Analysis of hydrogenase 1 levels reveals an intimate link between carbon and hydrogen metabolism in *Escherichia coli* K-12

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Two of the three [NiFe]-hydrogenases (Hyd) of *Escherichia coli* have a hydrogen-uptake function in anaerobic metabolism. While Hyd-2 is maximally synthesized when the bacterium grows by fumarate respiration, Hyd-1 synthesis shows a correlation with fermentation of sugar substrates. In an attempt to advance our knowledge on the physiological function of Hyd-1 during fermentative growth, we examined Hyd-1 activity and levels in various derivatives of *E. coli* K-12 MC4100 with specific defects in sugar utilization. MC4100 lacks a functional fructose phosphotransferase system (PTS) and therefore grows more slowly under anaerobic conditions in rich medium in the presence of D-fructose compared with D-glucose. Growth in the presence of fructose resulted in an approximately 10-fold increase in Hyd-1 levels in comparison with growth under the same conditions with glucose. This increase in the amount of Hyd-1 was not due to regulation at the transcriptional level. Reintroduction of a functional *fruBKA*-encoded fructose PTS into MC4100 restored growth on D-fructose and reduced Hyd-1 levels to those observed after growth on D-glucose. Reducing the rate of glucose uptake by introducing a mutation in the gene encoding the cAMP receptor protein, or consumption through glycolysis, by introducing a mutation in phosphoglucose isomerase, increased Hyd-1 levels during growth on glucose. These results suggest that the ability to oxidize hydrogen by Hyd-1 shows a strong correlation with the rate of carbon flow through glycolysis and provides a direct link between hydrogen, carbon and energy metabolism.

Received 28 November 2011

Revised 23 December 2011

Accepted 10 January 2012

INTRODUCTION

Escherichia coli is a facultative anaerobic enterobacterium that can grow by either respiration or fermentation. By definition, during fermentation a bacterium relies exclusively on substrate-level phosphorylation to conserve energy. Paradoxically, however, under ostensibly fermentative growth conditions, i.e. without an added exogenous electron acceptor, bacteria such as *E. coli* synthesize a number of membrane-associated oxidoreductases that potentially could contribute to proton motive force generation, thus calling into question whether this process can really be classified as fermentation. These oxidoreductases include three membrane-associated, multi-subunit [NiFe]-hydrogenases (Hyd) (Sawers & Clark, 2004). Two of these hydrogenases, Hyd-1

and Hyd-2, catalyse hydrogen oxidation and both have their respective active site located facing the periplasm (Forzi & Sawers, 2007). Upon hydrogen oxidation, the released protons contribute to scalar generation of a proton gradient while the electrons are channelled into the quinone pool where they can be used to reduce endogenously generated fumarate (Ballantine & Boxer, 1985; Giel *et al.*, 2006; Sawers *et al.*, 1985). Of the two hydrogen-oxidizing hydrogenases, synthesis of Hyd-2 is enhanced during growth with glycerol and fumarate or hydrogen and fumarate, and the enzyme shows highest activity at alkaline pH (Ballantine & Boxer, 1986; Noguchi *et al.*, 2010). These findings are also consistent with the results of a previous study (Richard *et al.*, 1999) showing that synthesis of Hyd-2 is catabolite-repressed. Thus, although present when *E. coli* is growing by fermentation, Hyd-2 synthesis correlates strongly with anaerobic respiration using fumarate as an electron acceptor.

Synthesis of Hyd-1 occurs maximally during fermentation and generally correlates with the appearance of the third

Abbreviations: IMAC, immobilized metal affinity chromatography; PTS, phosphotransferase system; SEC, size-exclusion chromatography.

A supplementary figure and supplementary methods are available with the online version of this paper.

hydrogenase, Hyd-3, which forms part of the hydrogen-evolving activity of the FHL (formate hydrogenlyase) complex (Sawers *et al.*, 1985). Moreover, Hyd-1 appears to be synthesized preferentially in the stationary phase (Brøndsted & Atlung, 1994). Unlike Hyd-2, Hyd-1 is not essential for respiratory growth on fumarate and H₂ (Dubini *et al.*, 2002). Despite being the more abundant of the two hydrogen-uptake hydrogenases in *E. coli*, the precise role of Hyd-1 in the hydrogen metabolism of the bacterium has remained obscure, despite early suggestions of a role in hydrogen recycling (Sawers *et al.*, 1985). Such a role for this enzyme has been demonstrated in *Salmonella enterica* serovar Typhimurium (Zbell & Maier, 2009).

Recent electrochemical studies (Lukey *et al.*, 2010) have shown that *E. coli* Hyd-1 is an O₂-tolerant enzyme with a high affinity for hydrogen and it functions at more positive redox potentials than Hyd-2. This correlates with earlier observations (Laurinavichene *et al.*, 2002) and also with the recent structural analysis of a related oxygen-tolerant hydrogenase from *Ralstonia eutropha* (Fritsch *et al.*, 2011), which is required for hydrogen-dependent aerobic energy conservation. The oxygen tolerance of Hyd-1 furthermore suggests an energy-conserving role for the enzyme in *E. coli*, particularly at low growth rates, when substrate becomes limiting and possibly under microaerobic conditions. All of these conditions are met during stationary phase growth (Lukey *et al.*, 2010). Under carbon starvation upon entering stationary phase, expression of the *hya* operon, which encodes Hyd-1, is regulated by sigma-38 (Atlung *et al.*, 1997). Taken together, these findings suggest that Hyd-1 could have a hydrogen-scavenging function at the aerobic–anaerobic interface in the intestinal tract and during carbon-limitation, and thus it could contribute to energy conservation.

In order to examine whether Hyd-1 synthesis shows any correlation with carbon substrate utilization, we have conducted a series of experiments comparing Hyd-1 levels after fermentative growth on glucose or fructose as the carbon source. To aid these studies, we took advantage of the fact that the *E. coli* K-12 strain MC4100 has a deletion in the *fruB* gene, which encodes a component of the fructose PTS (phosphotransferase system) transporter (Fig. 1), and it lacks a portion of the *fruK* gene encoding the fructose-1-phosphate kinase (Peters *et al.*, 2003). MC4100, and strains deriving thereof, are therefore reliant upon an alternative route for fructose utilization and uptake. As well as being able to enter the cell via the fructose PTS, fructose can also be transported by a mannose PTS encoded by *manXYZ*, which can non-specifically transport fructose when concentrations of the sugar are greater than 2 mM. Once transported by the mannose PTS, D-fructose is phosphorylated by manno(fructo)kinase (Mak) and enters glycolysis as fructose-6-phosphate (Fig. 1) (Kornberg, 2001). Expression of the *manXYZ* operon is, however, strongly catabolite-repressed.

The aim of this study was therefore to determine whether a link exists between sugar substrate utilization and hydrogen metabolism. We demonstrate a strong inverse correlation between restriction of carbon flow through glycolysis and the synthesis of the hydrogen oxidizing Hyd-1 enzyme, which is consistent with the proposed role of this enzyme in energy conservation during carbon limitation. Moreover, our results suggest that an alternative route of fructose uptake and entry into glycolysis in anaerobically growing *E. coli* cells exists that is independent of both the mannose PTS/manno(fructo)kinase and fructose PTS pathways.

METHODS

Strains, plasmids and growth conditions. All bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains were routinely grown at 37 °C on LB agar plates or with shaking in LB broth (Miller, 1972). Plates were solidified by adding 1.5 % (w/v) agar to the media.

Anaerobic growth was performed at 37 °C in sealed bottles filled with anaerobic media and under a nitrogen gas atmosphere. Anaerobic growth rate and growth curves were determined by measuring OD₆₀₀ by using a Spectronic 20+ spectrophotometer (Ochs) directly in Hungate tubes with a 15.7 mm path length. Cultures for determination of hydrogenase processing or for enzyme activity measurements were grown in TYEP media (Begg *et al.*, 1977) containing 1 % (w/v) peptone, 0.5 % (w/v) yeast extract, 0.1 M potassium buffer pH 6.5 supplemented with 0.8 % (w/v) of glucose (TGYEP) or 0.8 % (w/v) fructose (TFYEP). When indicated, cAMP was added to the medium at a final concentration of 2 mM.

To determine hydrogen gas production qualitatively, strains were grown as standing cultures in capped tubes containing 10 ml TYEP medium, pH 6.5 supplemented with either 0.8 % (w/v) glucose or 0.8 % (w/v) fructose, and dihydrogen accumulation was monitored in inverted Durham tubes. The antibiotics kanamycin, ampicillin, chloramphenicol and tetracycline were added to the media at final concentrations of 50, 100, 12.5 and 15 µg ml⁻¹, respectively.

Defined deletion mutations were transduced from appropriate strains obtained from the Keio collection (Baba *et al.*, 2006). The *crp* mutation was transduced using a P1kc lysate derived from SA2777 (provided by S. Adhya, National Institutes of Health) and introduced into the MC4100 background according to (Miller, 1972).

Plasmid construction and DNA manipulations. Recombinant DNA work was carried out according to published methods (Sambrook & Russell, 2001). Cloning of the *hyaA* upstream regulatory region for $\Phi(hyaA'-lacZ)$ translational fusion analysis has been described (Pinske & Sawers, 2011). For the $\Phi(hyaA-lacZ)$ transcriptional fusion construction plasmid pTS101 (Schlensog *et al.*, 1994) was used, and the fusion was transferred to λ RS45 (Simons *et al.*, 1987) and introduced in single copy into the λ attachment site of MC4100 resulting in strain CP782. Construction of strains DJR10 $\Phi(hyaA-lacZ)$ and DJR100 $\Phi(hybO-lacZ)$, both carrying single copy transcriptional fusions, has been reported (Richard *et al.*, 1999).

Preparation of cell extracts and determination of enzyme activity. Anaerobic cultures were harvested at an OD₆₀₀ of approximately 0.8. Cells from cultures were harvested by centrifugation at 4000 g for 10 min at 4 °C, suspended in 1 % (v/v) of the culture volume of MOPS pH 7.0 buffer and lysed on ice by sonication (30 W power for 5 min with 0.5 s pulses). Unbroken cells and cell debris were removed by centrifugation for 15 min at 10 000 g at 4 °C.

Fig. 1. Metabolic routes and regulation of fructose in *E. coli*. Glucose can be taken up and phosphorylated by the PTS encoded by *ptsG* while fructose is transported by *fruAB* (fructose PTS) entering glycolysis after further phosphorylation (*fruK* 1-phosphofructokinase) or by the alternative routes encoded by *mak* and *manXYZ*. A hypothetical novel route of fructose uptake and entry into glycolysis at the level of glucose-6-phosphate is indicated by the dashed arrow. Several steps of glycolysis and subsequent reactions are regulated by the catabolite repressor protein (CRP) bound to cAMP and by FruR (fructose repressor) as shown by arrows in red (–) for repression and green (+) for induction. The genes encoding the proteins analysed in this study are shown in bold.

and the supernatant was used as the crude cell extract. Protein concentration of crude extracts was determined (Lowry *et al.*, 1951) with BSA as standard. Hydrogenase activity was measured according to the method of Ballantine & Boxer (1985), except that the buffer used was 50 mM MOPS buffer, pH 7.0. The detection wavelength used was 578 nm and an E_M value of $8600 \text{ M}^{-1} \text{ cm}^{-1}$ was assumed for reduced benzyl viologen. One unit of activity was defined as the oxidation of $1 \mu\text{mol}$ hydrogen min^{-1} .

Protein purification. Strain FTH004 (Dubini *et al.*, 2002) was grown anaerobically at 37°C in 10 l batches containing LB medium supplemented with either 0.8 % (w/v) glucose or 0.8 % (w/v) fructose. Cells were harvested by centrifugation at $5000 g$ at 4°C and washed in

50 mM Tris/HCl pH 7.5. Hyd-1 was then isolated either by using the method of Lukey *et al.* (2010) or by taking up the cell pellet in 50 ml B-PER solution (Thermo Scientific) supplemented with DNase I, lysozyme, a protease inhibitor cocktail (Calbiochem) and 50 mM imidazole. The resultant crude extract was subjected to immobilized metal affinity chromatography (IMAC) with a 5 ml HisTrap HP column (GE Healthcare) pre-equilibrated with 50 mM Tris/HCl pH 7.5, 200 mM NaCl, 50 mM imidazole, 0.05 % (w/v) *n*-Dodecyl- β -Maltoside (DDM). To elute bound protein, a linear gradient of 50–1000 mM imidazole, in the same buffer, was applied. Where described, size-exclusion chromatography (SEC) was carried out with a Superdex200 10/30 column (GE Healthcare) equilibrated in 50 mM Tris/HCl pH 7.5, 200 mM NaCl, 0.05 % (w/v) DDM.

Table 1. Strains and plasmids used in this study

Strain	Genotype	Reference
MC4100	F ⁻ , <i>araD139</i> Δ (<i>argF-lac</i>) <i>U169</i> λ^- <i>rpsL150</i> <i>relA1</i> <i>deoC1</i> <i>flhD5301</i> Δ (<i>fruK-yeiR</i>)725(<i>fruA25</i>), <i>rbsR22</i> , Δ (<i>fimB-fimE</i>)632(::IS1)	Casadaban (1976), Peters <i>et al.</i> (2003)
BW25113	F ⁻ Δ (<i>araD-araB</i>)567 Δ <i>lacZ</i> 4787(:: <i>rmB-3</i>) λ^- <i>rph-1</i> Δ (<i>rhaD-rhaB</i>)568 <i>hsdR514</i>	Baba <i>et al.</i> (2006), Datsenko & Wanner (2000)
DHP-F2	MC4100 Δ <i>hypF</i>	Paschos <i>et al.</i> (2002)
FTH004	MC4100 <i>hyaA</i> ^{His}	Dubini <i>et al.</i> (2002)
JW3985	BW25113 Δ <i>pgi</i> :: <i>aphA3</i> (Kan ^R)	Baba <i>et al.</i> (2006)
JW2155	BW25113 Δ <i>fruK</i> :: <i>aphA3</i> (Kan ^R)	Baba <i>et al.</i> (2006)
CP1021	MC4100 Δ <i>crp39</i> :: <i>cat</i>	This study
CP1029	MC4100 Δ <i>crp39</i> :: <i>cat</i> Φ (<i>hyaA'</i> -' <i>lacZ</i>)	This study
DJR10	MC4100 Φ (<i>hyaA-lacZ</i>)	Richard <i>et al.</i> (1999)
DJR100	MC4100 Φ (<i>hybO-lacZ</i>)	Richard <i>et al.</i> (1999)
CP782	MC4100 Φ (<i>hycA-lacZ</i>)	This study
CP612	MC4100 Φ (<i>hyaA'</i> -' <i>lacZ</i>)	Pinske & Sawers (2011)
CP460	MC4100 Δ <i>pgi</i> :: <i>aphA3</i> (Kan ^R)	This study
CP461	MC4100 Δ <i>fruK</i> :: <i>aphA3</i> (Kan ^R)	This study
CP462	MC4100 Δ <i>fruR</i> :: <i>aphA3</i> (Kan ^R)	This study
CP521	MC4100 Δ <i>mak</i> :: <i>aphA3</i> (Kan ^R)	This study
CP1118	MC4100 Δ <i>yeiC</i> :: <i>aphA3</i> (Kan ^R) <i>fruBKA</i> ⁺	This study
pTS101	Km ^R Ap ^R <i>lacY</i> ⁺ <i>lacA</i> ⁺ , Φ (<i>hycA-lacZ</i>)	Schlensoeg <i>et al.</i> (1994)

SDS-PAGE and immunoblotting. Aliquots of protein (25 μ g) from crude extracts were separated by SDS-PAGE in 10 % (w/v) gels (Laemmli, 1970) and transferred to nitrocellulose membranes as described by Towbin *et al.* (1979). Antibodies raised against Hyd-1 (1:10 000; Sawers & Boxer, 1986), Hyd-2 (1:20 000), HycE (1:3000; Sauter *et al.*, 1992) and PflB (1:3000; Sawers *et al.*, 1998) were used. Secondary antibody conjugated to horseradish peroxidase was obtained from Bio-Rad. Visualization was carried out using the enhanced chemiluminescent reaction (Stratagene). Densitometric evaluation of Western blots was carried out using ImageJ (NIH, Abramoff *et al.*, 2004).

Non-denaturing PAGE was performed using 7.5 % (w/v) polyacrylamide gels pH 8.5 including 0.1 % (w/v) Triton X-100 (Ballantine & Boxer, 1985). Samples (25 μ g protein) were incubated with 5 % (w/v) Triton X-100 prior to application to the gels. Hydrogenase activity-staining was done as described by Ballantine & Boxer (1985) except that the buffer used was 50 mM MOPS pH 7.0.

Analysis of gene expression. β -Galactosidase enzyme activity was determined as described by Griffith & Wolf (2002) and carried out at room temperature. Strains were grown for 6, 12 and 24 h. These times correspond to the late exponential phase when grown with glucose (6 h), the late exponential phase (12 h) and the stationary phase (24 h) when grown with fructose. Samples (100 μ l) were taken and permeabilized with CHCl₃-SDS. Activity was monitored via the hydrolysis of ONPG. Unless specifically stated in the figure legends or tables, all assays were performed in triplicate and the experiment was repeated independently at least three times.

RESULTS AND DISCUSSION

E. coli strain MC4100 exhibits reduced growth with fructose as carbon source

The laboratory strain MC4100 has a deletion in the *fruB* gene, encoding a fusion protein of the enzyme IIa and HPr components of the fructose-specific PTS. It also lacks a

portion of the *fruK* gene encoding fructose-1-phosphate kinase (Peters *et al.*, 2003; Reizer *et al.*, 1994). Comparing fermentative growth in rich medium supplemented with either glucose or fructose demonstrated that MC4100 had a higher growth rate ($\mu=1.39$ h⁻¹) when cultured anaerobically in the presence of glucose than in the presence of fructose ($\mu=1.05$ h⁻¹), which is in agreement with previous observations (Aristidou *et al.*, 1999). Upon entering the early stationary phase, MC4100 grown with fructose also failed to attain the same final OD₆₀₀ after fermentative growth on fructose compared with glucose (Fig. 2a). It should be noted, however, that fructose was clearly still used by MC4100 because in the absence of an added carbon source, growth was significantly poorer (Fig. 2a).

Transduction of the Δ *yeiC*::Kan^R allele from JW2153 into MC4100 simultaneously restored a wild-type copy of the adjacent *fruBKA* operon (see Methods). Analysis of the growth curve of the resulting strain CP1118 (*fruBKA*⁺) in the presence of fructose revealed that growth with fructose was indistinguishable from growth with glucose and it was identical to the growth of strain BW25113 (*fruBKA*⁺) with these sugar substrates (Fig. 2b). This finding indicates that the reason for the reduced growth rate of MC4100 with fructose is the absence of a functional fructose-PTS encoded by *fruBKA*. Moreover, this result indicates that an alternative means of fructose uptake and utilization is used by the bacterium under these conditions.

Manno(fructo)kinase is not the main route of fructose entry into glycolysis in MC4100

In the absence of the *fruBKA*-encoded PTS, fructose, when present at high concentrations, can enter the cell via the

Fig. 2. Growth curves of MC4100 and BW25113 derivatives in anaerobic rich medium supplemented with fructose or glucose. Strains and mutants derived from MC4100 (circles) or BW25113 (squares) were grown anaerobically in TYEP containing 0.8% (w/v) glucose (filled symbols) or 0.8% (w/v) fructose (open symbols) or without a sugar supplement (triangles). Growth curves for (a) wild-type MC4100, (b) BW25113 and CP1118 ($\Delta yeiC$ - $fruBKA^+$), (c) CP462 ($\Delta fruR$), (d) JW2155 ($\Delta fruK$) and CP461 ($\Delta fruK$), (e) JW3985 (Δpgi) and CP460 (Δpgi) and (f) CP521 (Δmak) were performed in a minimum of three independent experiments and data are the mean $\pm$ SD. The time points at which samples were removed from the cultures for enzyme analysis are marked by an arrow.

mannose PTS encoded by the *manXYZ* operon and it is subsequently phosphorylated by the manno(fructo)kinase, Mak (Kornberg *et al.*, 2000). Growth of a *mak* mutant in the presence of fructose was similar to that of MC4100 (Fig. 2f). Taken together, these data indicate that in the absence of a functional manno(fructo)kinase, a route other than one involving this kinase is responsible for fructose utilization in anaerobically growing MC4100 strains.

Strongly upregulated levels of hydrogenase 1 in MC4100 grown anaerobically on fructose compared with glucose

In order to determine whether there was any link between hydrogen metabolism and carbon source utilization, we made use of antibodies raised against the catalytic subunits of Hyd-1, Hyd-2 and Hyd-3 (HyaB, HybC and HycE, respectively), which made it possible to follow the levels of synthesis of all three [NiFe]-hydrogenases during growth on glucose or fructose. During biosynthesis of the hydrogenase enzymes, the C-terminus of the large subunit is proteolytically cleaved once active site biosynthesis is completed, which results in a faster migrating polypeptide species (labelled 2 in Fig. 3a, b); the unprocessed form of the large subunit (labelled 1 in Fig. 3a, b) results when no maturation reaction has taken place, for example as shown for DHP-F2 ($\Delta hypF$) (Fig. 3a, top panel). The HypF

protein is a carbamoyltransferase that functions together with the HypE maturase to provide the cyanide ligands that are attached to the iron atom in the [NiFe] cofactor (Böck *et al.*, 2006). The faster migrating polypeptide is generally taken to signify the presence of active hydrogenase (Böck *et al.*, 2006). The processed species of the large subunit of all three Hyd enzymes was maximally present around the mid-exponential phase of growth (4–8 h) when MC4100 was grown with glucose (Fig. 3a).

Hyd-3 synthesis is induced when the intracellular formate concentration is high (Rossmann *et al.*, 1991). Western blot analysis of the levels of pyruvate formate-lyase (PflB), which generates formate from the cleavage of pyruvate (Knappe & Sawers, 1990), revealed that PflB levels (Fig. 3a, bottom panel) correlated with the presence of the Hyd-3 large subunit (Fig. 3a). The double band in the Western blot of PflB is indicative of the presence of active, radical-containing enzyme in the cell at the time of cell breakage (Knappe & Sawers, 1990).

Extracts derived from cells grown with fructose showed similar results to those observed after growth with glucose, for HycE (Hyd-3) and for PflB (Fig. 3a). In contrast, the level of HyaB (the large subunit of Hyd-1) in fructose-grown cells was increased around 10-fold compared with glucose-grown cells, as determined by densitometric analysis (Fig. 3c) of the blots shown in Fig. 3(a). This

Fig. 3. Qualitative and quantitative analysis of anaerobic enzymes during growth on glucose and fructose. The wild-type MC4100 (a) and the hydrogenase-negative strain DHP-F2 ($\Delta hypF$) (b) were grown anaerobically in TYEP supplemented with 0.8% (w/v) glucose or fructose, and samples of 25 μ g protein taken at the indicated time points (from 2 to 24 h) were separated by 10% SDS-PAGE, transferred to nitrocellulose and analysed using the respective antibodies raised against Hyd-1, Hyd-2, Hyd-3 and pyruvate-formate lyase (PflB) as indicated. The arrows show unprocessed (1) and processed (2) hydrogenase large subunit polypeptides. The asterisk adjacent to bands on the autoradiogram signifies non-specific cross-reacting polypeptides. (c) Relative densitometric quantification of the unprocessed (1) and processed (2) forms of HyaB in MC4100. The 10 h level of HyaB was set at 100% for the quantification analysis. The data shown are for a single representative experiment that was repeated twice.

increased level of synthesis was observed throughout the growth phase. The amount of HybC (the large subunit of Hyd-2) polypeptide was also increased after growth in the presence of fructose compared to growth with glucose (Fig. 3a, second panel); however, the maximal level of Hyd-2 was observed after 4 h growth and the level slowly decreased during the later stages of growth.

To determine whether the higher level of detectable HyaB polypeptide after growth with fructose compared to growth with glucose was dependent on the presence of active Hyd-1 enzyme, the same experiment was repeated with extracts derived from the *hypF* mutant DHP-F2 (Fig. 3b). Although the absolute level of HyaB (Hyd-1) in fructose-grown DHP-F2 ($\Delta hypF$) cells was somewhat reduced compared with MC4100, increased amounts of Hyd-1 large subunit polypeptide were also evident when compared with glucose-grown cells. Strains DHP-F2 ($\Delta hypF$) and MC4100 had the same growth rate under the conditions analysed (data not shown).

The increase in cellular Hyd-1 levels observed during growth with fructose was corroborated by protein purification experiments. The FTH004 strain (Dubini *et al.*, 2002) is a derivative of MC4100 that carries a modified *hya* operon encoding a hexa-histidine-tagged HyaA Hyd-1 small subunit. This doubles as an epitope tag for Western blotting and allows facile isolation of the Hyd-1 enzyme. When grown in the presence of glucose, FTH004 produces Hyd-1 that can be isolated by IMAC (Fig. 4a). The enzyme was active with benzyl viologen as electron acceptor (Fig. 4b) and under these glucose-containing growth conditions, 8.5 g cell paste (wet weight) yielded 0.7 mg enzyme [0.08 mg (g cells)⁻¹].

When cultured in the presence of fructose, growth yields were greatly reduced in comparison with glucose fermentation. Typically, 5.5 g cell paste (wet weight) was obtained from 10 l fructose-containing culture. However, in this case, the amount of Hyd-1 eluted from the IMAC column was visibly increased (Fig. 4d), as was the relative hydrogenase activity in each column fraction (Fig. 4e). The yield of enzyme was more than doubled by growth in the presence of fructose, with 0.17 mg enzyme (g cells)⁻¹. Finally, SEC suggested that the Hyd-1 species produced under either glucose or fructose fermentations were structurally similar, as they had identical elution profiles (Fig. 4c, f).

Reintroduction of the *fruBKA* operon into MC4100 reduces Hyd-1 levels to those observed after growth with glucose

The findings presented above suggest that the levels of Hyd-1 inversely correlate with slower utilization of the sugar substrate. To provide further support for this correlation, we examined Hyd-1 levels in strain CP1118 (*fruBKA*⁺), which has a wild-type allele of the *fruBKA* operon. Extracts derived from CP1118 grown with fructose and analysed by non-denaturing PAGE showed a similar intensity of Hyd-1 activity bands regardless of whether the strains were grown with glucose or fructose (Fig. 5a). This contrasted with the more intense Hyd-1 enzyme activity observed in extracts of MC4100 grown on fructose (Fig. 5a). Western blot analysis of extracts derived from CP1118 also confirmed that after fermentative growth with fructose, a similar level of the HyaB antigen (Hyd-1 catalytic subunit) to the level determined in glucose-grown cells was observed (Fig. 5b). Taken together, these results indicate that the level of Hyd-1

Fig. 4. Isolation of Hyd-1 from glucose- or fructose-containing medium. Strain FTH004 was grown anaerobically in the presence of 0.8 % (w/v) glucose or 0.8 % (w/v) fructose. Extracts were separately subjected to IMAC, and eluted fractions from glucose-grown (a) or fructose-grown (d) cells were analysed for the presence of the Hyd-1 small subunit by immunoblotting using an anti-His-tag antibody (Qiagen). Relative hydrogenase enzyme activities in each 1 ml IMAC fraction ($\mu\text{mol H}_2$ oxidized μl^{-1}) were used to compare the amount of Hyd-1 activity recovered from glucose-grown (b) and fructose-grown (e) cultures. SEC was used to compare the elution profiles of the Hyd-1 species isolated from cells grown under glucose-rich (c) or fructose-rich (f) conditions. Data from a representative experiment are shown.

Fig. 5. Restoration of the *fruBKA* operon encoding the fructose PTS phenotypically suppresses fructose-dependent Hyd-1 induction in MC4100. Extracts (25 μg protein) of MC4100 and its derivative CP1118 ($\Delta\text{yeiC fruBKA}^+$) were either separated by native-PAGE and subsequently stained for Hyd-1 activity (a) or analysed by Western blotting (b) using anti-Hyd-1 antiserum.

shows an inverse correlation with the rate of anaerobic carbon source utilization.

Growth of MC4100 on fructose reduces total measurable hydrogenase enzyme activity

Analysis of the total hydrogenase enzyme activity (combined activities of Hyd-1, Hyd-2 and Hyd-3 measured as hydrogen-dependent reduction of benzyl viologen) present in crude extracts of MC4100 grown fermentatively with glucose showed that the activity decreased steadily from 2.4 U (mg protein)⁻¹ in the late-exponential phase of growth (6 h time point) to approximately 20 % of that value in late stationary phase cells (24 h time point) (Table 2). Under those conditions, about 90 % of the activity is due to Hyd-3 and only 1 % is due to Hyd-1 (Pinske *et al.*, 2011). This correlated with the appearance of hydrogen gas measured qualitatively, due to the activity of the hydrogen-evolving FHL complex (data not shown).

After growth in the presence of fructose, the total hydrogenase activity measured in cells growing in the mid-exponential phase (6 h time point) was 0.4 U (mg protein)⁻¹, which was only approximately 15 % of the activity measured compared with growth in the presence of glucose (Table 2). This activity remained fairly constant even up to 24 h of culture. Hydrogen gas production did not start until approximately 24 h (data not shown), indicating that the bulk of the enzyme activity measured was due to the

Table 2. Analysis of total hydrogenase activity in crude extracts of cells during fermentation of glucose or fructose in rich medium

Samples were taken and total hydrogenase enzyme activity was determined in crude extracts as described in Methods. Activities are given as U (mg protein)⁻¹; mean ± SEM.

Strain	TGYEP-medium			TFYEP-medium		
	6 h*	12 h	24 h	6 h	12 h	24 h
MC4100	2.43 ± 0.70	1.47 ± 0.56	0.45 ± 0.11	0.40 ± 0.08	0.90 ± 0.23	0.45 ± 0.07
DHP-F2	< 0.04	< 0.03	< 0.04	< 0.03	< 0.01	< 0.02
CP462 (<i>fruR</i> ⁻)	3.33 ± 0.23	1.45 ± 0.51	0.39 ± 0.07	0.47 ± 0.02	0.62 ± 0.20	0.71 ± 0.22
CP461 (<i>fruK</i> ⁻)	4.07 ± 0.91	1.04 ± 0.24	0.28 ± 0.08	0.23 ± 0.04	0.31 ± 0.04	3.36 ± 1.32
CP521 (<i>mak</i> ⁻)	2.70 ± 0.66	0.46 ± 0.22	0.99 ± 0.55	0.85 ± 0.26	0.76 ± 0.28	0.32 ± 0.02
CP460 (<i>pgi</i> ⁻)	0.15 ± 0.03	0.25 ± 0.12	0.24 ± 0.06	0.11 ± 0.02	0.09 ± 0.03	1.49 ± <0.01

hydrogen-oxidizing enzymes Hyd-1 and Hyd-2. As a control, extracts of the *hypF* mutant DHP-F2, which lacks [NiFe]-hydrogenase maturation (Paschos *et al.*, 2002), had essentially no hydrogenase enzyme activity under any of the conditions tested (Table 2). These results indicate that Hyd-3 activity and concomitant hydrogen production by MC4100 growing with fructose starts in the late stationary phase of growth, while Hyd-1 is present in both the exponential and stationary growth phases.

A direct correlation between the growth rate on sugar substrates and Hyd-1 levels

FruR is a global regulator that modulates the direction of carbon flow and has been shown to repress expression of a number of genes in glycolysis (*pgk*, *gpmI* and *pykF*) in a cAMP-independent manner (Ow *et al.*, 2007) (Fig. 1). In a *fruR* deletion mutant, therefore, a faster carbon flow from glucose results (Ow *et al.*, 2007; Ramseier *et al.*, 1995). Consequently, there is a more rapid availability of formate when cells are fermenting glucose and this was reflected in

a 40 % increase in total hydrogenase activity observed at the 6 h time point in the *fruR* mutant CP462 (Table 2). In fructose-grown cells, the absence of the FruR protein causes constitutive expression of the *fruBKA* operon (Postma *et al.*, 1993; Reiner, 1977); however, as the *fruB* gene is absent in MC4100, introduction of a *fruR* mutation should have no consequence for growth or hydrogenase activity during growth on fructose. Indeed, introduction of the $\Delta fruR$ allele into MC4100 (strain CP462) did not affect anaerobic growth on fructose (Fig. 2c); nor did it influence total hydrogenase activity in comparison with MC4100 (Table 2). In comparison with the wild-type, the levels of detectable Hyd-1 large subunit HyaB in the *fruR* mutant after growth with glucose were significantly decreased, possibly due to the increased carbon flow or due to enhanced turnover (Fig. 6). Increased levels of the large subunit, HyaB, of Hyd-1 were nevertheless still observed after fermentation in the presence of fructose.

Mutations in the *fruK* gene cause reduced growth in the presence of fructose due to the accumulation of toxic fructose-1-phosphate in the cytosol (Ferenci & Kornberg,

Fig. 6. Western blot analysis of the catalytic subunit (HyaB) of Hyd-1 during anaerobic growth with glucose and fructose. (a) Extracts (25 µg protein) of strains CP460 (Δpgi), CP461 ($\Delta fruK$) and CP462 ($\Delta fruR$) grown in TYEP with either glucose or fructose for the times indicated were separated by SDS-PAGE (10 %, w/v, polyacrylamide), transferred to nitrocellulose and analysed with antibodies raised against Hyd-1. The arrows indicate the migration positions for processed and unprocessed species of the HyaB polypeptide of Hyd-1. An extract from DHP-F2 ($\Delta hypF$) showing only the unprocessed form of HyaB was used as a control. The locations of the molecular size markers (Fermentas) are given to the right of the gel and the asterisk designates an unknown cross-reacting polypeptide species that correlated with Hyd-1. (b) In a similar experiment, extracts from CP1021 (Δcrp) grown under the same conditions but without or with addition of 2 mM cAMP were compared. Data from a representative experiment are shown.

1973; see Fig. 1). FruR-dependent repression of *fruBKA* operon expression, and consequently regulation of fructose utilization, can be counteracted by increased concentrations of fructose-1-phosphate and fructose-1,6-bisphosphate (Saier & Ramseier, 1996). Thus, increased fructose-1-phosphate concentrations in a *fruK* mutant reverse the FruR-mediated metabolic flux increase (Ramseier *et al.*, 1995). Introduction of the *fruK* allele from JW2155 (see Table 1 for genotype), generating strain CP461, restored an intact copy of the *fruB* gene on the chromosome of MC4100; however, analysis of CP461 ($\Delta fruK$) revealed that growth was still poor in the presence of fructose (Fig. 2d). Notably, after growth for 6 h in the presence of glucose, total hydrogenase activity in strain CP461 ($\Delta fruK$) was increased by approximately 70 % compared with MC4100 (Table 2). Activity decreased rapidly at later stages of the growth phase. While growth in the presence of fructose resulted in a 50 % decrease of hydrogenase activity in the late exponential (6 h) and early stationary (12 h) phases of growth, surprisingly, after 24 h, total hydrogenase activity increased by over sevenfold relative to the same time point for the wild-type (Table 2). Western blot analysis revealed that the HyaB (Hyd-1) large subunit polypeptide profile of the CP461 ($\Delta fruK$) (Fig. 6a) resembled that of the wild-type with fructose (see Fig. 3a). This indicated that the strong increase in hydrogenase activity probably resulted from increased levels of Hyd-3, presumably through increased intracellular formate concentrations (Rossmann *et al.*, 1991), while Hyd-1 levels remained unaffected by the mutation.

Analysis of the total hydrogenase activity in strain CP521 (Δmak), which lacks manno(fructo)kinase, after growth with glucose or fructose revealed a similar activity pattern to that in MC4100 (Table 2). The exception was the twofold increase in total hydrogenase activity in CP521 compared with MC4100 after 6 h growth with fructose. The Hyd-1 activity profile after non-denaturing PAGE and activity staining also essentially resembled that of MC4100 in both fructose- and glucose-grown cells (Supplementary Fig. S1, available with the online version of this paper). This finding substantiates the conclusion that the mannose PTS/manno(fructo)kinase route is not the main one used for fructose utilization in MC4100 under the conditions analysed (see also Fig. 2f).

Utilization of fructose by MC4100 depends on phosphoglucose isomerase activity

In an attempt to gain more insight into the requirements for this putative new route of fructose utilization in MC4100, we introduced a mutation in the gene (*pgi*) encoding phosphoglucose isomerase, which prevents isomerization of glucose-6-phosphate to fructose-6-phosphate (Fig. 1; Fraenkel, 1996). Fermentative growth of CP460 (MC4100 Δpgi) and JW3985 (BW25113 Δpgi) with glucose was poor (Fig. 2e), which was anticipated because *pgi* mutants metabolize glucose via the reductive pentose-phosphate

cycle (Fraenkel, 1996). Surprisingly, however, growth of CP460 (Δpgi) in the presence of fructose was equally as poor as it was with glucose and did not even attain the levels of growth observed for MC4100 grown under the same conditions (compare Fig. 2a). As a control, anaerobic growth of the *pgi* mutant JW3985, a derivative of BW25113, on fructose was tested and found to be unimpaired (Fig. 2e). This is because in this strain, fructose can be transported by the fructose PTS and it enters glycolysis at the level of fructose-1,6-bisphosphate, which thus bypasses phosphoglucose isomerase (Fig. 1). Previous studies in *Salmonella* demonstrated that a *pgi* mutation affected transcriptional levels and activities of Hyd-1 and Hyd-3 and that this phenotype could also be suppressed by anaerobic growth on fructose (Jamieson & Higgins, 1986). In MC4100, however, the *pgi* mutation prevented utilization of fructose, suggesting that in MC4100, fructose enters metabolism via glucose-6-phosphate during anaerobic growth. Notably, however, after 24 h of growth in the presence of fructose, the OD₆₀₀ of CP460 reproducibly reached similar levels to that of JW3985 (BW25113 Δpgi), suggesting that in the stationary phase fructose was taken up by the catabolite-repressible mannose PTS (Fig. 2e). As a further control, the $\Delta yeiC$ allele was introduced into CP460 (MC4100 Δpgi), which reintroduced the *fruBKA* operon into the strain. Indeed, reintroduction of a functional *fruBKA* operon into CP460 (Δpgi) resulted in a growth phenotype in the presence of fructose like strain JW3985 (BW25113 Δpgi) (data not shown). This result confirms that no other mutation in the genome of CP460 prevented growth with fructose and that in MC4100 derivatives a further route of fructose utilization requiring phosphoglucose isomerase exists.

Reduced rate of glucose metabolism also increases Hyd-1 levels

Total hydrogenase enzyme activity was reduced approximately 20-fold after 6 h of growth of CP460 (Δpgi) in the presence of glucose and fourfold after 6 h of growth in the presence of fructose (Table 2). Because both Hyd-2 and Hyd-1 enzyme activities were still detectable as determined by native PAGE (Supplementary Fig. S1 and data not shown) the bulk of this decrease in activity was presumably due to loss of Hyd-3 activity. Under the growth conditions analysed, Hyd-3 constitutes between 80 and 90 % of the total hydrogenase activity in MC4100 (Pinske *et al.*, 2011). Notably, however, Western blot analysis of HyaB in CP460 (Δpgi) (Fig. 6a) revealed that after fermentation with glucose, higher levels of the polypeptide were observed than after growth with fructose, which contrasts with what was observed in MC4100 (*pgi*⁺). This was particularly evident at the 6 h time point. Analysis of Hyd-1 after non-denaturing PAGE revealed that activity was reduced in the *pgi* mutant after growth with fructose (Supplementary Fig. S1). This result indicates that if glucose metabolism is

slowed down then this also results in increased Hyd-1 synthesis.

In enteric bacteria, CRP (cyclic AMP receptor protein) is a global transcriptional regulator mediating carbon catabolite repression (Deutscher *et al.*, 2006). No typical CRP-binding site is located in the regulatory region of the *hya* operon (Richard *et al.*, 1999) and neither binding of cAMP–CRP to the *hya* promoter nor induction of *hyaA*–*lacZ* fusions by cAMP could be shown (Brøndsted & Atlung, 1994). Nevertheless, if Hyd-1 levels are directly responsive to metabolism of sugar substrates then the amount of Hyd-1 would also be expected to be increased in a *crp* mutant, which has markedly reduced rates of PTS-dependent glucose uptake (Deutscher *et al.*, 2006). To test this, we introduced a deletion in the *crp* gene into MC4100, generating strain CP1021, and examined Hyd-1 levels after fermentative growth with glucose or fructose (Fig. 6b). Crude extracts derived from strain CP1021 (Δcrp) after growth in the presence of glucose revealed strongly increased levels of the Hyd-1 large subunit HyaB after growth for 6 and 12 h and this high level of HyaB dropped off dramatically after 24 h (Fig. 6b), presumably due to enhanced proteolysis (Gutierrez-Ríos *et al.*, 2007). Moreover, growth with fructose did not result in the high levels of HyaB normally observed after growth of MC4100 with fructose. Rather, barely detectable levels of the polypeptide could be visualized (Fig. 6b) and only low activity of Hyd-1 in native PAGE was observed (Supplementary Fig. S1). In contrast with other strains, the *crp* mutant CP1021 grew poorly on glucose, but even more poorly on fructose (data not shown). This is due to the lack of induction of the respective glucose PTS (Fig. 1) and the lack of induction of the *manXYZ* operon transcription in the absence of CRP (Plumbridge & Kolb, 1991). Notably, however, CP1021 (*crp*) was able to produce hydrogen gas from glucose as well as from fructose as determined by the qualitative hydrogen accumulation assay using Durham tubes. Growth in the presence of exogenously added cAMP, as anticipated (Deutscher *et al.*, 2006), did not restore HyaB levels to

those observed for MC4100 (Fig. 6b). This finding further supports the proposal that fructose must be taken up and metabolized via glycolysis to cause an increase in Hyd-1 protein and activity.

Transcriptional regulation of the *hya* operon does not account for the increased Hyd-1 levels during growth on fructose

To determine whether the increased level of Hyd-1 synthesis during growth on fructose resulted from regulation of the *hya* operon at the transcriptional level, expression of a single-copy *hya*–*lacZ* transcriptional fusion (Richard *et al.*, 1999) was analysed at different points during the growth phase with glucose or fructose (Table 3). Expression levels remained remarkably constant throughout the growth phase and were similar under both conditions.

Post-transcriptional regulation through differential translation of the *hya* operon mRNA is a possible means of explaining the higher levels of Hyd-1 in fructose-grown cells. To examine whether *hya* expression was regulated at the level of translation, a *hyaA*'–*lacZ* protein fusion was generated and introduced in single copy onto the chromosome of MC4100. Growth of the strain in the presence of glucose revealed a low β -galactosidase activity of around 100 units in the mid-exponential phase of growth (Table 3). This value was reduced by approximately threefold at the later stages of growth. Notably, the level of expression of the translational fusion was only approximately 5 % of that of the transcriptional fusion, suggesting strong downregulation of *hya* operon mRNA translation.

In comparison, growth of MC4100 containing the chromosomal *hyaA*'–*lacZ* protein fusion with fructose resulted in β -galactosidase expression levels that were twofold higher after 6 h of growth, nearly tenfold higher after 12 h and roughly threefold higher after 24 h (Table 3). Although the overall level of expression was higher with fructose, in particular at around 12 h, this did not account for the large difference in protein levels observed between fructose- and glucose-grown cells as shown in Fig. 3. As a control,

Table 3. Transcriptional response of $\Phi(hyaA$ –*lacZ*), $\Phi(hyaA$ '–*lacZ*), $\Phi(hybO$ –*lacZ*) and $\Phi(hycA$ –*lacZ*) to fructose or glucose utilization

	β -Galactosidase specific activity (Miller units $\pm$ SD)*					
	Glucose			Fructose		
	6 h	12 h	24 h	6 h	12 h	24 h
$\Phi(hyaA$ – <i>lacZ</i>)	2820 $\pm$ 369	2829 $\pm$ 266	3308 $\pm$ 157	3522 $\pm$ 536	4395 $\pm$ 400	4512 $\pm$ 232
$\Phi(hyaA$ '– <i>lacZ</i>)	97 $\pm$ 41	27 $\pm$ 12	34 $\pm$ 14	216 $\pm$ 42	306 $\pm$ 43	131 $\pm$ 22
$\Phi(hybO$ – <i>lacZ</i>)	340 $\pm$ 16	418 $\pm$ 15	445 $\pm$ 46	522 $\pm$ 10	609 $\pm$ 20	779 $\pm$ 31
$\Phi(hycA$ – <i>lacZ</i>)	134 $\pm$ 50	18 $\pm$ 6	23 $\pm$ 20	388 $\pm$ 151	349 $\pm$ 70	444 $\pm$ 91

*Strains were grown in TYEP supplemented with either glucose or fructose.

expression of *hybO-lacZ* was not increased above 1.5-fold at the evaluated time points during growth on fructose compared with growth on glucose (Table 3). The *hyc-lacZ* fusions (Table 3) reflect the course of total hydrogenase activity during growth on glucose and fructose (see Table 2), with expression remaining relatively constant during growth on fructose (Table 3). During growth on glucose, the expression of *hyc-lacZ* was highest at 6 h and strongly downregulated at later time points. Expression of *hyc* with fructose was threefold higher than with glucose; however, this does not reflect what was observed with the total hydrogenase activities. Because expression of *hyc* is dependent on the availability of formate (Rossmann *et al.*, 1991; Schlensog *et al.*, 1994), our results, therefore, clearly show that fructose must be metabolized to formate in MC4100. In addition, gas production in Durham tubes (Varenne *et al.*, 1975) could also be observed after growth with fructose, while no dihydrogen production was seen when cells were grown in TYEP without additional carbon source (data not shown).

Conclusions

This work demonstrates that Hyd-1 levels increased strongly when MC4100 was cultivated anaerobically in the presence of fructose compared with when glucose was added as carbon source. MC4100 lacks a complete *fruBKA* operon and grows more slowly in the presence of fructose. Restoration of a functional *fruBKA* operon to MC4100 improved growth in the presence of fructose and this caused a concomitant reduction in the levels of Hyd-1. Slowing the rate of glucose uptake by introducing a *crp* mutation, or its utilization by introducing a *pgi* mutation, also caused an increase in the level of Hyd-1. Moreover, our findings with the MC4100 *pgi* mutant indicate that in the absence of *fruBKA*, fructose is metabolized via glucose-6-phosphate and bypasses the mannose-PTS-manno(fructo)kinase route of entry into glycolysis. Carbon flux through glycolysis was abrogated in a *pgi* strain during growth in the presence of fructose, indicating that carbon starvation alone (Kornberg, 2001) is not sufficient to induce high levels of Hyd-1; rather, it appears that reduced carbon flux through glycolysis in fermenting cells is required. This suggests that a key glycolytic metabolite controls Hyd-1 levels during fermentation and these increased levels correlate with a role for the enzyme in enhancing energy generation (Lukey *et al.*, 2010). Finally, the increase in Hyd-1 in response to reduced growth rate or carbon flow through glycolysis is not due to enhanced transcriptional regulation of the *hya* operon but rather appears to be due to post-transcriptional and post-translational regulatory effects. Future studies will be directed towards: (1) elucidating how fructose is utilized by anaerobically growing MC4100 in the absence of a fructose PTS; (2) determining which glycolytic intermediate(s) controls the levels of Hyd-1 synthesis; (3) understanding the mechanism underlying post-transcriptional regulation of *hya* mRNA translation; and (4) identifying the factors

controlling Hyd-1 enzyme turnover in response to carbon flow through glycolysis and the cellular energy status.

ACKNOWLEDGEMENTS

This work was supported by the Deutsche Forschungsgemeinschaft (grant SA 494/3-1 to R.G.S.) and the Biotechnology and Biological Sciences Research Council (grant BB/H001190/1) to F.S.

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Edited by: R. J. Maier