, Δ <i>sthA::FRT ΔpntA::FRT</i> (SthA [−] soluble transhydrogenase-deficient, PntA [−] membrane-bound transhydrogenase-deficient)	Sauer <i>et al.</i> (2004)	
Plasmids			
pCP20	rep _{pSC101} ^{ts} Ap ^R Cm ^R <i>cI857</i> 1 P _R flp ⁺	Datsenko & Wanner (2000)	
Primers*			
v-pflB	tattgttactttattcgctgatg	aaaggagtgaatgcgaccaata	This study
v-aceF	gcgagagttcaatgggacag	cgctaataacctcaatacattcat	This study
v-zwf	cgtaatatcaggggaatgaccc	gggcaaagaatgtcaaaaacaa	This study
v-pta	ccaaccaacgaagaactggt	tagcgcaaatattcccttgc	This study
v-ldhA	gcttaaatgtgattcaacatcactgg	agaatagaggatgaaaggtcattg	This study
v-adhE	cgagcagatgatttactaaaaaag	atcggcattgcccagaagg	This study

*'v' indicates primer sequences (5'–3') used for verification purposes. The forward sequence is followed by the reverse sequence in each case. Genes deleted or cloned are apparent from primer names.

MOPS media (pH 7.4) supplemented with 5 g glucuronate l⁻¹, 10 g tryptone l⁻¹ and 5 g yeast extract l⁻¹.

Prior to use, strains (stored as glycerol stocks at –80 °C) were streaked onto LB plates (with appropriate antibiotics if required) and incubated overnight at 37 °C in an Oxoid anaerobic jar with an Oxoid Carbon Dioxide Gas Generating kit.

Analytical methods. OD₅₅₀ was measured in a BioMate 5 spectrophotometer (Thermo Scientific) and used as an estimate of cell mass (1 OD₅₅₀ unit=0.34 g dry weight l⁻¹). After centrifugation, the supernatants were stored at –20 °C for HPLC analysis using a Shimadzu Prominence SIL 20 system (Shimadzu Scientific Instruments) equipped with an HPX-87H organic acid column (Bio-Rad). Specific details about the quantification of glucuronate, organic acids, ethanol and hydrogen have been previously reported (Dharmadi *et al.*, 2006; Dharmadi & Gonzalez, 2005). The identity of fermentation products was verified via 1D 1H NMR, as previously described (Murarka *et al.*, 2008; Dharmadi *et al.*, 2006).

Enzyme activities. Actively growing cells were harvested by centrifugation, washed twice with a saline solution (9 g NaCl l⁻¹) and stored as cell pellets at –80 °C until use. Cell pellets were resuspended in 0.1 M potassium phosphate buffer to obtain ~3.4 mg dry cell weight ml⁻¹, and permeabilized with chloroform (Murarka *et al.*, 2008; Tao *et al.*, 2001; Osman *et al.*, 1987). The activity of PDH (toward pyruvate) was monitored spectrophotometrically by following the reduction of NAD⁺ at 340 nm (Snoep *et al.*, 1990; Danson *et al.*, 1978). The reaction mixture (1 ml, final pH 7.0) contained

50 mM potassium phosphate (pH 8.0), 2.0 mM sodium pyruvate, 2.5 mM NAD⁺, 1.0 mM MgCl₂, 2.6 mM cystine-HCl, 0.13 mM CoA, 0.2 mM thiamine pyrophosphate (Danson *et al.*, 1978) and 50 μ l permeabilized cells prepared as described above. Linearity of the reactions (protein concentration and time) was established for all preparations. All spectrophotometric measurements were conducted in a BioMate 5 spectrophotometer (Thermo Scientific). The non-enzymic rates were subtracted from the observed initial reaction rates. Enzyme activities are reported as μ mol pyruvate oxidized min⁻¹ (mg cell protein)⁻¹ and represent averages for at least three cell preparations. Total cell protein was assumed to represent 50 % of the cell dry weight.

NADH/NAD⁺ assay. Intracellular levels of NADH and NAD⁺ were measured via the alkaline extraction-reverse phase HPLC method (Stocchi *et al.*, 1985), as previously described (Murarka *et al.*, 2008; Levanon *et al.*, 2005). The fermentation samples were collected in ice-cold tubes (0 °C) and centrifuged at 3200 g for 10 min while maintained under the same conditions. Nucleotides were extracted using the following procedure. Cell pellets were resuspended in 1 ml ice-cold 0.5 M KOH, kept on ice for 10 min with intermittent vortexing, centrifuged for 10 min and the supernatant was collected. A 1 ml volume of 1 M KH₂PO₄ buffer (pH 6.5) was then added to the supernatant and the resulting solution was filtered through a 0.22 μ m pore-size syringe filter. A 100 μ l aliquot of this solution was analysed using a Spectra HPLC system (Thermo Separation Products) equipped with a SupelcosilTMLC-18-T column (Supelco). The samples were run with an inorganic mobile phase (0.1 M

KH₂PO₄, pH 6) for 20 min followed by an organic mobile phase (a mixture of 90 % 0.1 M KH₂PO₄, pH 6, and 10 % methanol). The resulting chromatogram was analysed using the software Chromquest 4.1 (Thermo Electron), thus obtaining the area of the peaks corresponding to concentrations of NADH and NAD⁺. The identity of the peaks was verified by analysing samples spiked with known nucleotides.

Calculation of fermentation parameters. Specific growth rates (μ ; h⁻¹) were estimated by plotting total cell concentration versus time in a log-linear plot. The slope of the curves thus obtained was used as the average specific rate. These curves become straight lines during exponential growth and the calculated specific rate reaches its maximum value (μ_m). Specific rates (mmol g⁻¹ cell⁻¹ h⁻¹) during the exponential growth phase were calculated from data for glucuronate consumption and product synthesis and used to demonstrate balanced growth and validity of the steady-state assumption, as previously described (Gonzalez *et al.*, 2003). Growth and product yields (weight or molar basis) were calculated as the amount of cell mass or product synthesized per amount of glucuronate consumed. In the above calculations, an average molecular mass of 25.1 was used, which corresponds to an average *E. coli* cell of a molecular formula CH_{1.81}O_{0.52}N_{0.21} (Nielsen *et al.*, 2003).

In silico and in vivo MFA. MFA was conducted using both *in silico* and *in vivo* approaches. Flux balance analysis (referred to here as *in silico* MFA) is a method based on the mass balance constraints in a metabolic network (Edwards & Palsson, 2000), which can be mathematically represented as:

$$S \bullet v = 0$$

where *S* is the *m* × *n* stoichiometric matrix, *m* and *n* being the number of metabolites and reactions in the network, respectively, and *v* is a vector containing all the fluxes in the metabolic network. The *E. coli* model used in this work is based on the pathways known to be active in *E. coli* during fermentative metabolism in general, as well as those specific to glucuronate utilization (Mandrand-Berthelot *et al.*, 2004; Sawers & Clark, 2004; Neidhardt *et al.*, 1990, 1996; Keseler *et al.*, 2005; Pramanik & Keasling, 1997; Stryer, 1995) (see the Reaction Network included in the Supplementary Information available with the online version of this paper). The number of fluxes in the model was 82 and the number of mass balances 74. A unique distribution of metabolic fluxes was obtained using linear programming (MetaFluxNet) (Lee *et al.*, 2003), whereby the biomass flux was maximized. A list of the reactions included in this analysis along with the details about the construction of the stoichiometric model, the metabolic network and metabolites considered, and the values of estimated fluxes are provided as Supplementary Information with the online version of this paper.

In vivo MFA was conducted using the technique of metabolite balancing as described elsewhere (Stephanopoulos *et al.*, 1998). The metabolic network, and therefore the stoichiometric model, was similar to that described above for *in silico* MFA, but with 81 reactions, thus resulting in a system with seven degrees of freedom. In this case the available fermentation data were used to calculate seven extracellular fluxes associated with the consumption of glucuronate and the synthesis of fermentation products and biomass. These extracellular fluxes are equivalent to specific rates, and were calculated as described in the section Calculation of fermentation parameters. The availability of extracellular fluxes made the system determined and allowed the calculation of intracellular fluxes without the use of an optimization routine. Three independent measurements were used in the calculation of fluxes, thus allowing the estimation of SDS.

RESULTS

Cell growth, glucuronate utilization and product synthesis in wild-type MG1655 and strains deficient in pyruvate- and AcCoA-dissimilating enzymes

The fermentation profiles of MG1655 (wild-type) and PDH- (Pdh⁻) and PFL-deficient (Pfl⁻) strains are shown in Fig. 2. No PDH activity was found in mutant Pdh⁻ (*aceF* disrupted), while the lack of fermentative growth on glucose of mutant Pfl⁻ (*pflB* disrupted) demonstrated that PFL was inactive. Both mutants grew much more slowly than the wild-type and reached a lower final cell concentration. Mutant Pdh⁻ also exhibited a well-defined increase in the time required to reach exponential phase. The maximum specific growth rates (μ_m) of both mutants were much lower than that of the wild-type: 0.206 and 0.237 h⁻¹ for Pdh⁻ and Pfl⁻, respectively, compared with 0.354 h⁻¹ for MG1655. The fact that mutant Pfl⁻ is able to grow under fermentative conditions without supplementation of acetate or CO₂ is the first such observation, to our knowledge, as *E. coli* strains devoid of PFL lack the ability to grow fermentatively on other substrates in the absence of the aforementioned supplements (de Graef *et al.*, 1999; Kaiser & Sawers, 1994; Guest *et al.*, 1989; Varenne *et al.*, 1975).

The profiles of substrate and product concentrations for each strain are also shown in Fig. 2. MG1655 and Pdh⁻ had similar product profiles, although it took twice as much time for the mutant to consume all the substrate and synthesize the products. In both cases a homoacetogenic-like fermentation with co-production of formate was observed, but negligible amounts of succinate and lactate and no ethanol. Significant changes in product profile occurred upon inactivation of PFL (Fig. 2). As expected, there was no accumulation of formate and the generation of acetic acid was significantly lower than in MG1655 and Pdh⁻. Moreover, there is significant accumulation of lactic acid, reaching levels about 15- and 8-fold higher than those found in the MG1655 and Pdh⁻ cultures, respectively.

No significant changes were observed when the lactate pathway was blocked (deletion of *ldhA*) in MG1655, which agrees with earlier reports of no obvious anaerobic growth defects in LDH-deficient mutants (Mat-Jan *et al.*, 1989). However, when *ldhA* was knocked out in strain Pfl⁻, no cell growth or glucuronate fermentation was observed. Pfl⁻-Ldh⁻ double mutants are known to be unable to grow fermentatively on glucose and other sugars (Mat-Jan *et al.*, 1989).

Acetate synthesis appears to be an important pathway for glucuronate fermentation, as can be inferred by the amounts of acetate produced by all strains (Fig. 2). Acetate is synthesized from AcCoA through the successive action of phosphotransacetylase (PTA; encoded by *pta*) and acetate kinase (ACK; encoded by *ackA*), a pathway that generates

Fig. 2. Cell growth, glucuronate utilization and synthesis of fermentation products in wild-type MG1655 (a) and PDH- and PFL-deficient strains Pdh- (b) and Pfl- (c). Symbols show the concentrations of: ●, glucuronate; ▲, cells; ■, acetate; *, lactate; +, succinate; ◆, formate. The exponential growth phase is indicated by vertical dotted lines and the maximum specific growth rate (μ_m) associated with it is also shown.

one molecule of ATP (Fig. 1). PTA-negative mutants do not grow by fermentation of glucose (Guest, 1979) but are able to ferment glycerol (Murarka *et al.*, 2008). In the case of glucuronate, a PTA-deficient mutant (Pta- strain) did not grow fermentatively on glucuronate and the same results were obtained in a Pta-Ack- double mutant.

While the growth defect of Pta- mutants on glucose is eliminated by a second mutation of *adhE* (encoding acetaldehyde/alcohol dehydrogenase, Adh) (Gupta & Clark, 1989), the double mutant Pta-Adh- did not ferment glucuronate. Alcohol dehydrogenase mutants (Adh-) are also impaired in both glucose and glycerol fermentation (Guest, 1979; Gupta & Clark, 1989; Murarka *et al.*, 2008), but strain Adh- exhibited a phenotype very similar to MG1655 during glucuronate fermentation.

***In vivo* metabolic flux analysis of wild-type MG1655 and PDH- and PFL-deficient mutants**

Metabolite balancing (Stephanopoulos *et al.*, 1998; Romeo & Snoep, 2005; Nielsen, 2003), referred to here as *in vivo* MFA, was used to further investigate the differences between MG1655, Pdh- and Pfl-. The resulting metabolic flux maps for the three strains during exponential growth are shown in Fig. 3.

The flux from pyruvate to AcCoA in MG1655 was split between PFL and PDH. This was a surprising finding, since PDH is an enzyme primarily involved in respiratory metabolism (Sawers & Clark, 2004). Indeed, the *in vivo* activity of PDH in anaerobic *E. coli* cultures has been reported as either very low or undetectable (Smith & Neidhardt 1983; de Graef *et al.*, 1999; Snoep *et al.*, 1993; Lawford & Rousseau, 1997), and its role in the fermentative metabolism of *E. coli* has not been established. During fermentation of glucuronate, PDH could serve as an important source of AcCoA, reducing cofactor NADH or CO₂, all products of the dissimilation of pyruvate via this enzyme. The inactivation of pyruvate-dissimilating enzymes leads to significant redistribution of fluxes throughout the metabolic network, including pathways 'metabolically distant' from the dissimilation of pyruvate (Fig. 3). Since the nature of the metabolic response to the PDH and PFL deficiencies was different, we have categorized the changes as those occurring in either glycolytic and gluconeogenic pathways (i.e. upstream of pyruvate) or fermentative pathways (i.e. downstream of pyruvate) (see Figs 1 and 3).

The fluxes through the glycolytic and gluconeogenic pathways were very similar in strains MG1655 and Pfl-. Most of the glyceraldehyde 3-phosphate generated from the hexuronate dissimilation pathway is channelled to phosphoenolpyruvate/pyruvate, with only a small fraction of the carbon directed through the gluconeogenic and pentose phosphate (PP) pathways (Fig. 3). There are also significant differences between MG1655 and the Pfl- mutant in the pathways downstream of pyruvate (Fig. 3). The inactivation of PFL is compensated by a 3.5-fold increase in the flux through PDH. The increase in PDH flux in Pfl- appears to generate large quantities of NADH, whose reoxidation is facilitated by the production of the reduced metabolite lactate (Fig. 3).

The absence of PDH led to significant flux redistribution in the glycolytic and gluconeogenic pathways (Fig. 3). There

Fig. 3. *In vivo* distribution of metabolic fluxes for wild-type MG1655 (in bold type) and mutants *Pdh*⁻ (in italic type) and *Pfl*⁻ (underlined). Estimated fluxes represent the molar percentages of the average specific glucuronate uptake rates, which were 7.052 mmol (g dry weight)⁻¹ h⁻¹ (MW1655), 5.085 mmol (g dry weight)⁻¹ h⁻¹ (*Pdh*⁻) and 7.429 mmol (g dry weight)⁻¹ h⁻¹ (*Pfl*⁻). The fluxes were calculated for three independent samples taken during the exponential growth phase and means ± SDs are shown. Fluxes in larger-size font highlight differences between the strains. Arrowheads indicate the direction of fluxes shown as positive (negative fluxes are in the opposite direction to that of arrowheads). Broken lines represent multiple steps. The complete set of fluxes is provided as Supplementary Information. See Fig. 1 and legend for details of the pathways and abbreviations.

was a significant flux originating from glyceraldehyde 3-phosphate toward gluconeogenic and PP pathways, which is in contrast with the negligible flux through these pathways in MG1655 and *Pfl*⁻. However, a major fraction of the oxidative pentose phosphate pathway (ox-PPP) flux in mutant *Pdh*⁻ cycled back to glyceraldehyde 3-phosphate and fructose 6-phosphate via the non-oxidative pentose phosphate pathway (non-ox-PPP). The significant increase in flux through the ox-PPP in mutant *Pdh*⁻ is worth investigating, since the ox-PPP, like PDH (which is inactivated in strain *Pdh*⁻), generates CO₂ and reducing equivalents.

The transhydrogenase flux shown in Fig. 3 corresponds to the interconversion of NADPH and NADH catalysed by the soluble and membrane-bound transhydrogenases SthA and PntAB, respectively (Sauer *et al.*, 2004; Canonaco *et al.*, 2001). This flux is positive in MG1655 and in *Pfl*⁻,

implying that the reduced cofactor NADH is converted to the biosynthetic reducing power NADPH. The NADH required for this reaction is met by the flux through PDH. Accordingly, higher flux through PDH in strain *Pfl*⁻ led to a 54 % increase in the transhydrogenase flux (Fig. 3). This interconversion, however, is working in the opposite direction in strain *Pdh*⁻, where the PDH pathway is inactive and excess NADPH generated via the ox-PPP requires conversion to NADH.

***In silico* MFA suggests the basis for the differences in flux distribution among strains MG1655, *Pdh*⁻ and *Pfl*⁻**

Metabolic fluxes calculated via *in vivo* MFA portrayed major flux redistributions in response to the PFL and PDH deficiencies (Fig. 3). To further analyse this redistribution

of fluxes we used flux balance analysis (Edwards & Palsson, 2000), a technique widely applied to the study of microbial metabolism (Downs, 2006; Romeo & Snoep, 2005; Nielsen, 2003), and referred to here as *in silico* MFA. To this end, simulations were conducted in different scenarios by selectively relaxing or enforcing redox (R) and ATP (A) constraints (see Table 2 and Supplementary Information for details).

Key features of the distribution of metabolic fluxes obtained via *in silico* MFA are summarized in Table 2. The $A_C R_C$ 'simulated scenario' indicates that the system is subjected to both ATP and redox constraints (all scenarios are subjected to carbon constraints). Accordingly, simulations conducted under $A_C R_C$ scenarios should resemble the flux distributions calculated via *in vivo* MFA (Fig. 3). A comparison of the *in vivo* fluxes (Fig. 3) with the *in silico* fluxes shown in Table 2 ($A_C R_C$ simulated scenarios) validates the ability of the *in silico* approach to reveal key characteristics of glucuronate fermentation in wild-type and PDH- and PFL-deficient strains. Further simulations were conducted in scenarios in which redox (R) and/or ATP (A) constraints were relaxed and the results are discussed below.

For the wild-type network, most fluxes remained unchanged, including the cell yield, if the redox balance constraint was relieved (R_U scenario, unconstrained redox) (Table 2). In contrast, the cell yield increased more than fivefold upon relaxing the constraint on ATP availability (A_U scenario, unconstrained ATP), which then increased further upon relaxing the redox constraint ($A_U R_U$ scenario, unconstrained ATP and redox). In all simulated scenarios there was a large flux through PDH, while the ox-PPP flux was observed only in the A_U case (Table 2). This analysis suggests that the growth of wild-type cells is limited by ATP availability, and that the requirements for reducing equivalents are fulfilled by adjusting metabolism to use either PDH or the ox-PPP. Our experimental results suggested that the ATP required for cell growth is provided by the synthesis of acetate through the PTA-ACK pathway; i.e. a Pta- strain, which is unable to synthesize acetate, did not ferment glucuronate. The *in silico* analysis of a Pta-deficient mutant further supported this hypothesis, as this strain did not grow or ferment glucuronate but recovered the ability to grow on an unconstrained ATP scenario (see Supplementary Information).

As discussed in the sections above, the inactivation of PDH led to an increase in the ox-PPP flux, a pathway that like PDH generates reducing equivalents and CO_2 (but no ATP is involved in these pathways). *In silico* and *in vivo* MFA (Table 2, $A_C R_C$ scenario, Fig. 3) show that this additional flux through the ox-PPP in strain Pdh- is cycled back to the glycolytic intermediates glyceraldehyde 3-phosphate and fructose 6-phosphate. Consequently, it can be inferred that the high ox-PPP flux does not benefit the cells by providing PPP intermediates. One possible explanation for the increase in the ox-PPP flux is that it fulfils metabolic

requirements for either CO_2 or NAD(P)H. However, since our simulations show that there is a net generation of CO_2 in all strains and conditions (shown in Supplementary Information), it can be hypothesized that the cells increase the ox-PPP flux to generate reducing equivalents. Even with the increase in the ox-PPP flux, the cell yield in Pdh- is lower than in the wild-type. However, relaxing the constraint of redox balance in mutant Pdh- leads to equalization of fluxes with those in the wild-type (Table 2, R_U scenario). Also, relaxing the ATP constraint in Pdh- leads to a lower cell yield than a similar situation in the wild-type (A_U scenarios for MG1655 and Pdh-).

The analysis of mutant Pfl- in an unconstrained redox scenario (R_U) predicts a biomass yield equal to the wild-type, exclusive dissimilation of pyruvate via PDH, no lactate co-production, and a higher acetate flux (Table 2). These results suggest that the presence of PFL in wild-type *E. coli* enables the cells to maintain high flux through an ATP-generating pathway (phosphotransacetylase-ACK), while generating the required NADH via PDH. In the absence of PFL, pyruvate is dissimilated to AcCoA via PDH, generating extra reducing equivalents and thus requiring the synthesis of lactate to maintain redox poise. However, lactate is a suboptimal route in terms of ATP production, hence the lower cell yield.

The transhydrogenase fluxes for the ' $A_C R_C$ ' and ' A_U ' scenarios in strain Pdh- are negative (Table 2), indicating that the NADPH generated via the ox-PPP is more than that required for biosynthetic functions and that the excess is converted to NADH. The cell yield for these simulations is lower than the wild-type under the same simulated scenario, implying that the ox-PPP is unable to completely compensate for the loss of PDH. Since the ox-PPP, like PDH, generates reducing equivalents, we simulated the effect of inactivating this pathway by deleting the gene *zwf*, which encodes the enzyme that catalyses the first step of the ox-PPP (glucose-6-phosphate dehydrogenase). The performance of mutant Zwf- is predicted to be indistinguishable from that of MG1655, exhibiting similar growth yield and distribution of metabolic fluxes (Table 2, $A_C R_C$ scenario). However, the ox-PPP becomes essential for cell growth upon deletion of PDH (Table 2, double mutant Pdh-Zwf-, $A_C R_C$ scenario). Additionally, this double mutant is predicted to regain wild-type growth characteristics when redox constraints are relaxed (Table 2, R_U scenario). In contrast, relaxing the constraints on ATP availability does not lead to a recovery of Pdh-Zwf- (Table 2, A_U scenario).

Experimental evidence supporting the role of PDH in the fermentative metabolism of glucuronate

The *in silico* and *in vivo* analyses conducted in previous sections led to the hypothesis that the role of PDH in the fermentative metabolism of glucuronate is to generate reducing equivalents. Although PDH generates both CO_2 and reducing equivalents, the *in silico* analysis predicted

Table 2. *In silico* MFA of wild-type *E. coli* and mutants deficient in PDH (Pdh[−]), PFL (Pfl[−]) and glucose-6-phosphate dehydrogenase (Zwf[−]) enzymes

Several metabolic scenarios are simulated for each strain. In the base case, A_CR_C, both ATP ('A') and redox ('R') constraints are enforced (subscript 'c' indicates the constrained nature of the simulation). In other simulated scenarios, either one (A_U or R_U) or both (A_UR_U) of the constraints are relaxed (the subscript 'u' indicates the unconstrained nature of the simulation). Synthesis of ethanol and succinate was predicted to be zero in all the simulations. Abbreviations: GLR, glucuronate; GALP, glyceraldehyde 3-phosphate; PYR, pyruvate; PEP, phosphoenolpyruvate; DHAP, dihydroxyacetone phosphate; FRU6P, fructose 6-phosphate; GLC6P, glucose 6-phosphate; RL5P, ribulose 5-phosphate; R5P, ribose 5-phosphate; X5P, xylose 5-phosphate; E4P, erythrose 4-phosphate. See Fig. 1 for more details about pathways. Fluxes were normalized with respect to the glycerol consumption flux (on a molar basis).

Pathway or reaction	Strain and simulated scenario												
	Wild-type MG1655				Pdh [−]			Pfl [−]		Zwf [−]	Pdh [−] Zwf [−]		
	A _C R _C	R _U	A _U	A _U R _U	A _C R _C	R _U	A _U	A _C R _C	R _U	A _C R _C	A _C R _C	R _U	A _U
Glucuronate utilization													
GLR + ATP + NADH → GALP + PYR	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Embden–Meyerhof–Parnas													
GALP → → → PEP + NADH + ATP	0.947	0.947	0.536	0.653	0.894	0.947	0.471	0.961	0.947	0.947	1.000	0.947	1.000
PEP → PYR + ATP	0.847	0.847	0.000	0.000	0.800	0.847	0.000	0.889	0.847	0.847	1.000	0.847	1.000
Gluconeogenesis													
DHAP + GALP → FRU6P	0.020	0.020	0.285	0.130	0.074	0.020	0.372	0.014	0.020	0.020	0.000	0.020	0.000
FRU6P → GLC6P	0.004	0.004	0.555	0.026	0.171	0.004	0.000	0.003	0.004	0.004	0.000	0.004	3.000
ox-PPP													
GLC6P → RL5P + CO ₂ + NADPH	0.000	0.000	0.534	0.000	0.167	0.000	0.837	0.000	0.000	—	—	—	—
Non-ox-PPP													
R5P + X5P → E4P + FRU6P	−0.004	−0.004	0.158	−0.024	0.052	−0.004	0.262	−0.003	−0.004	−0.004	0.000	−0.004	0.000
X5P + E4P → GALP + FRU6P	−0.011	−0.011	0.120	−0.071	0.046	−0.011	0.228	−0.008	−0.011	−0.011	0.000	−0.011	0.000
Pyruvate dissimilation													
PYR → AcCoA + FORMATE	1.496	0.000	0.000	0.000	1.747	1.791	0.736	—	—	1.496	2.000	1.791	2.000
PYR → AcCoA + CO ₂ + NADH	0.295	1.791	0.699	0.633	—	—	—	1.032	1.791	0.295	—	—	—
Fermentation													
PYR + NADH → LAC	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.817	0.000	0.000	0.000	0.000	0.000
AcCoA → AC + ATP	1.712	1.712	0.270	0.112	1.672	1.712	0.359	0.974	1.712	1.712	2.000	1.712	2.000
Transhydrogenases													
NADH + NADP ⁺ → NAD ⁺ + NADPH	0.313	0.313	0.618	2.053	−0.039	0.313	−0.193	0.228	0.313	0.313	0.000	0.313	0.000
Cell growth													
Precursor metabolites → Biomass	0.135	0.135	0.726	0.884	0.128	0.135	0.637	0.098	0.135	0.135	0.000	0.135	0.000

that the cultures are not limited by CO₂, hence the hypothesized role. Experiments in which sodium bicarbonate was included in the medium resulted in fermentation profiles essentially indistinguishable from those presented in Fig. 2 (data not shown).

Given the proposed role of PDH in the generation of reducing equivalents, we assessed the internal redox state of MG1655, Pdh[−] and Pfl[−] by measuring the NADH:NAD⁺ ratio (Table 3). As can be inferred from the results, the internal redox state of the cells is significantly affected by the choice of pyruvate-dissimilating enzyme. The disruption of PDH was associated with a 30 % decrease in the NADH:NAD⁺ ratio, when compared with that measured in MG1655. The knockout of PFL, on the other hand, was accompanied by a more than 40 % increase in this ratio. Overall, the NADH:NAD⁺ ratio was twofold higher in mutant Pfl[−] when compared with Pdh[−]. The changes in NADH:NAD⁺ ratio (Table 3) correlated well with the observed changes in PDH fluxes (Fig. 3).

In the absence of PDH (i.e. mutant Pdh[−]), the flux analysis indicates that the ox-PPP produces the reducing equivalents otherwise generated by PDH, although the slower growth of Pdh[−] appears to indicate that the ox-PPP is less efficient in fulfilling this function. This led to the prediction that a deficiency in the ox-PPP would have no effect on cell growth in the wild-type background but would be detrimental when combined with a PDH deficiency (see Table 2, single and double mutants Zwf[−] and Pdh[−]Zwf[−]). Strains Zwf[−] and Pdh[−]Zwf[−] were then constructed and their fermentative growth on glucuronate was evaluated. While strain Zwf[−] grew at a maximum specific growth rate equivalent to that observed for MG1655, a 32 % reduction in this parameter was observed when *zwf* was disrupted in the PDH-deficient strain (Table 3). This double mutant grew very slowly (Table 3), exhibiting a lag time in fermenters of about 5 h and taking 28 h to reach stationary phase (data not shown). Neither the activity of PDH nor the internal redox state of the cells (NADH:NAD⁺ ratio) was significantly affected by the inactivation of Zwf in MG1655 (Table 3). However, a significant decrease in NADH:NAD⁺ ratio was observed upon Zwf inactivation in strain Pdh[−]. Note that PDH inactivation in MG1655 also led to a low

NADH:NAD⁺, although the decrease was less pronounced than in double mutant Pdh[−]Zwf[−] (Table 3). As in the case of mutant Pdh[−], supplementation of the medium with bicarbonate did not improve the performance of strain Pdh[−]Zwf[−], which suggests no CO₂ limitation (data not shown). These results indicate that both PDH and the ox-PPP are major sources of reducing equivalents, although the ox-PPP appears to make a significant contribution only in the absence of PDH.

If the reducing equivalents generated by PDH (i.e. NADH) were to play an important role as a source of biosynthetically useful reducing power (i.e. NADPH), they would need to be converted to NADPH by the action of transhydrogenases. In agreement with this reasoning, transhydrogenase-deficient strain SthA[−]PntA[−] (SthA[−], soluble transhydrogenase-deficient; PntA[−], membrane-bound transhydrogenase-deficient) exhibited a 33 % decrease in μ_m (0.229 h^{−1}) when compared with MG1655. While this decrease in μ_m was significant, it was not as large a decrease as that observed for Pdh[−] (45 %). Moreover, strain SthA[−]PntA[−] did not exhibit a significant delay in its transition into the exponential phase, although PDH-deficient strains did.

DISCUSSION

In the work reported here, *in silico* and *in vivo* MFA were used to investigate the fermentative metabolism of glucuronate in *E. coli*, with emphasis on pyruvate-dissimilation pathways. This analysis revealed that both PFL and PDH mediate the dissimilation of pyruvate. This is in contrast with previous reports of low (or undetectable) *in vivo* activity of PDH in anaerobic *E. coli* cultures grown on other carbon sources (Sawers & Clark, 2004; Smith & Neidhardt, 1983; de Graef *et al.*, 1999; Snoep *et al.*, 1993; Lawford & Rousseau, 1997). An even more surprising result was the finding that PDH can support fermentative growth on glucuronate in the absence of PFL. This was unexpected because PFL-deficient strains are unable to ferment glucose and other substrates in the absence of CO₂ or acetate (Hasona *et al.*, 2004; Sawers & Clark, 2004; de Graef *et al.*, 1999; Varenne *et al.*, 1975). Another interesting finding was that the homoacetogenic nature of glucuronate

Table 3. Maximum specific growth rate (μ_m), internal redox state (NADH:NAD⁺ ratio) and activity of PDH in wild-type MG1655 and mutants deficient in PDH (Pdh[−]), PFL (Pfl[−]) and glucose-6-phosphate dehydrogenase (Zwf[−]) enzymes

Cells were grown in Hungate tubes using MOPS minimal medium with 5 g glucuronate l^{−1}, as described in Methods.

Strain	μ_m (h ^{−1})	NADH:NAD ⁺ (ratio)	PDH activity [$\mu\text{mol (mg cell protein)}^{-1} \text{ min}^{-1}$]
MG1655	0.354 ± 0.008	0.123 ± 0.022	0.38 ± 0.04
Pdh [−]	0.206 ± 0.005	0.089 ± 0.011	0.00
Pfl [−]	0.237 ± 0.004	0.175 ± 0.022	0.34 ± 0.03
Zwf [−]	0.331 ± 0.007	0.136 ± 0.019	0.34 ± 0.03
Pdh [−] Zwf [−]	0.130 ± 0.003	0.063 ± 0.004	0.00

fermentation in the wild-type strain shifted to a mixed lactic and acetic acid fermentation upon deletion of the PFL pathway. While both *Pdh⁻* and *Pfl⁻* exhibited lower specific growth rates than MG1655, a slower transition into the exponential phase was only observed in the case of *Pdh⁻*. Finally, a significant redistribution of metabolic fluxes was found upon perturbation of pyruvate metabolism by PFL and PDH disruptions. Since no physiological role had been proposed for PDH in the fermentative metabolism of *E. coli*, we further investigated the metabolic differences between strains MG1655 and *Pdh⁻*.

In silico and *in vivo* MFA were used to help establish the role of PDH. *In vivo* MFA revealed an increase in the ox-PPP flux in mutant *Pdh⁻*, which was postulated to be the compensatory mechanism that the cells use to generate reducing equivalents in the absence of PDH; i.e. PDH and the ox-PPP are the two primary sources of reducing equivalents. Consequently, the *in silico* MFA predicted that blocking the ox-PPP would make the negative effect of the PDH deficiency more pronounced (Table 2). This analysis led to the hypothesis that the role of PDH was to supply reducing equivalents. The decrease in NADH:NAD⁺ ratio in the PDH-deficient strain (Table 3) indicates that PDH indeed plays an important role in maintaining the internal redox state. Moreover, a *zwf* deletion (which blocked the ox-PPP) had no growth phenotype when introduced in MG1655 but made the characteristic phenotype of a PDH deficiency more pronounced when introduced into strain *Pdh⁻* (i.e. slower transition into the exponential phase and lower specific growth rate). A *Pdh⁻*-*Zwf⁻* double mutant exhibited a NADH:NAD⁺ ratio even lower than that of *Pdh⁻* (Table 3).

The importance of the reducing equivalents generated by PDH is twofold. First, the dissimilation of pyruvate through PDH leads to an internal redox state favourable to the conversion of glucuronate to the glycolytic intermediates glyceraldehyde 3-phosphate and pyruvate, a pathway that consumes reducing equivalents (Fig. 1). The metabolic link between the PDH-mediated dissimilation of pyruvate and glucuronate utilization can be revealed through the analysis of the proposed role of PDH and the kinetic properties of D-mannonate reductase, a key enzyme in glucuronate utilization (Fig. 1) (Mandrand-Berthelot *et al.*, 2004). D-mannonate reductase exhibits typical Michaelis-Menten kinetics with respect to NADH ($K_m=0.03$ mM) and is inhibited by NAD⁺ (Mandrand-Berthelot & Lagarde, 1977). By generating the appropriate internal redox state (i.e. sufficiently high NADH and/or low NAD⁺), PDH ensures the functioning of D-mannonate oxidoreductase and therefore the utilization of glucuronate. On the other hand, given the redox-neutral nature of the overall pathway that converts glucuronate into pyruvate (Fig. 1), the generation of reducing equivalents by PDH could enable biosynthetic functions such as the synthesis of building blocks, macromolecules and ultimately cell mass. In this role, PDH works in partnership with transhydrogenases, which convert the

NADH generated by PDH into biosynthetically useful NADPH (Sauer *et al.*, 2004; Fuhrer & Sauer, 2009).

While the role of PDH as a source of reducing equivalents is supported by the results presented here, one might question why PDH is preferred for this purpose over the ox-PPP. The equations below compare the overall stoichiometry of each pathway, assuming equal NAD(P)H yields and the interconversion of NADH and NADPH via transhydrogenases:

PDH:

ox-PPP:

As can be seen, for the same amount of reducing equivalents generated upon consuming an equal amount of glucuronate, PDH generates more ATP (energy) and more AcCoA (carbon). Cells utilize both energy and carbon to generate the building blocks of biomass, and our *in silico* analysis suggests that glucuronate fermentation is limited by ATP availability. Therefore, PDH is more energy- and carbon-efficient than the ox-PPP, and this could be the reason why wild-type MG1655 prefers PDH to fulfil its needs for reducing equivalents and why PDH-deficient strains grow more slowly even when the ox-PPP is available to substitute for this function. An important role for PDH in the fermentative metabolism of *Enterococcus faecalis* during the utilization of a similarly oxidized substrate, pyruvate, has been reported (Snoep *et al.*, 1990).

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