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Metabolic selective pressure stabilizes plasmids carrying biosynthetic genes for reduced biochemicals in *Escherichia coli* redox mutants

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Abstract Several biotechnological processes rely on the utilization of high-copy-number plasmids for heterologous gene expression, and understanding the interactions between plasmid DNA and bacterial hosts is highly relevant for bioprocess optimization. We assessed metabolic modifications and physiological changes exerted by expression of a plasmid-encoded alcohol-acetaldehyde dehydrogenase from *Leuconostoc mesenteroides* (*adhE*$_{Lm}$) in *Escherichia coli* redox mutants. Plasmid pET$_{Lm}$, a pBluescript II KS(–)-derivative carrying *adhE*$_{Lm}$, was introduced in *E. coli* CT1061 [*arcA creC*(Con)]. This recombinant was able to attain a higher ethanol concentration in glycerol cultures compared to the parental strain. pBluescript II KS(–) was rapidly lost in 72-h bioreactor cultures (7.8±1.2% of plasmid-bearing cells), while pET$_{Lm}$ was present in 92.4±7.2% of the cells. In *E. coli* CT1061 carrying pBluescript II KS(–) the plasmid copy number steadily diminished in bioreactor cultures to reach 334±45 copies per chromosome at 72 h, while pET$_{Lm}$ was stably maintained, reaching 498±18 copies per chromosome at the end of the cultivation. Plasmid pETΩ$_{Lm}$, bearing a defective copy of *adhE*$_{Lm}$ interrupted by *cat*, reached 293±62 copies per chromosome, implying a functional role of *adhE*$_{Lm}$ on plasmid maintenance. The intracellular NADH/NAD$^+$ content suggest that regeneration of oxidized co-factors by the heterologous bioreaction might play a relevant role in plasmid maintenance.

Keywords *Escherichia coli* · Plasmid copy number · Redox mutants · ArcAB · CreBC · Microaerobiosis

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Introduction

Advances in biocatalysis development are determinant in reducing the environmental footprint of chemical processes, especially petroleum-based technologies (Demain 2009). Traditional methods for high-cell-density cultivation, such as enhancement by stirring and aeration or introduction of pure or air-enriched oxygen, are usually characterized by high costs and relative efficiency (Shiloach and Fass 2005). Therefore, biotechnological fermentations conducted under conditions with restricted oxygen supply have been increasingly explored. In the same framework, there was a worldwide increase in sustainable production of alternative fuels (Bai et al. 2008; Vertès et al. 2008; Clomburg and Gonzalez 2010; Peralta-Yahya and Keasling 2010; Solomon 2010). In an early attempt to improve bioprocesses aimed at ethanol production, the ethanol synthesis pathway of *Zymomonas mobilis* was cloned and expressed in *Escherichia coli*, enabling the recombinants to produce ethanol from hexoses and pentoses (Jarboe et al. 2007). Genes encoding pyruvate decarboxylase (*pdc*) and alcohol dehy-

drogenase (*adhB*) were combined in the *pet* operon (Ingram et al. 1987), which gave rise to a family of pUC-based ethanologenic plasmids.

Maintenance of recombinant plasmids supposedly imposes a severe metabolic burden on the bacterial host (Bentley et al. 1990; Friehs 2004; Rozkov et al. 2004; Ow et al. 2006). Loss of plasmid DNA will lead to the absence of desired phenotypes and plasmid-free bacterial variants would grow faster during fermentation, outcompeting plasmid-bearing cells during prolonged cultivation (Zielenkiewicz and Cegłowski 2001; Węgrzyn and Węgrzyn 2002). No matter how innately productive the organism is, it will not be possible to harness its full industrial potential unless efficient plasmid maintenance is attained. One way to ensure the stable presence of plasmid DNA is selective pressure, usually achieved by including an antibiotic in the culture medium. This method is effective but often impractical, especially when the recombinant strain is to be grown in large fermenters. These problems have been partially circumvented by developing antibiotic-independent strategies (Cranenburgh et al. 2001; Zielenkiewicz and Cegłowski 2001; Durany et al. 2005; Kroll et al. 2009). Optimization of plasmid vectors and host strains has received an important interest in biotechnology, especially due to novel applications of plasmids (Bower and Prather 2009).

Glycerol is the main by-product of the transesterification reaction of vegetable oils and animal fats that conduces to biodiesel synthesis, thus converting glycerol into an attractive biotechnological substrate (da Silva et al. 2009). Processes for the synthesis of reduced biochemicals using glycerol as the main carbon source have been described (Nikel et al. 2008b, 2010). Manipulation of central metabolic pathways were accomplished by means of mutations in the two-component signal transduction systems ArcAB (*a*erobic *r*espiration *c*ontrol) and CreBC (*c*arbon source *re*sponsive), responsible for modulation of the intracellular redox state and carbon source utilization (Avison et al. 2001; Pettinari et al. 2008; Nikel et al. 2009). *E. coli* CT1061 [an *arcA* and *creC*(Con) double mutant] has elevated intracellular NADH levels and high carbon source consumption rates, making it adequate as a host for reduced biochemicals synthesis (Nikel et al. 2008a). As a proof on concept, introduction of $adhE_{Lm}$ (encoding a bifunctional alcohol-acetaldehyde dehydrogenase from *Leuconostoc mesenteroides*) cloned in a multicopy plasmid in strain CT1061 resulted in enhanced ethanol synthesis from glycerol (Nikel et al. 2010). Since this plasmid bears *bla*, mediating β-lactam antibiotics resistance, ampicillin was used to suppress the possible rising of plasmid-free cells. Contrary to expectations, accumulation of unproductive plasmid-free segregants was not detected even in absence of selective pressure. This plasmid was rapidly lost under aerobic growth conditions, while it was stably maintained under conditions with restricted oxygen supply. As the maintenance behavior of this plasmid seemed to be dependent on metabolic traits directly related to the redox state of the cells, we decided to further study the phenomenon of plasmid stability. The intracellular redox state was determined and manipulated in different ways to follow the kinetic of plasmid loss, and a significant correlation was found between the ability of cells to reoxidize reducing equivalents and plasmid presence in redox mutants of *E. coli*.

Materials and methods

Bacterial strains and plasmids *E. coli* strains and plasmids used in this study are listed in Table 1. Plasmid $pADH_{Lm}$ (Koo et al. 2005) was the source of *adhE* from *L. mesenteroides* ($adhE_{Lm}$).

Molecular biology procedures and plasmid construction Unless otherwise indicated, all DNA procedures followed standard protocols (Sambrook and Russell 2001) and specific recommendations from manufacturers. *E. coli* DH5α was used as the host for molecular biology constructs. In order to construct a pET_{Lm} derivative with a defective copy of $adhE_{Lm}$, a 1,386-bp DNA fragment roughly corresponding to half the $adhE_{Lm}$ coding sequence was excised from pET_{Lm} by *Kpn*I digestion. A 963-bp DNA fragment encompassing *cat* (cloramphenicol acetyltransferase) was isolated from plasmid pKD3 by *Xba*I digestion. This DNA fragment and the *Kpn*I-treated pET_{Lm} were made blunt-ended by using the Klenow fragment of DNA polymerase I (Promega, Madison, WI, USA) in a reaction mixture containing 50 mM Tris–HCl (pH=7.2), 15 mM $MgSO_4$, 0.1 mM dithiothreitol, and 0.25 mM each dNTP. After 15 min incubation at room temperature, the reaction was stopped by adding EDTA to a final concentration of 15 mM and heating at 75°C for 20 min. Both Klenow-treated DNA fragments were purified and ligated to obtain $pET\Omega_{Lm}$. Successful disruption of $adhE_{Lm}$ was confirmed by DNA sequencing at the facility of Instituto de Investigaciones Biotecnológicas, Universidad Nacional de San Martín (San Martín, Buenos Aires, Argentina).

Culture conditions and bioreactor cultivation For routine growth and during molecular biology manipulations, strains were grown in LB medium (Sambrook and Russell 2001). M9 minimal medium contained (in grams per liter): Na_2HPO_4, 6.0; KH_2PO_4, 3.0; $(NH_4)_2SO_4$, 1.4; NaCl, 0.5; $MgSO_4{\cdot}7H_2O$, 0.2; and thiamine·HCl, 0.005 (initial pH adjusted to 7.2 with 1 M NaOH or HCl). SGA medium, suitable for cultivation of *arc* strains, contained the same

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

Strain, plasmid, or oligonucleotide	Relevant characteristics	Source or reference
E. coli		
DH5α	Φ80*lacZ*ΔM15 *recA1 endA1 gyrA96 thi-1 hsdR17*(r_K^- m_K^+) *supE44 relA1 deoR* Δ(*lacZYA-argF*)*U169*	Invitrogen, Carlsbad, CA, USA
K1060	F^- *fadE62 lacI60 tyrT58*(AS) *fabB5 mel-1*; considered wild-type in this study	Overath et al. (1970)
CT1061	As K1060, but *arcA*::IS*10*-L *creC*(Con); Tet^r	Nikel et al. (2006)
CT1061E	CT1061 carrying pET_{Lm}; Tet^r Amp^r	Nikel et al. (2010)
CT1061EΩ	CT1061 carrying $pETΩ_{Lm}$; Tet^r Amp^r Cm^r	This work
CT1061L	As CT1061, but Δ*ldhA*::*kan*; Tet^r Km^r	Nikel et al. (2010)
CT1061LE	CT1061L carrying pET_{Lm}; Tet^r Km^r Amp^r	Nikel et al. (2010)
CT1061LEΩ	CT1061L carrying $pETΩ_{Lm}$; Tet^r Km^r Amp^r Cm^r	This work
Plasmids		
pBluescript II KS(–)	High-copy-number phagemid vector used for cloning; T3 and T7 promoters, *lacPOZ*', ColE1 ori; Amp^r	Fermentas, Glen Burnie, MD, USA
pET_{Lm}	pBluescript II KS(–)-derivative carrying *adhE* from *Leuconostoc mesenteroides* ($adhE_{Lm}$); Amp^r	Nikel et al. (2010)
pKD3	Template plasmid for gene disruption, FRT::*cat*::FRT *bla oriRγ*; Cm^r Amp^r	Datsenko and Wanner (2000)
$pETΩ_{Lm}$	pET_{Lm}-derivative in which $adhE_{Lm}$ is interrupted by *cat*; Amp^r Cm^r	This work
Oligonucleotides		
bla-F	5′-CTACGATACGGGAGGGCTTA-3′	This work
bla-R	5′-ATAAATCTGGAGCCGGTGAG-3′	This work
polA-F	5′-GCGAGCGATCCAGAAGATCT-3′	This work
polA-R	5′-GGGTAAAGGATGCCACAGAC-3′	This work
dxs-F	5′-CGAGAAACTGGCGATCCTTA-3′	This work
dxs-R	5′-CTTCATCAAGCGGTTTCACA-3′	This work

Km^r kanamycin resistance; *Tet^r* tetracycline resistance; *Amp^r* ampicillin resistance; *FRT* Flp (*Saccharomyces cerevisiae* site-specific recombinase)-recognition target

E. coli K1060 was obtained through the *E. coli* Genetic Stock Center (CGSC 5040), University of Yale, New Haven, CT, USA

salts as M9 as well as 0.3 g l^{-1} casein amino acids (Diagnostic Systems, Sparks, MD, USA) and 5 ml l^{-1} of a trace element solution (Nikel et al. 2006). Solid media also contained 30 g l^{-1} agar. Cultures were amended with *pro analysi* glycerol (Anedra, Buenos Aires, Argentina), glucose, or sodium gluconate at the concentrations indicated in the text and tetracycline at 10 μg ml^{-1}. In some experiments, 100 μg ml^{-1} ampicillin, 50 μg ml^{-1} kanamycin, or 34 μg ml^{-1} cloramphenicol were also added whenever needed. Magnesium sulfate, thiamine, antibiotics, and carbon sources were added separately as filter-sterilized concentrated solutions after autoclaving and cooling of the medium.

Each cultivation was started using isolated colonies from a freshly streaked LB plate containing the appropriate antibiotics. Microaerobic shaken-flask experiments were conducted in 250-ml Erlenmeyer flasks completely filled with SGA medium for 72 h at 37°C. Gentle agitation was provided by a magnetic stirrer (approximately 75 rpm) to prevent biomass sedimentation. Aerobic cultures were conducted at 37°C in 250-ml Erlenmeyer flasks containing 25 ml of culture medium at 250 rpm. In some experiments, other medium-to-flask volume ratios and rotary agitation speeds were used as detailed in the text. All cultures were inoculated at 0.1 g l^{-1} initial cell dry weight (CDW) with an overnight culture grown in SGA medium at the oxygen availability condition to be used in the experiment.

Bioreactor cultivations were carried out in a 5.6-liter stirred tank reactor equipped with six flat-bladed disk turbines (BioFlo 110, New Brunswick Scientific Co., Edison, NJ, USA), with a 4-liter working volume and pH controlled at 7.20±0.05 by automatic addition of 3 M KOH or 1.5 M 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 microaerobic cultivation, air input was suppressed and the agitation speed was set at 75 rpm to avoid cell sedimentation. Oxygen concentration in the culture broth, estimated as the percentage of air saturation, was followed during each run by using a polarographic Ag/AgCl oxygen probe (Mettler-Toledo, Greifensee, Switzerland).

Screening of plasmid-bearing bacteria and stability of host chromosomal mutations Culture samples were withdrawn at selected times and immediately chilled at 0°C by placement in an ice bath. After being aseptically diluted in cold saline (9 g l^{-1} NaCl), suspensions were spread onto LB agar plates and incubated for 24 to 48 h at 37°C, and colonies were replicated with a sterile toothpick onto LB agar supplemented with 100 μg ml^{-1} ampicillin (Weber and San 1989). Parallel seeding of bacterial suspensions onto LB plates and LB supplemented with ampicillin plates was also used to estimate the percentage of plasmid-bearing cells. This method gave results similar to those obtained with the colony replica method above described. Plasmid-bearing cells were scored as the percentage of ampicillin-resistant clones over total viable counts in the antibiotic-free plates. Stability of chromosomal mutations was checked by plating cells onto LB agar containing 200 μg ml^{-1} toluidine blue O (Arc) or M9 minimal medium containing acetate as the sole carbon source (Cre). $LdhA^-$ phenotype was also checked by absence of anaerobic growth of cells plated onto M9 minimal medium containing 5 g l^{-1} sodium lactate as the sole carbon source. Sequencing of chromosomal targets was also used in order to confirm genetic stability of the *arcA*, *creC* and *ldhA* mutations (data not shown).

Determination of plasmid copy number by qPCR PCR primers, listed in Table 1, were designed using the Primer Express software package, version 3.0 (Applied Biosystems, Foster City, CA, USA). Detection of plasmid DNA was targeted at the *bla*-encoded β-lactamase using oligonucleotides *bla*-F and *bla*-R, which give rise to an 81-bp DNA amplification fragment. The chromosomal target was an 87-bp fragment in the promoter region of the *E. coli* DNA polymerase I gene amplified by oligonucleotides *polA*-F and *polA*-R. In some cases, a 113-bp long amplicon was included as an additional chromosomal target control amplified using oligonucleotides *dxs*-F and *dxs*-R, designed for the single-copy 1-deoxy-D-xylulose-5-phosphate synthase gene. For determinations, samples from the bioreactor were promptly taken over 25% (*v*/*v*) CH_3OH precooled at −20°C in order to stop metabolism. Cells were spun at 8,000 rpm for 5 min at 0°C, washed twice with chilled deionized water, and resuspended to appropriate densities in PCR-grade water.

Amplifications were performed in 20 μl mixtures. The mixture for one reaction contained 1× SYBR Green PCR Master Mix (Applied Biosystems), 3.5 mM $MgCl_2$, 250 nM each of forward and reverse primers, and 2 μl of sample (corresponding to 2.5×10^4 to 5×10^5 bacterial cells, the optimal working range previously determined). Separate reactions were prepared for detection of chromosomal and plasmid specific amplicons, each in triplicate, using ABI Prism optical 384-well reaction plates sealed with an adhesive cover. Appropriate negative and positive control reactions were routinely included in the experimental runs. Serial 10-fold dilutions of purified plasmid and genomic DNA were conducted in triplicates to establish standard curves, in a plot of the threshold cycle (Ct) as a function of log(Co), the concentration of template. Reactions were run on an ABI Prism 7900HT Sequence detection system (Applied Biosystems) using the following universal cycling conditions for all amplicons: 2 min at 50°C and 10 min at 95°C (AmpliTaq Gold DNA polymerase activation), followed by 45 cycles consisting of 15 s at 95°C and 1 min at 60°C. Upon completion of the amplification cycles, a dissociation stage was added (95°C for 15 s and ramping temperature from 60°C to 95°C at 0.1°C s^{-1}) while fluorescence signal was continually monitored for melting curve analysis. Ct values were determined after automatic adjustment of the baseline and manual adjustment of the fluorescence threshold, using SDS 2.2 software (Applied Biosystems). Amounts of plasmid or genomic DNA were obtained by interpolation of the sample Ct against the corresponding standard curve. Plasmid copy number was calculated as indicated in Eq. 1.

$$\text{Plasmid copy number per chromosome} = \frac{\text{Size of chromosomal DNA (bp)} \times \text{Amount of plasmid DNA (pg)}}{\text{Size of plasmid DNA (bp)} \times \text{Amount of genomic DNA (pg)}} \quad (1)$$

In vitro enzymatic determinations 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 mM ethanol and 8.3 mM NAD^+ in 50 mM sodium phosphate buffer (pH=6.5) in a final volume of 1 ml. NADH generation was followed from the change in absorbance at 340 nm in a Beckman DU 650 spectrophotometer (Beckman Coulter, Fullerton, CA, USA). One unit of Adh activity was defined as the amount of enzyme capable of catalyzing the generation of 1 μmol NADH min^{-1} under the conditions specified. Protein concentration was determined as described by Bradford (1976), using crystalline bovine serum albumin as standard.

Intracellular NADH and NAD^+ concentrations were estimated by using in vitro procedures based on rapid inactivation of the metabolism of growing cells followed by metabolite extraction. Culture broth samples (10 ml) were transferred to precooled plastic tubes and the metabolic activity was quenched by immersion of the tubes in liquid N_2. Samples were stored for no longer than 24 h at −70°C until enzymatic analysis. Aliquots of 1 ml of thawed

samples were treated with 300 µl of either 0.2 M HCl (NADH extraction) or 0.2 M NaOH (NAD^+ extraction). Acid/alkaline extraction was carried out at 50°C for 10 min, and samples were rapidly placed on ice to cool them at 0°C afterwards. Suspensions were neutralized by dropwise addition of 1 M HCl or NaOH, and cellular debris was removed by centrifuging at 12,000 rpm for 5 min. Supernatants were then transferred to new tubes and immediately used for co-factor measurements. Determinations used in this study followed the spectrophotometric cycling assay originally developed by Bernofsky and Swan (1973) [which involves 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide as the final electron acceptor] with the modifications described by Gibon and Larher (1997) and Nikel et al. (2008c).

Analytical procedures and statistical analysis Biomass concentration was determined as the CDW of washed pellets dried in Falcon tubes at 80°C for at least 36 h. Dried samples were allowed to cool and held in vacuo until weighed. Ethanol was determined with an enzymatic kit, and residual glycerol concentration was obtained by using a colorimetric assay (Nikel et al. 2008c). The degree of reduction (κ) of the carbon sources was calculated according to Nielsen et al. (2003). Fermentation parameters for cell growth, glycerol consumption, and metabolite synthesis were used to calculate yields on substrate ($Y_{E/S}$, gram of ethanol per gram of consumed glycerol). Significance of the differences when comparing results was evaluated by ANOVA and the Tukey–Kramer test (α=0.01 and 0.05).

Results

The kinetic behavior of plasmid lost in *E. coli* redox mutants depends on both carbon source oxidation state and oxygen availability

Previous results indicated that plasmids which increase the synthesis of reduced metabolites were maintained in *E. coli* in conditions requiring the ability of the cells to reoxidize NADH produced during central catabolism (Hespell et al. 1996; Nikel et al. 2006, 2010; Singh et al. 2009). The amount of reduced NADH depends on a number of factors, including electron acceptor availability and the oxidation state of the carbon source (Neidhardt et al. 1990). Thus, the degree of reduction of the substrate used should also affect plasmid maintenance. Carbon sources with different oxidation states were analyzed as a simple way to manipulate the intracellular $NADH/NAD^+$ ratio and test this hypothesis. The degrees of reduction of the carbon sources used are $\kappa_{glycerol}$=4.7, $\kappa_{glucose}$=4.0 and $\kappa_{gluconate}$=3.5, and all these substrates ultimately enter into the Embden–Meyerhof–Parnas glycolytic pathway. As deduced from the corresponding degrees of reduction, glycerol produces more reducing equivalents in the form of NADH than glucose while gluconate produces the least amount of NADH, since half of every gluconate molecule is directly converted to pyruvate, skipping the NADH-producing step in glycolysis. Shaken-flask cultures of *E. coli* CT1061E were conducted under microaerobic conditions using glycerol, glucose or gluconate (Fig. 1a). Glycerol cultures had a significantly higher percentage of plasmid-bearing cells (97.8±1.2%) when compared to

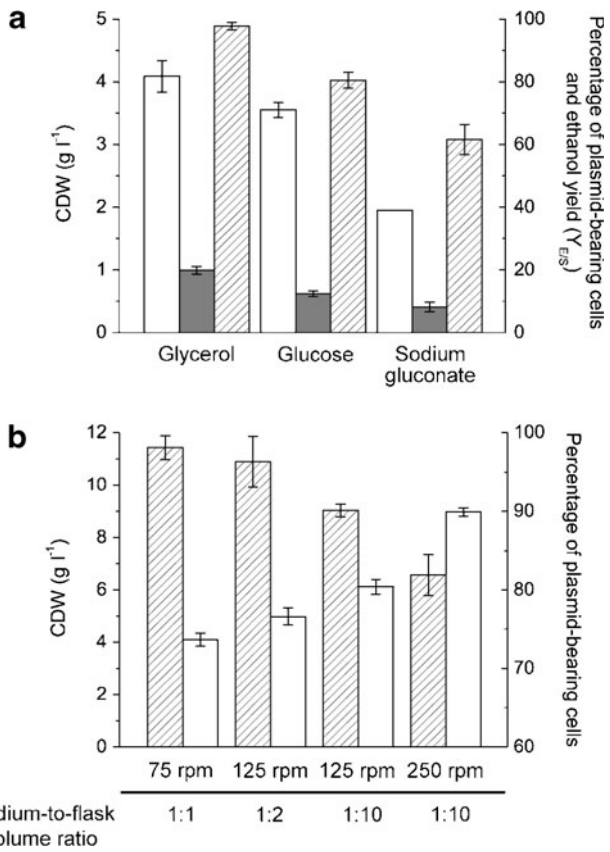

Fig. 1 Fermentation parameters in shaken-flasks cultures of *E. coli* CT1061E [*arcA*::IS*10*-L *creC*(Con) carrying pET_{Lm}] in absence of ampicillin selective pressure. **a** Growth (expressed as *CDW* cell dry weight, *open bars*), ethanol yield on carbon substrate ($Y_{E/S}$, *gray bars*), and plasmid maintenance (*hatched bars*) under microaerobic growth conditions (medium-to-flask volume ratio = 1:1, 75 rpm). Cultures were conducted in SGA medium supplemented with 1 C-mol l^{-1} of glycerol (κ=4.7), glucose (κ=4.0), or sodium gluconate (κ=3.5). **b** Growth (expressed as *CDW* cell dry weight, *open bars*) and plasmid maintenance (*hatched bars*) in shaken-flask cultures under different conditions of oxygen availability, varied according to the medium-to-flask volume ratios and rotary agitation speed. Cultures were developed in SGA medium supplemented with 30 gl^{-1} glycerol. In all cases, samples were taken at 72 h for determinations and the results represent the mean values for each parameter±standard deviation of duplicate measurements from at least two independent cultures

cultures developed on glucose (80.5±2.5%) or gluconate (61.6±4.8%), with $P<0.05$. The oxidation state of each carbon source was also reflected in the final biomass concentration and the yield of ethanol on substrate ($Y_{E/S}$).

Dependence of pET$_{Lm}$ maintenance on oxygen availability was qualitatively estimated in shaken-flasks cultures in which aeration conditions were manipulated through the medium-to-flask volume ratio and rotary agitation speed. Percentage of plasmid-bearing cells significantly decreased from 98.1±1.5% under microaerobic conditions up to 81.9±2.6% in the most aerated condition with $P<0.05$ (Fig. 1b), a trend also followed by ethanol concentrations and ethanol yields on glycerol (data not shown). At the same time, the biomass concentration steadily increased accompanying oxygen availability to reach 8.97±0.09 g l^{-1} under the condition with highest oxygen availability (which implies a 2.2-fold increase in biomass concentration when compared to microaerobiosis, $P<0.01$). Parallel experiments in which ampicillin was added as a selective pressure for plasmid maintenance showed that recombinant biomass was ≥90% throughout the cultivation irrespective of the oxygen availability.

The metabolic effects of plasmid maintenance are different in wild-type *E. coli* and its redox mutant derivatives

We asked the question whether the plasmid maintenance trait was strain-specific or plasmid-specific, and *E. coli* CT1061 was compared to K1060 under microaerobic growth conditions (<10% of air saturation throughout the run) by following the kinetic of plasmid loss in bioreactor cultures. Previous results showed that redox environment (as measured by the intracellular NADH/NAD$^+$ ratio) is much more reduced in *E. coli* CT1061, the unregulated redox mutant, than in K1060, the wild-type parental strain (Nikel et al. 2008c). In *E. coli* K1060 both pBluescript II KS(–) and pET$_{Lm}$ were rapidly lost in absence of selective pressure (Fig. 2a). Even when both plasmids were lost following similar kinetics, at 72 h a 37.3±5.9% of the cells retained pET$_{Lm}$ while only 2.6±0.9% maintained the empty vector, suggesting that its maintenance could be related to the ability of plasmid-bearing cells to regenerate NAD$^+$. This behavior was even more evident in *E. coli* CT1061, in which pBluescript II KS(–) was rapidly lost and at 72 h viable counts showed only a 7.8±1.2% of plasmid-bearing cells (Fig. 2b). In sharp contrast, pET$_{Lm}$ was present in 92.4±7.2% of the cells at the same cultivation time. This value was stably maintained during the whole cultivation period.

In order to evaluate the metabolic differences elicited by different plasmids on the physiology of the strains under study, shaken-flask cultures were carried out under microaerobic conditions and fermentation parameters were analyzed (Table 2). Interestingly, all strains derived from *E. coli* CT1061 carrying plasmids showed a higher specific

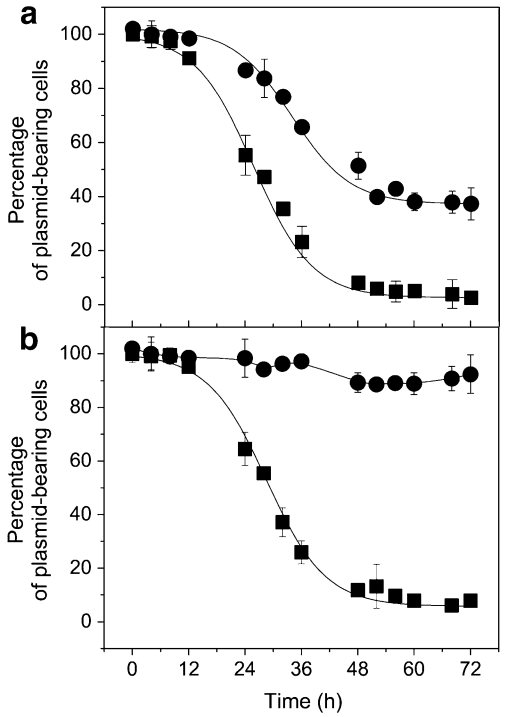

Fig. 2 Time-course plots for plasmid maintenance in different *E. coli* strains grown in microaerobic bioreactor cultures using SGA medium supplemented with 30 g l^{-1} glycerol. All cultures were conducted without ampicillin, and the *symbols* represent the mean values for each parameter±standard deviation of duplicate measurements from at least three independent cultures. **a** *E. coli* K1060 (wild-type strain) carrying pET$_{Lm}$ (*adhE$_{Lm}$*, *circles*) or pBluescript II KS(–) (empty vector used as a control, *squares*). **b** *E. coli* CT1061 [K1060-derivative, *arcA*::IS*10*-L *creC*(Con)] carrying pET$_{Lm}$ (*circles*) or pBluescript II KS(–) (*squares*)

consumption rate of glycerol when compared to the parental strain. Ethanologenic derivatives of CT1061 (carrying pET$_{Lm}$) have the highest specific growth rates under microaerobic conditions, a further proof of the positive effect of *adhE$_{Lm}$* expression on the physiology of these redox mutants. Moreover, a Δ*ldhA*-derivative of *E. coli* CT1061 (strain CT1061L) showed the highest growth rate. In such a Δ*ldhA* mutant, extra reducing equivalents are available since the NADH-dependent reduction of pyruvate to D-lactate is lacking. All the chromosomal mutations in *E. coli* CT1061L were stably maintained in prolonged cultures.

Plasmid copy number is affected by the presence of *adhE$_{Lm}$* under microaerobic conditions of growth

Plasmid replication is generally controlled in the bacterial cell, so that the copy number of a particular plasmid stays within a narrow range in a given host under defined growth conditions (Summers 1998; Škulj et al. 2008). However, the relationship between plasmid copy number and intracellular redox state was not extensively studied, and we decided to analyze if the enhanced plasmid maintenance in *E. coli* CT1061 is related to changes in

Table 2 Fermentation parameters for shaken-flasks cultures developed in SGA medium containing 30 g l^{-1} glycerol

E. coli strain	Relevant characteristics	q_S (mmol g $CDW^{-1}h^{-1}$)	μ (h^{-1})	$Y_{E/S}$ (g g^{-1})	Percentage of plasmid-bearing cells
K1060	Wild type	2.5±0.2	0.11±0.01	2.5±0.4	NA
CT1061	*arcA*::IS*10*-L *creC*(Con)	3.2±0.1	0.19±0.02	13.1±1.5	NA
CT1061/pBluescript II KS(–)	*arcA*::IS*10*-L *creC*(Con) carrying the empty vector	3.7±0.1	0.23±0.02	15.9±0.7	8.2±0.9
CT1061E	*arcA*::IS*10*-L *creC*(Con) carrying pET_{Lm}	4.2±0.2	0.31±0.03	22.3±0.9	96.5±1.9
CT1061LE	*arcA*::IS*10*-L *creC*(Con) Δ*ldhA*::*kan* carrying pET_{Lm}	4.0±0.2	0.33±0.01	28.5±1.3	97.3±1.3
CT1061EΩ	*arcA*::IS*10*-L *creC*(Con) carrying $pET\Omega_{Lm}$	3.9±0.3	0.22±0.02	15.1±1.9	12.7±0.8
CT1061LEΩ	*arcA*::IS*10*-L *creC*(Con) carrying $pET\Omega_{Lm}$	3.5±0.2	0.24±0.03	18.3±0.7	15.1±1.9

Specific glycerol consumption rates and growth rates were calculated during exponential growth. Ethanol yield on glycerol is expressed as a percentage. Ethanol yield and percentage of plasmid-bearing cells were calculated at the end of the cultivation period (72 h)

NA not applicable

plasmid copy number. *E. coli* CT1061E had a higher specific growth rate (μ=0.38±0.05 h^{-1}) in microaerobic bioreactor cultures than *E. coli* CT1061 carrying the empty vector (μ=0.26±0.03 h^{-1}, Fig. 3a), similar to results previously observed in shaken-flask cultures (Table 2). The growth curves showed no significant qualitative differences, even when *E. coli* CT1061E attained a ca. 1.3-fold higher cell density than the other strains at the end of the cultivation period. Interestingly, *E. coli* CT1061EΩ (carrying a defective copy of $adhE_{Lm}$) had a growth curve almost indistinguishable to that of *E. coli* CT1061/pBluescript II KS(–), with μ=0.24±0.05 h^{-1}.

Kinetics of plasmid copy number during bioreactor batch cultivations was followed by quantitative PCR (qPCR) analysis (Fig. 3b). Results were similar for the strains studied during the first 24 h of cultivation. In all cases, a rapid decrease in copy number was observed during the first 4 h, which probably responds to the fact that cells are quickly dividing during exponential growth thus diminishing the relationship between plasmid and chromosome copy numbers. Copy number sharply increased during the following 20 h for all plasmids. However, quite a different result was observed for the period comprised between 24 and 72 h among the tested strains. While in *E. coli* CT1061/pBluescript II KS(–) the ratio between plasmid copies and chromosomes number steadily diminished to reach 334±45 copies per chromosome at 72 h, *E. coli* CT1061E showed an almost-constant pET_{Lm} copy number, reaching 498±18 copies per chromosome at the end of the cultivation, significantly different to that of the control experiments (1.5-fold increment, $P<0.05$). Moreover, $pET\Omega_{Lm}$ had a curve of copy number kinetics very similar to that of pBluescript II KS(–) (293±62 copies per chromosome at 72 h).

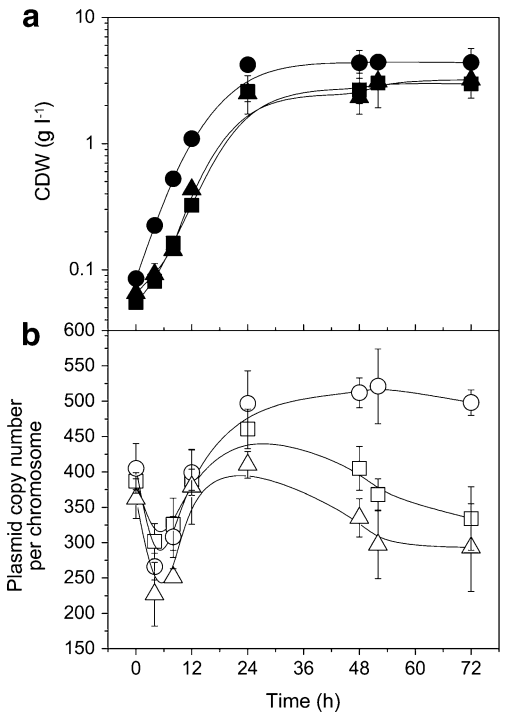

Fig. 3 Microaerobic bioreactor cultures using SGA medium supplemented with 30 gl^{-1} glycerol for *E. coli* CT1061 [*arcA*::IS*10*-L *creC* (Con)] recombinants carrying pBluescript II KS(–) (empty vector used as a control, *squares*), pET_{Lm} ($adhE_{Lm}$, *circles*) and $pET\Omega_{Lm}$ (Δ$adhE_{Lm}$::*cat*, *triangles*). All cultures were conducted without ampicillin, and the *symbols* represent the mean values for each parameter±standard deviation of triplicate measurements from at least two independent cultures. **a** Representative growth curves for the recombinants. **b** Time-course plots for plasmid copy number per chromosome, determined by quantitative PCR as detailed in "Materials and methods"

Efficient maintenance of plasmid pET_{Lm} is directly related to the presence and expression of $adhE_{Lm}$ and the NADH/NAD^+ ratio

Nicotinamide adenine dinucleotides were determined at the end of batch microaerobic cultures in order to analyze the intracellular redox state of each strain (Table 3). All strains had a similar content of total co-factors (ca. 4.5 μmol g CDW^{-1}), but the distribution of the oxidized and reduced forms varied significantly, thus affecting the NADH/NAD^+ ratio. For *E. coli* CT1061 carrying the empty vector, a NADH/NAD^+ ratio close to the unit clearly indicates the reducing nature of the intracellular environment, as expected for such an unregulated mutant. This parameter diminished ca. 2.3-fold in *E. coli* CT1061E ($P<0.05$), a result possibly related to the reoxidation of NADH by the overexpressed $AdhE_{Lm}$. In contrast, *E. coli* CT1061EΩ, with a defective copy of $adhE_{Lm}$, showed a intracellular redox state comparable to that of *E. coli* CT1061 carrying pBluescript II KS(–). The intracellular NADH/NAD^+ ratio also correlated with the enzymatic activity of $AdhE_{Lm}$. While the control strain and *E. coli* CT1061EΩ had a low specific Adh activity (which can be accounted for by the endogenous AdhE of *E. coli*), *E. coli* CT1061E showed a 2-fold increase in the specific Adh activity.

Discussion

It was generally recognized that plasmids impose a metabolic burden to the cell in a wide variety of growth conditions (Bentley et al. 1990; Węgrzyn and Węgrzyn 2002; Friehs 2004; Ow et al. 2006; Wang et al. 2006). As nonessential DNA molecules, plasmids require additional intracellular metabolic precursors and energy to exist within the host cell, thus usually reducing the growth rate of bacterial cells. Although faster-growing organisms will normally be selected over plasmid-bearing cells, the process by which this selection operates is not clear or easy to understand, as it lays hidden somewhere behind extremely complicated biological mechanisms linked to cellular metabolism and depends on the nature of the metabolic alterations induced by plasmids. Previous results, such as those obtained by Rozkov et al. (2004) with *E. coli* HB101 carrying plasmids that contain a therapeutic DNA sequence (a hepatitis B antigen), indicated that cells carrying recombinant plasmids are subjected to a severe metabolic burden, reflected in lower biomass yields and specific growth rates when compared to plasmid-free cells. However, Díaz-Ricci et al. (1995) later showed a positive effect of pUC-derived plasmids in different *E. coli* strains, and explained it in terms of changes in the intracellular cAMP concentrations and the cAMP-binding protein. Apart of these physiological and metabolic effects, it was shown that the presence of plasmid DNA in *E. coli* produces a fast drop of the extracellular and intracellular pH as a consequence of high accumulation of lactic, acetic, formic and succinic acids (Díaz-Ricci and Hernández 2000). Some results in our present study pointed to quite a different situation, highlighting the metabolic advantage of *E. coli* redox mutants in carrying ethanologenic plasmids, possibly due to the enhanced capacity to reoxidize NADH.

Several factors affect plasmid maintenance in a bacterial host of both genetic and metabolic nature. In this work, we were mainly interested in the metabolic traits conferred by the presence of plasmids. During catabolism, cells oxidize a carbon source utilizing NAD^+ as a co-factor and producing reducing equivalents in the form of NADH. Cells have to regenerate NAD^+ from NADH to achieve redox balance. Under oxygen-restricted conditions, such as those used in this work, NADH recycling is achieved through fermentative bioreactions. Since the heterologous bioreaction encoded by pET_{Lm} makes use of NADH as a co-factor for the synthesis of ethanol, it is expected that factors affecting the intracellular redox state also affect the extent of activity

Table 3 Alcohol dehydrogenase (Adh) specific activity, nicotinamide adenine dinucleotides content and intracellular redox state for *E. coli* CT1061 and its derivatives

E. coli strain	Relevant characteristics	Adh activity (μmol min^{-1} mg $protein^{-1}$)	NADH (μmol g CDW^{-1})	NAD^+ (μmol g CDW^{-1})	NADH/NAD^+ (mol mol^{-1})
CT1061/pBluescript II KS(–)	*arcA*::IS*10*-L *creC*(Con) carrying the empty vector	0.49±0.04	2.13±0.09	2.32±0.08	0.92±0.07
CT1061E	*arcA*::IS*10*-L *creC*(Con) carrying pET_{Lm}	1.15±0.09	1.34±0.15	3.26±0.09	0.41±0.06
CT1061EΩ	*arcA*::IS*10*-L *creC*(Con) carrying $pET\Omega_{Lm}$	0.54±0.03	1.87±0.13	2.18±0.07	0.86±0.09

Cells were grown under microaerobic conditions in batch bioreactor cultures using SGA medium supplemented with 30 gl^{-1} glycerol as the sole carbon source and samples were taken at 72 h. Total alcohol dehydrogenase activity was determined in permeabilized cells by following the ethanol-dependent reduction of NAD^+. NADH and NAD^+ were determined by an in vitro cyclic assay as described in "Materials and methods." Results shown in this table represent the mean value±standard deviation for triplicate measurements from at least two independent cultures

of $AdhE_{Lm}$. Both $Y_{E/S}$ and CDW values increased proportionally with respect of the degree of reduction of each carbon source, which is directly related to the NADH amount needed for biosynthetic reactions and ethanol synthesis. Experiments conducted using glycerol as the sole carbon source gave significant differences regarding plasmid maintenance when compared to other substrates. As the redox state is directly influenced by the oxygen availability, especially in an $ArcA^-$ metabolic background, these results would suggest that maintenance of pET_{Lm} in *E. coli* CT1061 is related to the ability conferred by this plasmid to reoxidize NADH. Comparison of *E. coli* K1060 and CT1061 was a helpful way to test the redox-dependent kinetic of plasmid loss since, as expected, all plasmids were more stably maintained in the unregulated redox mutant (Nikel et al. 2008c). Moreover, a Δ*ldhA*-derivative of *E. coli* CT1061 (strain CT1061L) showed the highest growth rate. In such a Δ*ldhA* mutant, extra reducing equivalents are available since the NADH-dependent reduction of pyruvate to D-lactate is lacking, and can be considered overstrained regarding NAD^+-regeneration pathways (Gokarn et al. 2001). These results clearly suggest that pET_{Lm} is maintained under conditions in which NAD^+ regeneration is an important trait for growing cells.

Plasmid copy number seemed to be also affected by the intracellular redox state. While no significant differences were observed during active growth in the control strain and redox mutants, a 1.5-fold higher plasmid content was observed in *E. coli* CT1061E carrying pET_{Lm}. The values obtained for each plasmid copy number are within the range reported for pBluescript vectors, which are normally present in *E. coli* cells at 300–500 copies per chromosome, similar to other plasmids containing a ColE1 origin of replication (Teich et al. 1998; Grabherr and Bayer 2002; Wang et al. 2006). It was recently demonstrated that the relationship between growth rate and plasmid copy number in several recombinant *E. coli* strains is dependent on particular interactions between plasmids and hosts (Yau et al. 2008), and therefore difficult to predict. In this study, we found the most significant differences at stationary phase, potentially implying an important influence of metabolic effects on plasmid copy number rather than growth rates. Even when disruption of $adhE_{Lm}$ (plasmid $pET\Omega_{Lm}$) resulted in loss of Adh activity and significantly influenced both the intracellular redox state and plasmid copy number, we cannot rule out the possibility that differences in plasmid DNA length or sequence could also play a role on the phenotype observed in *E. coli* CT1061EΩ.

It is to be noticed that we observed a similar behavior regarding plasmid stability in *E. coli* redox mutants while studying the heterologous accumulation of poly(3-hydroxybutyrate) under conditions with restricted oxygen supply on glycerol (Nikel and Méndez, unpublished). In fact, 91±2% of cells accumulating poly(3-hydroxybutyrate) under microaerobic conditions retained a recombinant plasmid carrying the *phaBAC* genes from *Azotobacter* sp. strain FA8 (Nikel et al. 2006), while the same plasmid was present in only 78±1% of the cells under aerobic conditions. Our results thus suggest that the redox nature of the biochemical pathway encoded in the plasmid could ultimately determinate plasmid stability and, as a direct consequence, product titer and yield. Ethanologenic *E. coli* strains described in this work should not require antibiotics in the growth media to maintain positive selective pressure for cells containing pET_{Lm} because loss of the plasmid would be a conditionally lethal event. A previous report also hypothesized that plasmids containing the *pet* operon would offer a physiological complementation for anaerobic growth of *pflB* (pyruvate-formate lyase) mutants of *E. coli*, since expression of the *pdc* and *adhB* genes allow conversion of pyruvate into ethanol and regenerate oxidized pyridine nucleotides which would otherwise become toxic for the cells (Hespell et al. 1996). This is an important phenotypic trait with potential applications in biotechnology, as it was previously demonstrated that *E. coli* B (ATCC 11303) carrying pLOI297 (*pdc* and *adhB* from *Z. mobilis*) and the chromosomally integrated recombinant KO11 exhibited dramatic loss of ethanologenicity during growth in a selective (antibiotic-supplemented) medium with different fermentation substrates (Lawford and Rousseau 1995, 1996; Dumsday et al. 1999). Finally, spreading of antibiotic resistances resulting from excessive antibiotics utilization in bioprocesses is a current concern to be taken into account.

The main goal of a successful heterologous expression system based on plasmids is to achieve the highest tolerable protein or metabolite yield and thus to walk the thin line between high levels of heterologous gene expression and the metabolic capacity of the host. Considering this important industrial objective, understanding the physiological interactions between plasmid DNA and its host is highly relevant for vector design and host optimization. Taken together, our results suggest a delicate balance between the metabolic burden imposed by plasmid replication and the expression of genes carried in the plasmid, providing evidence for the beneficial effect of the reactions enabled by the functions of plasmid-encoded genes.

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