Glycerol has become an inexpensive and abundant carbon source due to its occurrence as a by-product of biodiesel production particularly in Europe. With every 100 tons of biodiesel produced via transesterification of vegetable oils or animal fats, about 10 tons of crude glycerol is produced. The tremendous growth of the biodiesel industry has generated a glycerol surplus, resulting in a sharp decrease in glycerol prices between 2005 and 2007 (16, 33). Therefore, the development of processes to convert crude glycerol into higher-value products is an urgent need. One possibility is the production of thermoplastics, like polyhydroxyalkanoates (PHAs).

PHAs are bacterial storage compounds for carbon and energy. They are synthesized and intracellularly deposited as granules in many bacteria when the cells are cultivated in the presence of an excess of a carbon source and if one other nutrient limits growth. PHAs exhibit a high degree of polymerization. Molecular masses of up to several million Da have been reported previously (14). They are biodegradable, insoluble in water, nontoxic, biocompatible, piezoelectric, and/or elastomeric. These features make PHAs suitable for several applications in the packaging, medicine, pharmacy, agriculture, and food industries, as well as for raw material for production of enantiomerically pure chemicals and for production of paints (1, 28). One interesting polymer is poly(3-hydroxypropionate) (poly(3HP)). It combines the properties of poly(3-hydroxybutyrate) (poly(3HB)) and poly(2-hydroxypropionate) (poly(2HP)), which is also referred to as polyactic acid. Poly(3HP) has a higher stability than poly(2HP) with regard to hydrolytic cleavage but is more easily enzymatically cleaved than poly(3HB) due to the lacking methyl groups at the polymer backbone (7). Until now, poly(3HP) could be synthesized only chemically via the ring-opening polymerization of β-propiolactone (2) or biotechnologically by a propionyl-coenzyme A (CoA) synthase, like PrpE from Salmonella enterica, and a PHA synthase, like PhaC1 from Ralstonia eutropha, with 3-hydroxypropionate (3HP) as a precursor molecule (27, 31). In addition, poly(3HB-co-3HP) copolyesters with a very low 3HP content not exceeding 2.1 mol% of the constituents were obtained from structurally unrelated carbon sources in recombinant strains of R. eutropha expressing enzymes of the 3HP cycle of Chloroflexus aurantiacus (5). Furthermore, poly(3HB-co-3HP) copolyesters were obtained from R. eutropha and Alcaligenes latus when the cells were cultivated on structurally related carbon sources, like 3-hydroxypropionate and various α,ω-alkanediols (7, 17).

The aim of this study was to engineer a novel, non-naturally existing pathway for the biotechnological conversion of glycerol into poly(3HP) in Escherichia coli (Fig. 1). For this, we used (i) the glycerol dehydratase DhaB1 from Clostridium butyricum, with 3-hydroxypropionate (3HP) as a precursor molecule (27, 31). In addition, poly(3HB-co-3HP) copolyesters with a very low 3HP content not exceeding 2.1 mol% of the constituents were obtained from structurally unrelated carbon sources in recombinant strains of R. eutropha expressing enzymes of the 3HP cycle of Chloroflexus aurantiacus (5). Furthermore, poly(3HB-co-3HP) copolyesters were obtained from R. eutropha and Alcaligenes latus when the cells were cultivated on structurally related carbon sources, like 3-hydroxypropionate and various α,ω-alkanediols (7, 17).

Bacterial strains and plasmids. All strains and plasmids used in this study are listed in the supplemental material. For routine cloning, plasmids were introduced into E. coli TOP10 or E. coli XL1-Blue. For poly(3HP) production, the plasmid was transferred into E. coli HMS174(DE3).

Media. To produce poly(3HP) in fed-batch fermentation, we used Riesenberg medium (Rb) at 37°C, with an initial glycerol (Caldic) concentration of 300 mM (8). The feeding solution for the anaerobic production phase contained, in addition to the ingredients described by Korz et al. (8), 0.5 M di-Na+-fumarate (Carl Roth), 0.5 M K+-Na+-tartrate (Carl Roth), and 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). The cultivation was performed with a 2-liter Biostat B plus (Sartorius) fermenter. All other cultivations of E. coli or Salmonella enterica serovar Typhimurium LT2 were done under aerobic conditions at 37°C in lysogeny broth (LB) medium (21). The cultivation of Clostridium butyricum was done at 30°C under...
anaerobic conditions in 2× YT medium containing 2% (wt/vol) glucose (19). Antibiotics were added at appropriate concentrations as follows: ampicillin, 75 μg/ml; chloramphenicol, 25 μg/ml; kanamycin, 50 μg/ml; gentamicin, 20 μg/ml; and rifampin, 20 μg/ml.

**Plasmid construction.** The construction of pCOLADuet-1::dhaB1B2::pduP::phaC1 is described in the supplemental material.

**Testing of the expression plasmid.** All *in vitro* tests were done with crude extracts obtained by sonication from cells of *E. coli* HMS174(DE3)/pCOLADuet-1::dhaB1B2::pduP::phaC1 and cells of *E. coli* HMS174(DE3)/pCOLADuet-1 as a negative control. Expression of the cloned genes *dhaB1*, *dhaB2*, *pduP*, and *phaC1* was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) performed according to Laemmli (10) and by staining with Coomassie brilliant blue R-250 (32). The protein amount was determined by the method of Lowry et al. (13).

The glycerol dehydratase activity was measured as described by Raynaud et al. (19), and the propionaldehyde dehydrogenase activity was quantified according to Leal et al. (12). The basal activity was subtracted from the enzyme activity of 12.7 pmol·min⁻¹·mg⁻¹ protein. The addition of coenzyme A initiated the coenzyme A acylation of 3-hydroxypropionate with an average (± standard deviation) activity of 12.7 ± 1.3 μmol·min⁻¹·mg⁻¹ protein. The addition of coenzyme A initiated the coenzyme A acylation of the aldehyde group of 3HP by PduP (average [± standard deviation] of 6.5 ± 1.6 μmol·min⁻¹·mg⁻¹ protein). Levels for *E. coli* HMS174(DE3)/pCOLADuet-1 were not detectable. All assays were done in triplicate with crude extracts from different cultures.

**In vivo PHA accumulation in fed-batch cultures.** For poly(3HP) accumulation, *E. coli* HMS174(DE3)/pCOLADuet-1::dhaB1B2::pduP::phaC1 was cultivated in fed-batch fermentation for 92 h at 37°C in Rb (Table 1). Similarly to the microbial conversion of glycerol to 1,3-propanediol (24), the cultivation of our recombinant *E. coli* strain was performed in two different steps, with an aerobic growth phase and an anaerobic PHA production phase (Fig. 2). We used pure or crude glycerol, respectively, as the sole carbon source. In the first phase, an agitation of 400 rpm and an airflow of 2.5 ml/h were applied. The pH and the temperature were adjusted to 6.7 and 37°C, respectively. When the cells reached the early stationary growth phase, the aerobic fed batch was started and the Rb feeding solution, supplemented with 2 M glycerol, was added with a flow of 20 ml/h. After 45 h, the airflow was stopped, the culture was flushed with nitrogen, and the anaerobized system was closed. During the anaerobic production phase, di-Na fumarate, K Na-tartrate, and IPTG were added to the feeding solution and applied with a flow of 20 ml/h for 33 h to the fermentor to induce poly(3HP) production. The main fermentation products were succinate and ethanol. Detection of growth-inhibiting acetic acid was only marginal (Fig. 3; Table 1). Accumulation of PHAs within the cells was monitored by fluorescence microscopy (22), transmission electron microscopy (TEM) (30), and gas chromatography-mass spectrometry (GC-MS) (4, 23). For cultures with pure glycerol, accumulation of PHAs started about 6 h after protein expression was induced with IPTG; for cultures with crude glycerol, it started at 9 h. After 92 h of incubation, the cells were harvested by centrifugation for PHA analysis and isolation. The appearance of the accumulated poly(3HP) within the cells is typical for PHA synthesis in recombinant strains without any phasins (15, 25). Often, one big granule was seen in the middle of the cell (Fig. 4).

**PHA isolation and identification.** PHAs were extracted from lyophilized cells with hot chloroform in a Soxhlet apparatus. The extract was concentrated with a rotary evaporator, and the PHAs were precipitated by pouring this solution into 10 vol of ice-cold ethanol (11). This procedure was repeated at least four times to obtain highly purified polymer samples. Dried precipitate appeared as a white powder. Identification of the polyester was done by GC-MS (4, 23) and electrospray ionization-MS (ESI-MS) (see the supplemental material). Methyl esters of the extracted polymer eluted at a retention time of 7.045 min, which was identical to the retention time of 3-hydroxypropionate methyl esters. This particular peak was fractionated by electron impact (EI) to record the GC-MS spec-
Again, the extracted peak spectrum of the produced PHAs corresponded with the extracted peak spectrum of 3-hydroxypropionate. The ESI-MS analysis of the monomer, achieved by hydrolysis of the extracted polymer, showed a mass of 89 kDa, which corresponds to the mass of ionized 3-hydroxypropionate. The same peak occurred when commercially purchased 3-hydroxypropionate was injected. Collision-induced dissociation of the respective peaks led to nearly identical masses, i.e., 59 and 43 kDa (data not shown).

With pure glycerol as the sole carbon source, the final poly(3HP) content of the cells amounted to 11.98% (wt/wt [cell dry weight]), whereas cultures with crude glycerol accumulated the polymer only up to 5.2% (wt/wt [cell dry weight]) (Table 1). This could be due to growth- and/or enzyme activity-inhibiting residues like methanol, methyl ester, or ash from the transesterification of vegetable oils or animal fats.

Compared to that for other PHA-producing bacteria, this content is low. This is probably caused by a loss of pCOLADuet-1::dhaB1B2::pduP::phaC1. To verify plasmid stability, appropriately diluted samples of the fermentation broth were spread on LB agar plates in the presence or absence of kanamycin and incubated overnight at 37°C. The number of colonies on the plates without antibiotics was set to 100%. After 92 h of fermentation, only 56% of the cells possessed the expression plasmid. To achieve more stable production, a plasmid addiction system as recently described (9, 29) could be established. The advantages of such systems are numerous: (i) they provide antibiotic-independent selection, (ii) they result in higher plasmid stability, and (iii) due to the higher copy number, the expression level is higher than in the case of a chromosomal insertion. Addiction systems may therefore contribute to a reduction in production cost. Studies with such a system will be done during further strain optimization.

One further problem with poly(3HP) production is the redox balance (see the supplemental material) and the disposal of reducing equivalents. During polymer accumulation, large amounts of NADH were formed. A solution for that could be the coexpression of an NADH-consuming enzyme and cop-
roduction of a reduced product. It is obvious to use the 1,3-propanediol dehydrogenase (DhaT) from C. butyricum (19). This enzyme has two major advantages: (i) the surplus of NADH will be oxidized to NAD⁺, and (ii) another valuable product will be generated from glycerol.

In summary, the recombinant E. coli strain HMS174(DE3)/pCOLADuet-1::dhaB1B2::pduP::phaC1 can accumulate poly(3HP) at up to 11.98% of the cell dry weight. For an industrially relevant large-scale production of poly(3HP), further developments are necessary to obtain a high polymer yield on glycerol.

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FIG. 4. TEM of poly(3HP)-accumulating cells of E. coli HMS174(DE3)/pCOLADuet-1::dhaB1B2::pduP::phaC1. The fermentation was divided into an aerobic growth phase and an anaerobic production phase and was carried out using Riesenberg medium (19). The feeding solution for the anaerobic production phase was supplemented with 0.5 M di-Na-fumarate, 0.5 M K2Na-tartrate, and 1 mM IPTG. Bars, 100 nm.


