APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1994, p. 1198–1205
0099-2240/94/$04.00+0

Vol. 60, No. 4

# Production of Polyhydroxyalkanoates in Sucrose-Utilizing Recombinant *Escherichia coli* and *Klebsiella* Strains

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Received 31 August 1993/Accepted 31 January 1994

**The cloned poly-3-hydroxybutyrate (PHB) synthesis pathway from *Alcaligenes eutrophus* has been introduced into sucrose-utilizing strains of *Escherichia coli*, *Klebsiella aerogenes*, and *Klebsiella oxytoca*. The plasmid-borne genes were well expressed in these environments and were able to mediate the production of significant amounts of PHB when the bacteria were grown with sucrose as the sole carbon source. The molecular weight of the PHB polymer made in *K. aerogenes* and *E. coli* was approximately $1 \times 10^6$ to $2 \times 10^6$. Sucrose uptake in *K. aerogenes* was measured and found to be similar to that found for other *Klebsiella* strains, but sucrose uptake in the *E. coli* strain was not detectable. *K. aerogenes* is able to utilize sugarcane molasses as the sole carbon source to accumulate PHB at the rate of approximately 1 g of PHB per liter of culture fluid per h. A *K. oxytoca fadR* strain was able to incorporate 3-hydroxyvalerate into a poly-(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHB-co-V) polymer to levels as high as 56 mol% when grown in a medium containing propionate. Total PHB-co-V levels could be enhanced by adding propionate at the beginning of stationary phase rather than at the time of inoculation.**

Polyhydroxyalkanoates (PHAs) are a class of bacterial storage compounds that have received considerable attention in recent years because of their potential use as biodegradable thermoplastics (8). Within this family, a large amount of research has been conducted on the homopolymer poly-(3-hydroxybutyrate) (PHB) and the copolymer poly-(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHB-co-V) (2, 23, 43). The former polymer was discovered in 1926 (24) and has been the prototypical PHA, while the latter was more recently discovered, in 1983 (17), and has attracted interest because of its enhanced flexibility over PHB.

Both polymers have been commercially developed and marketed (8, 19, 23, 47). However, the widespread use of these polymers has been hindered by the high cost of production. Currently, the cost of the PHB-co-V resin is approximately $30/kg which compares most unfavorably with a functionally similar plastic, polypropylene, at approximately $2/kg. Many strategies have been developed in the last several years with the goal of decreasing the resin production cost. One of the foremost of these is to isolate naturally occurring bacterial strains which utilize less expensive carbon sources (9, 31, 32). One such carbon source is molasses, obtained either from sugar beets or from sugarcane. Molasses normally sells for about 33 to 50% of the cost of glucose, the carbon source usually employed for PHB production.

We have previously described the cloning of the *Alcaligenes eutrophus* PHB biosynthesis genes and high-level expression of PHB in recombinant *Escherichia coli* (20, 41). This system can be used to obtain intracellular polymer accumulations to levels as high as 95% polymer per cell dry weight when the bacteria are grown on glucose, lactose, or whey. In addition, the power of *E. coli* genetics has allowed us to develop strains which synthesize the copolymer PHB-co-V (40), can be lysed by osmotic shock (21), and contain plasmids that need not be stabilized by antibiotics in the medium (12). Taken together, these developments should facilitate a substantial decrease in production costs if implemented in a commercial system.

An additional enhancement to this system would be the ability to produce PHA with sucrose or molasses as the sole carbon source. Theoretically, this is possible since there are *E. coli* strains that are known to metabolize sucrose (5, 25, 33, 36, 37, 42), and some appear to be useful in commercial production strategies (46). A major advantage of using a *suc*$^+$ *E. coli* strain would be that the large amount of prior genetic work that has been done with laboratory strains of *E. coli* should be directly applicable to this strain. Alternatively, other members of the family *Enterobacteriaceae* are known to metabolize sucrose and may, because of their genetic similarity to members of the genus *Escherichia*, exhibit high-level expression of the PHB biosynthesis genes. Members of the genus *Klebsiella* are likely candidates because they grow well on sucrose (42), grow rapidly to high cell density (13), and have exhibited significant genetic similarity to *E. coli* (15, 26, 28, 30, 34, 44).

We have initiated a research program, based on these two strategies, aimed at developing a recombinant bacterial strain that synthesizes PHAs with sucrose or molasses as the carbon source. This paper describes three such strains: an environmentally isolated *E. coli* strain, *Klebsiella aerogenes* 2688, and *Klebsiella oxytoca* M5A1.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** *E. coli* HMS174 ($F^-$ *recA* $r^-_{K12}$ $m^+_{K12}$ $Rif^r$) carrying pJM9131 was used as a positive control for PHB production and has been described elsewhere (40, 45). *K. aerogenes* 2688 *hutC515* Δ[*bla*]-2 was provided by Brian Janes in the laboratory of Robert Bender at the University of Michigan. *K. oxytoca* was provided by Gary Roberts at the University of Wisconsin. *E. coli* RS3040 *fadR*::Tn*10* was provided by Paul Black at the University of Tennessee Medical Center. *E. coli* JMU213 was isolated from a 50-ml sample of water taken from Black's Run (a creek) in Harrisonburg, Va. Briefly, dilutions of the creek water were plated onto MacConkey agar-sucrose plates (Baltimore Biological Laboratory) which were incubated overnight

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at 37°C. Thirty sucrose-positive clones were picked and streaked onto mfc Endo agar plates (Baltimore Biological Laboratory) to screen for members of the *Enterobacteriaceae* family. Approximately 23 positive colonies were found, and these were stabbed into Simmons citrate agar tubes (Baltimore Biological Laboratory). Two isolates gave negative results. These were characterized and found to be gram-negative, rod-shaped, facultatively anaerobic, sucrose-positive coliforms. The two clones were subjected to biochemical characterization with the Enterotube II system from Roche Diagnostic Systems, by using a laboratory strain of *E. coli* as the positive control. Both clones were essentially the same as the positive control. One of these was chosen and given the strain designation JMU213 and used in further studies. The strain was characterized by fatty acid profile analysis (Acculab, Newark, Del.) and was confirmed to be *E. coli*. *E. coli* JMU213 was tested for antibiotic resistances by plating out the bacteria on agar plates containing the specific antibiotic and analyzing for growth. The clone was resistant to tetracycline at 10 μg/ml but was sensitive to kanamycin at 10 μg/ml and chloramphenicol at 20 μg/ml. It was variably resistant to ampicillin at 100 μg/ml.

Plasmid p4A, carrying an ampicillin resistance determinant, has previously been described (20). Plasmid pJM9131 is a derivative of p4A. Briefly, p4A DNA was digested with *Dra*I restriction endonuclease (this excises the majority of the ampicillin resistance gene), and then a blunt-ended kanamycin Genblock from Pharmacia Biochemicals was ligated, by standard techniques (27), with the p4A fragment that contained the replication origin and the PHB biosynthesis genes (approximately 6 kb). Plasmids were introduced into bacterial strains by electroporation (27).

Unless otherwise stated, all growth took place in Luria-Bertani medium (LB) or M9 minimal medium (27) at the carbon source concentration specified. In some experiments, a minimal medium that contained 10.5 g of $K_2HPO_4$, 4.5 g of $KH_2PO_4$, 3 g of $(NH_4)_2SO_4$, 0.1 g of $MgSO_4$, and 2 ml of trace element solution (11) per liter was used. Chemically inverted sugarcane molasses was provided by the Sugar Research Laboratory at Louisiana State University and contained 25 g of glucose, 23 g of fructose, and 14 g of sucrose per liter, in addition to unspecified amounts of ash, nitrogenous compounds, and various other minor constituents. Plasmids were stabilized by adding kanamycin at 100 μg/ml to the medium. All shake-flask experiments were done with baffled flasks shaken at a speed of 225 rpm in an orbital shaker-incubator set at a temperature of 30°C for *Klebsiella* strains or 37°C for *E. coli* strains.

**P1 transduction.** P1 transduction was done by standard procedures (29). First, *E. coli* RS3040 *fadR*::Tn*10* was crossed with *E. coli* MM294 (ATCC 33625) to produce *E. coli* JMU170 *fadR*::Tn*10*. A transducing lysate of *E. coli* JMU170 *fadR*::Tn*10* was used to transduce *K. oxytoca* M5A1 as per the published protocol except that, in order to allow time for kanamycin resistance to be expressed in the transductants, the *K. oxytoca* M5A1 cells were pelleted by centrifugation, resuspended in LB containing 50 mM sodium citrate, pelleted by centrifugation again, and finally resuspended in LB containing 50 mM sodium citrate. The culture was incubated for 1 h at 32°C with gentle shaking in an orbital shaker-incubator, and then aliquots were plated onto Luria agar plates containing 10 μg of kanamycin per ml. Both transductants and spontaneous $Kan^r$ mutants grew, but transductants could be selected because they were usually much larger colonies and they could be transferred to, and grew on, Luria agar plates containing 100 μg of kanamycin per ml. Spontaneous mutants manifested smaller colonial growth and would not grow at kanamycin concentrations of 100 μg/ml.

**PHA analysis.** Microscopic analysis of heat-fixed cells was done after staining with crystal violet for 10 s and destaining with running water for 10 s.

Quantitative determination of PHB and PHB-co-HV was performed by gas chromatographic analysis of PHA methyl esters obtained by methanolysis of dried cell pellets (7). Analyses were done with a Shimadzu GC-14A gas chromatograph, with a 0.53-μm-diameter DB-17 (J & W Scientific) column 15 m in length. Chromatographic conditions were as previously described (40). PHB and PHB-co-V amounts were calculated from a standard curve generated by using known quantities of PHB and PHB-co-V (both obtained from Aldrich Chemical Company) in the same analyses.

Molecular weight of PHB was calculated by measuring the intrinsic viscosity of different concentrations of PHB dissolved in chloroform (4). The constants $K$ and $a$ were obtained from the Polymer Handbook by J. Bandrup and E. H. Immergut (3). Comparison of the molecular weight of PHB made in recombinant organisms was made with PHB obtained from *A. eutrophus* and *Alcaligenes latus* obtained from Imperial Chemical Industries (Billingham, United Kingdom) and Petrochemica Danubia (Linz, Austria), respectively.

**Sucrose uptake.** Cells were inoculated into 3 ml of Luria broth containing kanamycin (50 μg/ml) and 0.5% sucrose (for sucrose uptake assay) or 0.5% glucose (for glucose uptake). The tube culture was then incubated overnight at 225 rpm and 30°C (for *Klebsiella* strains) or 37°C (for *E. coli*). A total of 1 ml of the overnight culture was transferred into 50 ml of M9 medium supplemented with thiamine (0.5 mg/liter)–Casamino Acids (1 g/liter)–kanamycin (50 μg/ml)–0.5% sucrose or 0.5% glucose and then incubated at 225 rpm and 30°C (for *Klebsiella* strains) or 37°C (for *E. coli*) in 250-ml baffled shake flasks for about 4 h. One milliliter of the culture was removed and centrifuged at 2,000 × *g* for 5 min. After removal of the supernatant, the pellet was washed with 1 ml of M9 medium and then resuspended in M9 medium to a final optical density at 600 nm of 1. One-half milliliter of this cell suspension in M9 medium was incubated at 30°C. A total of 2 μCi of [$^{14}$C]sucrose (632 mCi/mmol, 0.5 μCi/μl [New England Nuclear]) or 1 μCi of [$^{14}$C]glucose (320 mCi/mmol, 0.5 μCi/μl [New England Nuclear]) was added to the culture. At indicated time intervals, 80-μl samples were withdrawn and immediately applied to membrane filters (0.2-μm pore size) in a vacuum apparatus to remove the medium containing radioactive material. The membranes were then washed twice with 1 ml of M9 medium and air dried. The radioactive material retained by the membrane was measured in 5 ml of Scintiverse (Fisher Scientific) in a Beckman LS 5000TA scintillation counter. For specific activity measurements, a Bio-Rad protein assay kit (Bio-Rad Laboratories) was used to determine protein concentration in a 100-μl aliquot from the pelleted and washed cells.

**Analysis of extracellular sucrose, fructose, and glucose levels.** Five- to fifteen-microliter samples of cell culture supernatants were spotted onto a silica gel thin-layer chromatography (TLC) plate (Whatman silica gel, 10 by 230 cm, 250-μm layer). The samples were resolved in acetonitrile-$H_2O$ (85:15 ratio) for approximately 40 min until the solvent front reached the top of the plate. The plate was then air dried in a chemical fume hood. The bands were visualized by spraying the plate with 20% $(NH_4)_2SO_4$ and baking the plate at 80°C overnight. If necessary, the amount of radioactivity in each band (sucrose, glucose, or fructose) was measured by scraping the silica gel off the plate at the appropriate spot and mixing the gel powder

with 2 ml of scintillation fluid (Scintiverse) and counting in a Beckman LS 5000TA liquid scintillation counter.

**Fed-batch growth of *K. aerogenes* 2688(pJM9131).** A total of 12.5 ml of an overnight culture of *K. aerogenes* 2688(pJM9131) (LB–100 μg of kanamycin per ml) was used to inoculate a 250-ml culture (in a 1-liter baffled flask) of the same medium. This culture was incubated at 30°C and 225 rpm on an orbital shaker until it reached an optical density (at 600 nm) of approximately 0.8. The entire contents of this culture were then transferred to a Braun Biostat E fermentor containing 5 liters of minimal medium containing 5% sugarcane molasses (wt/vol) and 100 μg of kanamycin per ml. The culture was incubated at 30°C, and pH and dissolved oxygen were controlled at 7.0 and 80%, respectively. The pH was controlled by the addition of 6 M NaOH. The substrate feed was composed of 50 g of $(NH_4)_2SO_4$, 2.5 g of $MgSO_4$, 0.5 ml of trace element solution, and 500 ml of sugarcane molasses in a total volume of 1 liter. Because glucose and sucrose uptake in *K. aerogenes* occur at similar rates, the need for addition of substrate feed was estimated by monitoring only glucose levels with Chemstrips (Baxter Scientific Products). Total carbon source concentration in the fermentor was controlled by the addition of substrate feed to a final concentration between 0.5 and 1%. At appropriate times, samples (30 ml) were removed and analyzed for optical density (600 nm), PHB content, and cell dry weight.

**Cell dry weight measurements.** A total of 5 to 10 ml of cells was pelleted by centrifugation at 2,000 × *g* for 10 min. The culture supernatant was aspirated, and the pellet was resuspended in 5 ml of 0.85% saline. The resuspended bacteria were pelleted by centrifugation again, and the supernatant was aspirated. The final pellet was resuspended in 5 ml of water, transferred to preweighed aluminum weigh boats, dried for 24 h at 80°C, cooled, and weighed.

**Chemicals.** All chemicals were reagent grade and came from Sigma Chemical Company unless otherwise noted.

## RESULTS

**Production of PHB in *K. aerogenes* 2688(pJM9131) and *E. coli* JMU213(pJM9131) grown on sucrose.** To determine whether recombinant *K. aerogenes* 2688(pJM9131) and *E. coli* JMU213(pJM9131) produced PHB at levels comparable to those for recombinant *E. coli* strains used in previous studies (20, 40), a shake-flask study was done with *E. coli* HMS174 (pJM9131) as a positive control. The two test organisms were grown in M9 minimal medium containing sucrose as the sole carbon source, while the positive control was grown in the same medium containing glucose as the sole carbon source. All three strains accumulated PHB to comparable levels when measured in stationary phase (Fig. 1). However, *K. aerogenes* 2688(pJM9131) produced PHB at a slightly faster rate than the other two species. At 10 h postinoculation, the amount of PHB in *K. aerogenes* 2688(pJM9131) was roughly twice that of the other two strains, and at 13 h postinoculation, it was approximately 60% greater.

**Molecular weight of PHB produced in *K. aerogenes* 2688(pJM9131) and *E. coli* JMU213(pJM9131).** PHB from *K. aerogenes* 2688(pJM9131) possessed molecular weights (three different isolates) ranging between $9.0 \times 10^5$ and $1.8 \times 10^6$. Similarly, PHB from *E. coli* JMU213(pJM9131) had a molecular weight of $9.0 \times 10^5$. In comparison, PHB obtained from *A. latus* and *A. eutrophus* had molecular weights of $3.3 \times 10^5$ and $2.4 \times 10^5$, respectively.

**Sucrose and glucose uptake.** The mode of sucrose and glucose uptake by *K. aerogenes* 2688 and *E. coli* JMU213 was of some interest because, ultimately, this determines the rate of growth and the rate of PHB production. Uptake studies done on *K. aerogenes* 2688 and *E. coli* JMU213 (Fig. 2) revealed two different uptake strategies. *K. aerogenes* 2688 takes up both glucose and sucrose at similar rates. In contrast, *E. coli*

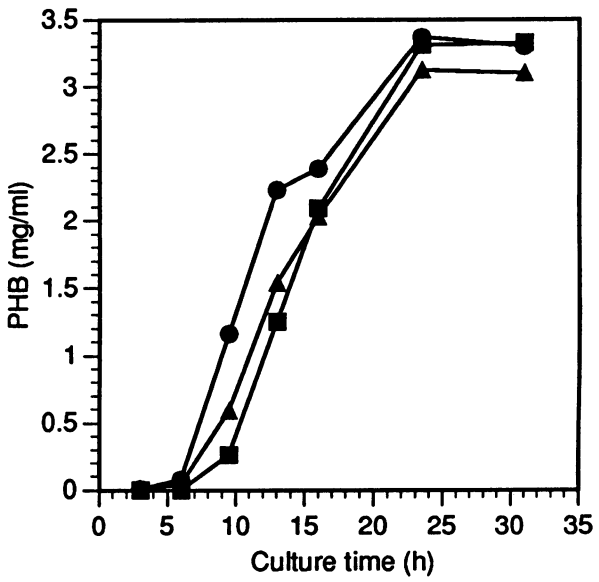


FIG. 1. PHB production of strains grown in minimal medium plus sucrose. Bacterial strains to be tested were grown in 1-liter shake flasks containing 300 ml of M9 minimal medium–2% carbon source (wt/vol)–appropriate antibiotics and grown at 30°C (*Klebsiella* sp.) or 37°C (*E. coli*). Fifteen-milliliter samples were removed from the culture at specified times and analyzed for PHB content. ■, *E. coli* HMS174 (pJM9131); ▲, *E. coli* JMU213(pJM9131); ●, *K. aerogenes* 2688 (pJM9131).

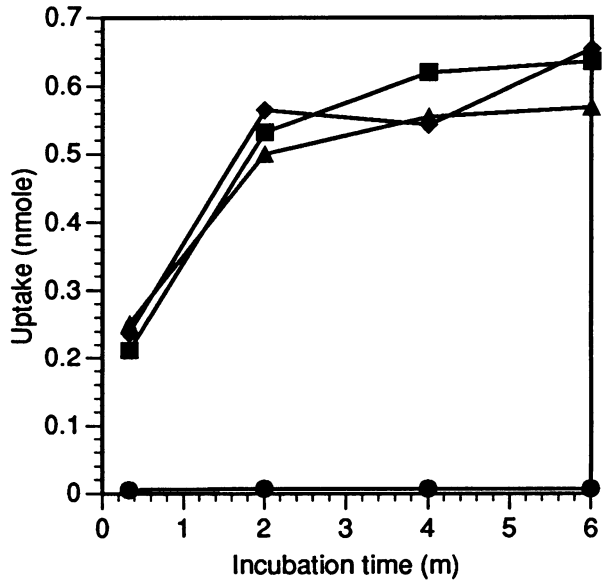


FIG. 2. Sucrose and glucose uptake by *E. coli* JMU213 and *K. aerogenes* 2688. Shown are glucose uptake of *E. coli* JMU213 (◆) and *K. aerogenes* 2688 (▲) and sucrose uptake of *E. coli* JMU213 (●) and *K. aerogenes* 2688 (■).

JMU213 takes up glucose at a rate similar to that of *K. aerogenes* 2688 but does not take up sucrose. This result was confirmed in three different trials.

Carbohydrate uptake was also examined by observing the disappearance of individual carbohydrate bands from TLC analyses of the extracellular culture fluid taken at different times during a growth experiment in which the carbon source was inverted sugarcane molasses (data not shown). The TLC studies confirmed that for *K. aerogenes* 2688, sucrose and glucose disappeared from the culture fluid at approximately the same rate (presumably taken into cell). Fructose was taken into the cell much more slowly. Chromatographic studies conducted with *E. coli* JMU213 and molasses, in which radioactive sucrose was added, indicated that the sucrose was not taken up directly but was first split into glucose and fructose before it was taken up. This was concluded from the fact that radioactive label that was initially found migrating with unlabeled sucrose on the TLC plate was subsequently found migrating with the unlabeled fructose on the TLC plate at later time points (radioactive label is on C-1 of fructose moiety). However, this association was transient, and at later time points, the vast majority of the radioactive label was found migrating on the TLC plate at a position that did not correspond to that of glucose, fructose, or sucrose. The exact nature of this uptake system remains to be elucidated.

In a separate set of experiments in which five time points were taken within the first 2 min of incubation (linear uptake), the specific uptake of sucrose by *K. aerogenes* 2688 was found to be 4 nmol/mg/min with an extracellular sucrose concentration of 1.5 μM.

**Growth of *K. aerogenes* 2688(pJM9131) on molasses.** To determine whether PHB levels from *K. aerogenes* 2688 (pJM9131) and *E. coli* JMU213(pJM9131) grown on molasses were comparable to those for the same strains grown on sucrose, the strains were grown in shake-flask studies in minimal medium containing 6% (wt/vol) molasses as the sole carbon source. *E. coli* HMS174(pJM9131) was used as a negative control. Of the three bacterial species, the *Klebsiella* strain exhibited superior PHB production (Fig. 3A), accumulating PHB to a final level of approximately 3 mg/ml, representing 50% of the cell dry weight. In addition to the fact that *K. aerogenes* 2688(pJM9131) accumulated the most PHB, it could also be observed that it did this at a comparatively rapid rate. Within 5 h of inoculation, the intracellular accumulation levels of PHB had reached approximately 50% (wt/wt). Since *E. coli* HMS174 is not able to metabolize sucrose, the low levels of PHB accumulation (0.15 mg/ml at 36 h) were not surprising. However, *E. coli* JMU213(pJM9131) accumulated much less PHB (0.18 mg/ml at 36 h) than one might expect of an organism able to utilize sucrose.

**Fed-batch growth of *K. aerogenes* 2688(pJM9131).** *K. aerogenes* 2688(pJM9131) was grown in a 10-liter Braun Biostat E fermentor with molasses as the sole carbon source. The amount of PHB accumulated after 32 h of growth was 24 mg/ml, with a final polymer content of 70% PHA per cell dry weight (Fig. 4). This percentage is somewhat deceiving in that the strain exhibited plasmid instability that was apparent upon observation of the cells by light microscopy (Fig. 5). The cells either contained no PHB at all (Fig. 5, $PHB^-$ cell) or were grossly distended by the inclusion of many granules (Fig. 5, $PHB^+$ cell). The instability was confirmed by plating individual colonies obtained from the fermentation on medium containing no antibiotic and on medium containing kanamycin.

A significant aspect of these experiments was the large increase in size of *K. aerogenes* packed with PHB granules (Fig. 5). The volumetric increase (estimated by measuring diameter and length in photo) between the $PHB^-$ cell and the $PHB^+$ cell in Fig. 5 is approximately 20-fold.

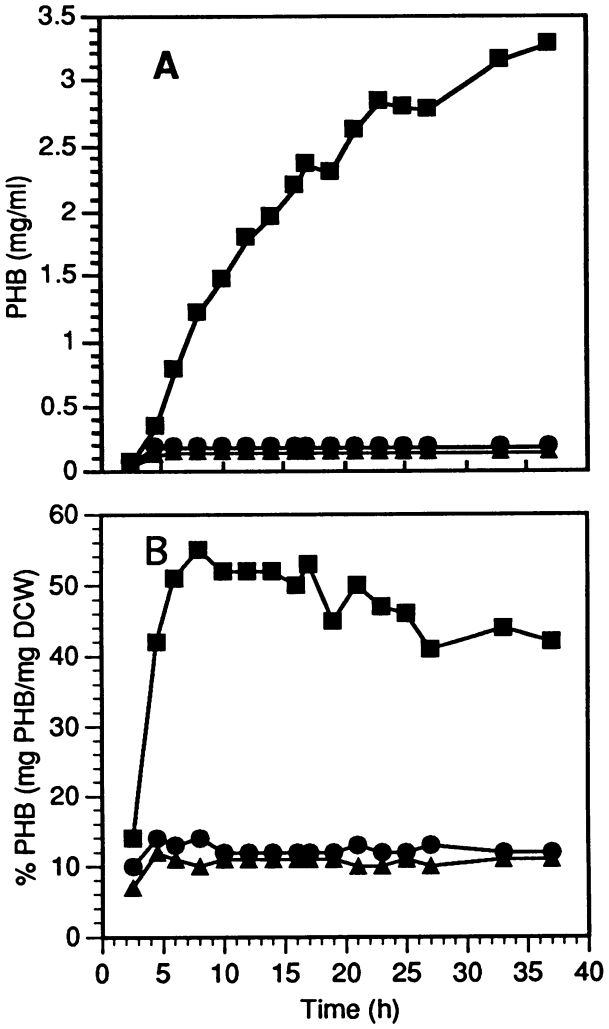


FIG. 3. PHB production of strains grown in minimal medium plus molasses. The experiment was done as described in the legend to Fig. 1, except that an optimized minimal medium containing 6% sugarcane molasses was used as the sole carbon source. Shown are PHB accumulation (A) and percent PHB (B) in *K. aerogenes* 2688 (pJM9131) (■), *E. coli* JMU213(pJM9131) (●), and *E. coli* HMS174 (pJM9131) (▲). DCW, dry cell weight.

**Copolymer production of *Klebsiella* strain.** A *fadR* mutation was introduced into *K. oxytoca* carrying pJM9131 by P1 transduction, and this strain was subsequently tested as to its ability to synthesize PHB-co-V. *K. oxytoca fadR* (pJM9131) grown in minimal medium plus glucose plus different levels of propionate (1 to 10 mM) proved to be very efficient at incorporating 3-hydroxyvalerate (3-HV) into the polymer (Fig. 6). The 3-HV incorporation increased from 11 mol% at 1 mM propionate to greater than 56 mol% at 10 mM propionate. The increase in moles percent was partly due to a rise in 3-HV incorporation from 0.47 mg/ml at 1 mM propionate to 1.2 mg/ml at 10 mM propionate and partly due to a decrease in 3-hydroxybutyrate (3-HB) incorporation from 4.4 mg/ml at 0 mM propionate to 0.97 mg/ml at 10 mM propionate. This decrease in 3-HB incorporation indicates that higher levels of

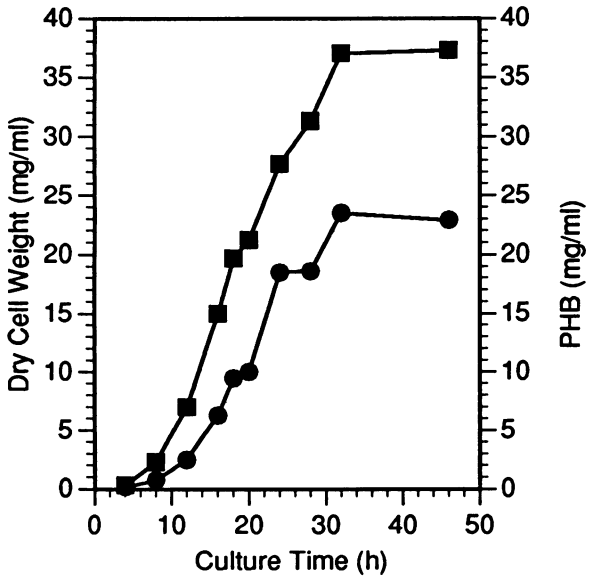


FIG. 4. PHB production of *K. aerogenes* 2688(pJM9131) in fed-batch fermentation. ■, cell dry weight; ●, PHB.

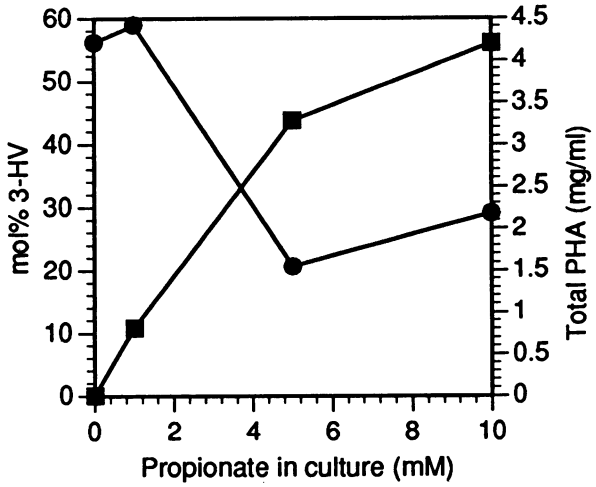


FIG. 6. 3-HV incorporation into copolymer as a function of propionate concentration in the medium. *K. oxytoca fadR* (pJM9131) was grown in 50-ml cultures (250-ml shake flasks) containing M9 minimal medium–1% glucose–100 μg of kanamycin per ml–different concentrations of propionate (0, 1, 5, and 10 mM). Duplicate 3-ml samples were taken from each flask after 30 h of growth and analyzed for 3-HB and 3-HV content. ■, 3-HV moles percent; ●, total PHA.

propionate in the medium may be detrimental to 3-HB production and, therefore, total polymer production in *K. oxytoca* M5A1(pJM9131).

In an attempt to minimize the propionate toxicity, a second experiment was conducted in which *K. oxytoca fadR* (pJM9131) was grown as described above, except that propionate was added at different times during the growth of the culture (Fig. 7). If propionate was added to the culture at the time of inoculation (Fig. 7, bar B) or in increments throughout the growth of the culture (Fig. 7, bar C), it resulted in a reduction of total polymer of approximately 27% compared with a culture in which propionate was not added. If propionate was

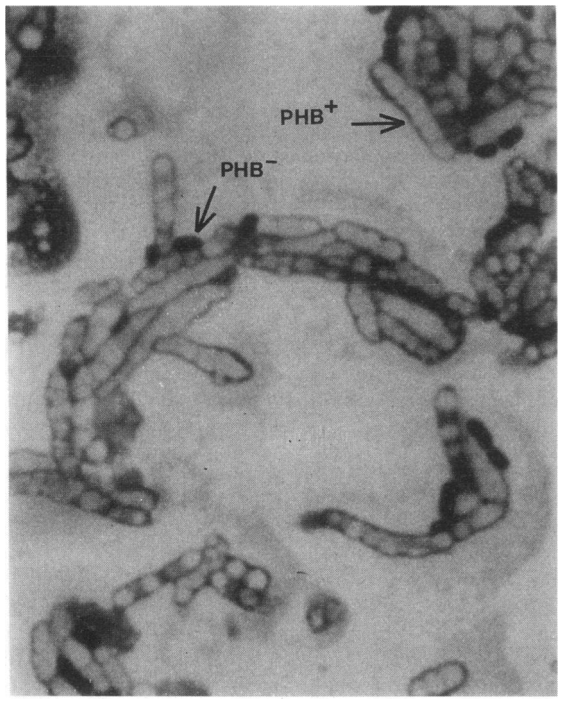


FIG. 5. Micrograph of cells from fermentation. PHB granules are seen as white (unstained) areas inside the cell. Total magnification, ×2,300.

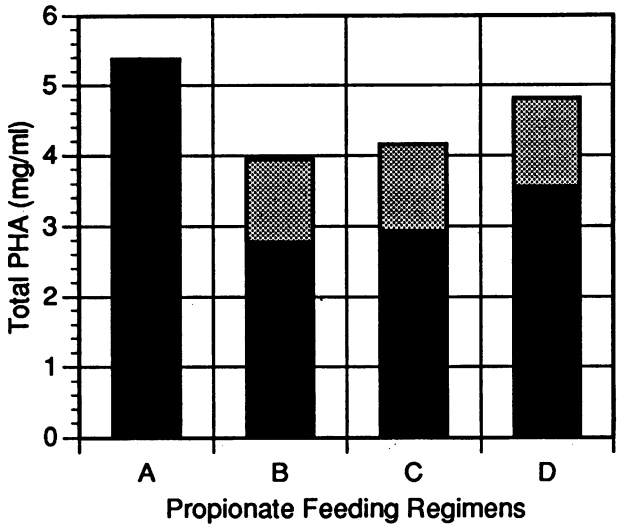


FIG. 7. Effect of different propionate feeding regimens on total polymer and 3-HV moles percent. *K. oxytoca fadR* (pJM9131) was grown in shake flasks containing M9 minimal medium–100 μg of kanamycin per ml–1% glucose. At the times indicated below, propionate was added to the cultures to a final concentration of 5 mM. After 48 h of growth, samples were taken and analyzed for 3-HB and 3-HV content. (A) No propionate added; (B) propionate added at time of inoculation; (C) propionate added in 1 mM increments at optical density (600 nm) of 0, 0.5, 1, 1.5, and 2.0; (D) propionate added at optical density (600 nm) of 2.0. Solid areas of bars indicate 3-HB content; shaded areas indicate 3-HV content.

added to the culture in early stationary phase (Fig. 7, bar D), total PHA synthesis decreased only 9% compared with the culture not containing propionate, and there was no decrease in the moles percent of 3-HV incorporated in comparison with the other two propionate addition regimens (approximately 23 mol% of 3-HV).

## DISCUSSION

In this paper, we have studied the PHA production capabilities of recombinant enterobacterial species that are able to utilize sucrose as their sole carbon source. When endowed with PHB plasmids, these strains, *E. coli* JMU213(pJM9131), *K. aerogenes* 2688(pJM9131), and *K. oxytoca* M5A1(pJM9131), produce polymer to about the same level as recombinant *E. coli* strains that have been used previously (between 3 and 4 mg/ml in shake-flask studies [16, 20, 40]). However, these strains possess the economic advantage of being able to use sucrose or sugarcane molasses as their sole carbon source. In addition, these strains grow to much higher cell densities than the laboratory strains of *E. coli* that we have previously employed, and they do this on a simpler medium.

Because it is important that the polymer have a high molecular weight in order to retain its strength during processing, we investigated the molecular weight of PHB produced in *K. aerogenes* 2688(pJM9131) and *E. coli* JMU213(pJM9131). In both strains, the molecular weight was approximately $10^6$, which was two to three times greater than that of naturally occurring PHB from *A. eutrophus* and *A. latus*. This difference could be significant, or it could be due to the fact that our figures were obtained from shake-flask-grown cultures and that the figures for naturally occurring PHB were obtained from cells grown in large fermentors. Further experiments are currently under way to determine whether PHB from fermentor-grown recombinant *Klebsiella* strains has a molecular weight above $10^6$.

The sucrose uptake of *K. aerogenes* KC2688 was commensurate with previously published figures for other *Klebsiella* species (42), but we were unable to detect sucrose uptake in *E. coli* JMU213 even though it was apparent that the strain could grow well in minimal medium containing sucrose as the sole carbon source. In TLC studies, we observed that first *E. coli* JMU213 splits the sucrose into fructose and glucose and then another reaction occurs which processes the fructose into another chemical form (as judged by movement of radioactive label on the TLC plate). Though the extracellular cleavage of sucrose has been observed previously (38, 39), we do not know of any bacteria that carry on subsequent rearrangement of the fructose. This process may account for the lag in PHB production that was seen in comparison with PHB production in *K. aerogenes* 2688(pJM9131), which takes up sucrose directly (42).

When grown on molasses, the *K. aerogenes* strain exhibited excellent growth characteristics and PHB production. However, *E. coli* JMU213(pJM9131) manifested much-reduced growth and PHB production, possibly because of toxic substances in the molasses as a result of the partial inversion of sucrose to glucose and fructose by hydrolysis with sulfuric acid. On the basis of these results, it is likely that *suc*$^+$ *E. coli* strains will face a major impediment in further development. Moreover, the strategy of using a *suc*$^+$ *E. coli* because of its genetic similarity to laboratory *E. coli* strains appears to be unnecessary since *K. aerogenes* 2688(pJM9131) grown in medium containing molasses demonstrated PHB production characteristics that were better than those of laboratory strains of recombinant *phb*$^+$ *E. coli* that had been previously tested.

Fed-batch studies done on *K. aerogenes* KC2688(pJM9131) indicated that this organism has excellent potential for production of large amounts of polymer. In our initial experiment, we obtained 24 g of PHB per liter in 32 h of growth. In terms of productivity, this is 0.65 g of PHB per liter per h. In studies with similar-size fermentation vessels, Ramsay et al. (35) obtained a productivity of 0.65 g of PHB per liter per h for *A. eutrophus* grown on glucose (24 g of PHB in 37 h), and Page (32) obtained a productivity of approximately 0.5 g of PHB per liter per h for *Azotobacter vinelandii* UWD grown on sugar beet molasses (20 g of PHB in 40 h). If rate of PHB production is examined only during the active production phase (discounting the lag phase), the productivity for *K. aerogenes* 2688 (pJM9131) was 1.04 g of PHB per liter per h compared with approximately 1.0 g of PHB per liter per h for *A. eutrophus* and 1.09 g of PHB per liter per h for *A. vinelandii* UWD. These figures indicate that PHB production in *K. aerogenes* 2688 is at least commensurate with that in bacterial strains that naturally produce PHB. Further fed-batch work is currently being done to optimize PHB production in *K. aerogenes* 2688(pJM9131).

We have been able to show that *K. oxytoca* M5A1 *fadR* can very efficiently make the copolymer PHB-co-V when grown in the appropriate medium. This copolymer production is superior to the copolymer production that was developed in *E. coli fadR atoC*(Con) strains because it produces copolymer in a simpler medium, to higher levels, and with less sensitivity to propionate levels in the culture. In shake-flask studies with an *E. coli fadR atoC*(Con) strain, the addition of propionate to a final concentration of 5 mM resulted in the incorporation of 3-HV to a concentration of 5 mol% (total PHA was 3.3 mg/ml [40]). In comparison, we grew *K. aerogenes* 2688(pJM9131) in medium containing a final concentration of 5 mM propionate to obtain the incorporation of 3-HV to 23 mol% (total PHA was 4.8 mg/ml). These results also compare well with *A. eutrophus* and *A. latus*, for which, in one shake-flask study, a final concentration of 40 mM propionate in the medium facilitated 3-HV incorporation to 45 mol% (*A. eutrophus*), and a final medium concentration of 1.3 mM propionate facilitated 3-HV incorporation to 3 mol% (*A. latus* [35]). In a separate study, *A. latus* and *A. eutrophus* were able to incorporate 3-HV into the copolymer to final concentrations of 22 mol% and 5%, respectively, when the culture contained 10 mM propionate (9).

Higher levels of propionate were toxic to the growth of *K. aerogenes* 2688 and PHA production. This was not an unexpected result. The toxicity of propionate to PHB production in *A. eutrophus* is well documented (8). It is also well-known that the toxicity of propionate can often be circumvented by adding the propionate to the culture in early stationary phase. This strategy worked well in *K. aerogenes* 2688 *fadR*(pJM9131) cultures. However, the same strategy was not successful for *E. coli fadR atoC*(Con) strains carrying similar plasmids (40). In this case, the propionate addition at the beginning of the culture or in mid-logarithmic phase resulted in PHA levels between 2 and 4 mg/ml, but addition of propionate to stationary-phase culture almost completely inhibited PHA production. The reason for this difference is not apparent at this time.

Before recombinant *Klebsiella* species can be considered for scale-up strategies, a *Klebsiella* strain which contains a stable PHB$^+$ plasmid must be developed. This is because antibiotics, even at bulk prices, would increase the cost of production to prohibitive levels. *K. aerogenes* 2688(pJM9131) and other *Klebsiella* strains that we have tested suffer from plasmid instability, even when grown in the presence of antibiotics. This was easily seen in micrographs of cells from the fermentation in which there were generally two types of cells, those appearing to be normal *K. aerogenes* and those that were greater than

90% PHB. Upon centrifugation, these separated into two distinct layers, a tan layer representing the PHB-filled cells and a darker brown layer representing $PHB^-$ cells.

It is likely that the plasmids we use (ColE1 replicon) are segregationally unstable. Bacterial cells that have lost the plasmid possess a large growth advantage over cells carrying the metabolic burden of PHB production, and so they rapidly overgrow the culture (6, 10). This metabolic burden can easily be envisioned by observing the $PHB^+$ cells which are often 10 to 20 times larger in volume than $PHB^-$ cells and by noting that in shake-flask culture the cells accumulated PHB to more than 50% of their dry weight within 5 to 6 h after inoculation. It may be possible to counter segregational instability with runaway expression vectors carrying stabilization genes, such as the *parB* locus (14, 18, 22). At noninducing temperatures, these vectors maintain their copy number below 10, thereby limiting the production of PHB and its toxic effect. In addition, segregational instability is suppressed by the existence of the *parB* locus, which encodes a protein which kills cells that have lost their plasmid (18).

In further studies, we are examining the possibility of employing *Klebsiella* strains as production systems for not only PHB and PHB-co-V, but for other PHAs. Species of the genus *Klebsiella* offer many immediate advantages in that they are well characterized and are already employed in commercial strategies for the production of chemical products (1, 48). Moreover, *Klebsiella* spp. are able to grow on a diverse group of carbon sources that includes molasses and cellobiose. With a significant amount of research being conducted in this area, it is conceivable that PHAs could be produced with cellulose as the carbon source. In addition, we have recently found that a cloned PHA synthase from *Nocardia corralina* can be expressed in *Klebsiella* species even though it is not expressed in *E. coli* (unpublished data). We are particularly interested in this phenomenon, which implies a type of transcriptional control for PHA synthases of some species that differs from that found in the PHA synthase of *A. eutrophus*, which is well expressed in *E. coli*. In addition, it provides us with a recombinant host to employ synthases with broader substrate specificities for the production of polymers other than PHB and PHB-co-V.

## ACKNOWLEDGMENTS

We thank Dorothy Connelly for her assistance in the preparation of the manuscript and Cassie Paup and John Kidwell for conducting the characterization of the molecular weight of the polymers. We also thank Brian Janes, Robert Bender, Gary Roberts, and Paul Black for the bacterial strains that were sent.

This work was supported by a grant from Tredegar Industries.

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