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Elimination of D-Lactate Synthesis Increases Poly(3-Hydroxybutyrate) and Ethanol Synthesis from Glycerol and Affects Cofactor Distribution in Recombinant *Escherichia coli*[▽]

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The effect of eliminating D-lactate synthesis in poly(3-hydroxybutyrate) (PHB)-accumulating recombinant *Escherichia coli* (K24K) was analyzed using glycerol as a substrate. K24KL, an *ldhA* derivative, produced more biomass and had altered carbon partitioning among the metabolic products, probably due to the increased availability of carbon precursors and reducing power. This resulted in a significant increase of PHB and ethanol synthesis and a decrease in acetate production. Cofactor measurements revealed that cultures of K24K and K24KL had a high intracellular NADPH content and that the NADPH/NADP$^+$ ratio was higher than the NADH/NAD$^+$ ratio. The *ldhA* mutation affected cofactor distribution, resulting in a more reduced intracellular state, mainly due to a further increase in NADPH/NADP$^+$. In 60-h fed-batch cultures, K24KL reached 41.9 g · liter^{-1} biomass and accumulated PHB up to 63% ± 1% (wt/wt), with a PHB yield on glycerol of 0.41 ± 0.03 g · g^{-1}, the highest reported using this substrate.

Poly(3-hydroxybutyrate) (PHB) is the best-known and most common polyhydroxyalkanoate (PHA). PHAs are polymers with thermoplastic properties that are totally biodegradable by microorganisms present in most environments and that can be produced from different renewable carbon sources (38). Accumulated as intracellular granules by many bacteria under unfavorable conditions (1, 21), PHAs are carbon and energy reserves and also act as electron sinks, enhancing the fitness and stress resistance of bacteria and contributing to redox balance (12, 30). *Escherichia coli* offers a well-defined physiological environment for the construction and manipulation of various metabolic pathways to produce different bioproducts, such as PHB, from cost-effective carbon sources.

In recent years, a significant increase in the production of biodiesel has caused a sharp fall in the cost of glycerol, the main by-product of biodiesel synthesis. As a result, glycerol has become a very attractive substrate for bacterial fermentations (10), specially for reduced products, such as PHB (36). The *E. coli* strain used in this work, K24K, carries *phaBAC*, the structural genes responsible for PHB synthesis, from *Azotobacter* sp. strain FA8 (23) (Table 1). The *pha* genes in K24K are expressed from a chimeric promoter and consequently are not subject to the genetic regulatory systems present in natural PHA producers. Because of this, it can be assumed that regulation of PHA synthesis in the recombinants is restricted by enzyme activity levels, modulated principally by substrate availability. In most natural producers, and also in PHB-producing *E. coli* recombinants, PHB is synthesized through the condensation of two molecules of acetyl-coenzyme A (acetyl-CoA), catalyzed by an acetoacetyl-CoA transferase or 3-ketothiolase, resulting in acetoacetyl-CoA. This compound is subsequently reduced by an NAD(P)H-dependent acetoacetyl-CoA reductase to *R*-(−)-3-hydroxybutyryl-CoA, which is then polymerized by a specific PHA synthase (34).

Cells growing on glycerol are in a more reduced intracellular state than cells grown on glucose under similar conditions of oxygen availability. This has a significant effect on the intracellular redox state, which causes the cells to direct carbon flow toward the synthesis of more-reduced products when glycerol is used than when glucose is used in order to achieve redox balance (31). When metabolic product distribution was analyzed in bioreactor cultures of K24K using glucose or glycerol as the substrate, product distributions with the two substrates were found to be different, as glycerol-grown cultures produced smaller amounts of acetate, lactate, and formate and more ethanol than those grown on glucose. However, PHB production from glycerol was lower than that from glucose, except under conditions of low oxygen availability (13).

Manipulations to enhance the synthesis of a metabolic product include several approaches to increase the availability of the substrates needed for its formation or to inhibit competing pathways. The effect of eliminating competing pathways on PHB production from glucose has been investigated through the inactivation of different genes, such as those encoding enzymes participating in the synthesis of acetate (*ackA*, *pta*, and *poxB*) or D-lactate (*ldhA*). A *pta* mutant, which produces very little acetate (6), and an *frdA ldhA* double mutant (40) had

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TABLE 1. *E. coli* strains, plasmids, and oligonucleotides used in this study

Strain, plasmid, or oligonucleotide	Relevant characteristics[b]	Reference or source
E. coli strains		
K1060[a]	F^- *fadE62 lacI60 tyrT58*(AS) *fabB5 mel-1*	29
K24	Same as K1060, carrying pJP24; Ap^r	23
K24K	Same as K1060, carrying pJP24K; Ap^r Km^r	23
ALS786[a]	F^- λ^- *rph-1* Δ*ldhA*::*kan*; Km^r	14
K24LT	Same as K1060 but Δ*ldhA*::*kan* by K1060 × P1(ALS786), carrying pJP24; Ap^r Km^r	This work
K24KL	Same as K1060 but Δ*ldhA* by allelic replacement, carrying pJP24K; Km^r	This work
TA3522[a]	F^- λ^- Δ(*his-gnd*)*861 hisJo-701*	2
TA3514[a]	Same as TA3522 but *pta-200*	19
TA3522L	Same as TA3522 but Δ*ldhA*::*kan* by TA3522 × P1(ALS786); Km^r	This work
TA3514L	Same as TA3514 but Δ*ldhA*::*kan* by TA3514 × P1(ALS786); Km^r	This work
Plasmids		
pQE32	Expression vector, ColE1 ori; Ap^r	Qiagen GmbH, Hilden, Germany
pJP24	pQE32 derivative expressing a 4.3-kb BamHI-HindIII insert containing the *phaBAC* genes from *Azotobacter* sp. strain FA8 under the control of a T5 promoter/*lac* operator element; Ap^r	23
pJP24K	pJP24 derivative; Ap^r Km^r	23
pCP20	Helper plasmid used for *kan* excision; *Saccharomyces cerevisiae FLP* λ *c*I857 λ P_R *repA*(Ts); Ap^r Cm^r	7
Oligonucleotides		
Δ*ldhA*-F	5′-TAT TTT TAG TAG CTT AAA TGT GAT TCA ACA TCA CTG GAG AAA GTC TTA TG**G TGT AGG CTG GAG CTG CTT C**-3′	This work
Δ*ldhA*-R	5′-CTC CCC TGG AAT GCA GGG GAG CGG CAA GAT TAA ACC AGT TCG TTC GGG CA**C ATA TGA ATA TCC TCC TTA G**-3′	This work

[a] Strain obtained through the *E. coli* Genetic Stock Center, Yale University, New Haven, CT.
[b] For oligonucleotides, the ATG codon of *ldhA* is underlined and the sequences with homology to FRT-*kan*-FRT in the template plasmid pKD4 (11) are shown in boldface.

increased PHB accumulation from glucose. A recent report using an *ackA pta poxB ldhA adhE* mutant under microaerobic conditions attained similar results (17). The inactivation of *ldhA* has also been shown to have an important effect on the metabolic product distribution in recombinant *E. coli* with glycerol as the carbon source, promoting ethanol synthesis (28). In the present work we analyzed the effect of *ldhA* inactivation in strain K24K using glycerol as the carbon source, with special emphasis on changes in carbon distribution and in the intracellular redox state, determined through cofactor levels.

MATERIALS AND METHODS

Bacterial strains, plasmids, and oligonucleotides. All *Escherichia coli* strains are listed in Table 1, along with plasmids and oligonucleotides used in this work.

DNA manipulations and mutant construction. Unless otherwise indicated, all DNA procedures followed standard protocols and specific recommendations from manufacturers. Fermentative D-lactate dehydrogenase (*ldhA*) mutant derivatives were constructed by P1 transduction (37) of the Δ*ldhA*::*kan* allele from *E. coli* ALS786 into different recipient strains. *E. coli* K24KL was constructed by allelic replacement (11) using a PCR fragment obtained with primers Δ*ldhA*-F and Δ*ldhA*-R. One Km^r isolate was selected, checked by PCR for *kan* insertion, and transformed with plasmid pCP20, encoding the *Saccharomyces cerevisiae* FLP recombinase (7). Km^s transformants were selected at 42°C, and *kan* excision and *ldhA* deletion were confirmed by PCR and DNA sequencing (data not shown).

Growth media and culture conditions. MYA medium contained 6.0 g · liter^{-1} Na_2HPO_4, 3.0 g · liter^{-1} KH_2PO_4, 1.4 g · liter^{-1} $(NH_4)_2SO_4$, 0.5 g · liter^{-1} NaCl, 0.2 g · liter^{-1} $MgSO_4 \cdot 7H_2O$, 10 g · liter^{-1} yeast extract, 5 g · liter^{-1} casein amino acids (Diagnostic Systems, Sparks, MD), and 30 g · liter^{-1} glycerol (Anedra, Buenos Aires, Argentina). Concentrations of antibiotics were 100 μg · ml^{-1} for ampicillin and 50 μg · ml^{-1} for kanamycin. Magnesium sulfate, antibiotics, and glycerol were added separately as filter-sterilized concentrated solutions after autoclaving and cooling the medium. Solid media also contained 30 g · liter^{-1} agar. Seed cultures for all experiments were prepared by dispersing a loopful of cells from a fresh LB plate into 50 ml of MYA medium in a 250-ml Erlenmeyer flask. Cultures were incubated in a rotary shaker overnight at 37°C and 250 rpm and used to inoculate the bioreactor at an initial cell dry weight (CDW) of approximately 0.05 g · liter^{-1}. Aerobic shaken-flask cultures were grown at 37°C in 250-ml Erlenmeyer flasks containing 25 ml of MYA medium and shaken at 250 rpm.

Bioreactor cultivation. 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). Batch cultures were grown in a 3-liter working volume, with agitation set at 500 rpm. The fermentor was sparged with 3 liter · min^{-1} of filter-sterilized air, and the pH was 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 · liter^{-1} Antifoam 289 (Sigma-Aldrich, St. Louis, MO) was manually added at the onset of each run. Dissolved oxygen was measured using an Ag/AgCl polarometric oxygen probe (Mettler Toledo, Greifensee, Switzerland). Fed-batch cultures were developed under conditions similar to those described for batch cultures, except that the stirrer speed was automatically adjusted up to 1,000 rpm to keep the dissolved oxygen level above 40% of air saturation. Glycerol concentration was maintained above 5 g · liter^{-1} by periodic addition of a feeding solution consisting of 500 g · liter^{-1} glycerol, 40 g · liter^{-1} casein amino acids, and 4 g · liter^{-1} $MgSO_4$.

Analytical procedures. Biomass concentration was determined as the CDW of washed pellets dried in 15-ml polypropylene centrifuge tubes at 80°C for at least 36 h. Dried samples were allowed to cool and held *in vacuo* until weighed. PHB content was determined gravimetrically after alkaline treatment of the biomass with 0.2 N NaOH (8) or by gas chromatography after methyl esterification (5). Extracellular metabolic products were determined by high-pressure liquid chromatography (HPLC) (13), and residual glycerol concentration was obtained by using a colorimetric assay (27). Intracellular NADH, NADPH, NAD^+, and $NADP^+$ contents were estimated by using *in vitro* procedures based on rapid inactivation of the metabolism of growing cells followed by acid or alkaline nucleotide extraction. Nucleotide determination was conducted by means of spectrophotometric cycling assays with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide as the final electron acceptor (3, 27).

RESULTS AND DISCUSSION

***ldhA* mutants have increased PHB synthesis from glycerol.** Growth and polymer synthesis in several PHB-producing isogenic *E. coli* strains were analyzed using glycerol. For this purpose, strain TA3522 and its *pta* variant, TA3514, were used, and *ldhA* derivatives of these strains (TA3522L and TA3514L, respectively) were constructed by P1 transduction (Table 1). Plasmid pJP24, carrying the *phaBAC* genes from *Azotobacter* sp. strain FA8 under the control of a T5 promoter/*lac* operator element (23), was introduced into all the strains. Growth and PHB accumulation in 24-h aerobic shaken-flask cultures of all plasmid-bearing isogenic strains growing on MYA medium supplemented with 30 g · liter^{-1} glycerol were analyzed. All mutants grew slightly less but accumulated more PHB than the *pta*$^+$ *ldhA*$^+$ strain. While TA3522/pJP24 reached 6.0 g · liter^{-1} and accumulated PHB up to 30% ± 1% (wt/wt), strain TA3514/pJP24 (*pta*) grew to 4.8 g · liter^{-1} and accumulated PHB up to 37% ± 1% (wt/wt), TA3514L/pJP24 (*pta ldhA* double mutant) grew to 4.6 g · liter^{-1} and accumulated 39% ± 1% (wt/wt) of PHB, and strain TA3522L/pJP24 (*ldhA*) grew to 4.9 g · liter^{-1} and accumulated the largest amount of polymer, 44% ± 1% (wt/wt). In order to investigate the effect of *ldhA* in a genetic background more suitable for the synthesis of bioproducts, K24LT, a derivative of strain K24, a prototrophic strain that we have used in previous work, was constructed by P1 transduction (Table 1). In 24-h bioreactor batch cultures, strain K24LT grew to a 1.5-fold-higher level than K24 (17.9 and 11.6 g · liter^{-1}, respectively) and produced more PHB (10.2 and 1.8 g · liter^{-1}, respectively), reaching a PHB content of 57% ± 3% (wt/wt).

Previous experience indicates that strains obtained through transduction may contain unexpected mutations that can affect the phenotype of the mutants, masking the effect of the studied alleles (25). In order to have an appropriate *ldhA* derivative, K24KL, a precise deletion derivative of K24K, was constructed by allelic replacement. The construction of a markerless *ldhA* mutant enabled the use of pJP24K, a Km^r variant of plasmid pJP24 with enhanced segregational stability (23) (Table 1).

***ldhA* affects growth and distribution of metabolic products in the recombinants.** In shaken-flask cultures, strain K24KL accumulated 1.4-fold more polymer than K24K (44% ± 1% versus 32% ± 2% [wt/wt], respectively) and it also grew more (Fig. 1A and B). These results were similar to those obtained in microaerobic cultures grown on glucose using a multiple-fermentation-pathway mutant according to a recent report (17). In order to further analyze changes in carbon distribution, major metabolic products were measured in the cultures. Levels of acetate, formate, and ethanol production were higher for K24KL than for K24K (1.2-, 1.6-, and 2.1-fold, respectively) at 24 h (Fig. 1C and D). The presence of these metabolites reflects a limitation in oxygen availability that prompted the accumulation of fermentation products, also observed in bioreactor cultures. Ethanol and PHB production started earlier in the *ldhA* mutant. As expected, lactate was produced only by K24K, and its production peaked at 10 h. The increases in acetate and formate observed in the *ldhA* derivative compared to the parental strain were proportional to that of biomass, but the increases in ethanol and PHB accumulation were much higher. This can be due to the elimination of lactate synthesis, resulting in a higher availability of reducing power and of acetyl-CoA, which can be redirected toward the synthesis of biomass and other metabolites. The intracellular contents of NADH, NADPH, NAD$^+$, and NADP$^+$ were determined at several times. The redox ratio of the cells (measured as the ratio [NADH + NADPH]/[NAD$^+$ + NADP$^+$]) was, as expected, much more reduced in the *ldhA* strain (Fig. 1A and B) than in the wild-type strain, favoring the production of reduced metabolites, such as ethanol and PHB.

To obtain a higher biomass concentration and more-controlled conditions, both strains were grown in 24-h bioreactor batch cultures. The *ldhA* derivative grew more, as observed in the shaken-flask cultures, but the specific growth rates were similar (Table 2). K24KL had increased amounts of all measured metabolites, except formate, perhaps as a result of increased flux of carbon toward acetyl-CoA and higher oxygen consumption than K24K. The increased availability of both carbon and reducing equivalents had the greatest effect on ethanol and PHB. This was reflected in the high ethanol-to-acetate ratio obtained for K24KL, which was twice the value obtained for K24K, indicating a reduced internal state, as verified by the redox ratio. Taking into account the specific production rates, PHB synthesis was the most affected, as a 1.7-fold increase was observed for K24KL compared to K24K (Table 2). The final PHB concentration for K24KL in the 24-h bioreactor batch culture was 6.1 g · liter^{-1}.

K24KL has high PHB and ethanol synthesis from glycerol in fed-batch cultures. Based on results obtained in the batch bioreactor experiments, fed-batch fermentations were performed to further increase biomass and PHB content to evaluate *E. coli* K24KL as a potential biocatalyst for PHB synthesis. Biomass and PHB concentration increased almost linearly during the whole cultivation period (Fig. 2A), attaining 41.9 and 26.4 g · liter^{-1} at 60 h, respectively. PHB accumulation increased 1.4-fold compared with that for batch cultures (Table 2) and was similar to that obtained in fed-batch cultures with glucose of an *E. coli* strain carrying the *phaCAB* genes from *Cupriavidus necator* and overexpressing a NAD kinase (20). The final PHB content achieved was 63% ± 1% (wt/wt), with a volumetric productivity of 0.45 ± 0.08 g · liter^{-1} · h^{-1}. This polymer content ranks among the highest reported in the literature with glycerol as the carbon source, second only to that reached by *Zobellella denitrificans* MW1 in 50-h fed-batch cultures (66.9% ± 7.6% [wt/wt]) (16). The PHB-specific production rate (164 ± 9 μmol · g cell dry weight [CDW]$^{-1}$ · h^{-1}) did not vary significantly compared to that for batch cultures (Table 2), and PHB yield on a carbon substrate at 60 h was 0.41 ± 0.03 g · g^{-1}, the highest reported when using glycerol as the carbon source. This value comes close to that reported by Mothes et al. (22) for nitrogen-limited fed-batch cultures of *C. necator* DSMZ 4058 (0.37 g · g^{-1}).

Major metabolic products besides PHB were measured throughout the experiment, and their accumulation kinetics (expressed as g · g CDW^{-1}) were analyzed (Fig. 2B). Interestingly, PHB content and ethanol accumulation followed similar linear patterns up to 36 h. This behavior can be explained by considering that both metabolites share acetyl-CoA as a precursor and both pathways use NAD(P)H as a cofactor. In line with this hypothesis, the redox ratio changed significantly over

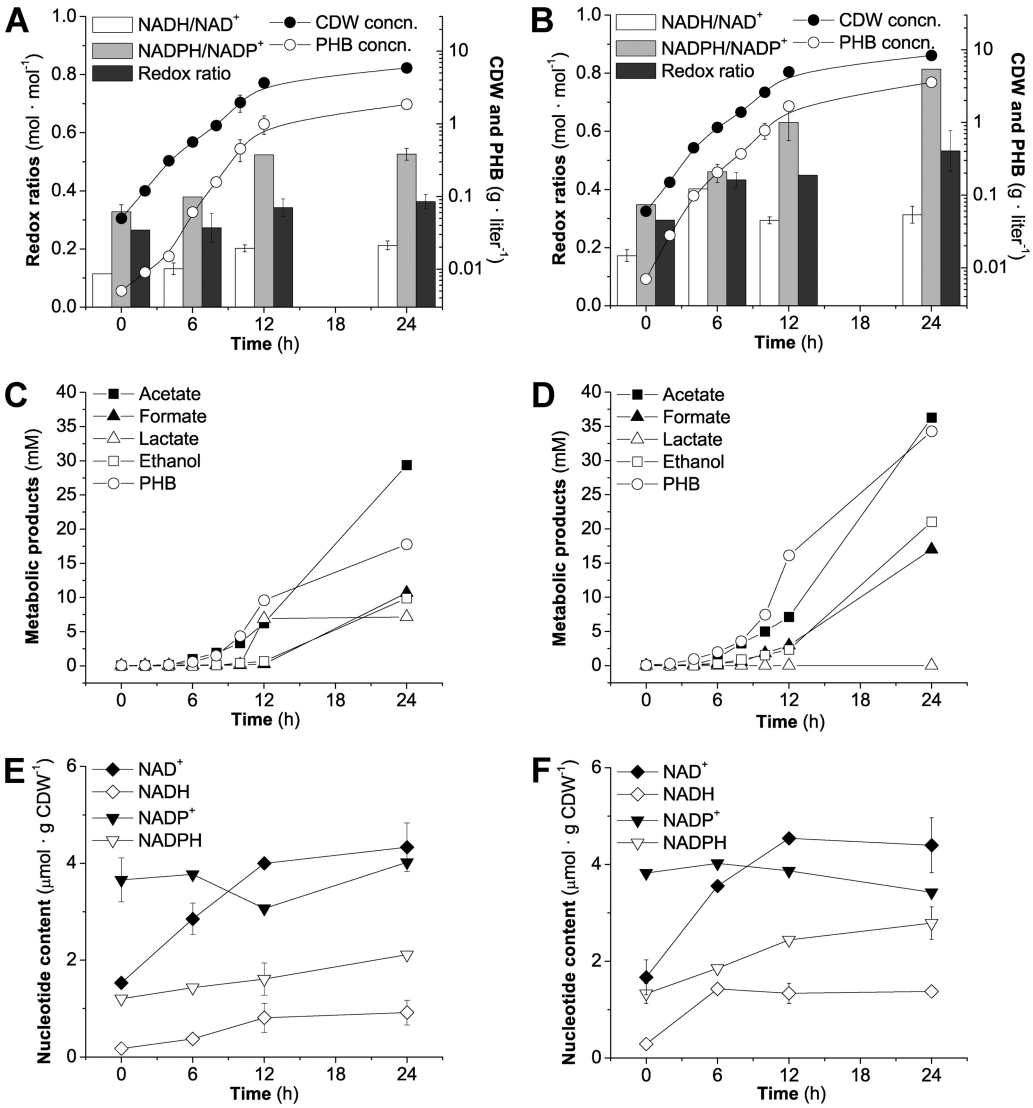

FIG. 1. Physiological characterization of *E. coli* K24K (A, C, and E) and K24KL (its *ldhA* derivative) (B, D, and F) in shaken-flask cultures in MYA medium supplemented with 30 g · liter^{-1} glycerol under aerobic conditions. (A and B) Growth, polymer synthesis, and redox ratios; (C and D) time course plots for the synthesis of relevant bioproducts; (E and F) intracellular content of reduced and oxidized nucleotides. The redox ratio was defined as the ratio of reduced to oxidized cofactors ([NADH + NADPH]/[NAD$^+$ + NADP$^+$]). Symbols and bars represent the mean values for each parameter ± standard deviations of duplicate measurements from at least three independent cultures. CDW, cell dry weight; concn, concentration.

this period, increasing from 0.25 ± 0.02 to 0.64 ± 0.03 mol · mol^{-1} (Fig. 2B). Acetate accumulation rose sharply during the first 12 h, perhaps as a consequence of the aerobic activation of the Pta-AckA pathway (composed of phosphotransacetylase and acetate kinase), observed when excess carbon source is present (9), a phenomenon also detected in glycerol cultures of ethanologenic *E. coli* strains (28). After 36 h, ethanol and acetate accumulation decreased while PHB content kept increasing, albeit at a lower rate. As these three metabolites compete at the acetyl-CoA branching point, the observed distribution could be accounted for by an increased flux of acetyl-CoA toward the overexpressed reducing-power-consuming PHB synthesis pathway, limiting its availability for the native Pta-AckA and AdhE pathways. An analogous effect was observed in experiments performed using a recombinant *E. coli* redox mutant, in which the activity of a heterologous plasmid-encoded alcohol dehydrogenase was observed to increase in glycerol cultures, probably as a strategy to maintain redox balance (28). Furthermore, this metabolic effect was more evident in an *ldhA* mutant.

In accordance with the expected funneling of carbon skeletons into reduced metabolites in strain K24KL, we observed a final ethanol-to-acetate ratio of 1.31 ± 0.07 mol · mol^{-1}, also reflected in the final redox ratio (0.76 ± 0.05 mol · mol^{-1}) at 60 h. Formate accumulation was the lowest among the measured extracellular products, and its value remained almost constant during the cultivation, even though a slight decrease was observed after 36 h, similar to results for acetate and ethanol. As expected, lactate was not detected in supernatants throughout the experiment.

TABLE 2. Fermentation parameters[a] for 24-h bioreactor batch cultures conducted under aerobic conditions in MYA medium with 30 g · liter^{-1} glycerol as the carbon source

Parameter	Value for *E. coli* strain:	
	K24K	K24KL
μ_{max}[e] (h^{-1})	0.64 ± 0.03	0.67 ± 0.02
Final CDW[b] concn (g · liter^{-1})	10.7 ± 0.8	13.9 ± 0.7
Final PHB content (% [wt/wt])	36 ± 2	44 ± 1
Specific consumption rate (mmol · g CDW^{-1} · h^{-1}) for:		
Glycerol	1.8 ± 0.5	1.6 ± 0.3
O_2	0.085 ± 0.009	0.113 ± 0.006
Specific production rate (μmol · g CDW^{-1} · h^{-1}) for:		
Acetate	52 ± 3	61 ± 2
Formate	77 ± 4	63 ± 5
Lactate	48 ± 5	ND[c]
Ethanol	149 ± 1	205 ± 1
PHB	103 ± 2	178 ± 3
Ethanol-to-acetate ratio (mol · mol^{-1})	0.36 ± 0.08	0.64 ± 0.02
Redox ratio[d] ([NADH + NADPH]/[NAD$^+$ + NADP$^+$])	0.34 ± 0.03	0.62 ± 0.04

[a] Maximum specific growth rates, as well as specific consumption and production rates, were calculated during exponential growth. Results represent the mean values ± standard deviations for duplicated measurements from at least two independent experiments.
[b] CDW, cell dry weight.
[c] ND, not detected.
[d] Nucleotide content was enzymatically determined at 24 h.
[e] μ_{max}, maximum specific growth rate.

Cofactor distribution is altered in the *ldhA* mutant. It has been proposed that PHB functions as an electron sink in natural producers (21) and promotes growth of *E. coli* redox mutants by consuming excess reducing power (30). Previous cofactor manipulations aimed at optimizing PHB synthesis from glucose in recombinant *E. coli* include the use of mutants that favor the use of the pentose phosphate pathway for catabolism, producing increased amounts of NADPH (18, 35). Transhydrogenases (32) and a NAD kinase (20) have also been manipulated to enhance PHB synthesis from glucose. On the other hand, increased PHB accumulation from glucose and glycerol has been observed in redox mutants defective in *arcA* (24, 26). In spite of the fact that PHB synthesis and the internal redox state of the cells are known to be associated, very few studies have analyzed the levels of the different cofactors in bacteria producing PHB (20, 32) and none used glycerol as a substrate. The distribution of reduced and oxidized cofactors in K24K and its *ldhA* derivative was studied to investigate possible relationships between cofactor distribution and metabolite production in PHB-synthesizing *E. coli* when grown on glycerol.

When the ratios of reduced to oxidized cofactors for K24K and K24KL were compared, it was found that values were higher for the *ldhA* derivative, as expected (Fig. 1A and B and 2B; Table 2). Furthermore, the highest ratio of reduced to oxidized cofactors observed for the *ldhA* derivative was due to differences in NADPH/NADP$^+$ ratios, which were higher than those observed for K24K. For both strains, the NADPH/NADP$^+$ ratios were higher than the NADH/NAD$^+$ ratios throughout growth in the shaken-flask experiments (Fig. 1A and B). This trend was also observed in the fed-batch (Fig. 2C) and in the batch fermentation experiments, in which the final NADPH/NADP$^+$ ratios were 0.55 and 1.13 for K24K and K24KL, respectively; while the NADH/NAD$^+$ ratios were 0.17 and 0.28 for K24K and K24KL, respectively. This is surprising, as studies of the enzymes involved in PHB synthesis indicate that NADPH is the preferred cofactor for the reductase (34). However, previous work using shaken-flask cultures with glucose have reported similar results for other PHB-synthesizing *E. coli* strains (32).

The relative amounts of the oxidized forms of both cofactors varied, gradually changing from a higher proportion of

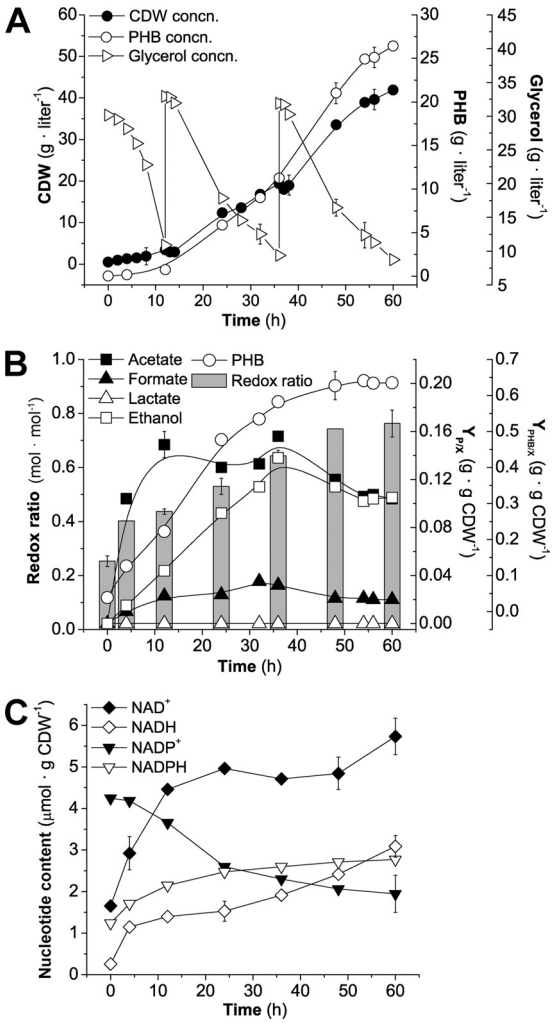

FIG. 2. Fed-batch bioreactor culture of *E. coli* K24KL. (A) Time course plots for glycerol, as well as biomass and polymer concentrations; (B) metabolic profile (expressed as the PHB content [$Y_{PHB/X}$] and metabolic product yield on biomass [$Y_{P/X}$]) and intracellular redox state; (C) intracellular contents of reduced and oxidized nucleotides. The redox ratio was defined as the ratio of reduced to oxidized cofactors ([NADH + NADPH]/[NAD$^+$ + NADP$^+$]). Symbols and bars represent the mean values for each parameter ± standard deviations of duplicate measurements from at least two independent cultures.

$NADP^+$ to a higher proportion of NAD^+ as growth and metabolite synthesis proceeded. The change from a larger amount of $NADP^+$ to a larger amount of NAD^+ occurred between 5 and 12 h of growth in shaken-flask cultures, so that NAD^+ was more abundant than $NADP^+$ during the stationary phase (Fig. 1E and F). A similar trend was observed in fed-batch cultures, in which $NADP^+$ levels continued decreasing, while the amounts of NAD^+ increased (Fig. 2C). In shaken-flask cultures, the amounts of NADPH increased with time, while NADH increased during the first hours of growth and then remained approximately constant (Fig. 1E and F). In contrast, NADH increased throughout growth in fed-batch cultures of strain K24KL, probably as a consequence of repeated substrate addition (Fig. 2).

The amount of NADPH was higher than that of NADH at all times in shaken-flask experiments for both PHB-synthesizing recombinants (K24K and K24KL), and until the last hours of growth (50 h) for the K24KL fed-batch culture. Previous work with other PHB-accumulating recombinant *E. coli* strains reported a higher level of NADH than of NADPH in similar conditions using glucose (32). These dissimilarities could be due to differences in recombinant strain and/or growth conditions. A recent report showed that, under conditions in which NADH contents were reduced, *E. coli* produced significantly more NADPH (15). Accordingly, the high rate of NADH consumption due to increased production of reduced metabolites in K24K and K24KL could account for the augmented NADPH content observed. One of the mechanisms used by *E. coli* to control cofactor balance involves transhydrogenases, encoded by *pntAB* and *udhA*, that interconvert NADPH and NADH and are differentially activated in different growth conditions (33). In a previous study, cultures grown on glycerol were observed to have high expression levels of *pntAB*, encoding the enzyme that catalyzes the formation of NADPH from NADH (33). Glycerol as a substrate yields more reducing equivalents than glucose, reportedly in the form of NADH; NADPH in these cultures is believed to be produced mainly through the isocitrate dehydrogenase step of the tricarboxylic acid cycle (39), so cultures grown on glycerol are thought to have an NADPH deficiency. However, based on the ability of *pntAB* mutants to grow on glycerol, it has been suggested that there are metabolic adaptations that enable the cells to obtain sufficient NADPH under these conditions (33). Additionally, the synthesis of PHB introduces profound changes in the central carbon metabolic pathways of the recombinants that could lead to the cofactor distribution observed.

E. coli adjusts its metabolism to optimize cell growth in each environmental condition by using different combinations of metabolic pathways. There is an intimate association between carbon and electron flow, as carbon will be directed toward the synthesis of more-reduced or more-oxidized metabolic products according to intracellular redox conditions (4, 31, 41). In the *ldhA* mutant used in this work, the increased availability of carbon and reducing power affected the distribution of cofactors and carbon partitioning among the metabolic products and resulted in a significant increase of PHB and ethanol synthesis. This work illustrates how *E. coli* metabolism can be manipulated to enhance the production of biotechnologically relevant products from a cheap and readily available substrate.

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