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Real-Time PCR Analysis of Metabolic Pathway of PHB in *Acidiphilium cryptum* DX1-1

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The time, yield, and related genes expression of PHB accumulation of *Acidiphilium cryptum* DX1-1 were investigated under four different initial C/N ratios, 1.2, 2.4, 7.5, and 24. The results of time and yield of poly-β-hydroxybutyrate (PHB) accumulation show that the initial C/N ratio of 2.4 was optimum for strain DX1-1 to accumulate PHB, but both higher and lower initial C/N ratios did not favor that process. Based on the genome of *Acidiphilium cryptum* JF-5, 13 PHB accumulation related genes in strain JF-5 were chosen and successfully cloned from strain DX1-1. The differential expressions of the 13 functional genes, in different C/N ratios as cited above, were then studied by real-time PCR. The results show that all the 13 genes were most upregulated when the initial C/N ratio was 2.4, and among which the gene *Acry_3030* encoding poly-β-hydroxybutyrate polymerase and *Acry_0626* encoding acetyl-CoA synthetase were much more upregulated than the other genes, which proved that they play the most important role for PHB accumulation, and acetate is the main initial substance for PHB accumulation for strain DX1-1. Potential regulatory motifs analysis showed that the genes related to PHB accumulation are regulated by different promoters and that the motif had weak similarity to the model promoters, suggesting that PHB metabolism in *Acidiphilium cryptum* may be mediated by a different mechanism.

Key words: *Acidiphilium cryptum*, PHB, real-time PCR

Poly-β-hydroxybutyrate (PHB), the most popular type of polyhydroxyalkanoate (PHA) that could be used to produce biodegradable plastics, the eco-friendly biopolymer materials, has been studied extensively in recent years [3]. PHB is generally produced by heterotrophic bacteria in the presence of excess organic carbon source, and it provides the cells with food, energy, and reducing power [18]. It was reported that under appropriate growth conditions, up to 88.8% of the cellular dry weight of PHB in *Ralstonia eutropha* [5] and 87% in recombinant *Escherichia coli* cells [11] were accumulated. However, the use of PHB produced by bacterial fermentation as a commodity polymer is limited by its high production cost compared with some widely used petroleum-derived plastics [8]. In consideration of reducing the cost of feed materials, CO_2 that can be fixed by green plants may be the most preferable. Therefore, PHB production in transgenic plants, by transferring its biosynthesis genes, has become the interesting topic for researching PHB production [15]. However, there are many drawbacks for plants to produce PHB, such as a relatively long period of growth cycle, low accumulation amount of PHB, and difficulty in processing the plants to isolate the PHB from other components.

Acidiphilium spp., which were almost isolated from acid mine drainages (AMD), could promote bioleaching by reducing Fe(III) [17]. Yukiko *et al.* [23] reported that *Acidiphilium rubrum* accumulated PHB granules. In the most recent years, we isolated from AMD of DeXing Copper Mine, Jiangxi Province, China, a new strain, *Acidiphilium cryptum* DX1-1, a facultative strain that could grow in either organic substance or inorganic sulfur energy substrate [22] and accumulate up to 88% cellular dry weight of PHB when it was cultured in excess of glucose substrate [21]. It was found more recently that strain DX1-1 could not accumulate PHB granules in culture medium with sulfur as the only energy, but it could restore the accumulation of PHB up to 88% cellular dry weight when 0.1% of glucose was added to the same sulfur-containing medium (data not yet published). It was evident in such a case that besides glucose, *A. cryptum* DX1-1 could fix CO_2 for accumulation of the cellular PHB. The price of sulfur is 60–100 dollars per ton, whereas it is 300–400 dollars per ton for glucose,

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suggesting a promise in reduction in raw material cost for industrial production of PHB by strain DX1-1. On the other hand, however, although high a content of PHB could be accumulated at that culture condition, it showed lower cells density and a longer period of growth than the reported recombinant *E. coli* cells, suggesting that before use in industry, *A. cryptum* DX1-1 needs to be improved. To reach this goal, the metabolism of PHB, CO_2, sulfur, and energy for that strain should be known. In this paper, the regulation of metabolism of PHB was chosen as the first step of the study.

The genome of *A. cryptum* JF-5 isolated by Küsel *et al.* [9] has been sequenced and most of the genes were annotated (http://genome.ornl.gov/cgi-bin/JGI_microbial/kegg_categories. cgi), which provided us the useful homologous genes to *A. cryptum* DX1-1. However, the regulation of metabolism of PHB is not clear, and there are no reports on the molecular mechanism of PHB accumulation by *Acidiphilium* spp. either. In this paper, in order to evaluate the functional genes related to accumulation of PHB and elucidate their regulation in *A. cryptum* DX1-1, differential expressions of 13 functional genes related to PHB metabolism that were derived from the genome of *A. cryptum* JF-5 were detected in *A. cryptum* DX1-1 by real-time PCR. Potential regulatory motifs of those genes were analyzed. This is the first time that the molecular mechanism of PHB accumulation of *Acidiphilium* spp. has been reported.

Materials and Methods

Strain, Culture Medium, and Determination Methods

A. cryptum DX1-1 (GenBank Accession No. DQ529311) used in the experiments was isolated from AMD of a reservoir in Dexing Copper Mine, Jiangxi Province, China, and conserved in our laboratory. Strain DX1-1 was maintained in a medium containing the following reagents (per liter): KCl 0.1 g, K_2HPO_4 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $Ca(NO_3)_2$ 0.01 g, pH 3.0. Glucose (40 g/l) and $(NH_4)_2SO_4$ (3, 10, 30, and 60 g/l) were chosen as the carbon source and nitrogen source, respectively. Cellular growth was monitored by direct cell counting (APHA 1989), and the PHB content was measured by the concentrated H_2SO_4 decomposition method [14].

Total RNA Extraction and cDNA Synthesis

Total RNA was isolated using the TRIzol reagent (Invitrogen Corporation, Carlsbad, U.S.A.) and purified with the RNeasy mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. On-column DNase digestion was performed with RNase-free DNase (Qiagen GmbH, Hilden, Germany) to remove genomic DNA. The integrality of total RNA was checked by 1.5% agarose gel electrophoresis and ethidium bromide staining, imaged by a BioSense SC-810 Gel Documentation System (Shanghai Bio-Tech Co., Ltd, China). Total RNA was quantified at OD_{260} and OD_{280} with the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, U.S.A.) and then served as the template to synthesize cDNA with SuperScript RNase H Reverse Transcriptase (Invitrogen Corporation, Carlsbad, U.S.A.). cDNA samples were diluted to a concentration of about 100 ng/μl for real-time PCR analysis.

Primers Design

Real-time PCR primers were designed by using Primer Premier 5.0 and then synthesized by Sagon Biotech, Shanghai, China. The annotation of *A. cryptum* DX1-1 genes and their putative cellular functions were from the Comprehensive Microbial Resource (CMR) of *A. cryptum* JF-5 (JGI; http://genome.ornl.gov/cgi-bin/JGI_microbial/kegg_categories.cgi). All primer pairs of the selected genes that encode proteins relating to PHB metabolism are listed in Table 1. 16S rRNA (DQ529311) of *A. cryptum* DX1-1 was used as the control gene.

The primers were diluted to obtain 10 mM working stock solutions. The specific fragments were amplified and purified by using the QIAquick 96-well purification kit (Qiagen GmbH, Hilden, Germany) and then checked by 1.5% agarose gel electrophoresis and ethidium bromide staining to detect whether PCRs contained a single product of the expected size. DNA sequencing was carried out by Sagon Biotech, and BLAST analysis in JGI was used to check the products.

Real-Time PCR Detection

Each real-time PCR mixture (final volume, 25 μl) contained 12.5 μl of SYBR Green Real-time PCR Master Mix (Toyobo Co., Ltd., Osaka, Japan), which contains *Taq* DNA polymerase, dNTP, $MgCl_2$, SYBR Green I dye, 1 μl of a 10 mM solution of sense/anti-sense primer, 5 μl of cDNA template, and 5.5 μl of nuclease-free water. The real-time PCR was carried out with the iCycler iQ Real-time PCR detection system (Bio-Rad Laboratories, Inc., Hercules, U.S.A.): 1 cycle of 95°C for 30 s, and then 40 cycles of 95°C for 15 s, 55–60°C (according to the optimal annealing temperature in Table 1) for 30 s, and 72°C for 30 s. At the completion of each run, melting curves for the amplicons were measured by raising stepwise the temperature by 0.5°C, from 55 to 95°C, while monitoring fluorescence. The specificity of the PCR amplification was checked by examining the melting curve for melting temperature T_m, its symmetry, and the lack of nonspecific peaks. PCR product standards were prepared from genomic DNA with an initial concentration of 10^7 copies/μl. Serial dilutions of 10^7 to 10^2 copies/μl were performed to prepare the standard curves.

The gene expression ratio was recorded as the fold difference in quantity from samples grown in the medium with an initial C/N ratio of 24. The results were normalized against the control gene, 16S rRNA, to correct sample-to-sample variation.

Computational Promoter Analysis and Motif Identification

To search for potential regulatory motifs upstream of genes related to PHB metabolism, the Neural Network Promoter Prediction software from the Berkeley *Drosophila* Genome Project (http://www.fruitfly.org/seq_tools/promoter.html), which combines neural networks with weight pruning, was used with time-delay neural networks for a complete promoter site prediction [4]. Based on the distance between the genes, the similarity of the cluster of orthologous groups of proteins (COG), functional classes assigned to each gene, the similarity of codon usage, and other factors, regions representing approximately 500 base pairs of the DNA sequences upstream of the translational start site of genes were extracted from the JGI genome database.

The multilevel consensus sequences for each promoter motif were then used to generate a sequence logo by AlignACE (http://weblogo.

Table 1. The primers for real-time PCR detection of expression of the PHB accumulation-related homologous genes in strain DX1-1.

Locus	Annotated function	Primer sequence	Annealing temp. (°C)	Amplicon length (bp)
DQ529311	16S rRNA	F: TGGTAGTCCACGCTGTAA R: AAATCCTGGTAAGGTTC TG	55	195
Acry_0626	Acetyl-CoA synthetase	F: TGAGCTTCGCTGAATTGTCCG R: CGCCAGCATCACCTCCCA C	60	131
Acry_2288	Acetyl-CoA synthetase	F: TCCGAACTACCCCGACACC R: CGACCGAACCCAGCACCC	58	163
Acry_1847	β-Ketoadipyl-CoA thiolase	F: GCCGTTTGGGCGTTATGG R: GCACTCCCCACCGCATC	60	264
Acry_2753	Acetoacetyl-CoA reductase	F: CGACGGGATGAAGGAGCG R: GGCGATGGCATTGACGG	56	175
Acry_3030	Poly-β-hydroxybutyrate polymerase	F: ACCTTCGTGCTGACCT R: TCGTGGATGTAGTGCC	50	278
Acry_1301	Polyhydroxyalkanoate synthase	F: GCTCGAACGACCTGATCTGG R: TGCGGGTGGAATCGGAAT	56	202
Acry_2255	Polyhydroxyalkanoate synthase	F: CCTCGTTCCTGCGTTTTCT R: CTCCAGCGCCCGTTCAAG	55	132
Acry_2759	Poly-β-hydroxyalkanoate depolymerase	F: TGCAGCTCGCCTCCTTCGT R: ATTCCGCCGTCAGGTCCA TC	57	156
Acry_0146	β-Hydroxybutyryl-CoA dehydrogenase	F: TGACGCTGTGCGATCTGGT R: GTCGGCGGGTTGGTGG	60	178
Acry_0494	β-Hydroxybutyrate dehydrogenase	F: CGAGTTTCCCTTTTCCG R: ACCGAGCCGCTTTTCTG	55	111
Acry_2355	β-Hydroxybutyrate dehydrogenase	F: TCTTGGCGGGGAGTTTGGTG R:TGAAATACGGCGGAAAGGTTGAG	60	216
Acry_0255	β-Hydroxyacyl-CoA dehydrogenase	F: GCGACCGGAGCGACAAAAT R: CGAAAGGCAGGGTGAAGGAAT	62	147
Acry_2779	β-Hydroxyacyl-CoA dehydrogenase	F: GTCAACCCGCCCTATCTCATT C R: TCCTGGCCCACGCTTTCC	62	107

Berkele.edu/logo.cgi) [6], which is a graphical representation of nucleic acid multiple sequence alignment. Each logo consists of stacks of symbols, one stack for each position in the sequence. The overall height of the stack indicates the sequence conservation at that position, whereas the height of symbols within the stack indicates the relative frequency of each amino or nucleic acid at that position [20].

RESULTS

Effects of the Initial C/N Ratios on PHB Accumulation in *A. cryptum* DX1-1

Table 2 lists the PHB yields and time of PHB accumulation in *A. cryptum* DX1-1 at different initial C/N ratios: 24, 7.5, 2.4, and 1.2, where $(NH_4)_2SO_4$ was chosen as the nitrogen source and glucose as the carbon source. It shows that the highest PHB yield, 0.88 g/1 g cellular dry weight, and the shortest PHB accumulation time in *A. cryptum* DX1-1 were attained at the case when the initial C/N ratio is 2.4. This indicates that PHB accumulation in strain DX1-1 needs a suitable initial C/N ratio, and both a too high and too low availability of N or C source does not favor PHB accumulation by that strain.

Quality of Total RNA and Testing of Primer Pairs

The total RNA ratio of OD_{260}/OD_{280} was about 1.95–2.05. The integrality of total RNA that was checked by 1.5% agarose gel electrophoresis and ethidium bromide staining is shown in Fig. 1. It shows that the total RNA extracted was in good integrality, and small RNAs (5S rRNA and tRNA) were removed in the purification process. The 23S/16S rRNA ratio was ≥1.50, based on the analysis of the brightness of strips in the gel, indicating that RNA was integral and in high quality.

The quality of the amplified products, which was checked by 1.5% agarose gel electrophoresis and ethidium bromide

Table 2. PHB production by strain DX1-1 under different initial C/N ratios.

Initial C/N ratio	PHB yield (g/g cellular dry weight)	Time of PHB accumulation (h)
24	0.60	72
7.5	0.68	61
2.4	0.88	48
1.2	0.72	56

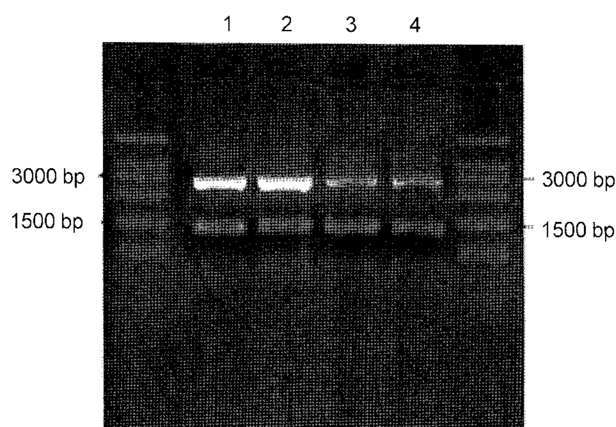


Fig. 1. Electrophoresis of total RNA, where samples are from cells incubated in culture media: 1: with initial C/N ratio 1.2 (incubated for 56 h); 2: with initial C/N ratio 2.4 (incubated for 48 h); 3: with initial C/N ratio 7.5 (incubated for 61 h); and 4: with initial C/N ratio 24 (incubated for 72 h).

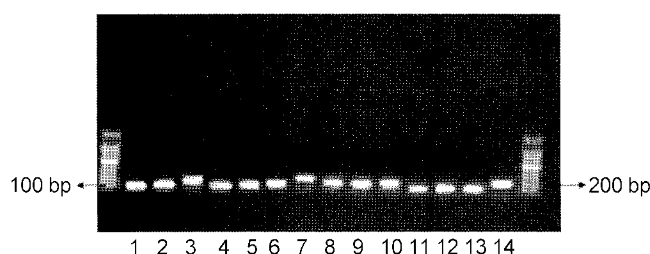


Fig. 2. Electrophoresis analysis of PCR products from positive plasmid with the designed primers for genes: 1: *Acry_3030*; 2: *Acry_1847*; 3: *Acry_0146*; 4: *Acry_0255*; 5: *Acry_2779*; 6: *Acry_0626*; 7: *Acry_2355*; 8: *Acry_2288*; 9: *Acry_2759*; 10: *Acry_2753*; 11: *Acry_1301*; 12: *Acry_2255*; 13: *Acry_0494*; 14: *DQ529311*.

staining, is shown in Fig. 2. It shows that PCRs contained single products of the expected size. BLAST analysis (data not shown) revealed that the sequence alignments for the PCR products were all in 100% of consistency with sequence alignments from *Acidiphilium cryptum* JF-5, which proved the functional genes from strain DX1-1 and strain JF-5 had the same sequence.

Differential Expression of Genes Related to PHB Metabolism of *A. cryptum* DX1-1 at Different Initial C/N Ratios

According to the PHB metabolism reported by Trainer and Charles [19], Reddy *et al.* [16], and Madison and Huisman [13], 13 genes that were related to metabolism of PHB in the genome of *A. cryptum* JF-5 were chosen as the research object. As shown in Table 1, these genes encode acetyl-CoA synthetase, β-ketoadipyl-CoA thiolase, acetoacetyl-CoA reductase, poly-β-hydroxybutyrate polymerase, polyhydroxyalkanoate synthase, poly-β-hydroxyalkanoate depolymerase, β-hydroxybutyryl-CoA dehydrogenase, β-hydroxyacyl-CoA dehydrogenase, and β-hydroxybutyrate dehydrogenase, respectively.

The differential expressions of the 13 chosen genes were checked with the nutritional states, the initial C/N ratios, and the results are listed in Table 3, where the expression of 16S rRNA was chosen as the control and the expressions of each gene at four different initial C/N ratios were compared with its expression in the case of initial C/N ratio of 24. It shows that all the 13 genes were basically upregulated, but in relatively lower level, when the initial C/N ratios were between 24 and 7.5, and were distinctly upregulated at the initial C/N ratio of 2.4, and were apparently downregulated at the initial C/N ratio of 1.2.

Table 3. List of the differential expressions of genes under different initial C/N ratios.

Locus	Annotated function	Initial C/N ratios			
		24	7.5	2.4	1.2
DQ529311	16S rRNA (control)	1	1	1	1
Acry_0626	Acetyl-CoA synthetase	1	5.89	1,070	206
Acry_2288	Acetyl-CoA synthetase	1	0.87	292	59.8
Acry_1847	β-Ketoadipyl-CoA thiolase	1	6.89	212	89.3
Acry_2753	Acetoacetyl-CoA reductase	1	8.56	302	139
Acry_3030	Poly-β-hydroxybutyrate polymerase	1	10.2	3,102	45.3
Acry_1301	Polyhydroxyalkanoate synthase	1	4.38	77.5	45.8
Acry_2255	Polyhydroxyalkanoate synthase	1	12.4	15.2	0.691
Acry_2759	Polyhydroxyalkanoate depolymerase	1	1.65	213	36.8
Acry_0146	β-Hydroxybutyryl-CoA dehydrogenase	1	5.73	135	70.2
Acry_0255	β-Hydroxyacyl-CoA dehydrogenase	1	7.89	92.1	34.8
Acry_2779	β-Hydroxyacyl-CoA dehydrogenase	1	3.21	73.6	23.1
Acry_0494	β-Hydroxybutyrate dehydrogenase	1	0.992	49.4	37.7
Acry_2355	β-Hydroxybutyrate dehydrogenase	1	9.39	694	98

The numbers in the table stand for the gene expression ratio, which was recorded as the fold difference in quantity from samples which in the medium with the initial C/N ratio of 24. The results were normalized against the control gene, 16S rRNA, to correct sample-to-sample variation.

Table 4. Motif sequence and the promoter score.

Locus	Promoter sequence	Score[a]
Acry_0626	TGATGGAGCAGCGCGCCGACAACAAGATCATCC	0.91
Acry_2288	TGTTGATACCGCGCGATCCATCGGCTATAGCGC	0.99
Acry_1847	TGTTTCACTTTGATTGCAATAGGTTTCCCCTATTG	0.99
Acry_2753	TTTCTTTTTGGCGAGACTTCACCCATATTGTAAAA	0.99
Acry_3030	CCGGGCGCGCGCCCCGGCATAATCCTCGAGCG	0.87
Acry_1301	GGGGGTATAACATCTGTGCCGCACCGCCGCGAC	0.99
Acry_2255	CCACGCCTTGCGACGAGGGGAAAGGGCGAAGA	0.83
Acry_2759	TTTTGGCGAGACTTCACCCATATTGTAAAAGGAA	0.99
Acry_0146	TTGACTTGCCGAACGACGCCCCATAGATCATCCA	0.83
Acry_0255	TTCGTCACCGCGCTCGGCGGCGCCGGGCTGCA	0.97
Acry_2779	CCCCCGCCTATCTCGACCGGCGGGGCCGGCCG	0.95
Acry_0494	ATCCTGATGTCATGATCGGCAACGGGACGATA	0.94
Acry_2355	CGATTCTTACGCATGATAATGCTTTTATCATG	0.91

[a]The promoter score, which is more near 1.0, where the result is more credible.

These phenomena were basically consistent with the PHB accumulation states at the same four initial C/N ratios as shown in Table 2, indicating that PHB accumulation was defined by the expression of the genes related to PHB metabolism. It is worthy to note that two genes, *Acry_0626* encoding acetyl-CoA synthetase and *Acry_3030* encoding polyhydroxybutyrate polymerase, were especially upregulated in expression, and *Acry_3030* was also more upregulated in expression than *Acry_2759* that encodes just the opposite degradation enzyme poly-β-hydroxyalkanoate depolymerase. Slightly more upregulated expression was observed for acetoacetyl-CoA reductase encoded by *Acry_2753* than its opposite degrading enzyme poly-β-hydroxybutyryl-CoA dehydrogenase encoded by *Acry_0146*. β-Hydroxybutyrate dehydrogenase is encoded by *Acry_0494* and *Acry_2355*. *Acry_2355* was upregulated 694 times, which was much higher than the expression of other genes related to PHB degradation. All the other genes showed relatively lower upregulated expressions than the genes *Acry_0626* and *Acry_3030*. These results indicate that the PHB accumulation was due to the increased upregulation in expressions of genes encoding the enzymes for synthesis than that for degradation, and the PHB accumulation process was mainly controlled by the enzymes acetyl-CoA synthetase and poly-β-hydroxybutyrate polymerase, which are possibly involved in the first and last steps of the PHB synthesis pathway.

Computational Prediction of the Consensus Motif in *A. cryptum*

To predict genes consensus motif, the DNA sequences upstream of heat-inducible genes were analyzed for potential regulatory motifs. Sequence analysis indicated that a few genes are likely encoded within operons, whereas others are divergently transcribed. A subset of 10 intergenic sequences (500-base each in length or the noncoding sequence ≤500 bp between two genes) containing putative promoter regions were searched using the Neural Network Promoter Prediction software for putative conserved DNA motifs associated with PHB metabolic genes. The results of analysis of consensus motifs are listed in Table 4 and shown in Fig. 3.

Discussion

Bacteria able to synthesize PHB can be divided into two groups [10]. The first group, accumulating PHB during the stationary growth phase, requires limitation of N, P, Mg, for example, and an excess of the carbon sources. The most important microorganism for industrial PHB production, *R. eutropha*, belongs to this group. The second group, accumulating PHB during the growth phase, includes *Alcaligenes latus*, a mutant strain of *Azotobacter vinelandii*, *Azotobacter beijerickii* [1], or recombinant strains of *E. coli* bearing the PHB operon of *R. eutropha*. Our strain, *Acidiphilium cryptum* DX1-1, belongs to the second group, because PHB accumulation decreased with the increase in initial C/N ratio. The strain DX1-1 needs a suitable initial C/N ratio to accumulate PHB.

Fig. 3. Motif model of the putative promoter.
The T-G -n- -n-G in the -35 region and n-G-A-n-n - A-C-A in the -10 region, with average 20 base pairs separating the two elements was predicted upstream of a number of PHB metabolism-related genes. The motif is represented by a sequence logo generated by the WebLogos software (http://weblogo.Berkele.edu/logo.cgi).

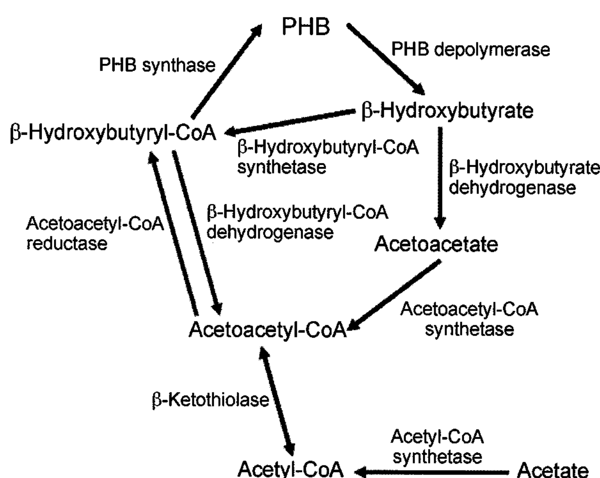
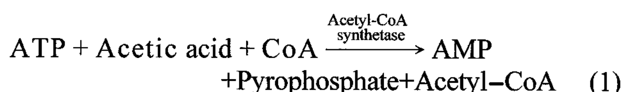


Fig. 4. Metabolic pathway of PHB in *Acidiphilium cryptum* DX1-1.

Enzyme action can be regulated on the levels of gene transcription and post-translation modification, including folding, sorting, and degradation of the protein. However, transcription is the most important factor to regulate the quantity of active protein. Real-time PCRs are particularly valuable in providing insight into the mechanism regulating a bacterial response to stress and provide useful data for generating computational models of stress response pathways. Although a few researches have reported that *Acidiphilium* spp. could accumulate PHB granules [23], there is not any report on the mechanism of PHB accumulation by *Acidiphilium* spp. In this paper, by investigation of the differential expression of the genes encoding the PHB accumulation-related proteins in response to a change in initial C/N ratios, it was found that acetyl-CoA synthetase and poly-β-hydroxybutyrate polymerase were the most important regulatory enzymes for PHB synthesis.

According to the functions of proteins encoded by those genes and the response to stress, the possible metabolic pathway of PHB was derived and is shown in Fig. 4. The biosynthetic pathway of PHB from *A. cryptum* DX1-1 is the same as other strains. However, *A. cryptum* DX1-1 uses acetate, instead of acetyl-CoA, as the main initial substance for PHB accumulation, which is the same as *Sinorhizobium meliloti* [2], but different from most other PHB-accumulating strains that use acetyl-CoA provided by decarboxylation of pyruvic acid as the initial substance for PHB accumulation [7]. The reason may be that *A. cryptum* DX1-1 is an acidophilic species and it has powerful glyoxylate and pyruvate metabolic pathways, which supply the acetate ceaselessly. Strain DX1-1 uses acetate to synthesize acetyl-CoA according to the following equation:



$$\text{ATP} + \text{Acetic acid} + \text{CoA} \xrightarrow{\text{Acetyl-CoA synthetase}} \text{AMP} + \text{Pyrophosphate} + \text{Acetyl-CoA} \quad (1)$$

In this step, the activity of acetyl-CoA synthetase is mainly regulated by the expression of *Acry_0626*. This step is also a key step owing to the particular upregulation in expression of *Acry_0626*.

β-Ketoadipyl-CoA thiolase is encoded by *Acry_1847*. In the PHB metabolism, this enzyme catalyzes the bidirectional reactions at the same time. Table 3 shows that the expression of *Acry_1847* also has higher expression for the case of initial C/N ratio of 2.4 than at the other three cases. However, considering the diplex effect of β-ketoadipyl-CoA thiolase, the gene *Acry_2753* is impossible as the key gene of PHB accumulation.

Poly-β-hydroxybutyrate polymerase catalyzes the last step of PHB synthesis. So far as is known, poly-β-hydroxybutyrate polymerase is encoded only by gene *Acry_3030* in *Acidiphilium* spp. When cells had the maximal yield of PHB, *Acry_3030* displayed the maximal change in expression, indicating it is the most important key gene to regulate PHB synthesis. There are no side pathways to polymerize β-hydroxybutyryl-CoA in the butanoate metabolism from the Web site (http://genome.ornl.gov/microbial/acry), which once more reveals the role of *Acry_3030* is important. Poly-β-hydroxyalkanoate depolymerase encoded only by gene *Acry_2759* catalyzes the degradation of PHB. There are no other enzymes to catalyze this reaction. In cells of *Acidiphilium* spp., poly-β-hydroxybutyrate polymerase and poly-β-hydroxyalkanoate depolymerase are the key enzymes for PHB synthesis and degradation, respectively. Therefore, increasing the expression of *Acry_3030* or decreasing the expression of *Acry_2759* by the molecular biology method may lead to a rise of the PHB yield. The expression of the two genes also show that in cells of *A. cryptum* DX1-1, PHB accumulation is a metabolic process, in which cells synthesize PHB and degrade PHB at the same time. The amount of PHB accumulation in cells depends on the dispersion of synthesis and degradation.

PHB is broken down to β-hydroxybutyrate, which is utilized as a carbon source for growth, by the enzyme poly-β-hydroxyalkanoate depolymerase. β-Hydroxybutyrate may be oxidized by β-hydroxybutyrate dehydrogenase, and it can also be catalyzed by β-hydroxybutyryl-CoA synthetase to form β-hydroxybutyryl-CoA, which will be oxidized by β-hydroxybutyryl-CoA dehydrogenase. β-Hydroxybutyryl-CoA dehydrogenase is encoded by gene *Acry_0146*. β-Hydroxybutyrate dehydrogenase is encoded by *Acry_0494* and *Acry_2355*. *Acry_2355* plays an important role in PHB degradation, because it was upregulated higher than other genes related to PHB degradation. There are many genes whose functions have not been confirmed for *A. cryptum*, despite that the sequencing has been completed. β-Hydroxybutyryl-CoA synthase and acetoacetyl-CoA synthase were just two of the functional undetected enzymes. Because of this, the main degradative pathway for β-hydroxybutyrate maintains unknown.

The regulation of the prokaryotes promoter as an initiator for transcription is one of the most complex processes in molecular biology. It has been shown that multiple functional sites in the primary DNA are involved in the polymerase binding process. These elements, such as the TATA-box, GC-box, CAAT-box, and the transcription start site are known to function as binding sites for transcription factors and other proteins that are involved in the initiation process. Both of them showed that the motif had weak similarity to the model promoter, suggesting that the PHB metabolism in *A. cryptum* may be mediated by a different mechanism. Genes encoding β-ketoadipyl-CoA thiolase, acetoacetyl-CoA reductase, and poly-β-hydroxybutyrate polymerase are in the same operon in many strains that accumulate PHB [12]. Those three genes are in three different operons for *A. cryptum*, which may be the reason for its high efficiency for PHB synthesis.

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