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Polymerase C1 Levels and Poly(*R*-3-Hydroxyalkanoate) Synthesis in Wild-Type and Recombinant *Pseudomonas* Strains

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A functional antibody highly specific for polymerase C1 of *Pseudomonas oleovorans* GPo1 was raised and used to determine polymerase C1 levels in in vivo experiments. The polymerase C1 antibodies did not show a cross-reaction with polymerase C2 of *P. oleovorans*. In wild-type *P. oleovorans* GPo1 and *Pseudomonas putida* KT2442, amounts of 0.075 and 0.06% polymerase relative to total protein, respectively, were found. *P. oleovorans* GPo1(pGEc405), which contained additional copies of the polymerase C1-encoding gene under the control of its native promoter, contained 0.5% polymerase C1 relative to total protein. Polymerase C1 reached 10% of total cell protein when the polymerase C1-encoding gene was overexpressed through the P_{alk} promoter in *P. oleovorans* GPo1(pET702, pGEc74). Amounts of poly(*R*-3-hydroxyalkanoate) (PHA) increased significantly under non-nitrogen-limiting conditions when additional polymerase C1 was expressed in *P. oleovorans*. Whereas *P. oleovorans* produced 34% (wt/wt) PHA under these conditions, a PHA level of 64% (wt/wt) could be reached for *P. oleovorans* GPo1(pGEc405) and a PHA level of 52% (wt/wt) could be reached for *P. oleovorans* GPo1(pET702, pGEc74) after induction, compared to a PHA level of 13% for the uninduced control. All recombinant *Pseudomonas* strains containing additional polymerase C1 showed small changes in their PHA composition. Larger amounts of 3-hydroxyhexanoate monomer and smaller amounts of 3-hydroxyoctanoate and -decanoate were found compared to those of the wild type. Two different methods were developed to quantify rates of incorporation of new monomers into preexisting PHA granules. *P. oleovorans* GPo1 cells grown under nitrogen-limiting conditions showed growth stage-dependent incorporation rates. The highest PHA synthesis rates of 9.5 nmol of C_8/C_6 monomers/mg of cell dry weight (CDW)/min were found during the mid-stationary phase, which equals a rate of production of 80 g of PHA/kg of CDW/h.

Pseudomonas oleovorans is capable of synthesizing a fully biodegradable plastic as intracellular storage material, poly(*R*)-3-hydroxyalkanoate (PHA). This was first discovered by de Smet et al. in 1983 (4) and was later found to be a common feature in fluorescent pseudomonads (18). Interest in PHAs has recently grown and is based on its biodegradability, hydrophobic and elastomeric properties, and the high molecular weight of the polymer. Future applications will use these characteristics in paper coatings or food packaging (3) or in possible medical technology such as drug release capsules (25).

As a general feature, PHA is produced as storage material when *Pseudomonas* strains are grown in the presence of an excess carbon source and under nutrient limitation. Different nutrient limitations, such as phosphorus, nitrogen, sulfur, or magnesium (23), can enhance PHA production. High-level productions of up to 74% PHA can be reached by *P. oleovorans* cells grown on *n*-octane and under nitrogen limitation (14). Synthesis of PHA by *P. oleovorans* GPo1 begins at the end of the exponential growth phase when nitrogen limitation starts, in contrast to *Pseudomonas putida* KT2442, in which PHA synthesis starts during exponential growth (19). When grown on fatty acids, PHA synthesis in *P. oleovorans* and *P. putida* KT2442 is directly related to the fatty acid oxidation pathway, as reflected by the incorporated monomers (8). Although *P. oleovorans* is in fact a *P. putida* biotype A strain (28), there are considerable differences between *P. oleovorans* and other *P. putida* strains. Thus, *P. putida* KT2442 is able to produce PHA from substrates such as glucose (15), citrate (13), or gluconate (32), whereas *P. oleovorans* is unable to do so; in this case, *P. putida* KT2442 evidently derives PHA precursors from the fatty acid synthesis pathway or fatty acid elongation pathway (16). PHAs made in both strains are heteropolymers composed of medium-chain-length (*R*)-3-hydroxy fatty acids, ranging from C_6 to C_{12} fatty acids. When octanoate is used as a carbon source in *P. oleovorans*, PHA normally consists of 88 to 92.5% (mol/mol) 3-hydroxyoctanoate monomers and of 7.5 to 12% 3-hydroxyhexanoate monomers (18, 19). The only two homopolymers that have been reported for *P. oleovorans* are poly-3-hydroxyheptanoate (18) and poly-3-hydroxy-5-phenylvalerate (10).

In *P. oleovorans*, the genes involved in PHA biosynthesis have been cloned and sequenced (20). They are chromosomally located in the *pha* operon. Clustering of the genes for PHA synthesis is found for many different PHA-producing strains (29). A homologous *pha* operon has also been found for *Pseudomonas aeruginosa* (31). The operon in *P. oleovorans* consists of two polymerase genes, *phaC1* and *phaC2*; a depolymerase gene, *phaZ*, which is located in between the two polymerase-encoding genes; and a fourth gene, *phaD*, which encodes a protein of unknown function.

Polymerases C1 and C2 have a homology of 53.6%, and as was shown by complementations of the PHA-negative mutant *P. putida* GPp104, the presence of one polymerase is sufficient for PHA production (20). Polymerases of *P. oleovorans* are very unspecific, as has been shown by the broad range of substrates that can be converted into PHA in vivo. Over 60 different monomer units, including those containing halogenated side groups or double bonds, have already been introduced in PHA by *P. oleovorans* (30), permitting the creation of PHAs with special characteristics or the use of introduced functional groups for further processing of PHA.

There are small differences between polymerase C1 and C2, as shown by studies of *P. putida* GPp104 *phaC* recombinants.

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TABLE 1. Bacterial strains and plasmids with relevant genotype and phenotype

Strain or plasmid	Relevant genotype or phenotype[a]	Source or reference
Strains		
P. oleovorans GPo1	OCT PHA^+	27
P. putida KT2442	Rif^r PHA^+ *hsdR1 hsdM*$^+$	1
P. putida GPp104	NTG mutant of KT2442, PHA^-	20
E. coli JM109	F′ *rec*A1 *end*A1 *hsd*R17 (r_K^- m_K^+) *rel*A *sup*E	35
Plasmids		
pGEc404	*phaC2* and *phaD* in pJRD215 Km^r Sm^r	20
pGEc405	*phaC1* and *phaZ* (59% of the N terminus) in pJRD215 pGEM-7Zf(+) Km^r Sm^r Ap^r	19
pET702	P_{alk}-*phaC1*-VSV G tag in pVLT35 RP4 *ori* Sm^r Ap^r	Q. Ren
pGEc74	*alkST* in pLAFR1 RK2 *ori* Tc^r	9
pRK2013	*mob*$^+$ *tra*$^+$ *colE1 ori* Km^r	5

[a] OCT, natural plasmid enabling growth on C_6 to C_{12} alkanes; NTG, *N*′-methyl-*N*′-nitro-*N*′-nitrosoguanidine; VSV G tag, 12-amino-acid tag based on VSV G to which monoclonal antibodies are available (21); Ap, ampicillin; Km, kanamycin; Rf, rifampin; Sm, streptomycin; Tc, tetracycline.

PHA monomer compositions changed towards more C_6 when the polymerase C1-encoding gene was expressed and to incorporation of more C_8 monomers when the polymerase C2-encoding gene was expressed (19).

In this paper, the effect of the amount of polymerase C1 in relation to PHA production and its composition were studied for *P. oleovorans* GPo1 and *P. putida* KT2442 in order to determine whether an increase of polymerase C1 can improve PHA productivity. Polymerase C1 of *P. oleovorans* was purified, and antibodies specific for the protein were raised. The antibodies were used as a tool for quantification of polymerase C1 levels in vivo for *Pseudomonas* strains grown under different conditions.

MATERIALS AND METHODS

Bacterial strains and media. Table 1 lists the bacterial strains and plasmids used in this study. The *P. oleovorans* GPo1, *P. putida* GPp104, and *P. putida* KT2442 strains were transformed with plasmids containing either the polymerase C1- or polymerase C2-encoding gene. The recombinants were made by three parental matings with donor strain *Escherichia coli* JM109(pGEc404 or pGEc405), *E. coli* JM109(pRK2013) as the helper strain (5), and *P. oleovorans* GPo1, *P. putida* GPp104, or *P. putida* KT2442 as the acceptor strain. Transconjugants were isolated on Luria broth (LB) plates with the appropriate antibiotic (kanamycin, 50 μg/ml; streptomycin, 50 μg/ml; tetracycline, 12.5 μg/ml; and rifampin, 25 μg/ml) and stored as stabs. Polymer synthesis levels of these recombinants were reproducible for 3 months when stored as stabs, in contrast to the recombinant stocks, which were stored in 15% glycerol at −80°C. *P. oleovorans* GPo1(pET702, pGEc74) (24, 26a) was used as an overexpression system for polymerase C1 and constructed by electroporation (7).

LB containing 5 g of NaCl per liter, 5 g of yeast extract (YE) per liter, and 10 g of tryptone per liter, E medium (33), E_2 medium, and $0.2NE_2$ medium (containing 20% of the total nitrogen in E_2 medium) (23) were used. MT trace elements (23) were added to all minimal media. For all *P. oleovorans* GPo1 recombinants and *P. putida* KT2442 strains, 0.1% YE was added to the minimal media. The following different carbon sources were added. Four percent (vol/vol) nonane was added as a second phase; 15 mM sodium octanoate (1.5 M stock solution) or 15 mM nonanoic acid (stock solution of 0.25 M dissolved in 10% Brij 58 [Sigma], adjusted to pH 7.1 and sterilized) was added to minimal medium. Precultures were made on E medium for all minimal medium main cultures of *P. oleovorans* GPo1; a droplet of nonane was added to precultures when nonane was used as the carbon source to induce the *alk* system (2). When main cultures were grown on minimal medium with 0.1% YE, precultures were made on LB medium with the appropriate antibiotics.

Determination of cell growth. Cell growth was determined by optical density at 450 nm (OD_{450}) on a Pharmacia spectrophotometer (34). Cell dry weight (CDW) measurements were done to determine the actual cell mass formed. CDW values for recombinant strains of *P. oleovorans* GPo1 and *P. putida* KT2442 were based on OD_{450} values, with the following factor: OD_{450} of 1 = 0.26 mg of CDW per ml.

Incorporation of unlabeled monomers into preexisting PHA. *P. oleovorans* GPo1 was grown in 250-ml batch cultures on $0.2NE_2$ medium on 4% nonane or in 200-ml cultures on 15 mM nonanoic acid with or without 0.1% YE at 30°C. Recombinant strains of *P. oleovorans* GPo1 were grown in 200-ml batch cultures on $0.2NE_2$ medium with 15 mM nonanoic acid, 0.1% YE, and the appropriate antibiotics at 30°C. A pulse of 15 mM octanoate was added to the cultures at early, middle, and late stages of PHA synthesis. PHA synthesis stages are defined by hours after inoculation as early, mid, and late stages for 5 to 7, 8 to 12, and >12 h, respectively. PHA compositions were analyzed, and C_8 and C_6 monomer incorporation rates in preexisting $C_{odd\text{-}numbered}$ PHAs were determined.

Incorporation of 1-^{14}C-labeled monomers into preexisting PHA. *P. oleovorans* GPo1 and *P. putida* GPp104 were grown in 50-ml batch cultures at 30°C on 0.2 NE_2 with octanoate as a substrate. Substrate amounts of 5, 10, or 22.5 mM were added to cultures pulsed in the early, mid-, or late stationary phase, respectively, in order to start with a constant and small amount of octanoate versus [1-^{14}C]octanoate. Octanoate was measured with a Permabond Carbowax 20 M column (Machery-Nagel) on a gas chromatograph, and corrections were made for [1-^{14}C]octanoate dilutions. Four milliliters of the culture was removed at different PHA synthesis stages, incubated in a table shaker, and pulsed with 18.5×10^3 Bq/ml of [1-^{14}C]octanoate (18.5×10^3 Bq = 0.009 μmol of [1-^{14}C]octanoate). Samples of 0.5 ml were taken and centrifuged, and PHA was extracted from the cell pellet by the method of Hahn et al. (11). Thirty percent sodium hypochlorite (1.5 ml) and 10 μl of 5 N NaOH were added to the cell pellet, which was then incubated at room temperature for 10 min to separate free [1-^{14}C]octanoate from 1-^{14}C-labelled monomers incorporated into PHA. An equal volume of chloroform was added, and the solutions were stored on ice and vortexed four times for 10 s each. After phase separation, the radioactive content of the water and chloroform phase was measured. To 400 μl of each phase, 4 ml of Readysafe scintillant (Beckman) was added, and radioactivity was counted in a scintillation counter. For the incorporation of unlabeled C_6 monomers (derived from [1-^{14}C]octanoate), a correction was made; C_6 monomers were calculated to be 10% of the C_8 monomers incorporated (data not shown).

Determination of PHA. Cell samples containing on the average 2 to 5 mg of PHA were assayed according to the method of Lageveen et al. (23). The methanolyzed PHA monomers were analyzed with a CP-Sil 5 CB column (Chrompack) on a gas chromatograph.

Preparation of polymerase C1 antibodies. *P. putida* GPp104(pGEc405) was grown on nitrogen-limited medium with octanoate as a substrate for polymerase C1 production. After opening the cells in a French pressure cell, the PHA granules were isolated on a discontinuous sucrose gradient (15 and 20% [wt/wt]) in 50 mM Tris-HCl (pH 8.0). Gradients were spun at 110,000 × *g*, at 4°C for 3.5 h, and the white layer at 15% sucrose, containing PHA granules, was washed twice in 50 mM Tris-HCl (pH 8.0). Polymerase C1 was released from the granules by incubating 1 part granules with 2 parts Deriphate 160 (Henkel) for 30 min at room temperature. After 60 min of centrifugation (10,000 × *g* at 4°C), the polymerase C1 fraction was purified by high-performance liquid chromatography on a butyl nucleosil 100-7 RP column (Machery-Nagel) with a water–0.1% trifluoroacetic acid–acetonitrile–0.1% trifluoroacetic acid gradient. The nearly pure polymerase C1 fractions were concentrated and loaded on a sodium dodecyl sulfate–10% polyacrylamide gel (see below), the band was cut out, and polymerase C1 was eluted from the gel and dialyzed against 0.2 M sodium bicarbonate (12). A New Zealand rabbit was injected three times with 150 μg of protein with time intervals of 4 weeks, and after 10 weeks, the blood containing the antibodies against polymerase C1 was obtained.

Preclearing the polymerase C1-containing blood serum. In order to remove unspecific antibodies, 0.5 ml of blood serum was incubated with 35 mg (wet weight) of whole cells of *P. putida* GPp104 for 2 h at room temperature. The supernatant, obtained after centrifugation (10 min at 10,000 × *g*), contained the specific polymerase C1 antibodies and could be stored at −20°C for up to 4 weeks. Storage at 4°C leads to loss of activity within 5 days.

SDS-PAGE and Western blot analysis. Proteins of whole cell samples were separated by SDS-polyacrylamide gel electrophoresis (PAGE) with 10% polyacrylamide gels as described before (22). Staining of protein gels was done with Coomassie brilliant blue R-250 (12). For Western blotting, the proteins were transferred from unstained gels to 0.45-μm-pore-diameter Protran nitrocellulose membranes (Schleicher & Schuell, Kassel, Germany) according to the manufacturer, with a Bio-Rad Transblot SD semidry electrophoretic transfer cell. Proteins were transferred for 1.5 h at 100 V (10 mA). The membrane was washed three times in Tris-buffered saline (TBS [pH 8]) and treated with TBS with 3% milk powder to block nonspecific binding. Primary antibody (rabbit anti-PHA polymerase C1 [1:250] or monoclonal mouse anti-vesicular stomatitis virus glycoprotein (VSV G) tag [21] [1:100]) was applied in fresh blocking solution and incubated at 25°C for 2 h. Membranes were then rinsed three times in TBS, followed by the addition of horseradish peroxidase-conjugated donkey anti-rabbit antibody (1:5,000) or sheep anti-mouse antibody (1:10,000) in fresh blocking solution and incubated at 25°C for 1 h. Membranes were finally washed in TBS (three times for 5 min each) and detection of the antibody was done after

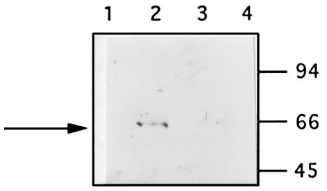

FIG. 1. Specificity of polymerase C1 antibody for PHA polymerases from *P. oleovorans*. The specificity of polymerase C1 antibodies towards polymerase C1 and polymerase C2 from *P. oleovorans* GPo1 was determined by Western blot analysis. The samples applied were cell extracts of *P. putida* GPp104 (PHA-negative mutant [lane 1]), *P. putida* GPp104(pGEc405) (polymerase C1 [lane 2]), *P. putida* GPp104(pGEc404) (polymerase C2 [lane 3]), and isolated PHA granules from *P. putida* GPp104(pGEc404) containing 0.5 μg of polymerase C2 (lane 4). In lanes 1 to 3, 25 μg of protein was loaded. Molecular mass marker sizes are given on the right in kilodaltons. The arrow indicates polymerase C1.

incubation with enhanced chemiluminescence kit reagents (Amersham Life Science) by exposure of the membrane to autoradiograms. Longer exposure times were needed for Western blot detections with polymerase C1 antibodies compared to VSV G tag antibodies due to a lower sensitivity of the antibody mentioned first. Amounts of polymerase C1 were estimated by comparison to known amounts of a polymerase C1 standard. For this comparison, PHA granules containing polymerase C1 were used as a standard and quantified by densitometric scanning with exact amounts of protein of a molecular weight standard.

RESULTS

Specificity of polymerase C1 antibodies. The specificity of the polymerase C1 antibody for PHA polymerases from different *Pseudomonas* strains was tested by Western blot analysis. A highly specific anti-polymerase C1 antibody was obtained, showing reactions with all polymerase C1-containing strains tested (Fig. 1 and 2). Although *P. oleovorans* GPo1 produced 44% (wt/wt) PHA, the amount of polymerase C1 was at the detection limit of ~0.75 mg of polymerase C1/g of protein, as determined by densitometric scanning. The polymerase from *P. putida* KT2442 was also detected at a level of ~0.6 mg/g of protein. *P. putida* GPp104, the PHA-negative mutant, did not show any cross-reaction with the antibody, but the protein was seen when the strain was complemented with the *phaC1* gene on plasmid pGEc405 (Fig. 1). The antibody raised against polymerase C1 did not cross-react with polymerase C2 of *P. oleovorans* GPo1. No reaction was found for whole cells of *P. putida* GPp104(pGEc404) or isolated PHA granules containing a 40-fold excess polymerase C2 compared to detectable levels of polymerase C1 (Fig. 1).

Effects of polymerase C1 amounts on PHA synthesis in different *Pseudomonas* strains under nitrogen-limiting and nonlimiting conditions. Additional copies of the polymerase C1-encoding gene in *P. oleovorans* GPo1(pGEc405) led to a maximum of 0.4% polymerase C1 per total protein under nonlimiting conditions, compared to *P. oleovorans* GPo1 containing only 0.075% polymerase C1 (wt/wt of protein). Batch cultivation of *P. oleovorans* GPo1(pGEc405) resulted in an increasing amount of polymerase C1 per total protein with time, as was detected by Western blot analysis, under both nitrogen-limited and nonlimited conditions (Fig. 2A). During batch cultivation of *P. putida* KT2442(pGEc405), a constant and significant amount of polymerase C1 was found during growth in both nitrogen-limited and non-nitrogen-limited media (ca. 2.5% polymerase C1 per total protein [Fig. 2B]). When *P. putida* KT2442 was grown on citrate or gluconate, no polymerase could be detected, even though some PHA was produced (data not shown).

Overexpression of the polymerase C1-encoding gene by *P. oleovorans* GPo1(pET702, pGEc74) (26a), in which the *phaC* gene is under control of the P_{alk} promoter and fused to a VSV G tag in the expression system, led to large amounts of polymerase C1. Addition of dicyclopropylketone (DCPK) activates the AlkS regulator protein encoded on pGEc74, which results in induction of the P_{alk} promoter and therefore regulates expression of the *phaC1* gene on plasmid pET702. Within 2 h after induction with 0.05% DCPK, polymerase C1 accounted for 10% of the total protein under nonlimited conditions (data not shown), while a maximum of 10% was found 6 h after induction with 0.05% DCPK for nitrogen-limited conditions. No polymerase C1 could be detected in uninduced cells, not even when the highly sensitive VSV G tag antibody was used (Fig. 2CD).

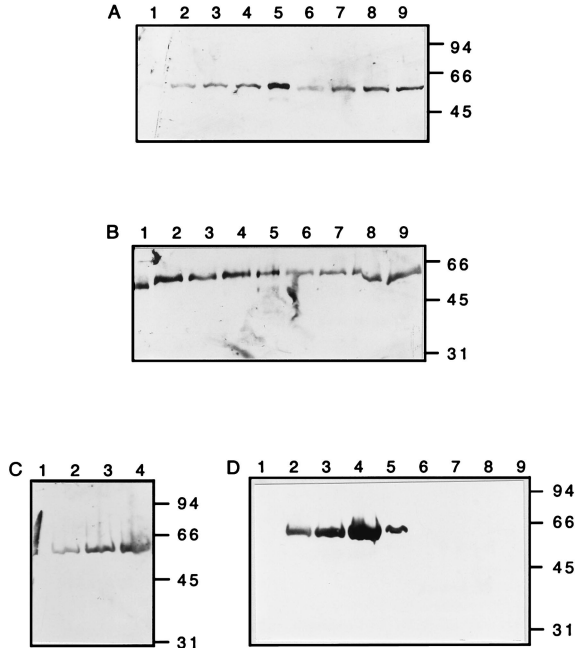

FIG. 2. Detection of polymerase C1 levels in cell extracts of recombinant *Pseudomonas* strains during different growth stages. (A and B) Western blot of cell extracts of *P. oleovorans* GPo1(pGEc405) (A) (20 μg of protein/lane, 2-min exposure time) and *P. putida* KT2442(pGEc405) (B) (25 μg of protein/lane, 20-s exposure time) exposed to anti-polymerase C1 antibodies. Lanes 1 to 4, samples from cultures grown under nitrogen limitation, taken at 6, 8, 10, and 14 h, respectively. Lanes 6 to 9, samples from cultures grown under non-nitrogen-limited conditions, taken at 6, 8, 10, and 14 h, respectively. Lane 5, PHA granules with 150 ng (A) and 0.5 μg (B) of polymerase C1. (C and D) Detection of polymerase C1 with polymerase C1 antibodies (C) and highly specific VSV G tag antibodies (D) in *P. oleovorans* GPo1(pET702, pGEc74) grown under nitrogen limitation. Induction was done with 0.05% DCPK after 6 h (C and D [lanes 1 to 4]); controls were not induced (D [lanes 6 to 9]). For lanes 1 to 4 and 6 to 9, samples were taken at 6, 7, 8, and 12 h, respectively. Lane 5 (D), PHA granules containing 0.5 μg of VSV G-tagged polymerase C1. In all other lanes, 25 μg of protein was loaded. Molecular mass marker sizes are given on the right in kilodaltons.

Figure 3 shows PHA production for the above recombinant *Pseudomonas* strains containing different amounts of polymerase C1. *P. oleovorans* GPo1 produced 44% (wt/wt) PHA under nitrogen-limiting conditions and 34% PHA under non-nitrogen-limiting conditions. Additional copies of the polymerase C1-encoding gene in *P. oleovorans* GPo1(pGEc405) led to a significant increase of PHA up to a maximum amount of 64% (wt/wt) under non-nitrogen-limiting conditions. Under nitrogen-limiting conditions, only a 3% increase was found compared to that in *P. oleovorans* GPo1. Additional copies of the polymerase C1-encoding gene in *P. putida* KT2442(pGEc405) did not lead to formation of larger amounts of PHA compared

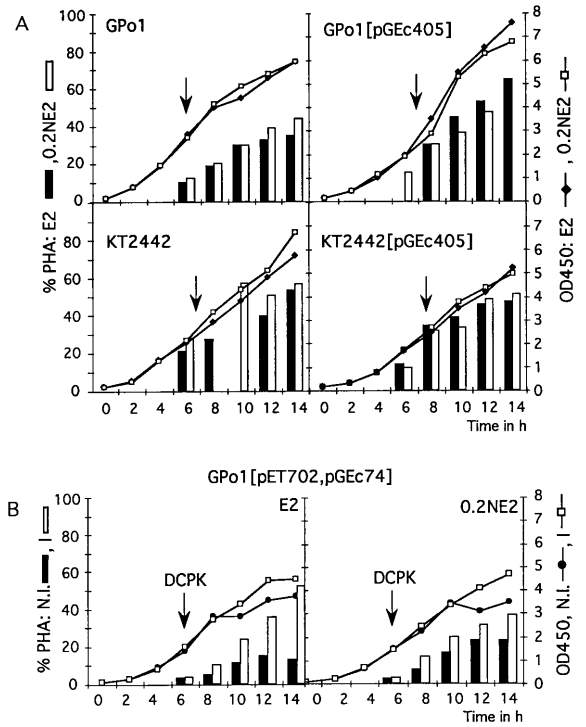

FIG. 3. Growth curves and PHA contents of *Pseudomonas* strains containing different amounts of polymerase C1. Growth curves and PHA contents are shown for batch cultivations on nitrogen-limited ($0.2NE_2$) and non-nitrogen-limited (E_2) medium with octanoate as the carbon source. (A) Cultures of *P. oleovorans* GPo1, *P. oleovorans* GPo1(pGEc405), *P. putida* KT2442, and *P. putida* KT2442(pGEc405) are shown. Arrows indicate the time of nitrogen exhaustion on 0.2NE2 medium. (B) Recombinant *P. oleovorans* GPo1(pET702, pGEc74) was induced (I) with 0.05% DCPK after 6 h (arrow), and control cultures were not induced (N.I.).

to those in *P. putida* KT2442, which already produced 57% PHA under nitrogen-limited conditions (Fig. 3A). Differences between nitrogen-limited and nonlimited conditions were small for both wild-type and recombinant *P. putida* strains (4% difference). Although large amounts of polymerase C1 were synthesized after induction in *P. oleovorans* GPo1(pET702, pGEc74), total amounts of PHA were smaller than or comparable to those found in wild-type *P. oleovorans* GPo1. The effect of hyperproduction of polymerase C1 under nitrogen limitation led to 14% more PHA compared to the level in noninduced cells. The difference was more obvious under non-nitrogen-limiting conditions, resulting in 52% PHA after induction compared to 13% PHA for noninduced cells (Fig. 3B).

Effects of amount of polymerase C1 on PHA composition. Polymerase C1 content was found to affect the PHA composition of *Pseudomonas* recombinants. Overproduction of polymerase C1 in *P. oleovorans* GPo1(pGEc405), *P. oleovorans* GPo1(pET702, pGEc74), and *P. putida* KT2442(pGEc405) led to a clear shift in monomer composition towards more C_6 and nearly the complete absence of C_{10} monomers compared to the level in control cultures (Table 2). The amounts of PHA produced by the different strains varied, but PHA compositions were constant during cultivation.

Incorporation of new monomers into preexisting PHA by *P. oleovorans* GPo1 and its recombinants. In order to quantify the effect of different growth stages and different amounts of polymerase C1 on PHA synthesis, rates of incorporation of new monomers into preexisting PHA were determined. A method was developed in which cells were grown in batch cultures under nitrogen-limiting conditions on either nonane or nonanoic acid, and newly synthesized PHA was determined after a pulse of octanoate. When *P. oleovorans* GPo1 was grown on nonane or nonanoic acid, the synthesized PHA consisted of C_9 and C_7 monomers; no C_8 or C_6 monomers were found. A subsequent pulse of octanoate led to detectable amounts of C_8 and C_6 monomers incorporated in the preexisting C_9/C_7 PHA. Incorporation of newly synthesized C_8 and C_6 monomers could be detected after 15 min and was shown to be linear for *P. oleovorans* GPo1 (Fig. 4A) and all strains tested over a period of 40 to 120 min. With this method, a highest monomer incorporation rate of approximately 3.3 nmol of C_8/C_6 monomers/mg of CDW/min into PHA was found for mid-stationary-phase cells of *P. oleovorans* GPo1. The incorporation of C_8/C_6 monomers was 30% lower during exponential growth and nearly threefold lower in the late stationary phase (Table 3 and Fig. 4A). Although a decrease in total amounts of PHA was found in the late stationary phase for *P. oleovorans* GPo1 grown on nonane, there clearly was still incorporation of C_8/C_6 monomers into PHA after the octanoate pulse was applied (Fig. 4A). The same method was used to quantify the effect of different amounts of polymerase C1 on rates of PHA synthesis. For this procedure, *P. oleovorans* GPo1 and recombinant strains were grown on minimal medium with 0.1% YE and nonanoic acid as a substrate. The maximum rate of octanoate

TABLE 2. Amounts of polymerase C1, PHA production, and monomer composition of *Pseudomonas* and its recombinants

Strain or recombinant[a]	Genotype[b]		Amt of polymerase C1 (mg/g of protein)[c]	% (wt/wt) PHA[c]	Monomer composition (mol%)[d]		
	phaC1	*phaC2*			C_6	C_8	C_{10}
GPo1	+	+	0.75	44	6.2	93.1	0.7
GPo1(pGEc405)	++	+	2.5	47[e]	11.7	88.0	0.3
GPo1(pET702, pGEc74)							
Noninduced	+	+	<0.5	23	6.7	92.4	0.9
Induced[f]	+++	+	100	37	10.6	89.4	ND[g]
KT2442	+	+	0.6	57	7.1	91.7	1.2
KT2442(pGEc405)	++	+	25	50	11.4	88.5	0.1

[a] Cultures were grown under nitrogen limitation with 15 mM octanoate as the carbon source.
[b] +, Chromosome encoded; ++, chromosome and vector encoded; +++, chromosome- and vector-encoded overexpression of polymerase C1.
[c] Levels of polymerase C1 and amounts of PHA were determined after 14 h.
[d] C_6, 3-hydroxyhexanoate; C_8, 3-hydroxyoctanoate; C_{10}, 3-hydroxydecanoate.
[e] Amount of PHA after 12 h.
[f] Induction after 6 h with 0.05% DCPK.
[g] ND, not detected ($P < 0.05$).

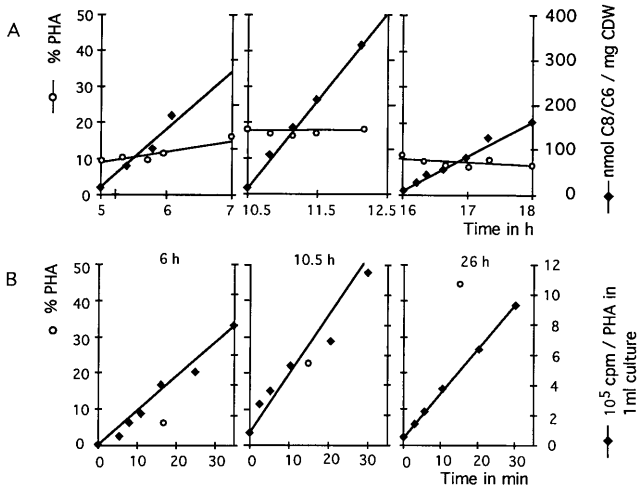

FIG. 4. Linear incorporation of new monomers into preexisting PHA by *P. oleovorans* GPo1. *P. oleovorans* GPo1 was grown on minimal medium ($0.2NE_2$), and the incorporation of new monomers into preexisting PHA and PHA percentages (wt/wt) was determined at different growth stages. (A) Nonane-grown cultures were pulsed with 15 mM octanoate at 5, 10.5, and 16 h, respectively. Incorporation of C_8/C_6 monomers into preexisting PHA is given in absolute amounts per milligram of CDW. (B) Octanoate-grown cultures were pulsed with [1-^{14}C]octanoate; incorporation of ^{14}C-monomers into preexisting PHA is shown for 30 min. Incorporated radioactive label, measured in total PHA extracted from 1 ml of culture, is expressed in counts per minute. The times after inoculation at which radioactive pulses were given are indicated in the graph.

incorporation into PHA during growth of *P. oleovorans* GPo1 on nonanoic acid with 0.1% YE was found to be 9.9 nmol of C_8/C_6 monomers/mg of CDW/min in the late stationary phase (Table 3). This rate was essentially the same when extra copies of the polymerase C1-encoding gene were introduced in *P. oleovorans* GPo1(pGEc405).

Overexpression of the polymerase C1-encoding gene under P_{alk} control in *P. oleovorans* GPo1(pET702, pGEc74) led to an approximately 130-fold increase of polymerase C1 after induction (10% of total protein), but had very little effect on the rate of incorporation of C_8/C_6 monomers into PHA.

To monitor monomer incorporation into preexisting PHA with much higher sensitivity, a second method was developed in which a radioactive substrate was pulsed into the culture. *P. oleovorans* GPo1 cells were grown in batch cultures on octanoate under nitrogen-limiting conditions and pulses of [1-^{14}C]octanoate were given during the early, mid-, and late stationary phases. Incorporation rates were determined by counting incorporated ^{14}C in the separated PHA fraction. This method confirmed the previous results determined by C_8 incorporation into C_9/C_7 PHA with a much higher sensitivity. Here also, the highest rates of 1-^{14}C-monomer incorporation into PHA in *P. oleovorans* GPo1 were found in the mid-stationary phase with a value of 9.5 nmol of C_8/C_6 monomers/mg of CDW/min. During exponential growth and in the late stationary phase, the rates of incorporation of 1-^{14}C-monomers were three- and sixfold lower, respectively (Table 3). The PHA-negative strain *P. putida* GPp104 showed no incorporation of radioactive monomers under the same conditions (data not shown). Incorporation of 1-^{14}C-monomers into PHA could be detected within 5 min after a given pulse and was linear for 30 min (Fig. 4B).

DISCUSSION

In this study, the first relationships between levels of polymerase C1 and amounts of PHA formed in vivo have been shown. Polymerase C1 levels were measured with antibodies

TABLE 3. Rates of incorporation of *P. oleovorans* GPo1 and recombinant strains containing additional polymerase C1

Strain	Medium, growth substrate[a]	Time (h)	CDW (mg/ml)	% (wt/wt) PHA[b]	Octanoate concn (mM)[c]	Incorporation rate of new C_8/C_6 monomers into C_9/C_7 PHA (nmol/mg of CDW/min)
GPo1	A, nonane	5	0.8	9.0		2.3
		10	1.5	17.8		3.3
		16	1.5	11.1		1.2
GPo1	A, octanoate, 1-^{14}C[d]	6	0.6	6.5	1.2	3.3
		10.5	0.8	22.0	3.6	9.5
		26.5	1.7	44.5	2.0	1.5
GPo1	A + YE, nonanoic acid	6	1.1	8.8		6.9
		9	1.4	34.4		7.6
		10	1.8	44.9		6.0
		12	2.0	32.8		6.8
		18	2.3	38.5		9.9
GPo1(pGEc405)	A + YE, nonanoic acid	6	1.0	27.2		8.7
		10	2.1	57.1		5.8
		12	2.6	54.5		10.5
GPo1(pET702, pGEc74)[e]	A + YE, nonanoic acid					
Induced		8	0.5	12.1		6.0
Uninduced		8	0.5	7.9		4.7

[a] A, N-limited medium; A + YE, N-limited medium plus 0.1% yeast extract.
[b] PHA percentages are given as weight percentage per CDW at the time of a given octanoate pulse.
[c] Remaining concentration of substrate at the time of a given pulse of [1-^{14}C]octanoate.
[d] Pulse substrate was [1-^{14}C]octanoate.
[e] Induced, induction with 0.05% DCPK after 6 h; uninduced, uninduced control.

raised against polymerase C1 of *P. oleovorans* GPo1, which are highly specific. Polymerase C1 levels in whole cells of wild-type *P. oleovorans* GPo1 are at the detection limit of approximately 0.075% of total protein when tested under the highest PHA-producing conditions, indicating that very little polymerase is needed for PHA production of up to 40 to 50% (wt/wt) PHA. Polymerases of *P. putida* KT2442 are also recognized by the antibody and show similar small amounts of 0.06% of total protein, comparable to those of *P. oleovorans* GPo1. Although the *pha* genes of *P. putida* KT2442 have not yet been sequenced completely, there is 93.6% identity for the 700-bp promoter region of *phaC1* (17) and 99% identity for the polymerase C2 of *P. putida* KT2442 and *P. oleovorans* GPo1 (32a). The polymerase C1 antibody did not cross-react with polymerase C2 of *P. oleovorans* GPo1, even though the overall identity between polymerase C1 and C2 is 53.6% (20).

It is assumed for *P. oleovorans* GPo1 and *P. putida* KT2442 that both the polymerase C1- and C2-encoding genes are located on the same operon, but not much is known about the mechanisms influencing transcription and translation. It is not yet known whether transcription of *phaC1* and *phaC2* is under control of one promoter. For *P. aeruginosa*, one operon is found, but two mRNAs are formed (31).

In bioreactor batch experiments, we have previously found that *P. oleovorans* GPo1 produces PHA only in the late exponential phase at the onset of a nutrient limitation, whereas *P. putida* KT2442 produces PHA in the early exponential phase independent of a nutrient limitation (19, 23, 26). Under nonlimiting conditions, less than 5% PHA is formed by *P. oleovorans* GPo1 (23, 26). In contrast to these earlier findings, this study showed PHA production by *P. oleovorans* of up to 34% under non-nitrogen-limiting conditions. PHA synthesis was first observed in the exponential phase, probably due to pH changes or lower oxygen-mixing rates in the shaking flask cultivations used here. Nevertheless, although differences in PHA productions seen in shaking flasks were smaller than those found in controlled batch experiments, *P. putida* KT2442 produced more PHA during earlier growth stages than did *P. oleovorans* GPo1 in both of these cultivation systems.

Polymerase C1 levels in *P. oleovorans* and *P. putida* recombinant strains were studied during growth. In *P. oleovorans* GPo1(pGEc405), an increase in the amounts of polymerase C1 was found during growth. We can see that *phaC1* under the control of the native promoter on the plasmid pGEc405 is not expressed constitutively at a constant level, as was proposed by Huisman (17). In contrast, a continuous high level of polymerase C1 was found in *P. putida* KT2442(pGEc405), indicating that transcription or translation seems to be under a host-dependent control.

In *P. putida* KT2442 and *P. putida* KT2442(pGEc405), little PHA and no polymerase could be detected when the strains were grown on substrates not related to PHA, such as citrate and gluconate, whereas under similar conditions with octanoate, large amounts of PHA and polymerase were measured. Catabolite repression might affect *phaC1* transcription in the case of nonrelated substrates. Similar results for substrate-dependent PHA production by *P. putida* KT2442 were shown for growth on glucose and decanoate (15). Although polymerase C1 is only needed in very small amounts for PHA production, as was shown for wild-type *P. oleovorans* GPo1, a considerably larger amount of 64% PHA was found under nonlimiting conditions after the introduction of additional polymerase C1 copies in *P. oleovorans* GPo1(pGEc405).

Two different assays showed that PHA synthesis in *P. oleovorans* GPo1 grown on minimal medium is growth stage dependent. The highest PHA synthesis rates are found in the mid-stationary phase, and about two- to sixfold-lower rates of incorporation were found in the early and late PHA synthesis stages. The highest incorporation rates of 9.5 nmol of C_8/C_6 monomers/mg of CDW/min were determined, which equals a production of 80 g of PHA/kg of CDW/h. Simultaneous PHA synthesis and degradation were observed for late-stationary-phase cultures of *P. oleovorans* GPo1 grown on nonane. This indicates that enzymes for the fatty acid oxidation and polymer synthesis are still active during the late stationary phases and can act simultaneously with depolymerase activity. The same was shown for polyhydroxybutyrate synthesis and degradation in *Alcaligenes eutrophus* under nongrowth conditions (6). The radioactive assay was very sensitive for in vivo determinations of newly synthesized PHA; within 5 min, newly incorporated monomers could be detected.

Whether enzyme activities and regulation of both polymerase C1 and C2 in *P. oleovorans* GPo1 are different could not be shown in these in vivo experiments. In vitro experiments are under way in which differences between the granule-bound activities are being investigated for polymerase C1 and C2. Comparisons of *P. oleovorans* GPo1 and the recombinant *P. oleovorans* strain GPo1(pGEc405), containing additional copies of the polymerase C1-encoding gene, showed essentially similar PHA synthesis rates. Therefore, the rate-limiting step in PHA synthesis is not the polymerase activity when grown under nitrogen limitation. More likely, intermediates are limiting. To overcome this, the amount of PHA precursors [(*R*)-3-hydroxyoctanoyl coenzyme A] might be increased by blocking the (*S*)-3-hydroxyacyl coenzyme A dehydrogenase or β-ketothiolase of the β-oxidation pathway.

When incorporation rates were determined with the P_{alk} expression system for polymerase C1 coupled to the VSV G tag, an approximately 130-fold increase in polymerase C1 amounts and a clear difference in final PHA amounts were found after induction (52 versus 13% for induced versus noninduced cultures). Simultaneously, only slightly higher incorporation rates were found for induced cells compared to the rates for noninduced control cultures. Most of the polymerase C1 might be produced in an inactive form due to incorrect folding. PHA amounts and incorporation rates for induced and uninduced recombinant cells were lower than those for *P. oleovorans* GPo1 cells, which can be explained by extra growth pressure due to antibiotics.

The monomer compositions of *P. oleovorans* GPo1 and *P. putida* KT2442 changed with additional copies of *phaC1* when grown on octanoate. A change was found towards smaller amounts of C_8 and C_{10} monomers and larger amounts of C_6 monomers. This phenomenon was also found by Huisman et al. (19) and Huijberts et al. (15) when *phaC1* was introduced into the PHA-negative mutant *P. putida* GPp104. In vitro work will be done to characterize the substrate specificity of both polymerases in more detail in order to better understand PHA synthesis and how polymer characteristics can be influenced. Protein engineering might be a future possibility for creation of polymerases with a more defined substrate specificity, which might lead to synthesis of special polymers or homopolymers.

An important tool for further PHA studies was found in antibodies specific for polymerase C1 of *P. oleovorans* GPo1. In vivo experiments showed that much higher levels of PHA production could be reached in recombinant *P. oleovorans* GPo1(pGEc405) cells containing additional polymerase C1. This may provide us with an important production strain for simultaneous high-cell-mass formation together with high-level PHA production independent of nitrogen limitation.

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