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■Full-Length Paper■

Cloning and Sequencing of the Poly (3-hydroxybutyrate)(PHB) Synthase Gene from Purple Non-Sulfur Bacteria *Rhodospirillum centenum* and Expression of the Gene in *Escherichia coli*

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Abstract: A 10-kb genomic fragment was isolated from *Rhodospirillum centenum* by Southern-hybridization and colony-hybridization, using a probe amplified by PCR with oligo-nucleotide primers constructed from a sequence conserved in poly (3-hydro xybutyrate)(PHB) synthase genes. After subcloning of an approximately 3-kb fragment (*Sma*I-*Eco*RV) that caused the production of PHB in *Escherichia coli* in the presence of β-ketothiolase (*phb*A), an acetoacety-CoA reductase (*phb*B) gene from *Ralstonia eutropha* H16 was obtained and sequenced. This fragment contained an open reading frame (ORF) whose amino acid sequence was highly similar to the sequences of other known PHB synthase genes, especially to a synthase from *Azospirillum brasilense* (74% identity).

Keywords: poly (3-hydroxybutyrate), PHB, PHB synthase, purple non-sulfur bacteria, *Rhodospirillum centenum*, *phb*C

Introduction

The production of intracellular polyesters belonging to the class of polymers known as polyhydroxyalka noates (PHAs) has been observed in a wide array of prokaryotic organisms[1]. The monomers composing the polyesters range in length from C4 (β-hydroxybutyrate) to C16 (β-hydroxyhexadecanoate)[2]. PHAs have attracted attention as a potential alternative to conventional petrochemical-derived plastics[3]. Poly (3-hydroxybutyrate) (PHB) is the simplest and most common PHA[1]. The metabolic pathways leading to the synthesis of PHB have been investigated in many bacteria. *Ralstonia eutropha*, a bacterium which accumulates PHB intracellularly at levels equivalent to about 70-90% of dry cell weight at maximum, has been extensively studied, and the genes of three enzymes involved in the synthesis of PHB have been cloned and sequenced[4, 5]. As in *R.eutropha*, in most bacteria, including *Zoogloea ramigera*, *Alcaligenes latus*, and *Rhodobacter sphaeroides*, a three-step metabolic pathway has been revealed. The first step is catalyzed by the enzyme β- ketothiolase (EC2.3.1.16), which condenses acetyl coenzyme A (acetyl-CoA) to acetoacetyl-CoA[6]. This intermediate is then reduced to D-(-)-β-hydroxy butyryl-CoA by an NADPH-dependent acetoacetyl-CoA reductase

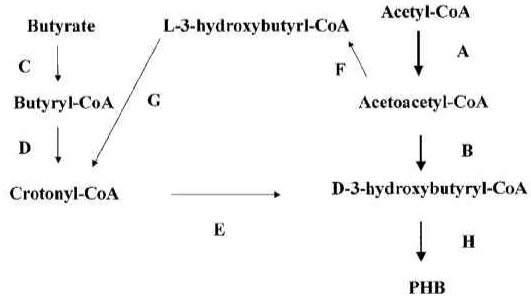

Fig. 1. Pathway of PHB synthesis and related reaction steps in PHB-accumulating bacteria. A, b-ketothiolase; B, acetoacetyl-CoA reductase (NADPH-dependent); C, Acyl-CoA synthetases; D, butyryl-CoA dehydrogenase; E, enoyl-CoA hydratase (forming D-3hydroxybutyryl-CoA); F, acetoacetyl-CoA reductase (NADH-dependent); G, enoyl-CoA hydratase (forming L-3hydroxybutyryl -CoA); H, PHB synthase. Thick arrows indicate the pathway of PHB synthesis in R. eutropha and thin arrows, the pathways in R. rubrum and R. centenum.

(EC 1.1.1.36). In the last step, PHB synthase catalyzes the polymerization of D(-)-3-hyd roxybutyryl-CoA to PHB. In *Rhodospirillu rubrum*, PHB is synthesized via a five-step pathway. An NADH-dependent acetoacety-CoA reductase (EC 1.1.1.35) catalyzes the formation of L-(+)-3-hydro xybutyryl-CoA, which is subsequently converted to D-(-)-3-hyoxybutyryl CoA by two stereospecific enoyl-CoA hydratases prior to polymerization[7] (Fig.1). *Rhodoiri-lum centenum* was isolated in 1987, and exbits a number of general properties typically observed in purple non-sulfur bacteria, but also displays a number of unusual characteristics as follows: (1) absence of any repression by O_2 of photo pigment synthesis; (2) synthesis of "R-bodies"; (3) swarming motility on agar surfaces; and (4) conversion of vibrioid/spiral cells to thick-walled cysts, and accumulation of PHB at cysts, under condition of aerobic growth in darkness on butyrate as a sole carbon source[8, 9]. Since among numerous PHB-accumulating bacteria, *R. centenum* is unique and limited in terms of the conditions it needs to accumulate PHB, investigation of the regulation of PHB synthesis in *R. centenum* may be important. As a first step in this process, we describe here the cloning and sequencing of the PHB synthase gene from *R. centenum* and its expression in *E. coli.*

Materials and Methods

Bacterial strains, plasmids, and culture

Bacterial strains and plasmids used in this study are listed in Table 1. All *Escherichia coli* strains were grown aerobically in Luria-Bertani (LB) medium or on solid LB agar (1.5%, wt/vol) plates at 37°C, or in M9 medium[10]. The following concentrations of antibiotics were used: ampicillin, 50 μg/ml; chloramphenicol, 34 μg/ml; tetracycline, 10 μg/ml. *R. centenum* was cultivated anaerobically under illumination (60-W incandescent bulb) at 30°C in 1927 CENS medium: in 1 liter, 2.2 g sodium pyruvate, 0.9 g K_2HPO_4, 0.6 g KH_2PO_4, 1 g NH_4Cl, 5 mg disodium EDTA, 200 mg $MgSO_4 \cdot 7H_2O$, 1 ml True Blue Trace Element solution (containing 2.5 g EDTA, 0.2 g $MnCl_2$, 0.1 g H_3BO_3, 0.1 g Na_2MoO_4, 50 mg $NiCl_2 \cdot 6H_2O$, 20 mg $CoCl_2 \cdot 6H_2O$, 10 mg $CuCl_2 \cdot 2H_2O$, 5 mg Na_2SeO_3, and 5 mg $NaVo_3 \cdot nH_2O$ per 250 ml deionized water), 75 mg $CaCl_2 \cdot 2H_2O$, 2 ml chelated iron solution (prepared by dissolving 1 g $FeCl_2 \cdot 4H_2O$ and 2 g dissodium EDTA in 1 liter deionized water, and adding 3 ml concentrated HCl), 20 μg vitamin B_{12}, 15 μg biotin, 0.5 g and $Na_2S_2O_3 \cdot 5H_2O$; pH was adjusted to 6.8 with NaOH[8].

DNA preparation and manipulation

Standard methods were used for the preparation and manipulation of DNA, PCR, Southern hybridi-

Table 1. Strains and plasmids

Strains	
E. coli	
JM109	*recA endA1 gyrA96 thi hsdR17 supE44 rela1 Δ(lac-proAB)/F'[traD36proAB+ lacI lacZΔM15]*
BLR(DE3)/pLysS	F- *ompT hsdS*$_B$(r$_B$ m$_B$) *gal dcm Δ(srl-recA)306*::Tn*10*(Tc^r)(DE3)/pLysS(Cm^r)
R. centenum	Wild type
Plasmids	
PUC18, 19	High copy cloning vector; Amp^r
pSTV29	Low copy cloning vector; Cm^r
pET23b	Expression vector; Amp^r
pET100	pET23b carrying *XbaI/EcoRI* fragment containing *R. eutropha phaCAB* PHB synthetic operon
pSTVReAB	pSTV29 carrying *Sse8387-EcoRI* fragment containing *R. eutropha phaAB*
pUCReC	pUC19 carrying *SmaI-StuI* fragment containing *R. eutropha phaC*
pET100Ce	pET100 carrying about 2-kbp fragment containing *R. centenum phaC* instead of *R. eutropha phaC*
pRcCP1	pUC18 carrying about 10-kbp *PstI* fragment containing *R. centenum phaC*
pRcCP2	pUC18 carrying about 10-kbp *PstI* fragment opposite direction containing *R. centenum phaC*
pRcCS1	pUC18 carrying about 4.5-kbp *SmaI* fragment opposite direction containing *R. centenum phaC*
pRcCS2	pUC18 carrying about 4.5-kbp *SmaI* fragment containing *R. centenum phaC*
pRcCE1	pUC18 carrying about 3-kbp *EcoRI-EcoRV* fragment opposite direction containing *R. centenum phaC*
pRcCE2	pUC18 carrying about 3-kbp *EcoRI-EcoRV* fragment containing *R. centenum phaC*

zation, and colony hybridization. Sequencing was performed with a SEQ-4×4 system and Thermosequenase Cy 5.5 (Amersham Biosciences, Tokyo, Japan), and with a BigDye Terminator v.3.1 Cycle Sequencing kit (ABI PRISM 310) as recommended by the manufacturer. Sequences were processed using the program GENETYX- MAC/ATSQ, version 4.2.0 (Software Development Co., Ltd., Tokyo, Japan).

Design of primers for cloning the PHB synthase gene

The primer of an inner part of the PHB synthase gene as a probe for Southern hybridization and colony hybridization was designed from consensus sequences based on comparisons with PHB synthase genes of purple non-sulfur bacteria, *Rhodosporillum rubrum* (accession number AF178117), and *Rhodobacter sphaeroides* (AY945501), and a root nodule bacterium, *Rhizobium meliloti* (U17227). A 500-bp fragment of the *R. centnum* PHB synthase gene was amplified by PCR with 5'-TGGATCAAY AARTTCT ACATAAT-3' as the forward primer and 5'-TTCCARTAGAGCAGRTCGAAG-3' as the reverse primer using genomic DNA of *R. centenum* as a template. The PCR product was labeled with [^{32}P]dCTP and used as a probe in Southern hybridization and colony hybridization. *R. centenum* genomic DNA was completely digested with *Pst*I. The resulting fragments were subjected to Southern hybridization.

Other analytical methods

PHB content was quantitated as the amount of crotonic acid by high-pressure liquid chromatography as described by Karr et al[11].

Results and Discussion

Cloning of a genomic fragment relevant to the PHB synthase gene

R. centenum genomic DNA was digested completely with *Pst*I. The resulting fragments were separated on a 1% agarose gel and transferred onto a nylon membrane. The DNA fixed on the nylon membrane was hybridized with a ^{32}P-labeled 500-bp probe prepared from PCR products with genomic DNA as a template (see Materials and Methods). The DNA corresponding to the positive signal, which was about 10-kbp long was extracted from the agarose gel, ligated to *Pst*I-digested pUC18, and introduced into *E. coli* JM109 by transformation. Of about 5,000 ampicillin-resistant recombinant colonies, two positive colonies were selected by colony hybridization. Both colonies were found to have about 10 kbp of foreign DNA, but oriented in the opposite direction to each other. One *Sma*I fragment (about 4.5 kbp) was isolated from one of two plasmids and ligated to pUC18 digested with *Sma*I. To confirm that the cloned 10-kbp and 4.5-kbp fragments have the PHB synthase gene, Southern hybridization and PCR were done using the same probe and same primers. Fig. 2 shows a restriction map of the 10-kbp fragment. According to this map, the *Sma*I-*Eco*RV fragment (about 3 kbp) contained the region for the PHB synthase gene, where the nucleotide sequence was analyzed. Within the 3-kbp fragment, one open reading frame (1,792 nucleotides) was found. It specified a protein with a deduced molecular mass of 66,962 Da (597 amino acids). The initiation codon was preceded by a putative Shine-Dalgarno sequence (Fig.3). The PHB synthase in *R. centenum* had about 74 and 64% identity to the PHB synthase in *Azospirillum brasilense* and *Rhodospirillum rubrum* in amino acid sequence, respectively (Fig.4).

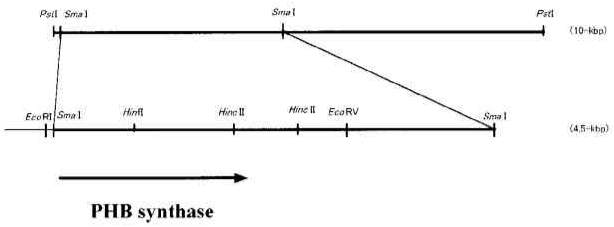

Fig. 2. Restriction map of the cloned fragment containing the PHB synthase gene of *R. centenum*.

Expression in *E. coli* of the PHB synthase gene from *R. centenum*

To confirm the cloning of the fragment having the ability to synthesize PHB, two systems which contain the β-ketothiolase (*phb*A) and acetoacety-CoA reductase (*phb*B) genes from *Ralstonia eutropha* H16 were constructed. One system consists of *E. coli* JM109 transformed with pSTVReAB carrying *R. eutropha pha*AB and pRcCP1 or pRcCE2 carrying *R. centenum pha*C. The other system consists of *E. coli* BLR transformed with pET100Ce which contains *R. eutropha pha*AB and

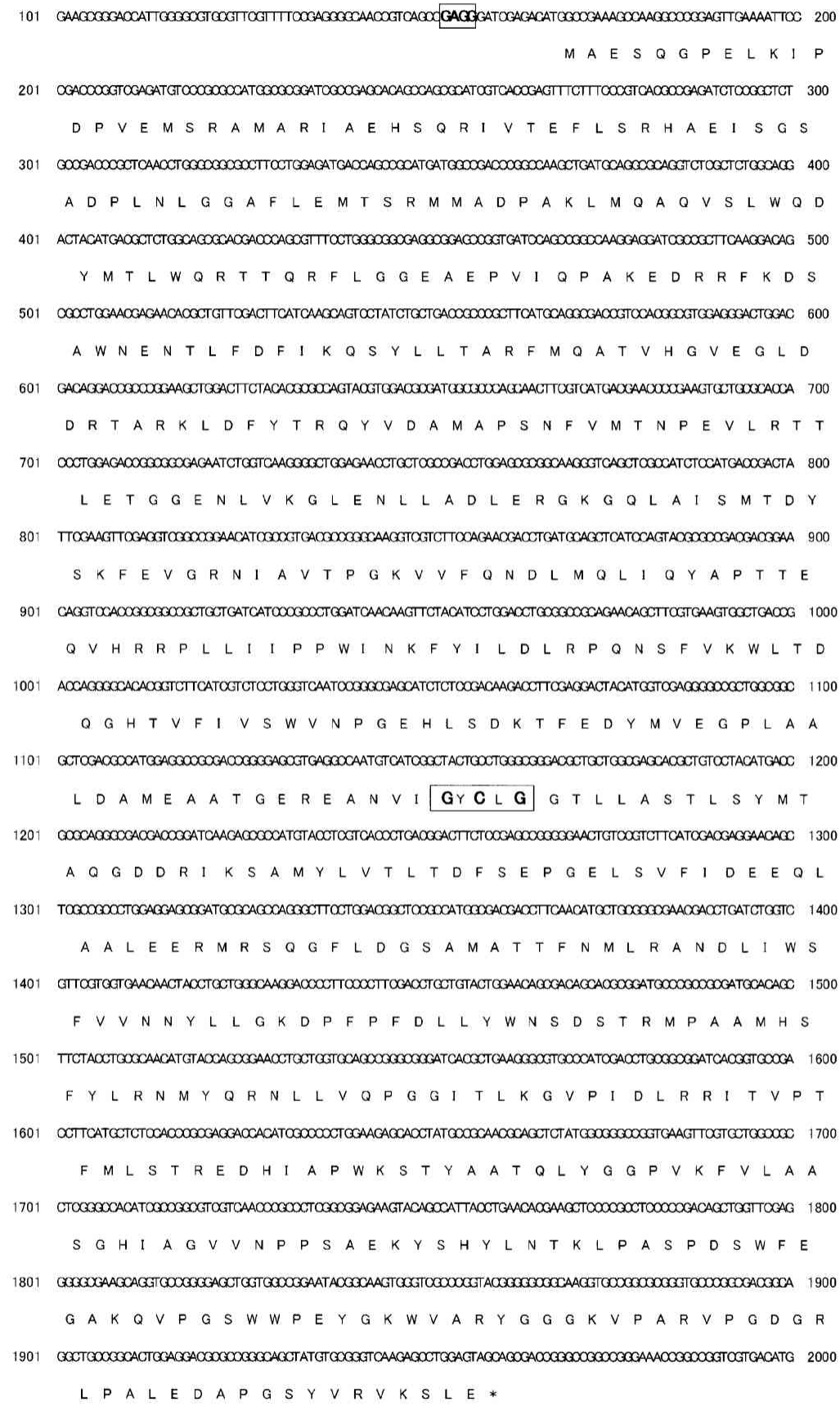

Fig. 3. Determined nucleotide sequence and deduced amino acid sequence of the R. centenum PHB syntheses gene. Boxed, bold, shaded "GAGG" is a putative Shine Dalgarno sequence, and boxed bold "C" is a putative center of an active site.

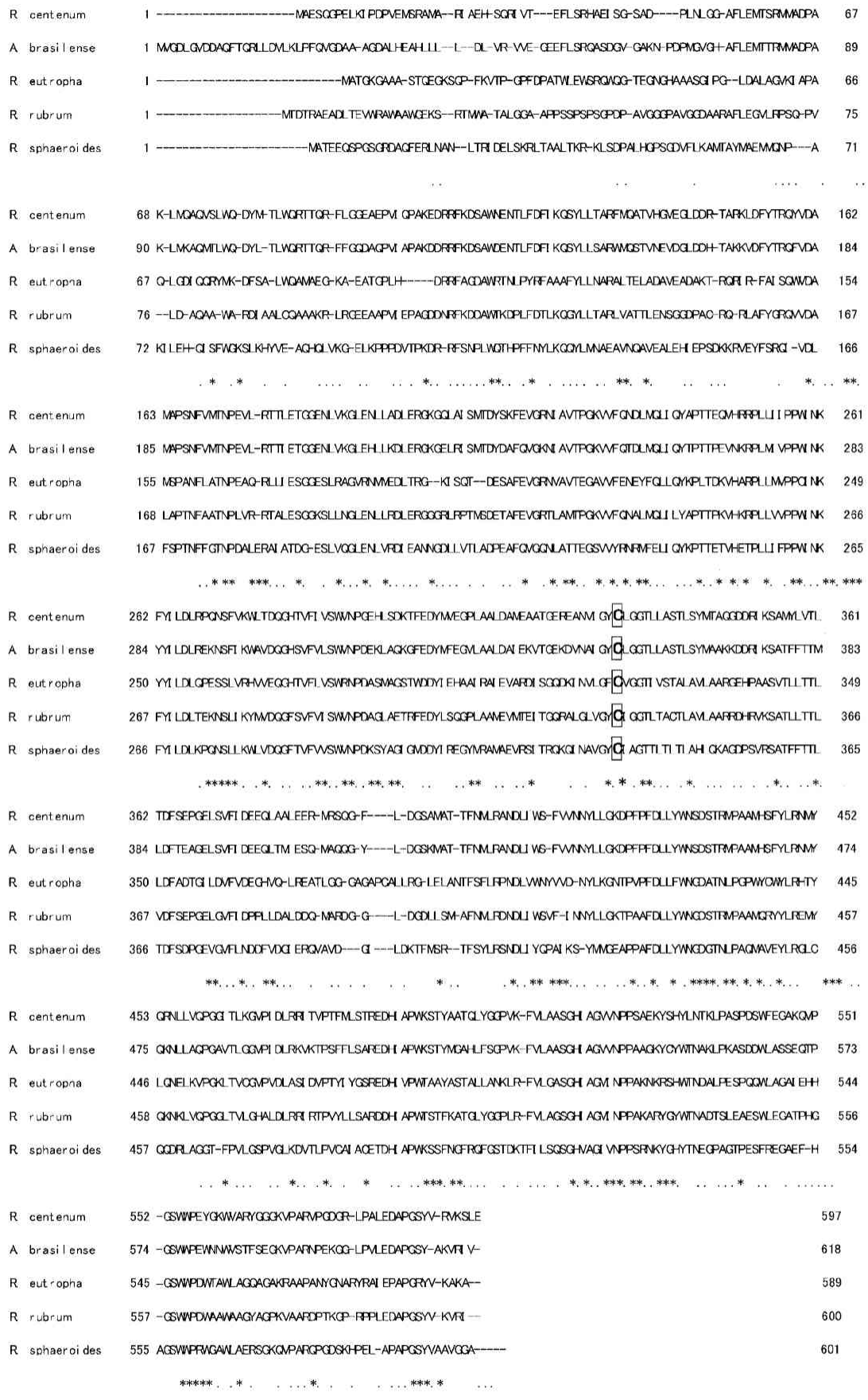

Fig. 4. Comparison of the amino acid sequence es R. centenum PHB synthase and other bacterial PHB synthases. Bold face "C" is a putative center of an active site and well conserved.

*R. centenum pha*C. A system with the same vectors but carring *R. eutropha pha*C instead of *R. centenum pha*C was used as a positive control, and JM109 harboring pSTVReAB and pUC18 was examined as a negative control. Fig. 5 shows that the cloned fragment containing the *R. centenum* PHB synthase gene synthesized PHB in *E. coli* on LB medium and M9 medium, but at only about 10% of the level produced by *R. eutropha pha*C. The reason why *R. centenum pha*C produces only a small amount of PHB in *E. coli* is not clear.

As it was only when *R. centenum* was cultivated under cyst-forming conditions with butyrate that PHB was produced, it is presumed that the synthesis of PHB is strictly controlled in *R. centenum*. *Azotobacter vinelandii*, a soil bacterium, which undergoes a process of cellular differentiation to form metabolically dormant cysts resistant to desiccation, produces the exopolysaccharide alginate, which is essential for the encystment process. Transcription of the algD gene, which codes for GDP-mannnose dehydrogenase, a key enzyme in the alginate biosynthetic pathway, is initiated at two promoters, one of which, p2, has the sigmaE consensus sequence. An AlgU, *A. vinelandii* sigmaE factor, mutant was impaired in alginate production, encystment, and transcription of the algD gene[12,13]. *R. centenum* PHB synthase may be controlled by a similar system. The control region upstream of the PHB synthase gene of *R. centenum* should be examined.

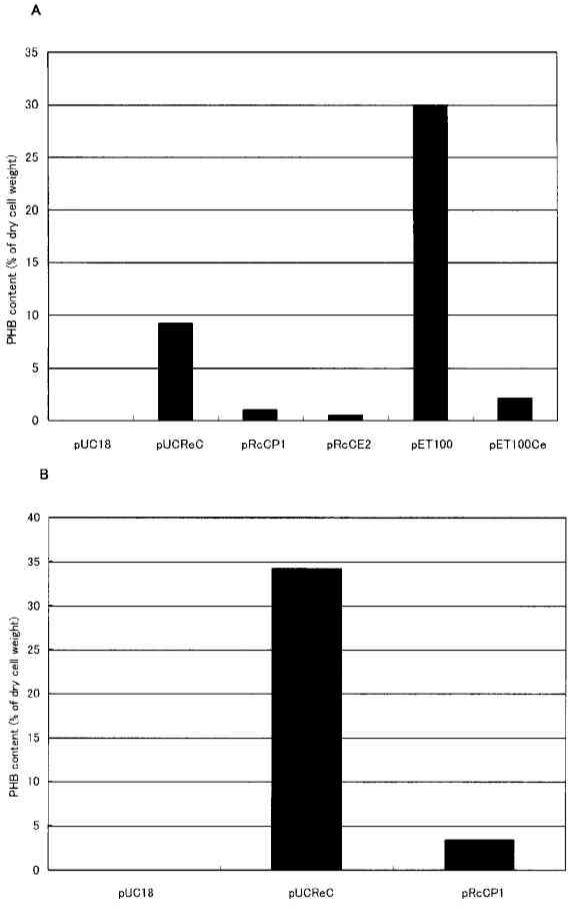

FIG. 5. Comparison of PHB accumulation in *E. coli* harboring various vectors. A) *E. coli* JM109 or BLR cultivated in LB medium with 2% glucose at 37 °C for 39 hours. PHB content was measured with a HPLC-based method. PUC18, pUCReC, pRcCP1, pRcCE2, *E. coli* JM109 harboring pSTVReAB with each vector, respectively; pET100, pET100Ce, *E. coli* BLR harboring each vetor. B) *E. coli* JM109 cultivated in M9 medium with 1% glucose at 37°C for 39 hours. PHB content was measured by HPLC. pUC18, pUCReC, pRcCP1, *E. coli* JM109 harboring pSTVReAB with each vector.

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