

■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-hydroxybutyrate)(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 (*phbA*), an acetoacetyl-CoA reductase (*phbB*) 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*, *phbC*

Introduction

The production of intracellular polyesters belonging to the class of polymers known as polyhydroxyalkanoates (PHAs) has been observed in a wide array of prokaryotic organisms¹. 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 plastics³. Poly (3-hydroxybutyrate) (PHB) is the simplest and most common PHA¹. 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-CoA⁶. This intermediate is then reduced to D-(-)- β -hydroxy butyryl-CoA by an NADPH-dependent acetoacetyl-CoA reductase

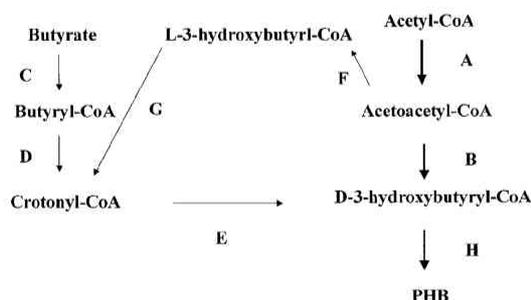


Fig. 1. Pathway of PHB synthesis and related reaction steps in PHB-accumulating bacteria. A, β -ketothiolase; B, acetoacetyl-CoA reductase (NADPH-dependent); C, Acyl-CoA synthetases; D, butyryl-CoA dehydrogenase; E, enoyl-CoA hydratase (forming D-3-hydroxybutyryl-CoA); F, acetoacetyl-CoA reductase (NADH-dependent); G, enoyl-CoA hydratase (forming L-3-hydroxybutyryl-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-hydroxybutyryl-CoA to PHB. In *Rhodospirillum rubrum*, PHB is synthesized via a five-step pathway. An NADH-dependent acetoacetyl-CoA reductase (EC 1.1.1.35) catalyzes the formation of L-(+)-3-hydroxybutyryl-CoA, which is subsequently converted to D(-)-3-hydroxybutyryl CoA by two stereospecific enoyl-CoA hydratases prior to polymerization⁷ (Fig.1). *Rhodospirillum centenum* was isolated in 1987, and exhibits 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 O₂ 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 medium¹⁰. 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 K₂HPO₄, 0.6 g KH₂PO₄, 1 g NH₄Cl, 5 mg disodium EDTA, 200 mg MgSO₄·7H₂O, 1 ml True Blue Trace Element solution (containing 2.5 g EDTA, 0.2 g MnCl₂, 0.1 g H₃BO₃, 0.1 g Na₂MoO₄, 50 mg NiCl₂·6H₂O, 20 mg CoCl₂·6H₂O, 10 mg CuCl₂·2H₂O, 5 mg Na₂SeO₃, and 5 mg NaVO₃·nH₂O per 250 ml deionized water), 75 mg CaCl₂·2H₂O, 2 ml chelated iron solution (prepared by dissolving 1 g FeCl₂·4H₂O and 2 g disodium EDTA in 1 liter deionized water, and adding 3 ml concentrated HCl), 20 µg vitamin B₁₂, 15 µg biotin, 0.5 g and Na₂S₂O₃·5H₂O; pH was adjusted to 6.8 with NaOH⁸.

DNA preparation and manipulation

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

Table 1. Strains and plasmids

Strains	
<i>E. coli</i>	
JM109	<i>recA endA1 gyrA96 thi hsdR17 supE44 relA1 Δ(lac-proAB)/F'[[traD36proAB+ lacI lacZΔM15]</i>
BLR(DE3)/pLysS	<i>F: ompT hsdS_λ(r_m me₆) gal dcm Δ(srl-recA)306::Tn10(Tc^r)(DE3)/pLysS(Cm^r)</i>
<i>R. centenum</i>	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 <i>Xba</i> I/ <i>Eco</i> RI fragment containing <i>R. eutropha phaCAB</i> PHB synthetic operon
pSTVReAB	pSTV29 carrying <i>Sse</i> 8387- <i>Eco</i> RI fragment containing <i>R. eutropha phaAB</i>
pUCReC	pUC19 carrying <i>Sma</i> I- <i>Stu</i> I fragment containing <i>R. eutropha phaC</i>
pET100Ce	pET100 carrying about 2-kbp fragment containing <i>R. centenum phaC</i> instead of <i>R. eutropha phaC</i>
pRcCP1	pUC18 carrying about 10-kbp <i>Pst</i> II fragment containing <i>R. centenum phaC</i>
pRcCP2	pUC18 carrying about 10-kbp <i>Pst</i> II fragment opposite direction containing <i>R. centenum phaC</i>
pRcCS1	pUC18 carrying about 4.5-kbp <i>Sma</i> I fragment opposite direction containing <i>R. centenum phaC</i>
pRcCS2	pUC18 carrying about 4.5-kbp <i>Sma</i> I fragment containing <i>R. centenum phaC</i>
pRcCE1	pUC18 carrying about 3-kbp <i>Eco</i> RI- <i>Eco</i> RV fragment opposite direction containing <i>R. centenum phaC</i>
pRcCE2	pUC18 carrying about 3-kbp <i>Eco</i> RI- <i>Eco</i> RV fragment containing <i>R. centenum phaC</i>

zation, and colony hybridization. Sequencing was performed with a SEQ-4×4 system and Thermo-sequenase 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, *Rhodospirillum rubrum* (accession number AF178117), and *Rhodobacter sphaeroides* (AY945501), and a root nodule bacterium, *Rhizobium meliloti* (U17227). A 500-bp fragment of the *R. centenum* 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 [³²P]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 ³²P-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

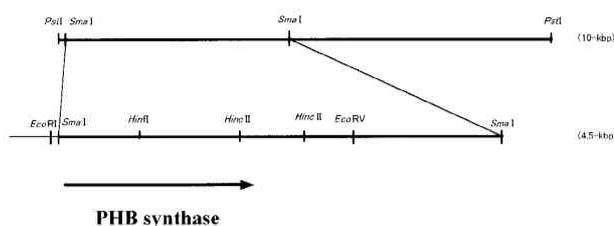


Fig. 2. Restriction map of the cloned fragment containing the PHB synthase gene of *R. centenum*.

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).

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 (*phbA*) and acetoacetyl-CoA reductase (*phbB*) genes from *Ralstonia eutropha* H16 were constructed. One system consists of *E. coli* JM109 transformed with pSTVReAB carrying *R. eutropha phaAB* and pRcCP1 or pRcCE2 carrying *R. centenum phaC*. The other system consists of *E. coli* BLR transformed with pET100Ce which contains *R. eutropha phaAB* and

101 GAAGCGGACCATGGGGGTGGTTGGTTTTGAGGGGCAACGGTCAAG**GAGG**GATCGAGACATGGCGAAAGCCAAAGCCCGAGTTGAAATTCG 200
M A E S Q G P E L K I P

201 OGACCCGGTGGAGATGTCCCGGCCATCGCGGGATCGCGGAGCACGCCAGCGGATCGTCAACGAGTTTCTTTCCCGTCAOCCGGAGATCTCCGGCTCT 300
D P V E M S R A M A R I A E H S Q R I V T E F L S R H A E I S G S

301 GCGCAOCCGCTCAACCTGGCGGGCCCTTCTCGAGATGAOCAGCCGATGATGGCCGACCCCGCCAAAGCTGATGCAGCCGAGGTCTCGCTCTGGCAGG 400
A D P L N L G G A F L E M T S R M M A D P A K L M Q A Q V S L W Q D

401 ACTACATGAGCTCTGGCAGCGCAGACCCAGCGTTTCTGGCGGGGAGCGGAGCCCGTGTATCCAGCCGCGCAAGGAGGATCCCGGCTTCAAGGACAG 500
Y M T L W Q R T T Q R F L G G E A E P V I Q P A K E D R R F K D S

501 GGCTGGAAAGAGAACCGCTGTTGACTTTCATCAAGCAGTCTATCTGTGACCCCGCGCTTCATGCAGCGACCCGTCACCGCGTGGAGGGACTGGAC 600
A W N E N T L F D F I K Q S Y L L T A R F M Q A T V H G V E G L D

601 GACAGGACCGCCCGAAGCTGGACTTCTACAGCGGCGAGTACGTGGACGGGATGGCGCCAGCCAACTTGTGATGACGAAACCCCGAAGTCTGGGCAOCC 700
D R T A R K L D F Y T R Q Y V D A M A P S N F V M T N P E V L R T T

701 CCCTGGACACCGCCCGGAGAACTCTGGTCAAGCGGCTGGAGAACCTGCTGGCGACCTGGAGCCCGCAAGCGTCAAGCTCGCCATCTCCATGACCGACTA 800
L E T G G E N L V K G L E N L L A D L E R G K G Q L A I S M T D Y

801 TTCCGAAGTTGAGGTCGGCCCGAACATCGCCGTGACCGCGGGCAAGGTGCTTCCAGAACGACCTGATGCAGCTCATCCAGTACGGCCCGACGACCGAA 900
S K F E V G R N I A V T P G K V V F Q N D L M Q L I Q Y A P T T E

901 CAGGTCACCGCCCGCCGCTGCTGATCATCCCGCCCTGGATCAACAGTCTTACATCTCGGACCTGGCGCCGAGAACAGCTTCTGAACTGGCTGAOCC 1000
Q V H R R P L L I I P P W I N K F Y I L D L R P Q N S F V K W L T D

1001 ACCAGCGGCACACCGCTTTCATGCTCTCTGGTCAATCCCGCGGAGCATCTCTCCGACAAAGCCTTCGAGGACTACATGGTGGAGCCCGCCCTGGCGCC 1100
Q G H T V F I V S W V N P G E H L S D K T F E D Y M V E G P L A A

1101 GCTCGACCCCATGGAGCCCGGACCCGGAGCGTGAGGCCAATGTGATCGGCTACTGCTGGCGGGACCGCTGCTGGCGAGCACGCTGTCTACATGACC 1200
L D A M E A A T G E R E A N V I **G Y C L G** G T L L A S T L S Y M T

1201 GCGCAGCCCGACCGCGATCAAGACCCCATGTACCTGTCACCCCTGAOCCACTTCTCCGAGCCCGGGAACTGTCCGCTTTCATGACGAGGAACAGC 1300
A Q G D D R I K S A M Y L V T L T D F S E P G E L S V F I D E E Q L

1301 TGGCCCGCTGGAGAGCGGATGGCGACCCAGCGCTTCTGGACCGCTCCCGCATCGCGACCCCTCAACATGCTGGCGCGGAAACGACCTGATCTGCTC 1400
A A L E E R M R S Q G F L D G S A M A T T F N M L R A N D L I W S

1401 GTTCGTTGTTGAACAACCTACCTGCTGGCCAAAGAACCCCTTCCCGTTCCGCTGCTGTACTGSAACAAGCACAGCAOCCGGATGCCCGCCCGATGCACAGC 1500
F V V N N Y L L G K D P F P F D L L Y W N S D S T R M P A A M H S

1501 TTCTAOCCTGGCAACATGTACACCGGAAOCTGCTGCTGCAGCCCGGGGGATCACCGCTGAAGGGCGTGCCCATGCAOCTGGCGGGATCACCGTCCOGA 1600
F Y L R N M Y Q R N L L V Q P G G I T L K G V P I D L R R I T V P T

1601 CCTTCATGCTCTCCACCCCGGAGGACACATCGCCCGCTGGAAGGACACCTATGCCGCAACGACGCTCTATGGCGCGCCGTTGAACTGCTGCTGGCCCG 1700
F M L S T R E D H I A P W K S T Y A A T Q L Y G G P V K F V L A A

1701 CTGGCGCACATCGCCCGGCTGCTCAACCCCGCCCTCGCGGAGAGTACAGCCATTACCTGAACACGAAAGCTCCCGCCCTCCCGGACAGCTGGTTGGAG 1800
S G H I A G V V N P P S A E K Y S H Y L N T K L P A S P D S W F E

1801 GGGCCGAAGCAGCTGCCCGGGAGCTGTTGGCCGGAATACGGCAAGTGGGTCCCGCGCTACGGCGGGCCAAAGCTGCCCGCGCGGTTGCCCGCGACCGCA 1900
G A K Q V P G S W W P E Y G K W V A R Y G G G K V P A R V P G D G R

1901 GGCTGCCCGCAGTGGAGGACCGCCCGGGCAGGTATGTGGCGTCAAGAGCCTGGAGTAGCAGGACCCCGCGCGCGGAAACCGCCCGGTTGTCGACATG 2000
L P A L E D A P G S Y V R V K S L E *

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

R. centenum	1	-----MAESGPELKI PDPVEMRAMA--RI AEH-SGRV VT---EFLSRHAEI SG-SAD----PLN.LGG-AFLEMTRMADPA	67
A. brasilense	1	MGDLGVDDAGFTGRLLDYLKLPFGVQDAA-AGDALHEAHL--L--DL--VR--VE--GEEFLSRGASDGV-GAKN-PDPVGVG+AFLEMTRMADPA	89
R. eutropha	1	-----MATGKAAA-STGEGKSGP-FKVTG--GFFDPATVLEVSQVGG-TEGNHAAASQI PG--LDALAGVKI APA	66
R. rubrum	1	-----MFDTRAEADL TEWRVAWAWEKGS--RTIWA-TALGGA-APPSSPSPSGPDP-AVGGGPAVGGDAARAFLEGLVFPSPG-PV	75
R. sphaeroides	1	-----MATEEGSGSGRFDAGFERLNN--LTRI DELSKRLTAALTKR-KLSDPALHQPSCGVFLKAMTAYMAEMQNP--A	71
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R. centenum	68	K-LMQAQLWQ-DYM-TLWQRTTGR-FLGGEAEPV GPAKEDRRFKDSAWMENTLFDRI KGSYLLTARFMGATVHGVEQLDDR-TARKLDFYTRQYVDA	162
A. brasilense	90	K-LMQAQLWQ-DYL-TLWQRTTGR-FFGQDAGPM APAKEDRRFKDSAWMENTLFDRI KGSYLLSARFMGATVNEVDGLDDH-TAKKVDYFTRQYVDA	184
R. eutropha	67	QH.GDI GGRYMK-DFSA-LWQAWAEG-KA-EATGPLH+-----DRRFAGDAWRITLPIYFFAAAFYLLNARALTELADAVEADAKT-RGRF R-FAL SQWDA	154
R. rubrum	76	--LD-AQAA-WA-FDI AALCGAAAKR-LRCEEAAPM EPAGDNRFRKDDAWTKDPLFDTLKGYLLTARLVATTLENSGGDPAC-RQ-RLAFYGRQWDA	167
R. sphaeroides	72	KI LEH-QI SFWCKSLKH-YVE-AQHQLVKG-ELKPPDVPDKDR-RFSNPLWTHPFFNYLKGQYLMAEVAWQAVEALEH EPSPDKRVEYFSRQI -VDL	166
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R. centenum	163	MPSNFVMTNPEVL-RTTLETGGENLVKQLENLADLERKGGQAI SMIDYSKFEVGRNI AVTPGKVVFGTDLMLLI QYAPTTEGMFRPLLI I PFW NK	261
A. brasilense	185	MPSNFVMTNPEVL-RTTLETGGENLVKQLEHLKOLERKGGELRI SMIDYDAFQVGNKI AVTPGKVVFGTDLMLLI QYPTTTEVNRKPLM VPPW NK	283
R. eutropha	155	MSPANFLATNPEAQ-RLLI ESGGESLRAGVRNMMEDLTRG-KI SQT--DESAFEVGRNAVTEGAVWFENEYFQLLQYPLTKVHARPLLMWPKI NK	249
R. rubrum	168	LAPTINFAATNPLVR-RTALESGGKSLNGLNLELRLDLERGGRLPFTMSDETAFEVGRILAMPKVVFGVALMLLI LYAPTTRKVKRPLLVPPW NK	266
R. sphaeroides	167	FSPTNFFGTNPDALERA ATDG-ESLVGQLENLVRDI EANNQILLVTLADPEAFQVGNLATTGSSVYRNRVFEI QYKPTTETVHETPLLI PFPW NK	265
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R. centenum	262	FYI LDLPKQNSLKVWLDQGHVFI VSWWNPGEHLSDKTFEDYMEQPLAALDAMEAATGEFEANM G C GGTLLASTLSYMTAGGDDRI KSAMMLVTL	361
A. brasilense	284	YVI LDLPKQNSFI KWAVDGGHVSFVLSWNPDEKLACKGFEDYMEGLAALDAI EKVTCEDVNAI G C GGTLLASTLSYMAAKKDDRI KSAFTFTTM	383
R. eutropha	250	YVI LDLPKQNSLKVWLDQGHVFI VSWWNPGEHLSDKTFEDYMEQPLAALDAMEAATGEFEANM G C GGTLLASTLSYMAAKKDDRI KSAFTFTTM	349
R. rubrum	267	FYI LDLPKQNSLKVWLDQGHVFI VSWWNPGEHLSDKTFEDYMEQPLAALDAMEAATGEFEANM G C GGTLLASTLSYMAAKKDDRI KSAFTFTTM	366
R. sphaeroides	266	FYI LDLPKQNSLKVWLDQGHVFI VSWWNPGEHLSDKTFEDYMEQPLAALDAMEAATGEFEANM G C GGTLLASTLSYMAAKKDDRI KSAFTFTTM	365
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R. centenum	362	TDFSEPGELSVFI DEEQLTMEESQ-MAQGG-Y----L-DGSKVAT-TFNMLRNDLI V6-FWVWNYLLGKDPFFDLYWNSDSTRMPAAMHSFYLRNMY	452
A. brasilense	384	LDFTEAGELSVFI DEEQLTMEESQ-MAQGG-Y----L-DGSKVAT-TFNMLRNDLI V6-FWVWNYLLGKDPFFDLYWNSDSTRMPAAMHSFYLRNMY	474
R. eutropha	350	LDFADTGI LDVFCVCEGHVQ-LREATLGG-GAGAPCALIRG-LELANTFSELRPNDLWVWVVD-NYLKGNTPVFPDLYWNGDNLNLPQWVQWYLR-ITY	445
R. rubrum	367	VDFSEPGELGVFI DFFLLDALDDQ-MARDG-G----L-DGDLI SM-AFNM.RDNDLI V6VF-I NNYLLGKTPAADFLLYWNGDNLNLPQWVQWYLR-IREMY	457
R. sphaeroides	366	TDFSDPCEGVFLNDDFVQI ERQVAVD--GI ---LDKTFMSR--TFSYLRSNDLI YGPAI KS-YMGEAPPADFLLYWNGDNLNLPQWVQWYLR-IREMY	456
* * * * *			
R. centenum	453	QRNLLVCPGGI TLKGVPI DLRRV TVPTFMLSTREDHI APWKSTYAATQLYGGPVK-FVLAASGHI AGWNPFSAEKYSHYLNTKLPASPDSPWFEKAGQMP	551
A. brasilense	475	QRNLLVCPGAVTLGGVPI DLKVKVTPSFTLSAREDH I APWKSTYMGALFSCPVK-FVLAASGHI AGWNPFAAGKYGYWTKLKPASDDWLASSEGTTP	573
R. eutropha	446	LQNELKVPQKLTVOGVPVQLASL DVPTVI YGSPREDHI VPMWAAVASTALLANKLR-FVLASGHI AGM NPPAKNRSHWITNDALPESPGQWLAGAI EHH	544
R. rubrum	458	QRNLLVCPGQLTVLGHALDLRRV RTPVYLLSARDHI APWSTFKATQLYGGPLR-FVLAASGHI AGM NPPAKARYGYWTKNADTSLEAESWLEGATPHG	556
R. sphaeroides	457	QQDRLAGGT-FPVLGSPVGLKDVTLVCAI ACETDHI APWKSSFNFRGFGSTDKTFI LSGSGHAGI VNPFSRNKYGYHTNEGPAGTPESEFREGAEF-H	554
* * * * *			
R. centenum	552	-GSWPEYKQWARYGGGKVPARVPCDGR-LPALEDAPGSYV-RKLSLE	597
A. brasilense	574	-GSWPEWNNWSTFSEKVPARNPEKGG-LPVALEDAPGSYV-AKVRV V-	618
R. eutropha	545	-GSWPDVNTAVLAGGAGAKRAAPANYGNARYRAI EPAPGRYV-KAKA-	589
R. rubrum	557	-GSWPDVNTAVLAGGAGAKRAAPANYGNARYRAI EPAPGRYV-KVRV-	600
R. sphaeroides	555	AGSWPFRAGANLAERSGKQVPRGQDSKH-PEL-APAPGSYVAAVGSA-----	601
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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 phaC. A system with the same vectors but carrying *R. eutropha phaC* instead of *R. centenum phaC* 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 phaC*. The reason why *R. centenum phaC* 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

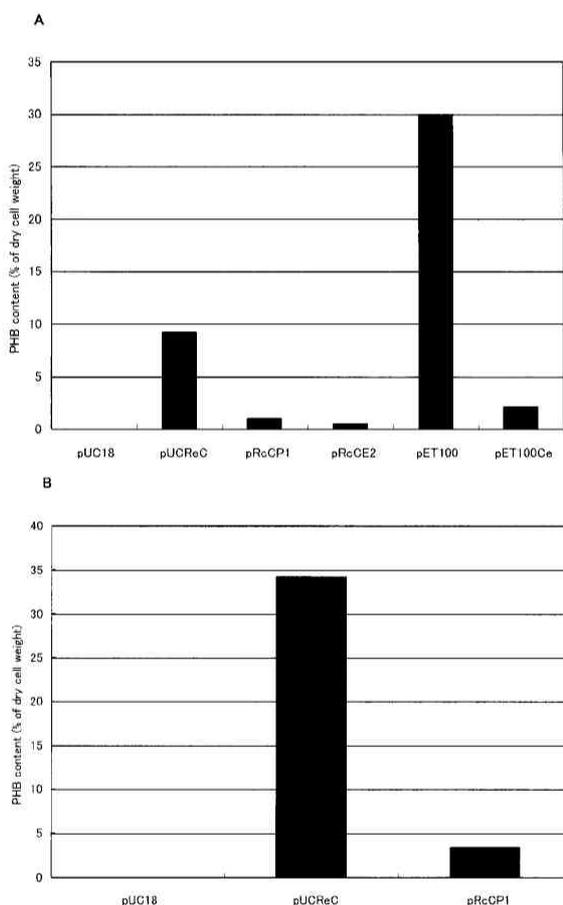


FIG. 5. Comparison of PHB accumulation in *E. coli* harboring various vectors. A) *E. coli* JM109 or BLR cultivated in LB medium with 2% glucose at 37 °C for 39 hours. PHB content was measured with a HPLC-based method. pUC18, pUCReC, pRcCP1, pRcCE2, *E. coli* JM109 harboring pSTVReAB with each vector,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

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-mannose 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.

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