Downloaded from orbit.dtu.dk on: Feb 07, 2025

DTU Library

New recombinant Escherichia coli strain tailored for the production of poly(3-hydroxybutyrate) from agroindustrial by-products

Nikel, Pablo Ivan; De Almeida, Alejandra; Melillo, Evelia C.; Galvagno, Miguel A.; Pettinari, M. Julia

Published in:
Applied and Environmental Microbiology

Link to article, DOI:
10.1128/AEM.00044-06

Publication date:
2006

Document Version
Publisher's PDF, also known as Version of record

Link back to DTU Orbit

Citation (APA):
Nikel, P. I., De Almeida, A., Melillo, E. C., Galvagno, M. A., & Pettinari, M. J. (2006). New recombinant *Escherichia coli* strain tailored for the production of poly(3-hydroxybutyrate) from agroindustrial by-products. *Applied and Environmental Microbiology*, *72*(6), 3949-3954. https://doi.org/10.1128/AEM.00044-06

General rights
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 2006, p. 3949–3954 Vol. 72, No. 6
0099-2240/06/$08.00+0 doi:10.1128/AEM.00044-06
Copyright © 2006, American Society for Microbiology. All Rights Reserved.

New Recombinant *Escherichia coli* Strain Tailored for the Production of Poly(3-Hydroxybutyrate) from Agroindustrial By-Products

Pablo I. Nikel,[1,2] Alejandra de Almeida,[1] Evelia C. Melillo,[1] Miguel A. Galvagno,[2,3] and M. Julia Pettinari[1]*

Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires,[1] Instituto de Investigaciones Biotecnológicas, Universidad Nacional de General San Martín,[2] and Departamento de Ingeniería Química, Facultad de Ingeniería, Universidad de Buenos Aires,[3] Buenos Aires, Argentina

Received 8 January 2006/Accepted 22 March 2006

A recombinant *E. coli* strain (K24K) was constructed and evaluated for poly(3-hydroxybutyrate) (PHB) production from whey and corn steep liquor as main carbon and nitrogen sources. This strain bears the *pha* biosynthetic genes from *Azotobacter* sp. strain FA8 expressed from a T5 promoter under the control of the lactose operator. K24K does not produce the lactose repressor, ensuring constitutive expression of genes involved in lactose transport and utilization. PHB was efficiently produced by the recombinant strain grown aerobically in fed-batch cultures in a laboratory scale bioreactor on a semisynthetic medium supplemented with the agroindustrial by-products. After 24 h, cells accumulated PHB to 72.9% of their cell dry weight, reaching a volumetric productivity of 2.13 g PHB per liter per hour. Physical analysis of PHB recovered from the recombinants showed that its molecular weight was similar to that of PHB produced by *Azotobacter* sp. strain FA8 and higher than that of the polymer from *Cupriavidus necator* and that its glass transition temperature was approximately 20°C higher than those of PHBs from the natural producer strains.

Polyhydroxyalkanoates (PHAs) are a group of polyesters produced by a large number of bacteria, which accumulate them in intracellular granules as a response to environmental stress and nutrient imbalance (7, 10). These thermoplastics have properties that vary according to their monomer compositions. A copolymer of 3-hydroxybutyrate and 3-hydroxyvalerate was commercialized in the 1980s, but high production costs have hindered the use of PHAs as commodity plastics, since their final price is considerably higher than that of petrochemical-based synthetic plastic materials (16). Growing concern about environmental pollution has renewed interest in the development of PHAs, which are totally biodegraded by microorganisms present in most environments (11). Also, these polymers can be produced from different renewable carbon sources (11).

Poly(3-hydroxybutyrate) (PHB) production costs can be reduced by several means, including the use of cheap substrates, such as whey, favored in countries with important dairy industries, or the enhancement of product yield, e.g., by using recombinant *Escherichia coli* (11, 18). *E. coli* is a suitable host as a heterologous expression background for foreign genes that can be easily manipulated and improved by means of recombinant DNA methodologies. Also, high-cell-density cultivation strategies for numerous *E. coli* strains are well established (15, 32). *E. coli* cells that accumulate large amounts of PHB become fragile, facilitating the isolation and purification of the biopolymer, and the bacterium does not express PHA-degrading enzymes (2).

PHB is the best known PHA and has been studied most often as a model product in the development of fermentation strategies. In the majority of PHB-accumulating species, it is synthesized in three sequential enzymatic steps: a 3-ketothiolase condenses two acetyl-coenzyme A (CoA) moieties to form acetoacetyl-CoA; a NADPH-dependent acetoacetyl-CoA reductase catalyzes the stereoselective reduction of acetoacetyl-CoA to D-(−)-3-hydroxybutyryl–CoA; and a PHB synthase links the 3-hydroxybutyryl–CoA monomers to the growing PHB chain by an ester bond (35).

Azotobacter sp. strain FA8 is a gram-negative, aerobic, nitrogen-fixing bacterium that accumulates PHB from several carbon sources (25), but it is not suitable for use in an industrial process, as it cannot be grown at sufficiently high densities. Also, it accumulates by-products, such as exopolysaccharides, that complicate oxygen transfer in the bioreactor and PHB extraction processes. The identification, cloning, and molecular analysis of the *pha* gene cluster of *Azotobacter* sp. strain FA8, coding for proteins involved in PHB synthesis, have been reported in earlier work (23, 24).

Genes responsible for PHB biosynthesis (*pha* or *phb* genes) from a number of microorganisms, such as *Cupriavidus necator*, formerly called *Alcaligenes eutrophus* (30, 36); *Pseudomonas aeruginosa* (14); *Alcaligenes latus* (6); *Thiocapsa pfennigii* (17); and *Streptomyces aureofaciens* (19), have been introduced into *E. coli*. In most cases, the biosynthetic genes were expressed under the control of their native promoters, and the resulting recombinants were able to accumulate PHA from different carbon sources.

In this paper, we present a recombinant *E. coli* strain that contains the *pha* biosynthetic genes from an *Azotobacter* species, specially designed for the production of PHB from milk whey. PHB was efficiently produced by the recombinant strain, and the polymer obtained was physically characterized.

* Corresponding author. Mailing address: Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Ciudad Universitaria—Pabellón 2, 1428 Buenos Aires, Argentina. Phone: 54-11-4576-3334. Fax: 54-11-4576-3342. E-mail: jul@qb.fcen.uba.ar.

Downloaded from http://aem.asm.org/ on June 13, 2017 by DTU Library

TABLE 1. Bacterial strains, plasmids, and primers

Strain, plasmid, or primer	Relevant characteristics	Reference or source
E. coli strains		
DH5α	F$^-$ λ$^-$ *endA1 hsdR17 hsdM*$^+$ *supE44 thi-1 recA1 gyrA96 relA1* Δ(*argF lacZYA*)*U169* ϕ*80d* Δ(*lacZ*)*M15*	Life Technologies
S17-1	*recA1 pro thi*; has the *tra* genes from plasmid RP4 integrated in the chromosome	33
K1060	F$^-$ *fadE62 lacI60 tyrT58*(AS) *fabB5 mel-1 supF58*	*E. coli* Genetic Stock Center
T1GP	*lacI203 fadR16 tyrT58*(AS) *atoC12 metE192 rha-11 cls-1 ilv-692*	*E. coli* Genetic Stock Center
DSM 4509	F$^-$ *thr-1 araC14 leuB6*(Am) Δ(*gpt-proA*)*62 lacY1 tsx-33 supE44*(AS) *galK2*(Oc) *hisG4*(Oc) *rfbD1 mgl-51 rpoS396*(Am) *rpsL31*(Str^r) *kdgK51 xylA5 mtl-1 argE3*(Oc) *thi-1 dam*	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
K24	As K1060; carrying pJP24	This work
K24K	As K1060; carrying pJP24K	This work
C. necator PHB-4	PHA-negative mutant of wild-type H16	29
Plasmids		
pRAC1	Cosmid pVK102 containing a 25-kb XhoI fragment containing the *pha* genetic region from *Azotobacter* sp. strain FA8	23
pRK404	Tc^r; IncP broad-host-range vector	8
pRH15	pRK404 carrying a 7.5-kb HindIII DNA fragment from *Azotobacter* sp. strain FA8 containing genes *phaR*, *ISAzsp1*, *phaB*, *phaA*, and *phaC*	This work
pBH15	pBluescript (Stratagene, La Jolla, CA) with the same insert as pRH15	This work
pGEM-T Easy	A/T cloning vector; Ap^r	Promega
pUC4K	pUC derivative; Ap^r Km^r	Pharmacia Biotech
pQE32	Expression vector; Ap^r	QIAGEN
pJP24	pQE32 derivative carrying a 3.9-kb BamHI-HindIII insert containing the *phaBAC* genes from *Azotobacter* sp. strain FA8; Ap^r	This work
pJP24K	pJP24 derivative; Ap^r Km^r	This work
pTZ18u-PHB	Plasmid carrying the three *phb* structural genes from *C. necator* (*phbCAB*)	26
Primers		
BACUp[a,b]	5′-CCG ACA AAA GGA AGG GAg ATC T**AT G**AC-3′	This work
BACLow[b,c]	5′-TTA AGC AAa AGC TtC TCA ACC CTT TAC GTA-3′	This work
KmUp[d]	5′-GCC TCC GGA CCT CAA GTC AGC-3′	This work
KmLow[d]	5′-TTC TCC GGA GTT ACA TTG CAC AAG-3′	This work

[a] Restriction site for BglII is underlined; initiation codon is in boldface.
[b] Letters in lowercase differ from the original gene sequence.
[c] Restriction site for HindIII is underlined.
[d] Restriction site for BspEI is underlined.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains, plasmids, and primers used in this work are summarized in Table 1.

DNA manipulations. Plasmid and genomic DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, and DNA ligations were performed by standard procedures (27) and following specific instructions from the manufacturers. Restriction enzymes were purchased from Promega (Madison, WI). Transformations were carried out as previously described (10). *E. coli* DH5α was routinely used as a host for most of the recombinant plasmids. *E. coli* S17-1 was used as a donor for the conjugative transfer of mobilizable plasmids. For amplification and maintenance of plasmids that were cut with *dam*-sensitive restriction endonucleases, *E. coli* DSM 4509 was used.

Construction of the *pha* expression vector. Primers BACUp and BACLow (Table 1) were used to obtain a 3,975-bp amplification fragment using genomic DNA from *Azotobacter* sp. strain FA8 as a template. This fragment was cut with HindIII and BglII and ligated to vector pQE32 cut with HindIII and BamHI, and the ligation mixture was used to transform *E. coli* DH5α competent cells using standard procedures. The resulting plasmid, pJP24, confers resistance to ampicillin and contains the genes *phaBAC* expressed from a promoter operator element consisting of the phage T5 promoter and two *lac* operator sequences. The insert is cloned in such a way that the amino-terminal end of PhaB is fused to the first six amino acids of LacZ, followed by six histidine residues, resulting in a fusion protein.

Introduction of kanamycin resistance in pJP24. In an attempt to enhance plasmid stability, resistance to kanamycin was added to pJP24. A 1.0-kbp fragment conferring kanamycin resistance was generated by PCR using plasmid pUC4K as a template. Oligonucleotides KmUp and KmLow (Table 1) were used, incorporating a BspEI restriction site at the 5′ ends of both primers. The PCR product and pQE32 were digested with BspEI and ligated, resulting in pQKm. pQKm was digested with HindIII (partial digestion) and XbaI (total digestion) to obtain a 1.9-kb fragment, which was ligated into pJP24 cut with the same enzymes, resulting in the ampicillin- and kanamycin-resistant plasmid pJP24K.

Culture conditions. For DNA manipulations and strain construction, cultures were grown at 37°C with reciprocal agitation (130 strokes · min^{-1}) in LB. For antibiotic selection, the concentrations of antibiotics were 100 μg · ml^{-1} for ampicillin and 50 μg · ml^{-1} for kanamycin. When necessary, media were solidified by the addition of 15 g · liter^{-1} agar. Cells were maintained as 20% (vol/vol) glycerol stocks at −20°C after growing overnight in LB. For shaken-flask experiments, either M9 minimal medium or LB (27), supplemented with the carbon sources indicated in the text, were used. When needed, 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma Chemical Co., St. Louis, Mo.) and 40 μg · ml^{-1} of required amino acids were added.

Seed cultures for bioreactor experiments were prepared by inoculating 500 μl of the bacterial glycerol stock into 50 ml of a semidefined medium (OM) with whey as the carbon source (21) in 250-ml Erlenmeyer flasks. The cultures were incubated in a rotary shaker overnight at 37°C and 250 rpm.

Downloaded from http://aem.asm.org/ on June 13, 2017 by DTU Library

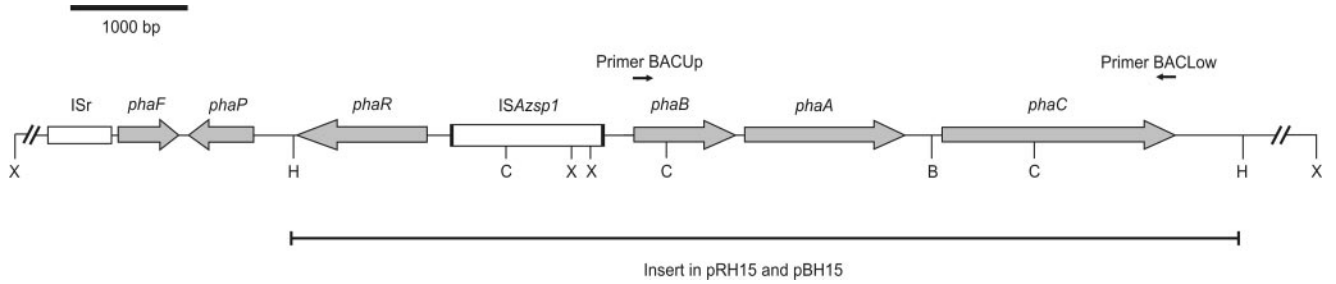

FIG. 1. Organization of the *Azotobacter* sp. strain FA8 genomic region containing the *pha* structural genes, *phaB*, *phaA*, and *phaC*, and genes *phaR*, *phaP*, and *phaF* flanked by IS*Azsp1* and IS*r*. Relevant restriction sites are indicated with capital letters (C, ClaI; H, HindIII; X, XhoI). The small black arrows indicate the positions of primers used for PCR amplification. The thick gray arrows indicate the orientation of transcription, and the white boxes indicate insertion sequences. The black line at the bottom of the picture represents the region cloned in pRH15 and pBH15.

Fed-batch fermentations. Fed-batch cultures were carried out at 37°C in a 5.6-liter fermentor (BioFlo110; New Brunswick Scientific Co., Edison, NJ) with a starting volume of 2.0 liters OM containing 100 $\mu g \cdot ml^{-1}$ ampicillin (for K24) or 50 $\mu g \cdot ml^{-1}$ kanamycin (for K24K). In the first stage of the cultivation, pH was controlled at 7.20 by automatic addition of 3 N KOH. The dissolved-oxygen concentration was maintained above 30% air saturation by automatic control of the agitation speed. Antifoam (0.02% [vol/vol] Antifoam 289; Sigma) was added at the onset of cultivation. The feeding solution used for fed-batch cultures was a concentrated and deproteinated whey solution containing 25% (wt/vol) lactose prepared as described by Ahn et al. (1). The pH-stat feeding strategy was employed. When the pH rose to a value higher than its set point (7.20) by 0.15 U due to carbon source depletion, an appropriate volume of feeding solution was added (up to 800 ml). Samples for biomass and PHB production and plasmid stability determination were withdrawn every 4 h.

Determination of plasmid stability. The number of plasmid-containing cells was determined by plating samples from the bioreactor cultures on LB plates with or without the appropriate antibiotics. Plasmid stability was recorded as the percentage of ampicillin- or kanamycin-resistant cells.

Biomass determination. The cell concentration, defined as cell dry weight (CDW) (in grams) per liter of culture broth, was determined by placing 10-ml samples into a previously dried and weighed 15-ml centrifuge tube. After centrifugation at 10,000 × *g* for 10 min at 4°C, the cell pellet was washed twice with deionized water. After the supernatant was decanted, the centrifuge tube was dried in an oven at 85°C for 36 h and then cooled in a desiccator and weighed.

Analysis of PHB production. For qualitative detection of PHB inclusion bodies, cells were observed by fluorescence microscopy after being stained with the basic oxazine Nile Blue A (22). The PHB content was quantitatively determined by gas chromatography using the method described by Braunegg et al. (5) modified as previously described (20). Pure PHB from *C. necator* was used as a standard. The PHB concentration was defined as g polymer per liter of culture broth. PHB content was defined as the PHB percentage of CDW.

Purification of PHB. PHB produced in bioreactor cultures was extracted from lyophilized cells with hot $CHCl_3$ using a Soxhlet apparatus, ethanol precipitated, and recovered by filtration. The precipitate was dried, dissolved in $CHCl_3$, filtered to remove particles, and dried on a glass petri dish to obtain a thin film. The resulting polymer was characterized by gas chromatography as described above.

DSC measurements. The glass transition temperature (T_g), melting temperature (T_m), and crystallinity of purified PHB were determined by differential scanning calorimetry (DSC) using a Mettler 822 calorimeter and STARe Thermal Analysis System v. 6.1 software (Mettler Toledo AG, Switzerland). The instrument was calibrated using standard compounds of known T_m and fusion enthalpy (indium, zinc, and lead). Measurements were made with 10- to 20-mg sample dry mass at a scanning rate of $10°C \cdot min^{-1}$ using hermetically sealed aluminum pans of 40-μl inner volume (Mettler), using an empty pan as a reference. Scans were performed at 30 to 220°C, and the reported data are the averages of three determinations. The T_g was taken as the midpoint of the heating capacity change. The crystallinity of PHB was estimated from the enthalpy of fusion obtained by DSC. The fusion enthalpy of a theoretical 100% crystalline sample was asssumed to be $146 J \cdot g^{-1}$ (4).

Molecular weight determinations. The relative molecular weight (M_r) of PHB was calculated by measuring the intrinsic viscosity (η) of 500-μl aliquots of a 1% (wt/vol) PHB solution in $CHCl_3$ using a Brookfield viscosimeter model DV-II+ (Brookfield Engineering Laboratories, Stoughton, MA). η was converted to M_r according to the Mark-Houwink equation (3): $[\eta] = 1.18 \times 10^{-4} M_r^{0.78}$.

The physical properties of the polymer produced by *E. coli* recombinants were compared with those of PHB purified from *C. necator* grown in MM-fructose (30) and *Azotobacter* sp. strain FA8 grown in Burk's medium supplemented with 3% (wt/vol) glucose (24).

RESULTS AND DISCUSSION

Expression of the *Azotobacter* sp. strain FA8 *pha* genes in *E. coli*. Although PHB biosynthesis genes from various natural producers have been successfully cloned and expressed from their natural promoters in *E. coli* recombinants using several plasmid vectors (6, 14, 19, 30), we could not detect polymer formation in *E. coli* DH5α containing the *Azotobacter* sp. strain FA8 *pha* region using different DNA fragments and vectors. Cosmid pRAC1 (23) carries a 25-kb insert with the whole *pha* region from *Azotobacter* sp. strain FA8 (Fig. 1) containing the three *pha* structural genes with their upstream region, including *phaR*, a gene that has been found to activate *pha* genes in *Azotobacter vinelandii*; *phaP*, coding for a granule-associated protein; and *phaF*. This cosmid was able to complement polymerase mutations in *C. necator* PHB-4 (24) and *Pseudomonas putida* but was unable to promote synthesis of PHA in *E. coli* (data not shown). Complementation tests performed with plasmid pRH15, constructed by cloning a 7.5-kb HindIII fragment from pRAC1 containing genes *phaBAC* and *phaR* (Fig. 1) into pRK404 (8), gave similar results (data not shown).

In order to rule out the possibility that the lack of PHB accumulation was due to low gene dosage (pRK404 is a medium-copy-number vector), the same HindIII fragment was cloned in pBluescript. The insert in the resulting plasmid, pBH15, was checked by recloning it in pRK404 and testing its ability to complement the polymerase mutation in *C. necator* PHB-4. Plasmid pBH15 was transformed into *E. coli* DH5α, and the recombinants were grown in LB supplemented with 2% (wt/vol) glucose and tested for accumulation of PHB by microscopic observation of cells stained with Nile Blue A. No PHB was observed in cells containing pRH15 or pBH15, while the polymer was detected in cells containing plasmid pTZ18u-PHB (26), which carries the *C. necator phb* genes (data not shown). As the cloned genes were able to complement polymerase mutants, it was thought that *phaC* was being expressed in the recombinants, but probably *phaA* and *phaB* were not

Downloaded from http://aem.asm.org/ on June 13, 2017 by DTU Library

TABLE 2. PHB accumulation from lactose in different *E. coli* strains carrying plasmid pJP24[a]

Host strain	CDW (g · liter^{-1})	% PHB[b]
S17-1	0.61	2.3
T1GP	0.47	1.2
K1060	1.00	6.2

[a] Cells were grown overnight in 10 ml M9 medium supplemented with 3% (wt/vol) lactose, 100 μg · ml^{-1} ampicillin, and the amino acids required by the auxotrophic strains. PHB was measured by gas chromatography as described in Materials and Methods. The reported data are the averages of at least three determinations.

[b] The amount of PHB is given as an average weight percentage of the CDW.

being adequately expressed, as previous research performed using genes from other PHA-synthesizing bacteria has shown that PHA polymerases alone cannot promote PHB accumulation in *E. coli* (13).

In order to ensure the correct expression of the three *pha* structural genes, they were cloned in an expression vector (pQE32) using a strong promoter, under the control of the *lac* operator. This expression system was chosen because it had already been used to efficiently express several recombinant proteins in *E. coli* in our laboratory and because, as we intended to use milk whey, a lactose-containing substrate, as the carbon source, addition of an inducer would be avoided.

A PCR fragment containing the structural genes for the synthesis of PHB from *Azotobacter* sp. strain FA8, *phaBAC*, was cut and ligated to pQE32 as indicated in Materials and Methods, and the ligation mixture was used to transform *E. coli* DH5α. The resulting plasmid, named pJP24, contained a *phaB-lacZ* gene fusion. Accumulation of PHB from gluconate was investigated in the transformants by microscopic observation of cells stained with Nile Blue A. The PHB content was determined by gas chromatography in overnight cultures of positive clones grown in LB supplemented with 1% (wt/vol) gluconate and 1 mM IPTG. Recombinants accumulated PHB to approximately 15% of the CDW.

Production of PHB from lactose. As *E. coli* DH5α is a *lac* mutant, pJP24 was transferred to several Lac$^+$ strains: *E. coli* S17-1, wild type for all *lac* genes, and two *lacI* strains, K1060 and T1GP, to analyze the production of PHB from lactose. The recombinants were tested for PHA accumulation in overnight cultures grown in M9 minimal medium containing 100 μg · ml^{-1} ampicillin and 3% (wt/vol) lactose as the sole carbon source and supplemented with methionine, valine, and isoleucine, required by strain T1GP for growth, and proline and thiamine, required by strain S17-1. The polymer composition and content were determined by gas chromatography. In all cases, the PHA accumulated was a homopolymer of 3-hydroxybutyric acid. A low level of PHB accumulation was observed for T1GP recombinants in this medium. K1060 recombinants produced the highest biomass and accumulated more PHB than the other strains (Table 2). Also, K1060 recombinants are prototrophs, so they can grow in minimal medium without amino acids. For this reason, one of the K1060 recombinants, strain K24, was used in further studies.

PHB and biomass production of K24 in fed-batch cultures. A semisynthetic medium (OM) that contains salts and two agroindustrial by-products, whey and corn steep liquor, as the major carbon and nitrogen sources, respectively, has been described in previous work. OM was statistically optimized in order to achieve maximum biomass and polymer accumulation in K24 batch cultures (21).

In this work, strain K24 was grown in 24-h fed-batch cultures in OM. Biomass, polymer accumulation, and plasmid stability were measured throughout the fermentation. Biomass production reached 58.2 g · liter^{-1} at 24 h, and PHB accumulation peaked at 20 h, reaching a value of 46.9% of CDW, after which the polymer content decreased, representing only 30.3% of CDW at 24 h (Fig. 2A). This decrease in polymer content can be attributed to plasmid loss, as the growing proportion of cells without the recombinant plasmid resulted in a lower PHB-to-biomass ratio. In fact, after 24 h, only 65% of the cells contained pJP24 (Fig. 3), and this number decreased to 19% after 36 h (data not shown). The volumetric productivity was 0.73 g PHB per liter per hour, which is higher than PHB productivity values previously reported in our laboratory for K24 batch cultures (21).

Improvement of plasmid stability. Plasmid loss in K24 fed-batch cultures can be partly due to the loss of selective pressure, as β-lactamase produced by the growing cells degrades the ampicillin present in the medium, allowing the growth of cells without the plasmid. In order to avoid this effect, a kanamycin resistance gene was added to pJP24. A DNA fragment

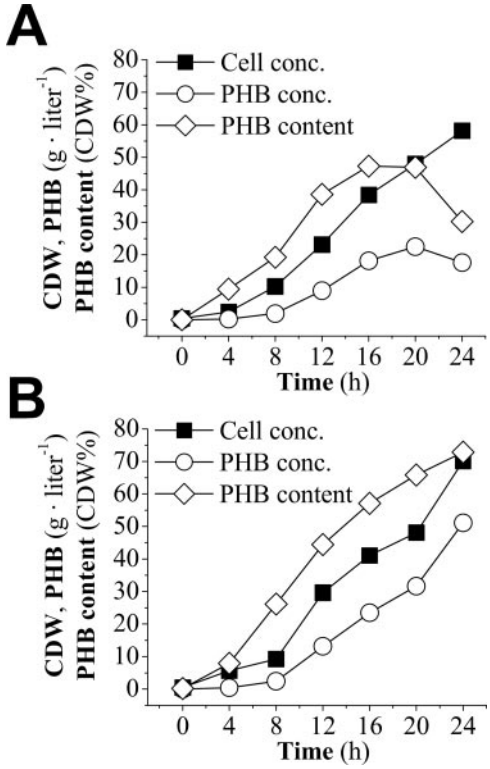

FIG. 2. Biomass and PHB accumulation in fed-batch cultures of strains K24 (A) and K24K (B). The cultures were grown in a 5.6-liter fermentor in OM medium. The feeding solution was a concentrated and deproteinated whey solution containing 25% (wt/vol) lactose. The fermentations were repeated at least twice with similar results. Means from triplicate representative experiments are shown. conc., concentration.

Downloaded from http://aem.asm.org/ on June 13, 2017 by DTU Library

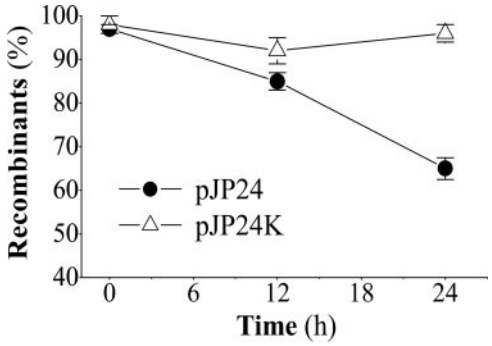

FIG. 3. Plasmid stability in strains carrying plasmids pJP24 (K24) and pJP24K (K24K) grown in fed-batch cultures in OM medium. Plasmid stability is expressed as the percentage of ampicillin- (K24) or kanamycin-resistant (K24K) cells. The reported data are the averages of at least three determinations.

obtained by PCR amplification of *aph* from plasmid pUC4K, coding for aminoglycoside 3′-phosphotransferase, was inserted into pJP24, giving rise to pJP24K. pJP24K was transformed into strain K1060, and the resulting strain, named K24K, was grown in fed-batch under the same conditions used for strain K24, replacing ampicillin by kanamycin. As seen in Fig. 2B, K24K accumulated much more polymer than K24 while producing only slightly larger amounts of biomass. K24K reached 70.1 g CDW and 51.1 g PHB per liter at 24 h in fed-batch culture, resulting in a PHB content of 72.9%. When the plasmid stability was analyzed, 96% of K24K cells were resistant to kanamycin after 24 h, showing a considerable increase in plasmid stability compared to K24 (Fig. 3). In contrast with results obtained for K24, PHB production and accumulation in K24K did not decrease after 20 h but continued to rise during the 24-h experiment (Fig. 2B). The PHB volumetric productivity for K24K attained at 24 h was 2.13 g PHB per liter per hour. This value is comparable to that reported by Ahn et al. for a 37.5-h fed-batch culture of a recombinant *E. coli* strain bearing the *A. latus pha* genes (1).

Physical characteristics of the PHB produced by the recombinant strains. In order to be suitable for industrial processing, the polymer must have a high molecular weight that enables it to retain its strength (28, 34). The physical characteristics of PHB obtained from *E. coli* K24 and *E. coli* K24K were compared with those of the polymer purified from *C. necator* and *Azotobacter* sp. strain FA8. The molecular weight of the polymer obtained from *C. necator* was similar to the values reported in the literature (9). PHB recovered from the *E. coli* recombinants had a molecular weight similar to that of the polymer obtained from *Azotobacter* sp. strain FA8, between 64 and 68% higher than that of *C. necator* (Table 3).

To analyze the morphological state of PHB obtained from recombinant *E. coli*, the melting temperature and the fusion enthalpy of PHB produced by the above-mentioned strains were measured. As shown in Table 3, PHB recovered from the recombinants and natural producers presented similar melting temperatures and percentages of crystallinity. The crystallinities of all PHB samples ranged between 63 and 64%, suggesting that a crystalline structure was developed during the recovery process.

The T_gs for PHBs isolated from the two natural producer strains were similar to previously reported values (18), but the polymers obtained from both recombinant strains showed higher values. Considering that the M_rs of the polymers were similar, as well as their crystallinity degrees, the differences in T_g values could be attributed to a higher chain flexibility or cross-linking characteristics of the natural polymers, possibly due to different genetic and metabolic backgrounds of the different bacteria studied.

Concluding remarks. The recombinant strains presented in this work produce PHB in a minimal-salts medium supplemented with two agroindustrial by-products, whey and corn steep liquor, by means of the constitutive expression of *Azotobacter* sp. strain FA8 *pha* genes from a strong heterologous promoter.

Processes for the production of PHA from whey using recombinant *E. coli* have been described previously, but in all cases, the *pha* genes were expressed from their natural promoters. There are no previous reports of PHB-producing recombinant *E. coli* strains expressing *pha* genes belonging to the genus *Azotobacter*, but two papers describe constructions in which the three *pha* structural genes of *C. necator* were expressed from nonnative promoters. In both cases, the PHB production levels were lower than those achieved with constructions that used the natural promoter. Kidwell et al. (12) used a temperature-dependent copy number plasmid carrying the *pha* genes expressed from a *tac* promoter. They induced PHB synthesis by a temperature upshift after the exponential growth phase but obtained poor accumulation results, so the authors concluded that constitutive expression was more suitable for PHB production. Shi et al. (31) used a different temperature-induced construction, involving phage lambda promoters and a temperature-sensitive repressor. A final PHB concentration of approximately 9 g · liter^{-1} was reported in their study for 40-h fed-batch cultures.

Strain K24K does not produce the lactose repressor. This trait is important when using lactose as a carbon source, as it ensures constitutive expression of the lactose transport and utilization genes. Absence of the lactose repressor is also useful when using a carbon source other than lactose, as it eliminates the need to add an inducer to promote the expression of *pha* genes. The *lac* catabolic repression sites are not present in pJP24K, so *pha* gene expression in this plasmid is not affected by catabolic repression.

Results obtained in fed-batch cultures of strain K24K at the

TABLE 3. Physical properties of purified PHB obtained from the natural producers and *E. coli* recombinants[a]

Source of purified PHB	M_r (10^6 Da)	T_g (°C)	T_m (°C)	% Crystallinity
C. necator	1.12 ± 0.04	2.0 ± 0.5	175.1 ± 0.5	62.8
Azotobacter sp. strain FA8	1.88 ± 0.08	1.8 ± 0.5	176.5 ± 0.5	64.3
K24	1.84 ± 0.07	21.8 ± 0.5	172.6 ± 0.5	62.8
K24K	1.86 ± 0.05	22.6 ± 0.5	172.6 ± 0.5	63.2

[a] *C. necator* was grown in MM-fructose, *Azotobacter* sp. strain FA8 was grown in Burk's medium supplemented with 3% (wt/vol) glucose, and K24 and K24K were grown in fed-batch cultures in OM medium with whey as the main carbon source. The reported data are the averages of at least three determinations.

Downloaded from http://aem.asm.org/ on June 13, 2017 by DTU Library

laboratory scale indicate it is a suitable strain for the production of PHB from milk whey.

ACKNOWLEDGMENTS

We thank Beatriz S. Méndez for helpful discussions. We are also indebted to Úrsula Böhm de Bordenave for viscosimetric determinations and Patricio R. Santagapita and M. del Pilar Buera for DSC analysis.

This work was supported by grants from UBA, Fundación Antorchas, and CABBIO. M.J.P. and M.A.G. are career investigators from CONICET. P.I.N. and A.D.A. have graduate student fellowships from CONICET.

REFERENCES

1. **Ahn, W. S., S. J. Park, and S. Y. Lee.** 2000. Production of poly(3-hydroxybutyrate) by fed-batch culture of recombinant *Escherichia coli* with a highly concentrated whey solution. Appl. Environ. Microbiol. **66:**3624–3627.
2. **Aldor, I. S., and J. D. Keasling.** 2003. Process design for microbial plastic factories: metabolic engineering of polyhydroxyalkanoates. Curr. Opin. Biotechnol. **14:**475–483.
3. **Bandrup, J., and E. H. Immergut.** 1989. Polymer handbook, 3rd ed. Interscience Publishers, New York, N.Y.
4. **Barham, P. G., A. Keller, E. L. Otum, and P. A. Holmes.** 1984. Crystallization and morphology of a bacterial thermoplastic: poly-β-hydroxybutyrate. J. Mater. Sci. **19:**2781–2794.
5. **Braunegg, G., B. Sonnleitner, and R. M. Lafferty.** 1978. A rapid gas chromatographic method for the determination of poly-β-hydroxybutyric acid in bacterial biomass. Eur. J. Appl. Microbiol. Biotechnol. **6:**29–37.
6. **Choi, J. I., S. Y. Lee, and K. Han.** 1998. Cloning of the *Alcaligenes latus* polyhydroxyalkanoate biosynthesis genes and use of these genes for enhanced production of poly(3-hydroxybutyrate) in *Escherichia coli*. Appl. Environ. Microbiol. **64:**4897–4903.
7. **Dawes, E. A., and P. J. Senior.** 1973. The role and regulation of energy reserve polymers in micro-organisms. Adv. Microb. Physiol. **10:**135–266.
8. **Ditta, G., T. Schmidhauser, E. Yakobson, P. Lu, X. W. Liang, D. R. Finlay, D. Guiney, and D. R. Helinski.** 1985. Plasmids related to the broad host range vector, pRK290, useful for gene cloning and for monitoring gene expression. Plasmid **13:**149–153.
9. **Hahn, S. K., Y. K. Chang, and S. Y. Lee.** 1995. Recovery and characterization of poly(3-hydroxybutyric acid) synthesized in *Alcaligenes eutrophus* and recombinant *Escherichia coli*. Appl. Environ. Microbiol. **61:**34–39.
10. **Hanahan, D.** 1985. Techniques for transformation of *Escherichia coli*, p. 109–135. *In* D. M. Glover (ed.), DNA cloning: a practical approach, vol. 1. IRL Press, Oxford, England.
11. **Khanna, S., and A. K. Srivastava.** 2005. Recent advances in microbial polyhydroxyalkanoates. Process Biochem. **40:**607–619.
12. **Kidwell, J., H. E. Valentin, and D. Dennis.** 1995. Regulated expression of the *Alcaligenes eutrophus pha* biosynthesis genes in *Escherichia coli*. Appl. Environ. Microbiol. **61:**1391–1398.
13. **Kim, J.-H., and J. Lee.** 1997. Cloning, nucleotide sequence and expression of gene coding for poly-3-hydroxybutyric acid (PHB) synthase of *Rhodobacter sphaeroides* 2.4.1. J. Microbiol. Biotechnol. **7:**229–236.
14. **Langenbach, S., B. H. Rehm, and A. Steinbüchel.** 1997. Functional expression of the PHA synthase gene *phaC1* from *Pseudomonas aeruginosa* in *Escherichia coli* results in poly(3-hydroxyalkanoate) synthesis. FEMS Microbiol. Lett. **150:**303–309.
15. **Lee, S. Y.** 1996. High cell-density culture of *Escherichia coli*. Trends Biotechnol. **14:**98–105.
16. **Lenz, R. W., and R. H. Marchessault.** 2005. Bacterial polyesters: biosynthesis, biodegradable plastics and biotechnology. Biomacromolecules **6:**1–8.
17. **Liu, S. J., and A. Steinbüchel.** 2000. A novel genetically engineered pathway for synthesis of poly(hydroxyalkanoic acids) in *Escherichia coli*. Appl. Environ. Microbiol. **66:**739–743.
18. **Madison, L. L., and G. W. Huisman.** 1999. Metabolic engineering of poly(3-hydroxyalkanoates): from DNA to plastic. Microbiol. Mol. Biol. Rev. **63:**21–53.
19. **Mahishi, L. H., G. Tripathi, and S. K. Rawal.** 2003. Poly(3-hydroxybutyrate) (PHB) synthesis by recombinant *Escherichia coli* harbouring *Streptomyces aureofaciens* PHB biosynthesis genes: effect of various carbon and nitrogen sources. Microbiol. Res. **158:**19–27.
20. **Nikel, P. I., M. J. Pettinari, M. A. Galvagno, and B. S. Méndez.** 2006. Poly(3-hydroxybutyrate) synthesis by recombinant *Escherichia coli arcA* mutants in microaerobiosis. Appl. Environ. Microbiol. **72:**2614–2620.
21. **Nikel, P. I., M. J. Pettinari, B. S. Méndez, and M. A. Galvagno.** 2005. Statistical optimization of a culture medium for biomass and poly(3-hydroxybutyrate) production by a recombinant *Escherichia coli* strain using agroindustrial by-products. Int. Microbiol. **8:**243–250.
22. **Ostle, A. G., and J. G. Holt.** 1982. Nile blue A as a fluorescent stain for poly-β-hydroxybutyrate. Appl. Environ. Microbiol. **44:**238–241.
23. **Pettinari, J. M., L. Chanetón, G. Vázquez, A. Steinbüchel, and B. S. Méndez.** 2003. Insertion sequence-like elements associated with putative polyhydroxybutyrate regulatory genes in *Azotobacter* sp. FA8. Plasmid **50:**36–44.
24. **Pettinari, M. J., G. J. Vázquez, D. Silberschmidt, B. Rehm, A. Steinbüchel, and B. S. Méndez.** 2001. Poly(3-hydroxybutyrate) synthesis genes in *Azotobacter* sp. strain FA8. Appl. Environ. Microbiol. **67:**5331–5334.
25. **Quagliano, J. C., P. Alegre, and S. S. Miyazaki.** 1994. Isolation and characterization of *Azotobacter* sp. for the production of poly-β-hydroxyalkanoates. Rev. Argent. Microbiol. **26:**21–27.
26. **Resch, S., K. Gruber, G. Wanner, S. Slater, D. Dennis, and W. Lubitz.** 1998. Aqueous release and purification of poly(β-hydroxybutyrate) from *Escherichia coli*. J. Biotechnol. **65:**173–182.
27. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1988. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
28. **Savenkova, L., Z. Gercberga, V. Nikolaeva, A. Dzene, I. Bibers, and M. Kalnin.** 2000. Mechanical properties and biodegradation characteristics of PHB-based films. Process Biochem. **35:**573–579.
29. **Schlegel, H. G., R. Lafferty, and I. Krauss.** 1970. The isolation of mutants not accumulating poly-β-hydroxybutyric acid. Arch. Microbiol. **71:**283–294.
30. **Schubert, P., A. Steinbüchel, and H. G. Schlegel.** 1988. Cloning of the *Alcaligenes eutrophus* genes for synthesis of poly(β-hydroxybutyric acid) (PHB) and synthesis of PHB in *Escherichia coli*. J. Bacteriol. **170:**5837–5847.
31. **Shi, H., K. Kyuwa, M. Takasu, and K. Shimizu.** 2001. Temperature-induced expression of *phb* genes in *Escherichia coli* and the effect of temperature patterns on the production of poly-3-hydroxybutyrate. J. Biosci. Bioeng. **91:**21–26.
32. **Shiloach, J., and R. Fass.** 2005. Growing *E. coli* to high cell density—a historical perspective on method development. Biotechnol. Adv. **23:**345–357.
33. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. Bio/Technology **1:**784–791.
34. **Steinbüchel, A.** 1995. PHB and other polyhydroxyalkanoic acids, p. 432–434. *In* G. R. H. J. Rehm, A. Pühler, and P. Stadler (ed.), Biotechnology: products of primary metabolism, 1st ed., vol. 6. Wiley-VCH, Weinheim, Germany.
35. **Steinbüchel, A., and B. Füchtenbusch.** 1998. Bacterial and other biological systems for polyester production. Trends Biotechnol. **16:**419–427.
36. **Vandamme, P., and T. Coenye.** 2004. Taxonomy of the genus *Cupriavidus*: a tale of lost and found. Int. J. Syst. Evol. Microbiol. **54:**2285–2289.

Downloaded from http://aem.asm.org/ on June 13, 2017 by DTU Library