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ORIGINAL ARTICLE

Ethanol synthesis from glycerol by *Escherichia coli* redox mutants expressing *adhE* from *Leuconostoc mesenteroides*

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acetyl-CoA availability, alcohol-acetaldehyde dehydrogenase, *Escherichia coli*, glycerol metabolism, heterologous gene expression, microaerobiosis, microbial physiology.

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Abstract

Aims: Analysis of the physiology and metabolism of *Escherichia coli arcA* and *creC* mutants expressing a bifunctional alcohol-acetaldehyde dehydrogenase from *Leuconostoc mesenteroides* growing on glycerol under oxygen-restricted conditions. The effect of an *ldhA* mutation and different growth medium modifications was also assessed.

Methods and Results: Expression of *adhE* in *E. coli* CT1061 [*arcA creC*(Con)] resulted in a 1·4-fold enhancement in ethanol synthesis. Significant amounts of lactate were produced during micro-oxic cultures and strain CT1061LE, in which fermentative lactate dehydrogenase was deleted, produced up to 6·5 ± 0·3 g l^{-1} ethanol in 48 h. *Escherichia coli* CT1061LE derivatives resistant to >25 g l^{-1} ethanol were obtained by metabolic evolution. Pyruvate and acetaldehyde addition significantly increased both biomass and ethanol concentrations, probably by overcoming acetyl-coenzyme A (CoA) shortage. Yeast extract also promoted growth and ethanol synthesis, and this positive effect was mainly attributable to its vitamin content. Two-stage bioreactor cultures were conducted in a minimal medium containing 100 μg l^{-1} calcium D-pantothenate to evaluate oxic acetyl-CoA synthesis followed by a switch into fermentative conditions. Ethanol reached 15·4 ± 0·9 g l^{-1} with a volumetric productivity of 0·34 ± 0·02 g l^{-1} h^{-1}.

Conclusions: *Escherichia coli* responded to *adhE* over-expression by funnelling carbon and reducing equivalents into a highly reduced metabolite, ethanol. Acetyl-CoA played a key role in micro-oxic ethanol synthesis and growth.

Significance and Impact of the Study: Insight into the micro-oxic metabolism of *E. coli* growing on glycerol is essential for the development of efficient industrial processes for reduced biochemicals production from this substrate, with special relevance to biofuels synthesis.

Introduction

Concern about energy security has stimulated recent interest in identifying sources for large-scale substitution of petroleum-based fuels. Besides, environmental deterioration resulting from over-consumption of petroleum-derived products could eventually threat human society sustainability (Demain 2009). Metabolic engineering of biological pathways for the synthesis of industrial chemicals offers an interesting alternative to fossil fuels and petroleum-derived chemicals (Vemuri and Aristidou 2005). Ethanol has potential as a biofuel and feedstock for the production of oxygenated fuels, having a positive environmental impact because of its low-polluting combustion (Stephanopoulos *et al.* 1998).

A great deal of research has been directed towards economical ethanol generation (Zaldivar *et al.* 2001; Lin and Tanaka 2006; Bai *et al.* 2008). Nowadays, *Saccharomyces*

cerevisiae is the preferred bioethanol production host, primarily as a result of its generally recognized-as-safe status, proven industrial process robustness and good physiological and genetic characterization (Vertès *et al.* 2008). There are a number of other candidate ethanol-producing micro-organisms (e.g. *Pichia stipitis*, *Pachysolen tannophilus*, *Klebsiella oxytoca*, *Erwinia chrysantemi*, *Lactobacillus* and *Clostridium* species, among many others) (Stewart *et al.* 1983), but none of them have all the desirable traits of an ideal ethanologen (Stephanopoulos 2007). Recombinant strains of *Escherichia coli*, a well-characterized and versatile micro-organism, could fulfil these requirements. Strain KO11, an ethanologenic derivative of strain ATCC 11303 containing chromosomally integrated *pdc* and *adhB* from *Zymomonas mobilis* (Ohta *et al.* 1991), and its derivatives efficiently convert pentose and hexose sugar mixtures derived from vegetal biomass to ethanol (Jarboe *et al.* 2007). However, large-scale utilization of these strains was mainly hampered by their sensitivity to high ethanol concentrations and metabolic inhibitors resulting from lignocellulosic materials hydrolysis (Ingram *et al.* 1998).

Availability of raw glycerol, a coproduct from biodiesel industry, has increased dramatically during the last 10 years, creating disposal problems. Even when part of the raw glycerol is upgraded to pharmaceutical quality, it is a cheap and easily available feedstock emerging as a potential fermentation substrate (da Silva *et al.* 2009). Fermentations using glycerol as substrate for bulk products [such as poly(3-hydroxyalkanoates) (Nikel *et al.* 2008a; Ibrahim and Steinbüchel 2010), 1,3-propanediol (Gonzáles-Pajuelo *et al.* 2004) and succinic acid (Lee *et al.* 2001)] have been increasingly explored. Moreover, *E. coli* can use glycerol as a carbon source under micro-oxic or anoxic growth conditions (Dharmadi *et al.* 2006; Nikel *et al.* 2008c).

ArcAB (*a*erobic *r*espiration *c*ontrol) and CreBC (*c*arbon source *re*sponsive) are two-component signal transduction systems of *E. coli* (Avison *et al.* 2001; Pettinari *et al.* 2008) responsible for modulation of the intracellular redox state and carbon source utilization, respectively. Metabolic flux analysis based on ^{13}C-labelling showed that both ArcAB and CreBC systems have a deep impact on central metabolic pathways of *E. coli* under micro-oxic growth conditions (Nikel *et al.* 2009). *Escherichia coli* strain CT1061, an *arcA* and *creC*(Con) mutant (Nikel *et al.* 2008b), has enhanced carbon source consumption as well as a reducing intracellular environment (characterized by a high NADH/NAD$^+$ ratio), making it adequate as a candidate host for reduced biochemicals synthesis.

We evaluated the physiological and metabolic changes exerted by the heterologous expression of *adhE* from *Leuconostoc mesenteroides* (*adhE*$_{Lm}$, encoding a bifunctional alcohol-acetaldehyde dehydrogenase) in *E. coli* CT1061. Glycerol was used as the main carbon source, and phenotypic and genetic manipulations were assayed to evaluate carbon fluxes partitioning in central metabolic pathways and reducing equivalents utilization. As acetyl-coenzyme A (CoA) availability was observed to play an important role on the metabolic distribution, two-stage bioreactor cultures were assayed to efficiently direct carbon fluxes towards ethanol synthesis.

Materials and methods

Bacterial strains and plasmids

All *E. coli* strains are K-12 derivatives and are summarized in Table 1, along with plasmids used in this study.

Media and culture conditions

MYA medium contained (in g l^{-1}): Na_2HPO_4, 6·0; KH_2PO_4, 3·0; $(NH_4)_2SO_4$, 1·4; NaCl, 0·5; yeast extract, 10·0; casein amino acids, 5·0; and $MgSO_4{\cdot}7H_2O$, 0·2; initial pH = 7·2 (Nikel *et al.* 2006). SGA medium contained the same salts as MYA, but yeast extract was suppressed and casein amino acids concentration was lowered to 0·3 g l^{-1}. Cultures were amended with *pro analysi* glycerol (Anedra, Buenos Aires, Argentina) and tetracycline at 30 g l^{-1} and 10 μg ml^{-1}, respectively; and 100 μg ml^{-1} ampicillin (Amp) or 50 μg ml^{-1} kanamycin (Km) whenever needed. Solid media also contained 30 g l^{-1} agar. In some experiments, organic acids, acetaldehyde, vitamins and complex nitrogen sources were included in SGA medium as filter-sterilized (0·22 μm) and neutralized stock solutions. Corn steep liquor [containing approx. 50% (w/v) solids] was a generous gift from Productos de Maíz S.A. (Buenos Aires, Argentina). The vitamin stock solution contained the following (in mg l^{-1}): calcium D-pantothenate, 100; folic acid, 100; pyridoxine, 100; *p*-aminobenzoic acid, 100; riboflavin, 100; and biotin, 20. Micro-oxic shaken-flask experiments were conducted in 250-ml Erlenmeyer flasks completely filled with the corresponding culture medium for 48–96 h at 37°C. Gentle agitation was provided by a magnetic stirrer (approx. 75 rev min^{-1}) to prevent biomass sedimentation. Oxic cultures were conducted at 37°C in 250-ml Erlenmeyer flasks containing 50 ml of culture medium at 250 rev min^{-1}. All cultures were inoculated at 0·1 g l^{-1} initial cell dry weight (CDW) with an overnight culture grown in the same medium and oxygen availability condition to be used in the experiment.

Two-stage bioreactor cultivations were carried out in a 5·6-l stirred tank reactor equipped with six flat-bladed

Table 1 *Escherichia coli* strains and plasmids used in this study

Strain or plasmid	Relevant characteristics*	Source or reference
E. coli		
K1060†	F^- *fadE62 lacI60 tyrT58*(AS) *fabB5 mel-1*	Overath *et al.* (1970)
ALS786†	F^- λ^- *rph-1* Δ*ldhA*::*kan*, Km^r	Gokarn *et al.* (2001)
CT1061	K1060 *arcA*::IS*10*-L *creC*(Con); Tet^r	Nikel *et al.* (2006)
DH5α	Φ80*lacZ*ΔM15 *recA1 endA1 gyrA96 thi-1 hsdR17*(r_K^- m_K^+) *supE44 relA1 deoR* Δ(*lacZYA-argF*)*U169*	Invitrogen Corp., Carlsbad, CA, USA
CT1061E	CT1061 carrying pET_{Lm}; Tet^r Amp^r	This work
CT1061L	CT1061 Δ*ldhA*::*kan* by CT1061 × P1(ALS786); Tet^r Km^r	This work
CT1061LE	CT1061L carrying pET_{Lm}; Tet^r Amp^r Km^r	This work
CT1061ER	CT1061E derivative with improved growth and ethanol tolerance	This work
CT1061LER	CT1061LE derivative with improved growth and ethanol tolerance	This work
Plasmid		
$pADH_{Lm}$	pGEM-T Easy (Promega, Madison, WI, USA) derivative, contains *adhE* gene from a natural isolate of *Leuconostoc mesenteroides* cloned as a single PCR amplicon; Amp^r	Koo *et al.* (2005)
pBluescript II KS(−)	Cloning vector; T3 and T7 promoters, *lacPOZ*′; Amp^r	Fermentas Inc., Glen Burnie, MD, USA
pET_{Lm}	pBluescript II KS(−)-derivative carrying *adhE* from *Leuc. mesenteroides*; Amp^r	This work

*Km, kanamycin; Tet, tetracycline; Amp, ampicillin.
†Strains obtained through the *E. coli* Genetic Stock Center, University of Yale, New Haven, CT, USA.

disk turbines (BioFlo 110; New Brunswick Scientific Co., Edison, NJ, USA) essentially as described by Nikel *et al.* (2006), with a 4-l working volume and pH controlled at 7·20 ± 0·05 by automatic addition of 3 mol l^{-1} KOH or 1·5 mol l^{-1} H_2SO_4. To prevent foam formation, 30 μl l^{-1} Antifoam 289 (Sigma-Aldrich, St Louis, MO, USA) was manually added at the onset of the run. During the first stage of the cultivation, the oxygen dissolved concentration was maintained above 40% of air saturation by automatically adjusting the agitation speed from 200 up to 800 rev min^{-1} while sparging the fermentor with 3 l min^{-1} air. At 12 h, air input was suppressed and the agitation speed was set at 75 rev min^{-1} to avoid cell sedimentation.

DNA manipulations

Preparation of plasmids, DNA enzymatic restriction and ligation and agarose gel electrophoresis followed standard procedures (Sambrook *et al.* 1989), and specific instructions from the manufacturers. *Escherichia coli* DH5α was routinely used for plasmid maintenance and amplification.

Plasmid pET_{Lm} was constructed by ligation of a 2697-bp *Eco*RI DNA fragment obtained from $pADH_{Lm}$ (Koo *et al.* 2005) into vector pBluescript II KS(−) digested with the same restriction enzyme. In plasmid pET_{Lm}, $adhE_{Lm}$ is cloned colinearly with P_{lac}. Plasmid DNA transformation of *E. coli* strains was made by using the standard $CaCl_2$ method (Hanahan 1985).

Construction of a Δ*ldhA* derivative of *Escherichia coli* CT1061

Fermentative lactate hydrogenase gene (*ldhA*) was disrupted by P1 transduction (Sternberg and Maurer 1991) of the Δ*ldhA*::*kan* allele from *E. coli* ALS786 to CT1061, resulting in strain CT1061L. Km^r-colonies were purified, and $LdhA^-$ phenotype was checked by absence of anoxic lactate synthesis from glucose as the sole carbon source (Bunch *et al.* 1997).

Isolation of ethanol-resistant derivatives of *Escherichia coli* CT1061E and CT1061LE by metabolic evolution

Escherichia coli CT1061E and CT1061LE were inoculated and serially transferred into prewarmed MYA medium in 18 × 150-mm culture tubes containing increasing concentrations of ethanol and/or 2-propanol. Incubation was performed at 37°C during 4–6 days intervals without agitation to allow metabolic evolution through competitive, growth-based selection. After every 3–4 liquid transfers, cultures were diluted and spread onto solid MYA medium containing ethanol (or 2-propanol) to check vigour and purity of the strains. The largest colonies (>2 mm) were selected for the next inoculation into fresh medium with a higher alcohol concentration. Mini-prep extraction of plasmid DNA followed by *Eco*RI digestion and gel electrophoresis was used to confirm integrity of pET_{Lm}. Ethanol-resistant strains (*E. coli* CT1061ER and CT1061LER)

were stored on MYA plates containing 25–35 g l^{-1} 2-propanol at 4°C and, for long-term storage, in MYA medium containing 40% (v/v) glycerol at −70°C.

Enzymatic measurements

Alcohol dehydrogenase activity was assayed by measuring the ethanol-dependent reduction of NAD^+. Cells were washed and permeabilized for enzyme determination as detailed by Mackenzie *et al.* (1989), and 10–30 μl of the crude cell extract was added to a reaction mixture containing 333 mmol l^{-1} ethanol and 8·3 mmol l^{-1} NAD^+ in 50 mmol l^{-1} sodium phosphate buffer (pH = 6·5) in a final volume of 1 ml. NADH generation was evaluated from the change in absorbance at 340 nm in a Beckman DU 650 spectrophotometer (Beckman Coulter Inc., Fullerton, CA, USA). One unit of Adh activity was defined as the amount of enzyme capable of catalyse the generation of 1 μmol NADH per min under the conditions specified. Protein concentration was determined as described by Bradford (1976), using crystalline bovine serum albumin as standard.

Analytical determinations

Biomass (expressed as CDW) was determined gravimetrically from the pellet fraction of appropriate culture aliquots, after two rounds of centrifugation (8000 ***g***, 10 min) and washing of the cells with 150 mmol l^{-1} NaCl (Nikel *et al.* 2006).

Ethanol concentration was measured by using an enzymatic kit based on alcohol dehydrogenase (Randox Laboratories Ltd., Oceanside, CA, USA). Acetate, formate, lactate, pyruvate, fumarate, malate, succinate and 2-ketoglutarate concentrations were determined by gas chromatography. Briefly, 1 ml of culture supernatants was treated with 2 ml of CH_3OH and 0·4 ml of 9 mol l^{-1} H_2SO_4 in teflon-stoppered vials at 60°C for 30 min. After methylation, 1 ml of each H_2O and $CHCl_3$ were added, and the reaction mixture was vortexed during 20 s. After settling of the layers, the lower (organic) phase was collected, dried over anhydrous Na_2SO_4, and the methyl-derivatives of the organic acids were detected in a Hewlett-Packard HP5890 gas chromatograph equipped with a Hewlett-Packard FFAP column (Hewlett-Packard Co., Palo Alto, CA, USA). Acetaldehyde was detected by the same method but omitting the derivatization procedure. Known amounts of authentic metabolites were treated as described earlier whenever needed and used as standards.

Residual glycerol in the culture broth was assayed by a specially developed colorimetric method (Nikel *et al.* 2008c). A 200-μl aliquot of culture supernatant (or an appropriate dilution containing up to 250 μg ml^{-1} glycerol) was mixed with 1 ml of 5 mmol l^{-1} $NaIO_4$ (in 20 mmol l^{-1} CH_3COOH/CH_3COONH_4 buffer) and 2·5 ml of 35 mmol l^{-1} 2,4-pentanodione in 2-propanol while vortexing. After incubation at 50°C for 30 min, absorbances were measured at 410 nm using the same reaction mixture (without added glycerol) as a blank. Calibration curves were constructed with known amounts of *pro analysi* glycerol.

Percentage of plasmid-bearing cells was determined by their ability to form colonies in Amp-supplemented plates (Weber and San 1989). Samples taken from the bioreactor were immediately diluted in cold saline (150 mmol l^{-1} NaCl), and dilutions were evenly spread onto MYA plates, with or without 100 μg ml^{-1} Amp. Each plate was triplicated to reduce experimental error. After 18 h of incubation at 37°C, the quantity of Amp^r- and Amp^s-colonies formed on the plates was compared and used to estimate the percentage of plasmid-containing cells in the culture.

Calculations and statistical analysis

Fermentation parameters for cell growth, glycerol consumption and metabolite synthesis were used to calculate yields on substrate (g of biomass or product per g of consumed glycerol) and volumetric productivities (g l^{-1} h^{-1}). Fermentation and redox balances, as well as theoretical yields, were estimated by using a similar procedure to that described by Gonzalez *et al.* (2008). For stoichiometric calculations of carbon recovery, it was assumed that moles of CO_2 plus moles of formate equals moles of acetate plus moles of ethanol. An empirical biomass composition of $CH_{1·9}O_{0·5}N_{0·25}$ was assumed for *E. coli* cells (Neidhardt *et al.* 1990). Significance of the differences when comparing results was evaluated by analysis of variance (ANOVA).

Results

Fermentative parameters of an *Escherichia coli* redox mutant expressing *adhE* from *Leuconostoc mesenteroides* during micro-oxic growth on glycerol

We had previously observed that the specific production rate for ethanol (from endogenous AdhE) was higher for *E. coli* CT1061 when compared to that of wild-type K1060 or its Δ*arcA* derivative (Nikel *et al.* 2008c), even under oxic growth conditions. We sought to increase ethanol synthesis by introducing *adhE* of *Leuc. mesenteroides* (expressed in plasmid pET_{Lm}) in strain CT1061, resulting in *E. coli* CT1061E. When compared to *E. coli* CT1061 carrying pBluescript II KS(−), ethanol synthesis by strain CT1061E was 1·4-fold higher ($P < 0·05$) in

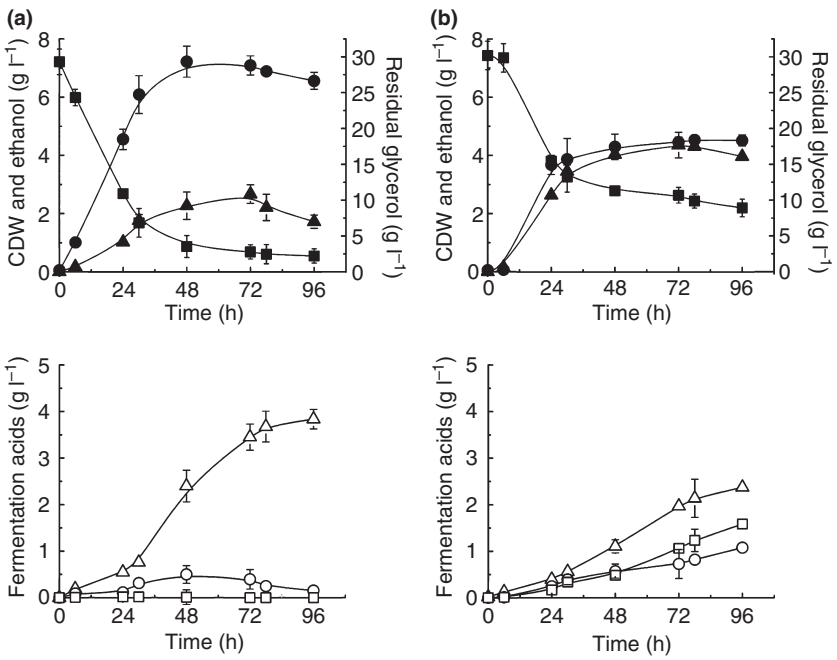

Figure 1 Growth, carbon source consumption and metabolic profile of *Escherichia coli* CT1061E [Δ*arcA creC*(Con), carrying *adhE* from *Leuconostoc mesenteroides*] during oxic (a) or micro-oxic (b) shaken-flasks cultivation in MYA medium containing 30 g l^{-1} glycerol. Shown are (●) biomass concentration (expressed as CDW, cell dry weight); (▲) ethanol synthesis and (■) residual glycerol concentration; as well as fermentation acids production [(○) formate; (△) acetate and (□) lactate]. Results represent the average ± standard deviation from duplicated measurements from at least two independent cultures.

micro-oxic shaken-flasks cultures. Organic acids production (namely, acetate, formate and lactate) was diminished in the cultures of *E. coli* CT1061E, suggesting a significant bias of carbon skeletons towards ethanol synthesis (data not shown).

Oxic and micro-oxic batch cultures of CT1061E were performed in shaken flasks. Growth was impaired under oxygen-restricted conditions, reaching a biomass concentration of 4·5 g l^{-1} after 72 h (Fig. 1b), compared to 7·2 g l^{-1} observed in oxic cultures (Fig. 1a). The specific growth rate was also higher in aerobiosis. Glycerol was almost completely consumed during oxic cultivation while in micro-oxic cultures its residual concentration was 8·9 g l^{-1} at 96 h with a significantly lower substrate consumption rate. Ethanol was detected under both oxic and micro-oxic conditions, peaking at 72 h in both cases (2·7 ± 0·2 and 4·5 ± 0·1 g l^{-1}, respectively). The significant bias towards ethanol synthesis under conditions with restricted oxygen supply was reflected in the ethanol yield coefficients on glycerol [0·11 g g^{-1} (aerobiosis) and 0·23 g g^{-1} (microaerobiosis)]. Accordingly, the percentage of the theoretical ethanol yield on glycerol increased from 20 to 45% ($P < 0·01$). After 72 h, we consistently detected a slight decrease in ethanol concentration attributable, at least in part, to loss by evaporation.

In aerobiosis the main fermentative metabolite besides ethanol was acetate (Fig. 1a), showing a steady increase during the cultivation up to 3·8 g l^{-1} at 96 h, probably as a consequence of oxic activity of the acetate biosynthesis pathway [composed of phoshotransacetylase (Pta) and acetate kinase (AckA)] when carbon source is in excess (Clark 1989). Lactate was not detected, and formate concentrations remained below 0·5 g l^{-1} (showing a concentration peak at 48 h). Acetate, formate and lactate were detected under micro-oxic growth conditions (Fig. 1b), and their concentrations reached 2·4, 1·1 and 1·6 g l^{-1} at 96 h, respectively.

Metabolic pattern of a *ldhA* mutant derived from *Escherichia coli* CT1061

Fermentative lactate dehydrogenase (LdhA) competes for glycolytic intermediates and reducing power at the pyruvate branch point (Bunch *et al.* 1997; Gokarn *et al.* 2001). We decided to delete *ldhA* rather than *pta-ackA* (which encode the above-mentioned Pta-AckA pathway) or *pfl* (encoding pyruvate-formate lyase) as it was suggested that the latter genes are necessary for efficient anoxic glycerol dissimilation by *E. coli* (Dharmadi *et al.* 2006; Gonzalez *et al.* 2008). Elimination of LdhA resulted in a 1·5-fold enhancement in ethanol concentration, reaching 6·5 ± 0·3 g l^{-1} at 48 h (Fig. 2a), while growth and carbon source consumption rate were not significantly affected. Lactate was no longer detected in supernatants of CT1061LE cultures (Fig. 2b). An increase in both acetate and formate synthesis was observed (1·2 and 1·7-fold, respectively), presumably because of the excess amount of carbon intermediates to be redistributed among fermentative pathways (Clark 1989). Nevertheless, the yield of ethanol on glycerol increased up to 0·29 g g^{-1} (approx. 59%

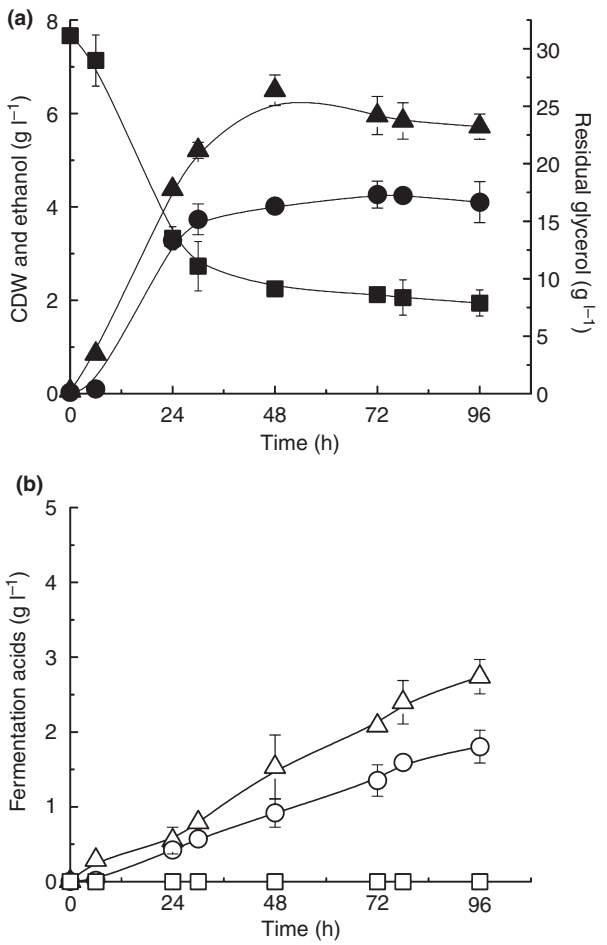

Figure 2 Growth, ethanol synthesis and carbon source consumption (a) and metabolic profile (b) of *Escherichia coli* CT1061LE [Δ*arcA creC*(Con) Δ*ldhA*, carrying *adhE* from *Leuconostoc mesenteroides*] during micro-oxic shaken-flasks experiments in MYA medium containing 30 g l^{-1} glycerol. Shown are (●) biomass concentration (expressed as CDW, cell dry weight); (▲) ethanol synthesis and (■) residual glycerol concentration; as well as fermentation acids production [(○) formate; (△) acetate and (□) lactate]. Results represent the average ± standard deviation from duplicated measurements from at least two independent cultures.

of the theoretical yield, $P < 0{\cdot}05$). Ethanol synthesis by a Δ*ldhA* derivative of wild-type K1060 carrying $adhE_{Lm}$ was also assayed as a control, peaking at 3·9 ± 0·6 g l^{-1} at 48 h.

Enrichment of ethanol-resistant derivatives of *Escherichia coli* CT1061E and CT1061LE

It was demonstrated that 2-propanol has a similar effect on *E. coli* physiology to that of ethanol (Ingram 1990), and we observed that strains tolerant to 2-propanol can also withstand similar ethanol concentrations. Ethanol is highly volatile and would be partially lost during long-term metabolic evolution, so it was replaced by 2-propanol in liquid media. Growth of *E. coli* CT1061E and CT1061LE inoculated into MYA medium containing 10 g l^{-1} ethanol was negligible and after 50 sequential transfers, the 2-propanol (or ethanol) concentration was increased stepwise up to 25 g l^{-1} by coselecting enhanced growth and productivity. Additional ethanol (up to 10 g l^{-1}) was produced during micro-oxic growth providing higher selective pressure. Further improvements were attained with *E. coli* CT1061LE, which was capable to withstand 2-propanol or ethanol concentrations up to 35 g l^{-1}. Dilution and plating on agarized media were performed after every 3–4 transfers to enrich for large and raised colonies (also indicating lower organic acids production). Cultures were maintained at the maximal ethanol concentration for at least 4 weeks before individual clones were selected after plating onto solid MYA medium, giving rise to strains CT1061ER and CT1061LER, with tolerances up to 25 and 35 g l^{-1} ethanol, respectively. Diluting cultures of these strains into fresh broth containing concentrations of 2-propanol or ethanol higher than 25 or 35 g l^{-1} did not yield strains with higher tolerance that also retained fast growth and efficient ethanol production.

The ethanol-tolerant phenotype was stably maintained after several rounds of growth without selective pressure. Moreover, this trait was also checked in strains retrieved from stocks conserved at −70°C after several months. Kinetic properties and total Adh activity of the strains under study were compared in micro-oxic shaken-flask cultures (Table 2). *Escherichia coli* CT1061E and CT1061LER presented the highest growth rates and, at the same time, enhanced Adh activity when compared to the corresponding parental strains. Indeed, total Adh activity in strain CT1061LER was significantly higher than that of the parental strain (5·6-fold increase, $P < 0{\cdot}01$). We therefore selected strain CT1061LER for further studies.

Effect of metabolic intermediates on micro-oxic growth and metabolic profile of *Escherichia coli* CT1061LER

Maximal ethanol productivity of *E. coli* CT1061LER would not be hampered by low alcohol tolerance, so we investigated the catalytic effect of externally added central metabolic intermediates on the fermentation pattern of this strain. Citrate, succinate, 2-ketoglutarate, malate, fumarate and oxaloacetate accounted for <10% of the total fermentation products (on a C-mol basis), while pyruvate was not detected in glycerol-containing cultures.

Pyruvate and acetaldehyde were almost completely metabolized after 72 h (Table 3). The highest ethanol

Table 2 Comparison of specific growth rate and total alcohol dehydrogenase (Adh) activity among experimental strains in 72-h shaken-flask cultures in MYA medium (containing 30 g l^{-1} glycerol) under micro-oxic growth conditions

Escherichia coli strain	Relevant characteristics	μ_{max}* (h^{-1})	Adh activity† (μmol min^{-1} per mg protein)
CT1061/pBluescript II KS(−)	*arcA*::IS*10*-L *creC*(Con)	0·35 ± 0·02	0·53 ± 0·02
CT1061E	*arcA*::IS*10*-L *creC*(Con) carrying pET_{Lm}	0·42 ± 0·03	1·09 ± 0·09
CT1061LE	*arcA*::IS*10*-L *creC*(Con) Δ*ldhA*::*kan* carrying pET_{Lm}	0·46 ± 0·01	1·54 ± 0·07
CT1061ER	*arcA*::IS*10*-L *creC*(Con) carrying pET_{Lm}, improved growth and ethanol resistance	0·51 ± 0·01	1·88 ± 0·01
CT1061LER	*arcA*::IS*10*-L *creC*(Con) Δ*ldhA*::*kan* carrying pET_{Lm}, improved growth and ethanol resistance	0·67 ± 0·02	2·97 ± 0·05

*Specific growth rates were calculated during exponential growth.
†Adh activity was enzymatically determined at the end of the cultivation as described in 'Materials and methods'. Results represent the mean values for triplicate measurements from two independent cultures.

Table 3 Effect of different additives on the fermentation pattern of *Escherichia coli* CT1061LER [ethanol-tolerant derivative of CT1061LE, Δ*arcA creC*(Con) Δ*ldhA*, carrying *adhE* from *Leuconostoc mesenteroides*] after 72 h of incubation in SGA medium (containing 30 g l^{-1} glycerol) under micro-oxic growth conditions

Additive	CDW (g l^{-1})	Fermentation product (g l^{-1})			Carbon recovery (%)
		Formate	Acetate	Ethanol	
None	3·89	1·85	2·98	2·76	91 ± 7
Sodium pyruvate	5·14	2·21	4·96	5·36	93 ± 6
Acetaldehyde	6·28	2·23	5·03	4·95	102 ± 1
Sodium citrate	4·07	1·85	3·85	3·18	94 ± 3
Sodium 2-ketoglutarate	4·91	3·74	3·67	4·17	89 ± 5
Sodium oxaloacetate	3·98	1·98	2·91	2·58	100 ± 3
Sodium malate	4·05	1·86	1·97	2·56	92 ± 4
Sodium fumarate	4·15	1·95	2·14	2·67	88 ± 7
Sodium succinate	4·24	2·56	3·09	2·63	93 ± 2

All additives were amended at 2 g l^{-1} at the beginning of the cultivation except for acetaldehyde, which was added twice at 0·25 g l^{-1} each (6 and 24 h) to avoid toxic effects. Redox balances were >90% for all experiments shown in this table. Results represent the mean values for triplicate measurements of growth (expressed as CDW, cell dry weight) and metabolic profile of two independent cultures. Standard deviations were <10% of the mean for all experimental values and were omitted here for clarity.

concentrations were obtained in cultures supplemented with either pyruvate or acetaldehyde (1·9- and 1·8-fold higher when compared to that of the control experiment without additives, $P < 0·05$). CDW concentrations followed a similar pattern, being 1·3- and 1·6-fold higher than in control experiments ($P < 0·05$). In these two cultures acetate production increased while formate synthesis was not significantly modified. Enhanced growth and higher ethanol synthesis by both pyruvate and acetaldehyde addition seem to be coincident with higher acetate excretion. As the Pta-AckA pathway is highly reversible, elevated extracellular levels of acetate in pyruvate- and acetaldehyde-supplemented fermentations may result in an increase in intracellular acetyl-CoA availability, allowing further growth and increasing ethanol production. Interestingly, addition of acetate gave similar results (data not shown).

Addition of tricarboxylic (TCA) cycle intermediates was also investigated (Table 3). Succinate was the only added metabolite not efficiently metabolized (<6% was used during the entire cultivation period). Addition of malate and fumarate resulted in small increases in fumarate levels, but did not stimulate growth or ethanol production. Addition of oxalacetate, malate, fumarate and succinate reduced ethanol synthesis but slightly increased growth. In sharp contrast, 2-ketoglutarate promoted growth and significantly stimulated ethanol production (1·3- and 1·5-fold, respectively; $P < 0·05$). TCA pathway intermediates that are immediate precursors of 2-ketoglutarate were not beneficial. Citrate was almost completely metabolized but had no significant effect on biomass or ethanol production, and growth on added citrate was accompanied by accumulation of fumarate and a high acetate/formate ratio (1·6 ± 0·2 mol mol^{-1}), similar to that observed with pyruvate (1·7 ± 0·1 mol mol^{-1}).

Effect of the nitrogen source and vitamins on micro-oxic growth and fermentation pattern of *Escherichia coli* CT1061LER

Maximal ethanol synthesis was observed in MYA medium, which contains significant amounts of both yeast extract and casein amino acids. However, addition of high levels of complex nutrients adds to the cost of ethanol production and waste treatment requirements (Lawford and Rousseau

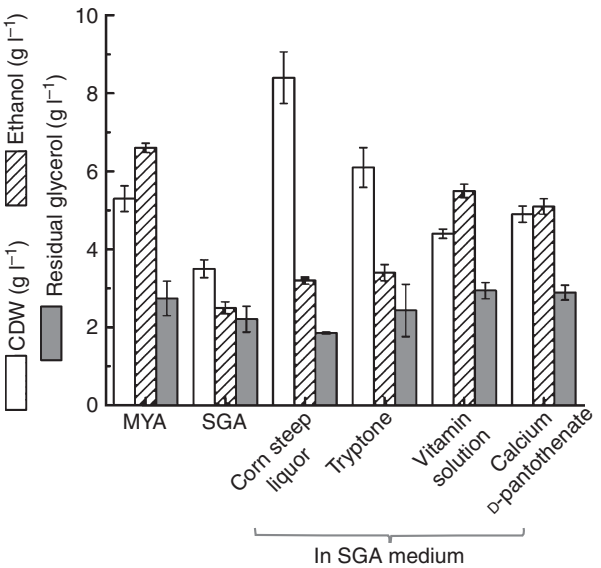

Figure 3 Effect of medium composition on growth (expressed as CDW, cell dry weight), ethanol synthesis and glycerol consumption by *Escherichia coli* CT1061LER [ethanol-tolerant derivative of CT1061LE, Δ*arcA cre*C(Con) Δ*ldhA*, carrying *adhE* from *Leuconostoc mesenteroides*] in 72-h micro-oxic shaken-flask experiments. Semi-synthetic SGA medium was used to assess the effect of different amendments to replace yeast extract present in complex MYA medium (the composition of both media is detailed in 'Materials and methods'). Corn steep liquor and tryptone were added to provide 1·55 g l^{-1} amino nitrogen, while vitamins and calcium D-pantothenate were included at catalytic concentrations, similar to those found in yeast extract. Results represent the average ± standard deviation from duplicated measurements from at least three independent cultures.

1996; Bai *et al.* 2008). We studied the addition of different nitrogen sources to SGA medium (which contains a very low concentration of casein amino acids to prime growth, required for *arcA* mutants). Each component was supplemented to provide approx. 1·55 g l^{-1} amino nitrogen, the amount supplied by 10 g l^{-1} yeast extract (Dahod 1999). Addition of 15 g l^{-1} corn steep liquor, the cheapest complex nutrient, had a more pronounced effect on growth (attaining the highest biomass concentration among the additives tested, $P < 0{\cdot}05$), but ethanol synthesis was similar to that observed in SGA medium (Fig. 3). Carbon source consumption was enhanced, being mainly funnelled into biomass synthesis rather than ethanol production. Addition of 7·5 g l^{-1} tryptone had a significant effect on cell growth ($P < 0{\cdot}05$), but ethanol synthesis and glycerol consumption were not modified when compared to those in SGA medium. Besides, elevated concentrations of corn steep liquor (>50 g l^{-1}) supported growth rates and levels of ethanol synthesis higher than those obtained in MYA medium (data not shown).

We next tried to identify components on yeast extract that could act as enhancers of ethanol synthesis. Apart from being a carbon and nitrogen source, yeast extract provides a significant amount of vitamins, so we replaced yeast extract by 1 ml l^{-1} of a vitamin solution (a vitamins supply similar to that of 10 g l^{-1} yeast extract). Vitamin-amended cultures in SGA medium gave similar results to those obtained in MYA medium regarding ethanol synthesis and glycerol consumption. Biomass concentration was slightly lower, which could be attributed to the fact that yeast extract also supplies carbon. We then assayed each vitamin separately at different concentrations (data not shown). D-Pantothenate was mainly responsible for enhanced ethanol synthesis. Indeed, addition of 100 μg l^{-1} calcium D-pantothenate gave similar ethanol synthesis and biomass concentrations to those obtained with the vitamin stock solution. D-Pantothenic acid is an important precursor in the acetyl-CoA biosynthetic pathway of *E. coli* (Neidhardt *et al.* 1990). In agreement with results obtained using other additives in SGA medium, it could be hypothesized that high ethanol synthesis levels result in an acetyl-CoA shortage which should be avoided to attain significant growth and ethanol synthesis in minimal media.

Two-stage bioreactor culture of *Escherichia coli* CT1061LER and plasmid stability

We assayed two-stage cultures, in which acetyl-CoA was accumulated during a first oxic stage and, after oxygen deprivation, excess acetyl-CoA not consumed in oxidative metabolism could be transformed into ethanol by $AdhE_{Lm}$. Bioreactor cultivations were conducted in SGA medium without antibiotics, and containing 30 g l^{-1} glycerol and 100 μg l^{-1} calcium D-pantothenate.

During the oxic phase, cells grew exponentially with a specific growth rate of 0·42 ± 0·04 h^{-1}, reaching a CDW concentration of 4·5 ± 0·2 g l^{-1} at 12 h (Fig. 4). During this phase 54 ± 2% of the total glycerol was consumed, but only 3·3 ± 0·1 g l^{-1} of ethanol was synthesized. This result can be expected as the carbon source was mainly catabolized through oxidative metabolism, in which pyruvate is converted into acetyl-CoA and CO_2 by the pyruvate dehydrogenase complex (Clark 1989; Neidhardt *et al.* 1990). Given the high CDW concentration (>5 g l^{-1}), at this point it was difficult to maintain highly oxic conditions, even with air supply and full agitation. Air supply was subsequently suppressed, and the dissolved oxygen concentration rapidly dropped below 5% of air saturation. CDW concentration did not significantly increase under oxygen-restricted conditions, which suggests a metabolic switch to fermentative conditions. Accordingly, ethanol synthesis was promoted under microaerobiosis, reaching 15·4 ± 0·9 g l^{-1} at 48 h. This represents an ethanol volumetric productivity of 0·34 ± 0·02 g l^{-1} h^{-1}. Taking into account that 93 ± 1% of the added glycerol

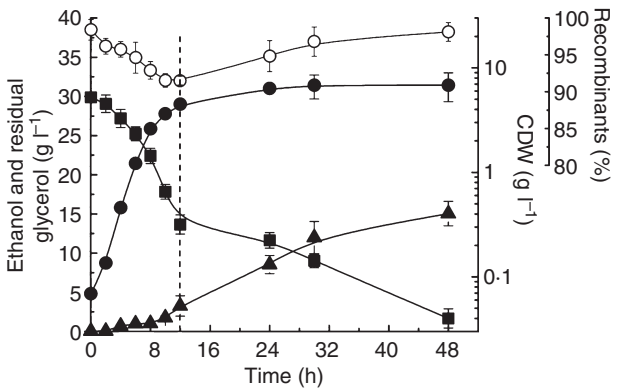

Figure 4 Two-stage culture of *Escherichia coli* CT1061LER [ethanol-tolerant derivative of CT1061LE, Δ*arcA creC*(Con) Δ*ldhA*, carrying *adhE* from *Leuconostoc mesenteroides*] in a benchtop bioreactor. Oxic conditions were maintained during the first 12 h by flushing the fermentor with 3 l min^{-1} of air and adjusting the agitation speed up to 800 rev min^{-1}. Air supply was suppressed thereafter (dashed line), and gentle agitation at 75 rev min^{-1} was applied to prevent biomass sedimentation. Shown are (●) biomass concentration (expressed as CDW, cell dry weight); (▲) ethanol synthesis and (■) residual glycerol concentration. Plasmid stability was estimated by plating adequately diluted culture samples onto MYA plates with or without ampicillin and was expressed as the percentage of plasmid-bearing cells (○). Results represent the average ± standard deviation from duplicated measurements from at least two independent bioreactor cultures.

was consumed at the end of the cultivation, the yield of ethanol on carbon source was 0·39 ± 0·01 g g^{-1} (approx. 81% of the theoretical yield, $P < 0·01$). Further extension of the cultivation time gave no significant differences regarding ethanol concentration but reduced ethanol volumetric productivity (data not shown). Acetate and formate were the main fermentation by-products, reaching 2·8 ± 0·2 and 3·6 ± 0·3 g l^{-1} at 48 h, respectively. A similar fermentation pattern on glycerol was reported for a *Klebsiella planticola* strain isolated from red deer rumen (Jarvis *et al.* 1997). The carbon recovery balance calculated at 48 h was 105 ± 8%, indicating a good carbon closure between ethanol, biomass, formate and acetate.

Plasmid stability in these cultures was tested by determining the percentage of Amp-resistant cells during the entire cultivation. During the oxic stage the percentage of plasmid-bearing cells steadily decreased reaching 92·1 ± 0·2% at 12 h, but under conditions of restricted oxygen supply pET_{Lm} was stably maintained. After the 48-h fermentation, 98·3 ± 0·9% of the cells maintained the plasmid.

Discussion

Anoxic fermentation of glycerol by *E. coli* has received much attention in the last few years, and its use in industrial bioprocesses was analysed (Yazdani and Gonzalez 2007, 2008; Gonzalez *et al.* 2008; Murarka *et al.* 2008; Trinh and Srienc 2009). Most of the results published regarding metabolic engineering of *E. coli* for ethanol synthesis describe experiments conducted under strict anoxic conditions. Only recently it was realized that micro-oxic conditions are better suited for efficient synthesis of reduced biochemicals such as ethanol (Durnin *et al.* 2009), a fact previously suggested and validated for poly(3-hydroxybutyrate) accumulation by recombinant *E. coli* (Nikel *et al.* 2008a). *Escherichia coli* CT1061, an *arcA creC*(Con) double mutant, responded to *adhE* overexpression by significantly increasing carbon fluxes towards ethanol synthesis while concomitantly decreasing carbon transformation into acetate, formate or lactate (Fig. 1). This is in accordance with previous results obtained with lactic acid bacteria (like *Oenococcus oeni*, *Lactobacillus brevis* and *Leuc. mesenteroides*), because alcohol-aldehyde dehydrogenase enzymes usually exhibit a lower Michaelis–Menten constant for acetyl-CoA than AdhE of *E. coli* (Zaunmüller *et al.* 2006). This metabolic alteration was also reported for ethanologenic *E. coli* strains carrying the *pet* operon (encompassing *pdc* and *adhB* of *Z. mobilis*) (Díaz-Ricci *et al.* 1992; Orencio-Trejo *et al.* 2008).

Metabolic evolution also contributed to isolate strain derivatives with higher ethanol tolerance, a trait previously shown to be mainly related to alterations in the lipid content of the plasma membrane when *E. coli* is challenged with increasing alcohol concentrations (Dombek and Ingram 1984). Besides, it was suggested that tolerance of *E. coli* to ethanol also involves increased metabolism of glycine and higher production of the osmoprotectant betaine. DNA microarray analysis also showed that *marAB*, which encode multiple antibiotic resistance proteins, are expressed at higher levels in an ethanol-tolerant strain of *E. coli* (Gonzalez *et al.* 2003).

Modification of the carbon partitioning at the pyruvate branch point by eliminating the competing LdhA pathway (Fig. 2) was assayed. This resulted in higher pyruvate (and, consequently, acetyl-CoA) availability and is also assumed to decrease NADH utilization. Apart from this genetic manipulation, which significantly improved ethanol synthesis (1·5-fold increase, $P < 0·05$), carbon fluxes distribution was also evaluated by assaying different additives in semi-synthetic medium (SGA). Beneficial effects of pyruvate and acetaldehyde supplementation on growth and ethanol synthesis by *E. coli* CT1061LER could result not only from higher acetyl-CoA availability for $AdhE_{Lm}$, but also from an increase in the flow of carbon skeletons into 2-ketoglutarate and subsequent biosynthesis intermediates. Pyruvate plays a dual role during fermentation (Clark 1989); it is both a source of carbon skeletons for

biosynthesis and a source of electron acceptors which allows energy production through the Embden-Meyerhof-Parnas pathway. During micro-oxic growth of *E. coli* on glycerol, two NADH molecules are produced per each pyruvate molecule (Dharmadi *et al.* 2006). Each NADH molecule must be oxidized by reducing an electron acceptor, such as acetaldehyde, or by biosynthetic reactions. Addition of pyruvate to media has been shown to increase the intracellular pyruvate pool in *E. coli* (Yang *et al.* 2001), which in turn increases the ratio of potential electron acceptors for oxidation of NADH from glycolysis. Acetyl-CoA, pyruvate and upstream metabolites in the Embden-Meyerhof-Parnas pathway are used for the biosynthesis of approx. one-half of all cellular constituents (Neidhardt *et al.* 1990). The availability of these upstream intermediates could be increased when either acetyl-CoA or pyruvate are added, accounting for the enhanced growth observed in these cultures. On the other hand, acetaldehyde addition could favour ethanol synthesis by directly providing more substrate for $AdhE_{Lm}$, as demonstrated for the homoethanol biosynthetic pathways of *S. cerevisiae* and *Z. mobilis* (Stanley *et al.* 1997). High acetyl-CoA levels also promote biosynthesis by relieving the NADH-mediated allosteric inhibition of citrate synthase (Weitzman 1966) and by serving as an allosteric activator of phosphoenolpyruvate carboxylase (Izui *et al.* 1981). High acetate-to-formate ratios were observed in pyruvate- and citrate-amended fermentations, while this ratio was near to 1 mol mol^{-1} when TCA intermediates other than citrate were used. Citrate lyase, an enzyme which cleaves citrate into an equimolar mixture of oxaloacetate and acetate (Schneider *et al.* 2000), is induced when citrate or isocitrate are directly added to the culture medium. Concentrations of both oxaloacetate-derived fumarate and acetate were higher in fermentations conducted with added citrate than in fermentations with 2-ketoglutarate and other TCA pathway intermediates, which would reflect the induction of citrate lyase, as reported by Underwood *et al.* (2002) for an ethanologenic *E. coli* strain when growing on xylose. Induction of this enzyme probably limits citrate beneficial effects on biosynthesis, which is in good agreement with the enhanced growth and ethanol synthesis observed when 2-ketoglutarate was added.

Wild-type strains of *E. coli* do not normally need D-pantothenate for growth even in minimal media, and the requirement of this vitamin for optimal ethanol synthesis by *E. coli* CT1061LER in SGA medium seems to be directly related to CoA-SH metabolism. Adh activity in heterofermentative lactic acid bacteria is very sensitive to D-pantothenate deprivation. Richter *et al.* (2001) showed that ethanol formation from acetyl-CoA in the heterofermentative pathway from glucose in *O. oeni* is completely inhibited during CoA-SH and/or D-pantothenate limitation, well before general growth and cell synthesis are inhibited. Acetyl-CoA availability would be negatively affected if there is not enough D-pantothenate-derived CoA-SH, thus resulting in lower ethanol productivity. From an industrial point of view, addition of small quantities of low-cost vitamins would be preferred over the use of expensive complex substrates which, at the same time, normally exhibit variable and hard-to-predict compositions.

Our results could also provide an interesting insight into the growth impairment of *arcA* mutants in minimal medium. In a Δ*arcA* background, central metabolic pathways are unregulated, and TCA cycle reactions are still operative under conditions of restricted oxygen supply (Pettinari *et al.* 2008). These mutants are unable to grow in minimal medium if it is not supplemented with high levels of complex nutrients, such as yeast extract (Nikel *et al.* 2006, 2008b), probably reflecting a regulatory deficiency in the partitioning of pyruvate-derived carbon skeletons between competing requirements for NAD(P)H oxidation and biosynthesis. However, we reported that a *creC*(Con) mutation allowed growth of *arcA* mutants even in semi-synthetic media (Nikel *et al.* 2008b). If the TCA cycle is still active a shortage of intermediate metabolites would take place, and this could be partially prevented if sufficient amounts of glycolytic intermediates are provided by the constitutive activity of Cre (Avison *et al.* 2001). In the presence of the highly expressed $AdhE_{Lm}$ pathway, central metabolites are mainly drained into ethanol rather than biomass constituents. Therefore, addition of metabolites or vitamins that replenish the acetyl-CoA pool and the TCA cycle intermediates is effective in promoting growth and ethanol synthesis.

Differences were observed in Adh activity between the different strains (Table 2). Previous studies indicated that it can be affected by substrate availability and redox conditions (Garrigues *et al.* 1997; Koo *et al.* 2005), so an increase in the Adh activity is expected for the *ldhA* mutant, as it has a higher availability of reducing power and acetyl-CoA. The ethanol-tolerant strains also had higher Adh activity, probably because of some of the uncharacterized changes that enhance solvent resistance.

During oxic conditions in two-stage bioreactor cultures, acetyl-CoA is being produced through the pyruvate dehydrogenase complex activity, and after a switch into fermentative conditions this key metabolic intermediate is converted into fermentation products to ensure efficient NAD(P)H oxidation, which is even more important in *E. coli arc* mutants when using a highly reduced substrate such as glycerol. Ethanol accumulated in the culture medium under conditions of restricted oxygen supply without any significant biomass increment, reflecting the

redirection of acetyl-CoA into ethanol instead of macromolecular biosynthesis. The ethanol concentrations and volumetric productivities attained were similar to those recently reported for different *E. coli* strains growing on glycerol under different conditions of oxygen availability (Durnin *et al.* 2009; Trinh and Srienc 2009). Also, we did not detect significant plasmid loss during the micro-oxic phase of the cultivation. This could be attributable to intrinsic plasmid properties or to the expression of the cloned $adhE_{Lm}$. Experiments that will shed light on this effect are currently under way in our laboratory.

Glycerol offers the combined advantages of being a cheap carbon source (da Silva *et al.* 2009) and producing more reducing equivalents during fermentation than hexoses (Dharmadi *et al.* 2006) because of its high degree of reduction per carbon atom, making it ideal for the synthesis of reduced bioproducts. Zhou *et al.* (2006) reported that D-(−)-lactate synthesis significantly increased when betaine was added to the culture medium as an osmoprotectant. It was likewise found that modification of the trehalose content in recombinant *E. coli* also resulted in higher growth (Purvis *et al.* 2005), and trehalose-dependent stress tolerance was identified as a relevant trait for industrial purposes involving baker's yeast (Attfield 1997). A compatible solute like glycerol could serve not only as the main carbon source, but also as a relevant osmoprotectant, thus enhancing the behaviour of the ethanologenic strains.

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