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Dissecting the roles of *E. coli* hydrogenases in biohydrogen production

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Abstract

Escherichia coli can perform at least two modes of anaerobic hydrogen metabolism and expresses at least two types of hydrogenase activity. Respiratory hydrogen oxidation is catalysed by two 'uptake' hydrogenase isoenzymes, hydrogenases -1 and -2, and fermentative hydrogen production is catalysed by hydrogenase-3. Harnessing and enhancing the metabolic capability of *Escherichia coli* to perform anaerobic mixed-acid fermentation is therefore an attractive approach for bio-hydrogen production from sugars. In this work, the effects of genetic modification of the genes encoding the uptake hydrogenases, as well as the importance of pre-culture conditions, on hydrogen production and fermentation balance were examined. In suspensions of resting cells pre-grown aerobically with formate, deletions in hydrogenase-3 abolished hydrogen production, whereas the deletion of both uptake hydrogenases improved hydrogen production by 37 % over the parent strain. Under fermentative conditions, respiratory H_2 uptake activity was absent in strains lacking hydrogenase-2. The effect of a deletion in *hycA* on H_2 production was found to be dependent upon environmental conditions, but H_2 uptake was not significantly affected by this mutation.

1. Introduction

Biological approaches to energy production are growing in importance as fossil-fuel resources verge on the limits of economical extraction (Holmes & Jones, 2003) and the environmental cost of carbon emissions gain recognition in financial terms (Hopkin, 2004; Klepper & Peterson, 2006).

Escherichia coli is attractive for biotechnological applications such as biohydrogen production. In contrast to other H_2-producing micro-organisms, such as the clostridia, *E. coli* is fast-growing, non-sporulating, and well-characterised in physiological and biochemical terms. Furthermore, metabolic engineering using 'crippled' strains such as K12 (and its derivatives) provides information relevant to the future modification of a wild-type strain, while also mitigating against accidental release. When turnover rate is considered, the H_2-producing Hyd-3 of *E. coli* (a NiFe hydrogenase) is significantly slower than Fe hydrogenases (e.g. of clostridia) (Hallenbeck & Benemann, 2002), although the superiority of different fermentative bacteria is controversial and a detailed comparison is beyond the scope of this paper.

Formate is the sole precursor of H_2 in *E. coli* (Ordal & Halvorson, 1939), being cleaved to H_2 and CO_2 by the formate hydrogenlyase complex (FHL) (Stephenson & Stickland, 1932; Sawers, 2005). Formate arises from the mixed acid fermentation of sugars (Fig. 1), with a maximum yield of 2 mol H_2/mol glucose (Clark, 1989). In practice, yields are typically ~1 mol H_2/mol glucose as several factors may affect the rate and yield of H_2 production (Stephenson & Stickland, 1932; Bisaillon *et al.*, 2006). In this study, the role of hydrogenases in the formation and uptake of H_2 during fermentation of glucose was addressed, with the aim to maximise the rate and yield of H_2 produced.

E. coli K-12 has the potential to express four hydrogenases (Hyd-1-4). With the exception of Hyd-4, which has never been biochemically characterised, the *E. coli* hydrogenases can operate reversibly *in vitro* but possess physiological directionality (Sawers, 1994). Hyd-3 is encoded by the *hyc* operon and forms, with a formate dehydrogenase (FDH_H) and numerous other electron transport proteins, the FHL complex, which is responsible for H_2 production from formate (Rossman *et al.*, 1991). The putative FHL-2 complex (homologous to FHL) is hypothesised to carry out energy conservation by formate-dependent proton translocation and contains Hyd-4 (*hyf* operon) (Andrews *et al.*, 1997; Skibinski *et al.*, 2002). Hyd-2 (encoded by the *hyb* operon) functions in anaerobic respiration as an uptake hydrogenase (Ballantine & Boxer, 1985; Sawers *et al.*, 1985). Hyd-1 (encoded by the *hya* operon) is also an uptake hydrogenase, and has been suggested to be expressed under fermentative conditions to recycle the H_2 produced from formate (Sawers *et al.*, 1985; Sawers & Boxer, 1986). The physiological role of Hyd-1 is not yet clear and it has also been suggested to function in energy conservation under acid stress (King & Przybyla, 1999).

Attempts to negate residual hydrogen uptake activity under fermentative conditions, which detracts from the net H_2 production, have been successful in a range of H_2 producing organisms including anoxygenic photosynthetic bacteria (Willison *et al.*, 1984; Toussaint *et al.*, 1991; Jahn *et al.*, 1994; Franchi *et al.*, 2004; Kim *et al.*, 2006), cyanobacteria (Happe *et al.*, 2000; Masukawa *et al.*, 2002; Yoshino *et al.*, 2006), and recently in *E. coli* (Bisaillon *et al.*, 2006; Penfold *et al.*, 2006). This approach, which relies on physiological uni-directionality of the isoenzymes, would not be appropriate for other fermentative H_2-producers (e.g. clostridia and thermophilic archaea) in which H_2 uptake and production are performed by the same (reversible) hydrogenases (Hallenbeck, 2005).

Genetic techniques have been employed previously to improve H_2 production by *E. coli*. In particular, strain HD701 (devoid of HycA, the *hyc* operon repressor) was capable of upregulating H_2 production more rapidly than the parent strain (MC4100) upon transfer to H_2 producing conditions (Sauter *et al.*, 1992; Sode *et al.*, 2001; Penfold *et al.*, 2003; Yoshida *et al.*, 2005). Inactivation of the *tat* (twin arginine transport) export system effected a similar improvement in overall H_2 production, comparable to the HycA deficiency (Penfold *et al.*, 2006). However, *tat* mutations are pleiotropic, causing defects in outer membrane biosynthesis and cell division, in addition to preventing the correct assembly of uptake hydrogenases and respiratory formate dehydrogenases (Sargent *et al.*, 1998; Stanley *et al.*, 2001). Therefore, the *tat*-deficient strains were assumed to exhibit the effects of reduced activity of the uptake hydrogenases (Hyd-1 and Hyd-2) although this was not proven. It was also assumed by Penfold *et al.* (2006) that H_2 uptake activity was not expressed by other isoenzymes. However, contrary to expectation, the superimposition of the *tat* deficiency phenotype onto the HycA deficiency phenotype did not result in any further increase in H_2 production, raising the possibility of a compensatory H_2 oxidation activity by Hyd-3 (Penfold *et al.*, 2006).

In the current work, the fermentation balances of strains genetically deprived of specific hydrogenase activities were analysed. Evidence is provided that the respiratory hydrogenase-2 contributes to the vast majority of H_2 recycling activity during fermentative hydrogen production, which supports the suggestion that it is the loss of this activity, rather than other pleiotropic effects, that leads to an increased H_2 yield when the Tat system is inactivated.

2. Materials and Methods

2.1 Bacterial strains

Strain HD701 was provided by Professor A. Böck (Lehrstuhl für Mikrobiologie der Universität, Munich, Germany) and was derived from MC4100 (Sauter *et al.*, 1992). All strains are listed in Table 1.

2.2 H_2 production experiments

E. coli stocks were maintained at –70 °C in 25 % glycerol (1 part overnight culture in nutrient broth, 1 part 50 % glycerol w/v) and revived on nutrient broth (Oxoid) at (37 °C, 8 h, 250 rpm) before plating on nutrient agar (Oxoid). Plates were stored at 4 °C for up to 1 week before use. For experiments, colonies were picked into 30 ml nutrient broth with added sodium formate (0.1 M) (6 hours, 37 °C, 250 rpm). Cells were harvested from late log phase cultures (4500 *g*, 10 min) consisting of 500 ml nutrient broth with added sodium formate (0.1 M) (0.001 % inoculum, 14-16 hours, 30 °C, 250 rpm). Cell pellets were washed twice in 100 ml phosphate buffered saline (PBS: 1.43 g Na_2HPO_4, 0.2 g KH_2PO_4, 0.8 g NaCl, 0.2g KCl per litre, pH 7.0) before resuspending in 10 ml PBS to produce a concentrate containing 30-40 mg dry weight/ml. Biomass concentration was estimated by reference to a previously determined conversion factor. An OD_{600} of 1 corresponded to a concentration of 0.48-mg dry weight/ml.

The cell concentrate was made anaerobic by purging with argon (30 min) before 1-2 ml was transferred into ~120 ml anaerobic bottles containing 100 ml test medium (made anaerobic by 3 cycles of 30 min under vacuum and argon purging) to give an initial cell concentration of 0.5 mg dry weight/ml. OD_{600} was measured at the end of the experiment to estimate biomass growth. Test medium consisted of 0.1 M MES buffer (2-(N-Morpholino)ethanesulfonic acid), pH 6.80 supplemented with 6.96 g/l NaCl (final concentration), 0-100 µl 2 M NH_4Cl and 0.3 ml trace elements solution given in (Hewitt *et al.*, 2000). Unless otherwise stated the final concentration of NH_4Cl was 1 mM.

Anaerobic bottles were connected to a gas collection apparatus (Macler *et al.*, 1979) and allowed to equilibrate (30 °C, reciprocal shaking at 130 rpm, 5 min) before the fermentation was initiated by the addition of 1 ml degassed 2 M glucose (to 20 mM). The volume of H_2 evolved was measured over a solution of 2 M NaOH containing universal indicator (Sigma, UK) for ~45 hours. A previous study confirmed that H_2 and CO_2 are the only gases evolved by *E. coli* (Penfold *et al.*, 2003). The presence of H_2 in the evolved gas was confirmed using a combustible gas meter (GMI, UK) and the removal of CO_2 (to <0.5 % v/v) was confirmed using a ThermoQuest gas chromatograph (TraceGC2000) fitted with a Shincarbon ST column (100/120 mesh, length: 2 m, ID: 2 mm, Shimazu, Japan). The GC operating conditions were split 60:1, 40 °C + 15 °C/min for 10 min, and the injection volume was 1 ml.

2.3 Chemical analyses

Samples (2 ml) were filtered (0.2 µm supor membrane) and filtrates were stored at -20 °C before analysis. Organic acids were measured by anion HPLC using a Dionex 600-series system as described previously (Redwood & Macaskie, 2006). Glucose was assayed using the colorimetric dinitrosalicylic acid assay (Chaplin, 1986) and ethanol was determined colorimetrically by monitoring the enzymatic reduction of NAD (A_{340}) using alcohol dehydrogenase (Sigma A-6338, assay concentration: 2.64 U/ml) after pre-removal of aldehyde by aldehyde dehydrogenase (Sigma A-7011, assay concentration: 16.18 U/l).

2.4 Analysis of fermentation balance

All products were measured as described above, with the exception of CO_2 which was calculated (as described below). Unknown quantities of fermentation balance were estimated according to equations 1-4, substituting values for products formed (e.g. mol product/mol glucose) from Table 1. As described previously (Sode *et al.*, 1999), equations 1-3 are derived from the metabolic pathway of mixed acid fermentation (Fig. 1).

H_2 uptake was estimated as the imbalance between the theoretical formate decomposed (acetate + ethanol – formate) and the measured H_2 ($H_{2formed}$) (equation 1), i.e. the difference between the expected H_2 and that found by measurement:

$$H_2uptake = acetate + ethanol - formate - H_{2\,formed} \qquad (1)$$

Succinate formation requires the incorporation of CO_2 (Fig.1), which in a gas-scrubbed medium devoid of added carbonate would be derived from the decomposition of formate (HCOOH = CO_2 + H_2). Therefore, the net production of CO_2 (CO_2est) was estimated by subtracting the succinate formed from the theoretical formate decomposed (equation 2).

$$CO_2est = acetate + ethanol - formate - succinate \qquad (2)$$

The carbon balance (C. bal.) was obtained by summing the carbon fractions of the products of fermentation. The carbon allocation to biomass formation was determined from the observed increase in OD_{600} and conversion to dry weight using 53.1 % as the carbon fraction of *E. coli* biomass (Harris & Adams, 1979; Pramanik & Keasling, 1997). Therefore, a factor of 0.044 was applied for the conversion from g biomass/mol glucose to mol carbon/mol glucose (equation 3).

$$C.\ bal.\ (\%) = \frac{3lact. + 2acet. + 2ethanol + 4succ. + form. + CO_2 + 0.044biomass}{6} \times 100 \qquad (3)$$

All experiments were replicated on at least 3 occasions (as stated) using independent cultures. Results are expressed as mean ± standard error of the mean.

3. Results

3.1 Effects of pre-growth conditions on H_2 production in E. coli MC4100 and HD701

For all tests on H_2 production, cultures were pre-grown before cells were harvested, washed and transferred to 100 ml reactors. The ability of resting cells to produce H_2 was independent of the availability of NH_4^+-nitrogen (0-10 mM NH_4Cl) during the test reaction (1 mM was used subsequently), but it was dependent upon the presence or absence of oxygen and sodium formate during pre-growth. As expected, cells pre-grown anaerobically produced H_2, whereas aerobically pre-grown cells failed to produce H_2 even after prolonged incubation (24 h), attributable to the lack of expression of the genes encoding the FHL complex responsible for H_2 production (Gest, 1954; Sauter *et al.*, 1992). Interestingly, the aerobic expression of the genes encoding FHL was induced by the addition of sodium formate (0.1 M) to the pre-growth medium. A significant difference was not observed (t-test, P>5 %) when the initial rate and total volume of H_2 production were compared, among cells pre-cultured with formate either anaerobically or aerobically. From a biotechnological point of view, therefore, this represents a significant advantage since the relative ease of biomass production and greater biomass yield/ml of aerobic pre-growth would certainly be more economically favourable for a scaled-up system. In previous studies H_2 was produced by aerobically grown formate-supplemented *Bact. coli* (Escherich) (Stephenson & Stickland, 1932) and significant *hyc* operon expression was measured during aerobic growth with sodium formate in *E. coli* MC4100 (Rossman *et al.*, 1991). In the present study the addition of sodium formate during aerobic growth caused a 10 % reduction in biomass yield relative to growth in unsupplemented broth, but upon the establishment of fermentative conditions (after washing to remove formate), strain MC4100 produced H_2 immediately and with an equal initial rate, endpoint and yield to the anaerobically-grown controls (see above). Thus, the aerobic-formate condition represents the preferred method for the preparation of an *E. coli* culture having high H_2-production activity.

3.2 Effects of Hyd-3 and HycA deletions on H_2 production and uptake

All strains expressing an active FHL complex (‘Hyd-3$^+$’) produced H_2 for approximately 45 h, during which at least 98 % of glucose (~2 mmol) was consumed and the pH decreased from 6.80 to 5.93 ± 0.06. The rates of H_2 production were identical in strains HD701 (HycA$^-$), and MC4100 (parent) (Fig. 2A), confirming that pre-growth (of the parent strain) in the presence of formate overcame the requirement for use of an FHL up-regulated strain. This would also rule out a pleiotropic effect of the HycA deficiency in a possible co-up-regulation of H_2 uptake, or, indeed any uptake hydrogenase activity of Hyd-3 (see below). Strains FTD147, FTD150 and HD705 (all devoid of Hyd-3), did not produce H_2 indicating that the remaining hydrogenases Hyd-1, Hyd-2 and Hyd-4 were not capable of H_2 production under these conditions. H_2 uptake by these strains was not investigated as it was shown previously that strain FTD89, which contains active Hyd-3 but no Hyd-1 or -2, and from which FTD147 and FTD150 were both derived, has no detectable *in vivo* fumarate-dependent H_2 uptake activity (Dubini *et al.*, 2002). This activity would not be restored upon the addition of further genetic modifications to produce strains FTD147 and FTD150. Similarly, strain HD705 (having Hyd-1 and Hyd-2 but no Hyd-3) possesses *in vivo* fumarate-dependent H_2 uptake activity similar to the parent strain (Sargent *et al.*, 1999).

3.3 Effects of Hyd-1 and Hyd-2 deletions on H_2 production and uptake

Strains FTD67 and FTD89 (which both lack Hyd-2) exhibited high rates of H_2 production, and were easily distinguishable from the parent strain from the outset, whereas a strain lacking Hyd-1 activity (FTD22) showed no significant change in H_2 production (Fig. 2B). The yields of H_2 from glucose (Table 1) mirrored this trend, both Hyd-2 deficient strains having significantly higher yields than the parent strain ($P < 0.001$), whereas no significant difference was found between the Hyd-1 deficient strain and the parent strain ($P = 0.15$), or between the Hyd-2 deficient strain FTD67 and the -1 and -2 double deficient strain FTD89 ($P = 0.35$).

The extent of H_2 recycling was calculated from the products formed (Table 1). For the parent strain (MC4100) hydrogen uptake activity resulted in a 31 % reduction in H_2 yield. Analysis of the activity of other, potentially competing, metabolic pathways shows very little variation in the proportions of lactate, succinate and ($H_{2formed}$ + H_2 uptake), indicating that the increased H_2 yield resulted from the removal of H_2 uptake, rather than from secondary effects on the activity of competing pathways.

However, a secondary effect on the fermentation balance was observed. The ratio of acetate/ethanol was significantly higher in strains FTD67 and FTD89 (devoid of Hyd-2) than in the parent strain (t-test, P<5 %, df=7). This can be attributed to the involvement of H_2 uptake in redox balance, whereby the oxidation of H_2 would increase the required disposal of reductant by the normal mechanism: the reduction of acetyl-CoA to ethanol. The increased ratio of acetate/ethanol would hypothetically result in an increased yield of ATP and hence growth, although the observed increases in growth for strains FTD67 and FTD89 (compared to the parent strain) was not statistically significant (t-test, P>5 %).

4. Discussion

In this study, the HycA deficient strain (HD701) and the parent strain (MC4100) produced H_2 with equal rate and yield if formate was present in the pre-growth medium. This is interesting in light of previous observations that HycA deficient strains of *E. coli* showed increased FHL expression and increased rate of H_2 production in comparison to parent strains (Sauter *et al.*, 1992; Sode *et al.*, 2001; Penfold *et al.*, 2003; Yoshida *et al.*, 2005). The HycA repressor is thought to control the expression of the *hyc* operon (encoding FHL complex structural components) by competing with formate for binding sites on the FhlA activator (Skibinski *et al.*, 2002). All data support the hypothesis that the absence of the HycA repressor results in a decreased threshold concentration of formate required to de-repress the expression of the FHL complex. The aerobic culture in a high sodium formate background (this study) is likely to have induced the expression to the maximum level, overcoming the effects of a deficiency in HycA, and permitting the rapid culture of cells possessing high H_2 production activity. Formate was shown previously to de-repress the *hyc* operon under aerobic conditions (Rossman *et al.*, 1991). Although the use of sodium formate would represent an additional cost upon scale-up, the excess would contribute to H_2 production, and the quantity necessary to produce a pre-adapted culture (expressing FHL activity) may be significantly less than that used here (0.1 M), as a previous study used only 0.03 M (Rossman *et al.*, 1991).

Increased H_2 production by *E. coli* in the absence of uptake hydrogenase activity has been observed by several authors (Sode *et al.*, 1999; Bisaillon *et al.*, 2006; Penfold *et al.*, 2006) but a compensatory uptake function of the residual hydrogenases has not been previously excluded. In the light of the analysis of fermentation balance (Table 1), the improvement can be attributed predominantly to the inactivity of hydrogenase-2. The calculation of H_2 uptake was based on the imbalance between estimates of formate decomposed (ethanol + acetate - formate) and $H_{2formed}$ (equation 1). As no oxidants were present, this imbalance cannot be attributed to the consumption of formate by respiratory formate dehydrogenases and the absence of H_2 uptake in the specific absence of Hyd-2 precludes any significant H_2 uptake activity by the remaining enzymes.

The H_2 yield reached only 52 % of the theoretical maximum (2 mol H_2/mol glucose). This was attributed to the formation of significant quantities of lactate and succinate, the quantities of which were not affected by deficiencies in Hyd-1 and Hyd-2. Therefore, the increased H_2 yield was due to decreased H_2 uptake and not to the decreased activity of lactate and succinate formation. The flow of carbon to these products, rather than through pyruvate formate-lyase (PFL) resulting in H_2 production, represented (on average) a loss of 37.9 % of potential H_2. Lactate and succinate formation can be controlled using strains defective in the fermentative lactate dehydrogenase and fumarate reductase, respectively (Mat-Jan *et al.*, 1989; Sode *et al.*, 1999; Sode *et al.*, 2001), and lactate formation can also be decreased through the control of pH and substrate supply (M.D. Redwood and L.E. Macaskie, unpublished).

During anaerobic fermentation under conditions studied here, *E. coli* exhibits no significant hydrogenase uptake activity by any factors other than hydrogenase-2. The deletion of the genes encoding respiratory hydrogenase 1 had no significant effect on H_2 production, whereas the yield was improved by more than one third through the deletion of uptake hydrogenase-2. For the industrial application of this work to bio-H_2 production the Hyd-1 and -2 deficient strain (FTD89) would be most advantageous. Further modifications to this strain to control lactate and succinate formation could result in yields close to 2 mol H_2/mol glucose.

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Table 1 Bacterial strains and fermentation balances

Strain ID	Genotype	Hyd-[a]			Products formed (mol/mol glucose)								Growth[d] (g/mol glucose)	Carbon balance (%)[e]	Strain source
		1	2	3	$H_{2formed}$	Formate	Acetate	Ethanol	Lactate	Succinate	CO_2*est*[b]	H_2 uptake[c]			
MC4100	Parental strain	+	+	+	0.764 (0.030)	0.149 (0.011)	0.499 (0.036)	0.649 (0.012)	0.318 (0.012)	0.494 (0.008)	0.504 (0.026)	0.236 (0.045)	2.61 (1.947)	100 (1.53)	f
HD701	Δ*hycA*	+	+	+	0.737 (0.023)	0.132 (0.013)	0.363 (0.036)	0.667 (0.015)	0.359 (0.009)	0.470 (0.009)	0.422 (0.035)	0.162 (0.025)	2.57 (0.281)	95 (2.54)	f
FTD147	Δ*hyaB*, Δ*hybC*, Δ*hycE*	-	-	-	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	g
FTD150	Δ*hyaB*, Δ*hybC*, Δ*hycE*, Δ*hyfB-R*	-	-	-	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	g
HD705	Δ*hycE*	+	+	-	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	f
FTD22	Δ*hyaB*	-	+	+	0.800 (0.012)	0.181 (0.032)	0.528 (0.052)	0.695 (0.025)	0.312 (0.013)	0.452 (0.036)	0.590 (0.070)	0.242 (0.062)	3.01 (1.040)	102 (3.97)	h
FTD67	Δ*hybC*	+	-	+	1.024 (0.040)	0.214 (0.026)	0.686 (0.016)	0.539 (0.005)	0.383 (0.030)	0.432 (0.024)	0.674 (0.061)	-0.001 (0.021)	2.52 (0.926)	104 (1.99)	i
FTD89	Δ*hyaB*, Δ*hybC*	-	-	+	1.043 (0.025)	0.176 (0.012)	0.640 (0.057)	0.583 (0.028)	0.372 (0.029)	0.436 (0.021)	0.611 (0.069)	0.004 (0.094)	3.62 (1.419)	104 (5.14)	h

[a] + present, - defective; [b] equation 2; [c] equation 1; [d] g bacterial dry weight; [e] equation 3, [f] (Sauter *et al.*, 1992); [g] (Sargent, F - unpublished), [h] (Sargent *et al.*, 1999); [i] (Dubini *et al.*, 2002)
Values are the means of at least 4 replicates (± S.E.M.).
ND, Strains FTD147, FTD150 and HD705 produced no detectable H_2 and were not studied further.

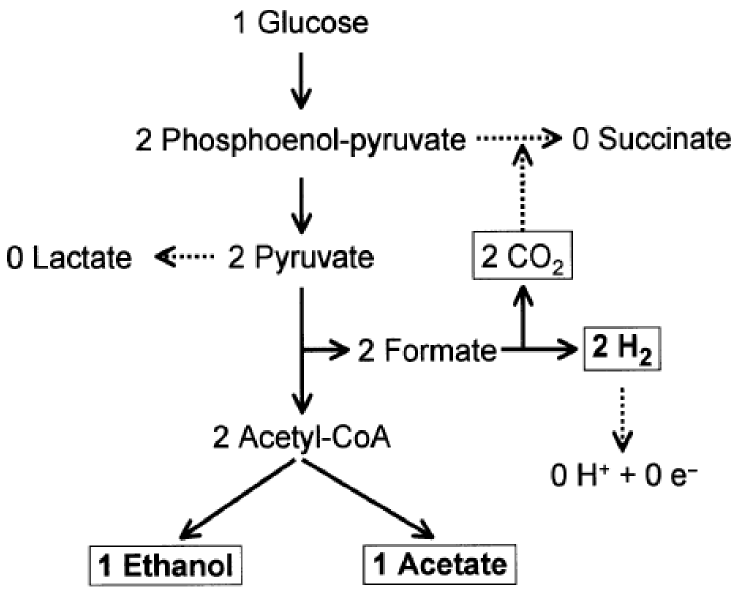

Fig. 1. Metabolic scheme for mixed acid fermentation. The solid lines represent pathways contributing to H_2 production. The broken lines represent pathways competing with H_2 production. The ideal products are boxed. Ideally the values of lactate formation, succinate formation and H_2 uptake would be zero, and hence there would be no recycling of produced CO_2 in the carboxylation of PEP.

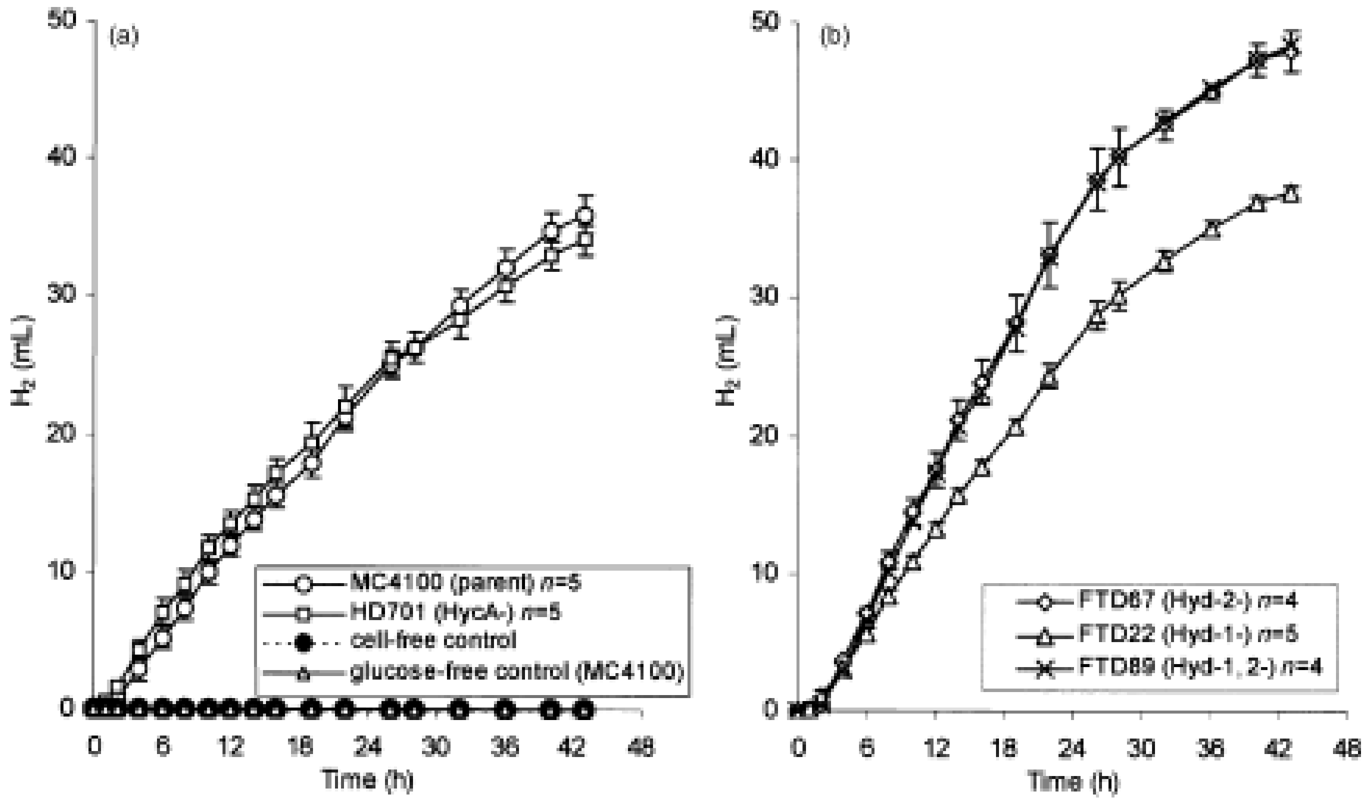

Fig. 2. H_2 production by *E. coli* strains deficient in HycA (A), and uptake hydrogenases (B). Bars represent standard errors.

Supplementary figure (not published)

Figure 3 was removed from the publication at the request of the editor and it is included here for clarity.

The figure illustrates that the sum of H_2 formed and H_2 uptake was reasonably constant, whereas Hyd-2 activity affected the distribution of potential H_2 between these two fates, whereas the effects on other aspects of fermentation balance were relatively minor.

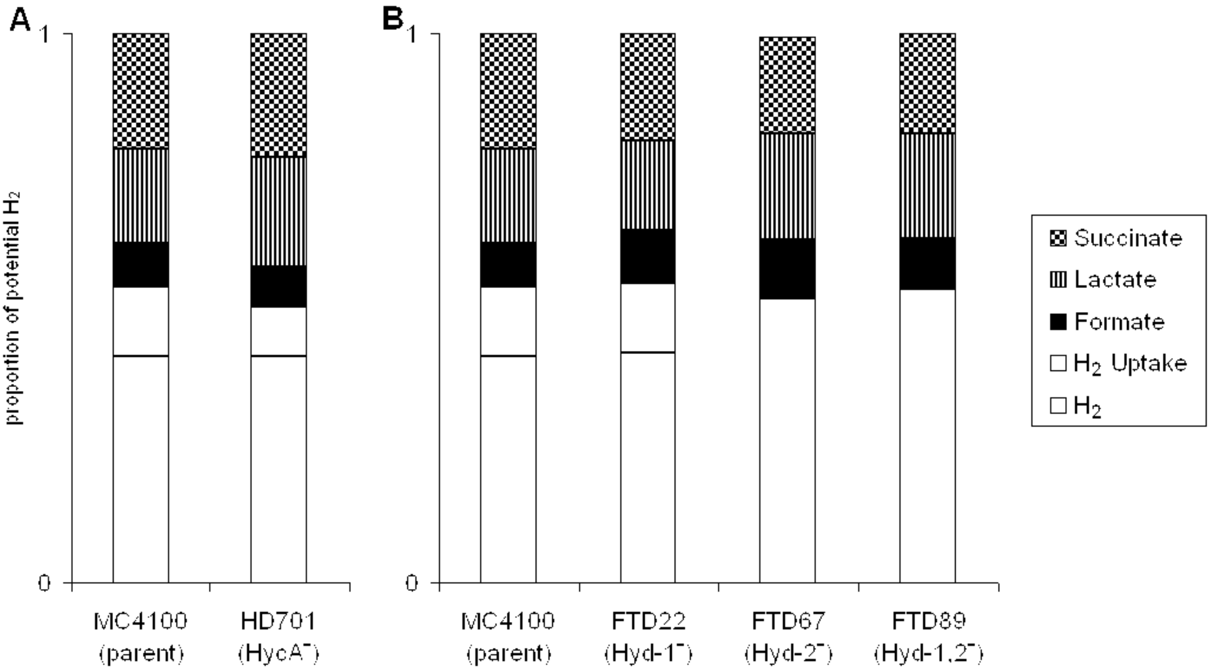

Figure 3. Fates of potential H_2 in *Escherichia coli* strains deficient in HycA (A) and uptake hydrogenases (B).

In accordance with the scheme of mixed acid fermentation (Fig. 1), one mole of lactate, succinate, formate or 'H_2 uptake' represents one mole of potential H_2 production, whereas acetate and ethanol are produced concomitantly with H_2. Data are the normalised means of at least four replicate experiments. Means and standard errors (pre-normalisation) are given in Table 1. For each strain the sums of potential H_2 and measured H_2 were not significantly different from 2 mol H_2/mol glucose and did not vary significantly between strains.