Chapter 9

Polyhydroxyalkanoate (PHA) Production from Carbon Dioxide by Recombinant Cyanobacteria

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Additional information is available at the end of the chapter

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1. Introduction

Global warming is the urgent issue of our time, and the carbon dioxide is a greenhouse gas of the major concern. There are various research activities for carbon dioxide mitigation, such as CO_2 recovery from the flue gas of industrial sites, underground and undersea CO_2 storage, and also chemical/biological conversion of CO_2 into the industrial materials [1].

On-site CO_2 fixation by bioprocess is based on the activities of photosynthetic organisms. The fixation of CO_2 by photosynthetic microorganisms can be an efficient system for the CO_2 mitigation, but one of the major problems of this system is the effective utilization of the fixed biomass. The biomass produced by photosynthetic microorganisms must be utilized as a resource, or it will be easily degraded by microorganisms into CO_2 again. Cyanobacteria are procaryotic photosynthetic microorganisms and can provide a simple genetic transformation system for the production of useful materials from CO_2. We have established an efficient vector-promoter system for the introduction and expression of foreign genes in the marine cyanobacterium *Synechococcus sp.* PCC7002, and examined the production of biodegradable plastic, polyhydroxyalkanoate (PHA), by genetically engineered cyanobacteria. The PHA is a biopolymer accumulated by various microorganisms as reserves of carbon and reducing equivalents. PHAs are linear head to tail polyesters composed of 3-hydroxy fatty acid monomers (Figure 1), have physical properties similar to those of polyethylene, and can replace the chemical plastics in some applications, such as disposable bulk materials in packing films, containers, and paper coatings [2]. PHA applications as implant biomaterials, drug delivery carrier, and biofuel have also been investigated [3]. The commercial mass production of PHA from corn sugar by using the genetically enigineered microorganism was started in 2009 in the United State and China [3].

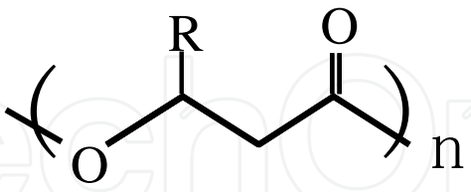

Figure 1. Chemical structure of PHAs

Assimilation and conversion of CO_2 into the biodegradable plastics (biopolymers) by photosynthetic microorganisms is an ideal bioprocess because it converts CO_2 directly into the useful bioplastics with solar energy. It also contributes to the low carbon society by substituting the environmentally unfriendly petroleum-based plastics with the carbon neutral bioplastics, and also by saving the fossil fuel resource required for the petrochemical plastics production. In addition, the biodegradable plastics reduce the burden of plastics waste on landfills and the environment.

2. Shuttle-vector construction

2.1. Construction of a shuttle-vector between *Escherichia coli* and cyanobacteria

Since most of the industrial CO_2 emission sites locate in the seashore area in Japan, we choose a marine cyanobacterial strain for the fixation and utilization of CO_2. The marine cyanobacterial strain *Synechococcus sp.* PCC7002 (*Agmenellum quadruplicatum* PR-6, ATCC 27264) [4] was obtained from the American Type Culture Collection, and cultured at 30 °C in medium A [5] under continuous illumination (50 μmol photons m^{-2} s^{-1}) by bubbling with 1% CO_2 in air.

For the construction of a shuttle-vector between *E. coli* and *Synechococcus sp.* PCC7002, we isolated and characterized the smallest endogenous plasmid pAQ1 of this cyanobacterium [6, 7]. The plasmid pAQ1 is 4809 bp long, and has four open reading frames (ORFs): ORF943, ORF64, ORF71, and ORF93 (numbers show putative amino acid number). The construction of the shuttle-vector was done by digesting pAQ1 plasmid and bacterial pUC19 plasmid with restriction enzymes which cleave each plasmid at a unique site, and by ligating the linearized plasmids. The plasmid pUC19 and the plasmid pAQ1 were linearized by *Sma*I and *Stu*I digestions, respectively, and were ligated to generate the shuttle-vector pAQJ6 (Figure 2; both *Sma*I and *Stu*I are blunt-end forming restriction enzymes). The effect of the four ORFs on the transformation efficiency was examined, and ORF943 was found to be important for the maintenance of the shuttle-vector. From this result, the simplified shuttle-vector pAQJ4 with the full ORF943 was designed (Figure 2).

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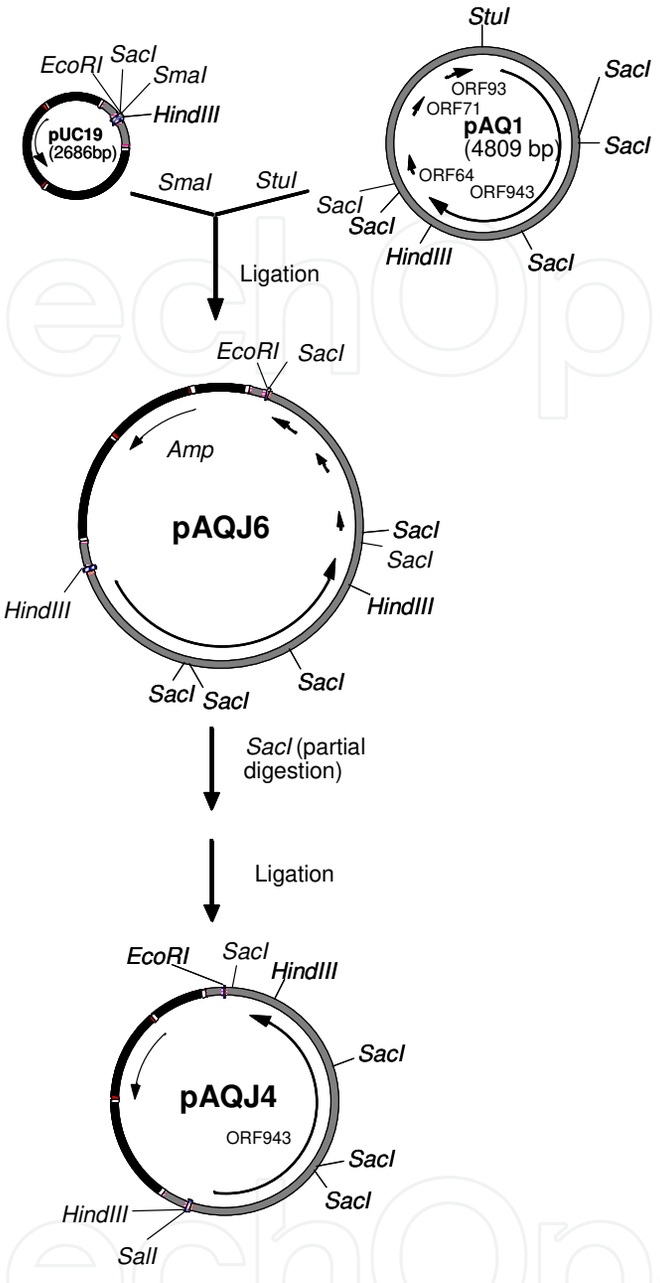

Figure 2. Construction of shuttle-vector between *E. coli* and *Synec hococcus* sp. PCC7002

The stability of the prototype shuttle-vector, pAQJ6, in cyanobacterial cells was relatively low, but that of the simplified shuttle-vector, pAQJ4, was much improved; this vector could be stably retained in cyanobacterial cells after 100 generations of growth without antibiotics selection [8]. This is probably because that there are several hot spots for the homologous recombination between endogenous pAQ1 plasmid and pAQJ6 vector, and these hot spots might have been eliminated in the simplified pAQJ4 vector. The transformation efficiency of the shuttle-vector pAQJ4 was about 4 x 10^5 (cfu / μg DNA), when we transformed 4 x 10^7 of cyanobacterial cells with 0.3 μg (0.1 pmol) of pAQJ4 vector in 1 ml solution. This transformation efficiency was 10 to 100 times higher than those of the shuttle-vectors for this cyanobacterium

previously reported [4, 9]. With this system, we can obtain several million of cyanobacterial transformant in one experiment, thus this shuttle-vector system can also be applied for the construction of cDNA libraries using cyanobacteria as host cells.

An example of the use of this shuttle-vector for cDNA library is the construction of cDNA library of the halotolerant marine green alga *Chlamydomonas* W80 for the isolation of anti-stress genes [10]. *C.* W80 shows a surprisingly high oxidative stress tolerance caused by methyl viologen (MV), which is reduced by the photosynthetic apparatus generating highly toxic superoxide (O_2^-). *C.* W80 tolerates up to 200 μM of MV [11, 12], while other oxygen-evolving photosynthetic organisms such as higher plants, algae and cyanobacteria usually tolerate only less than 5 μM of MV. This alga is a prominent genetic resource of anti-stress genes, and various unique anti-stress genes, such as ascorbate peroxidase [13], glutathione peroxidase [14], and the novel salt and cadmium stress related (*scsr*) gene [15], have been isolated from this alga. Using the cDNA library of *C.* W80 constructed in pAQJ4 shuttle-vector, we isolated anti-stress genes by a functional expression screening in cyanobacteria. The principle of the screening method was based on the acquisition of stress-tolerance of the cyanobacterial cells carrying the genes of *C.* W80, and a unique anti-stress gene (a new member of group 3 late embryogenesis abundant protein genes) was successfully isolated [10].

2.2. Promoter for the expression of the genes on shuttle-vector

An effective promoter is important for the expression of the genes on shuttle-vector. The promoter of the RuBisCO (*rbc*) gene of *S.* PCC7002 was chosen for the source of strong promoter, and the *rbc* gene was isolated by screening the genomic library of *S.* PCC7002. RuBisCO is one of the key enzymes of Calvin–Benson cycle (photosynthetic CO_2 fixation cycle), and the most abundant protein in photosynthetic organisms. Our genomic clone of the *rbc* gene (DDBJ Accession No. D13971) is 4234 bp long, and has 962 bp of five prime untranslated region (5' UTR) of *rbc* large subunit (*rbcL*). To determine the location of the promoter activity in the 5′-UTR of *rbc* gene, we constructed CAT (chloramphenicol acetyltransferase) reporter gene construct in pAQJ4 vector, and examined the promoter activities of the 5′-UTR of *rbc* gene by introducing various deletions into this region. The promoter activity was found to be located in the region close to the coding region of *rbcL*, and the 304 bp fragment of the 5' UTR containing the promoter region was used for the promoter for pAQJ4 vector. In addition to the promoter, we also introduced multiple cloning site (MCS) into pAQJ4 vector, and the expression shuttle-vector pAQ-EX1 (DDBJ Accession No. AB071392) was finally developed.

Figure 3 shows the map of the shuttle-vector pAQ-EX1. The transformation efficiency of pAQ-EX1 for *S.* PCC7002 cells was about 6 x 10^5 cfu / μg DNA [16].

The transformation of the fresh water cyanobacterium *S.* PCC7942 with pAQ-EX1 vector was also examined [16], and the *S.* PCC7942 cells were successfully transformed with this vector, although the transformation efficiency (4.0 x 10^2 cfu/ μg DNA) was much lower than that for *S.* PCC7002. Since *S.* PCC7942 cells do not have the pAQ1 plasmid, which is the origin of pAQ-EX1 vector, there is no possibility of homologous recombination between pAQ-EX1 and pAQ1 in *S.* PCC7942 cells. We can, therefore, expect a higher stability of pAQ-EX1 vector in *S.* PCC7942 cells than in *S.* PCC7002 cells, and actually the pAQ-EX1 plasmid was quite stably

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maintained in *S.* PCC7942 cells. The approximate copy numbers of the pAQ-EX1 plasmid in *S.* PCC7002 and *S.* PCC7942 estimated from the yield of plasmid are 15 to 30 copies/cell for *S.* PCC7002, and 5 to 15 copies/cell for *S.* PCC7942, depending on the growth phase of the culture. The *rbc* promoter on pAQ-EX1 vector worked well also in *E. coli* cells, thus the inserted gene on the pAQ-EX1 vector can be efficiently expressed in *E. coli*, in marine cyanobacterium *S.* PCC7002, and in fresh water cyanobacterium *S.* PCC7942.

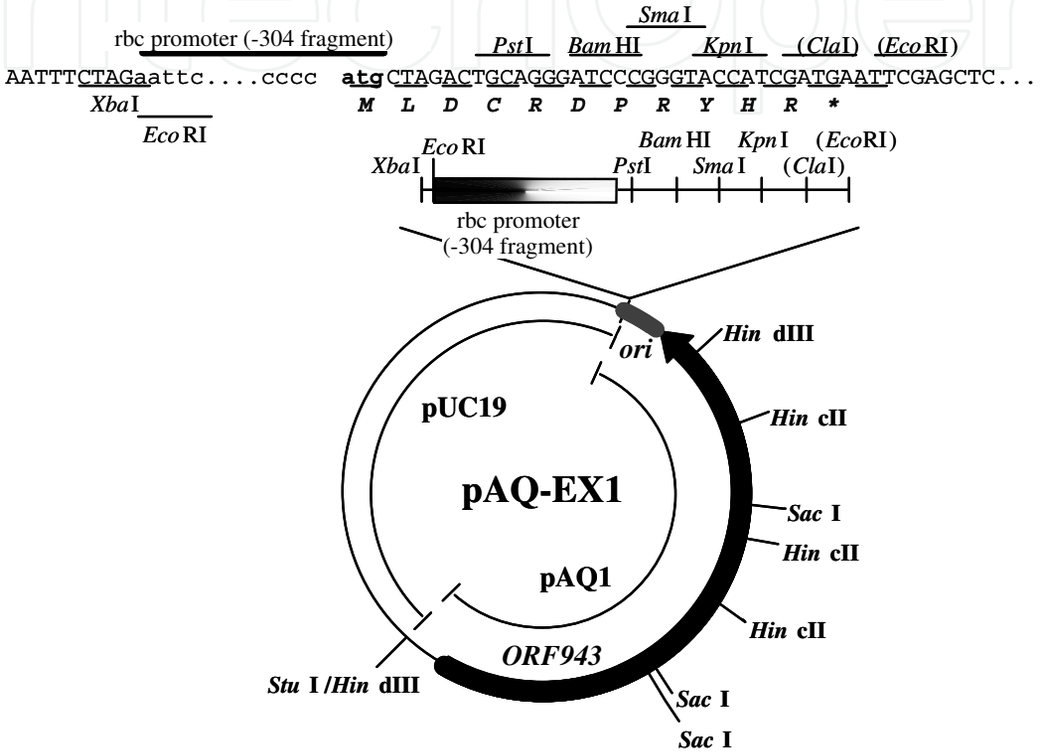

Figure 3. Map of the shuttle-vector pAQ-EX 1

2.3. CO_2 response element in *rbc* promoter

The changes in *rbc* promoter activity in response to CO_2 condition were examined because the down-regulation of *rbc* gene expression by elevated CO_2 concentration has been reported in several photosynthetic organisms. Table 1 shows the comparison of mRNA levels in *S.* PCC7002 cells cultured under various CO_2 conditions (0.03%, 1%, and 15%). The mRNA levels of *rbcL* gene were determined by RT-PCR, and compared to those of the reference gene (*ATPaseA* gene). The mRNA levels of the *rbcL* gene significantly decreased under the higher CO_2 conditions, suggesting the presence of some elements, which down regulates the transcription in response to CO_2 concentration. To examine the CO_2-regulatory element in the *rbc* promoter region, various deletions were introduced into this region, and cyanobacterial CAT assay was done by using pAQJ4-CAT vector [17]. Figure 4 shows the tested promoter fragments and the results of CAT assay. The core promoter region was shown to be located in the

-228 through -132 region, because the promoter activities were drastically decreased in the R3, R4, and R5 fragments, which lack this region. The promoter fragment, designated as R7, which contains whole -304 through -1 region, showed down regulation in promoter activity by elevated CO_2 condition (1% CO_2), while this down regulation was not observed in the R6 fragment lacking the -304 through -250 region. These results indicate that a CO_2-regulatory *cis* element exists in the -304 through -250 region, and a high expression level can be retained with R6 promoter fragment even under high CO_2 condition. The -304 through -250 region is quite A/T rich, and we also identified, by a DNA affinity precipitation assay, the 16-kDa protein which acts as a *trans*-element in CO_2 regulation [17].

Culture condition (CO_2 %)	*rbcL*	*ATPase A*	*rbcL* / *ATPase A*
	Relative mRNA level [a]		
0.03	1.0	1.0	1.0
1	0.80	1.0	0.80
15	0.57	2.2	0.26

[a]mRNA level is shown as the relative value to the mRNA level at 0.03% CO_2

Table 1. Transcript levels of *rbc*L and reference (ARPase A) genes of S. PCC7002 under various CO_2 conditions

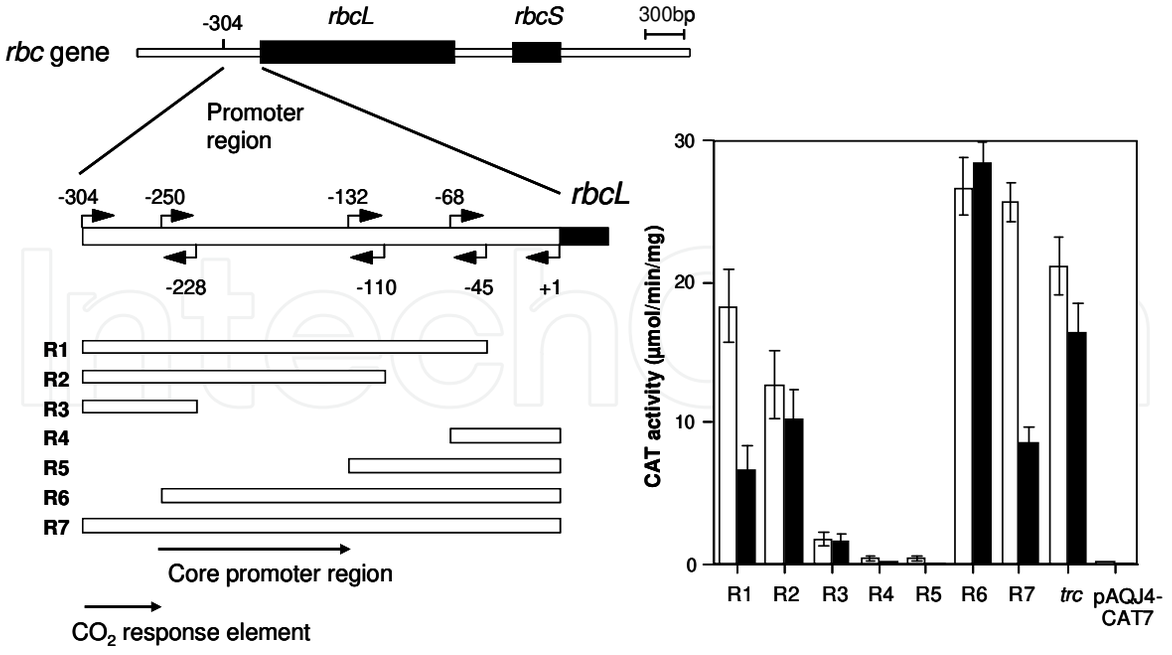

Figure 4. Promoter activities of various fragments of *rbc* promoter of *Synechococcus sp*. PCC7002

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3. *recA* complementation as a selection pressure for plasmid stability

There are two foreign gene expression systems for cyanobacteria [18, 19]; one is the plasmid vector system, as we describe in this chapter, and another one is the integration of the foreign DNA into the cyanobacterial genome through homologous recombination. The advantages of the plasmid system are i) the higher copy numbers of the foreign genes in cyanobacterial cells compared to the genome integration method, ii) the well established procedure for the modification of the genes on plasmid, such as point mutation, insertion and deletion, and iii) the wide range of expression host with a shuttle vector system. On the other hand the limitation of plasmid system is the necessity for antibiotics for the maintenance of plasmid. Especially when the genes on plasmid cause a heavy metabolic load, such as PHA production, to the host cells, the plasmids are easily excluded from the cells in the absence of antibiotics pressure. The use of antibiotics is, however, not realistic for the large scale cyanobacterial culture for CO_2 mitigation with respect to its cost. In *E. coli* cells, the *parB* (*hok*/*sok*) locus of plasmid R which mediates stabilization *via* post-segregational killing of plasmid-free cells is effective for the antibiotics-independent stable maintenance of the plasmid [20], but in cyanobacteria there has been no such a practical plasmid stabilization system reported. We developed a practical plasmid stabilization system by utilizing the *recA* complementation mechanism. RecA is a multifunctional protein that plays key roles in various cellular processes, such as recombination and DNA repair in bacteria [21, 22]. The amino acid sequences of RecA proteins from the different microorganisms are well conserved, and there are several reports on the complementation of *recA* mutation in some bacteria by *E. coli recA* gene. Murphy et al. [23] reported that a *recA* null mutation is lethal in the cyanobacterium, but the *E. coli recA* gene in trans on a plasmid can complement the function of *recA* resulting in segregation of cyanobacterial *recA* null mutant. We expected that this complementation mechanism can be used as a selection pressure to prevent the loss of the plasmid which causes a significant metabolic burden to the host cells.

Figure 5 shows the principles of the selective pressure for the maintainance of plasmid in conventional antibiotics selection (Figure 5A) and our *recA* complementation (Figure 5B) systems. In the conventional antibiotics selection system, the antibiotics-resistant (Ab-R) gene casette is introduced in plasmid, and the loss of the plasmid makes the host cells sensitive to antibiotics. In the *recA* complementation system, the *recA* gene in the genome of the host cells is inactivated by homologous recombination, and the function of genomic *recA* gene is complemented by the *recA* gene on the plasmid. In cyanobacteria, *recA* null mutation is lethal, and the host cell without plasmid can not survive. Generally, cyanobacteria have several copies of genome [24] and *recA* null mutant cells carrying a plasmid with *E. coli recA* gene were generated by three steps as follows (Figure 6); i) generation of *recA* partial mutant of *S.* PCC7002 by homologous recombination with kanamycin resistance gene (*km*) cassette, ii) introduction of shuttle-vector with *E. coli recA* gene into the *recA* partial mutant cells, and iii) conversion of the partial *recA* mutant into the *recA* null mutant by further homologous recombination. The use of *recA* complementation as a selection pressure is a simple and versatile method; only the *E. coli recA* gene on the plasmid, and the partial *recA* mutant host are required, and the *recA* null mutant can easily be obtained by subculturing the cells in the medium with kanamycin (Km). Unlike cyanobacteria, *recA* null mutation is not lethal in *E. coli*, thus this complementation system is not applicable for *E. coli*.

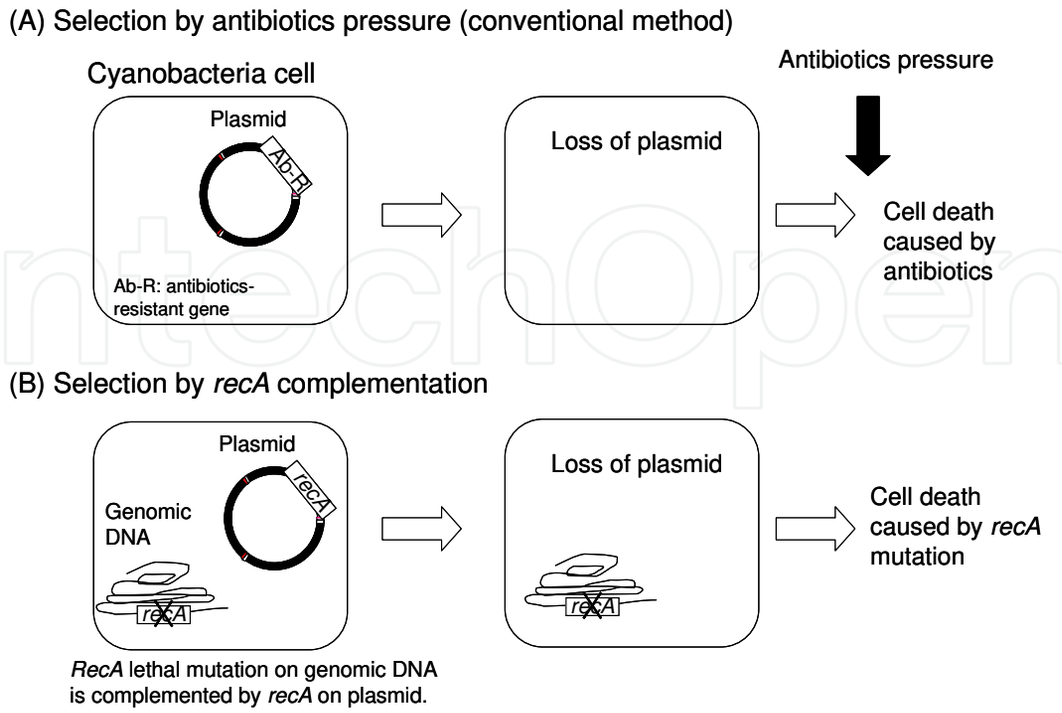

Figure 5. recA complementation as a selection pressure for plasmid stability

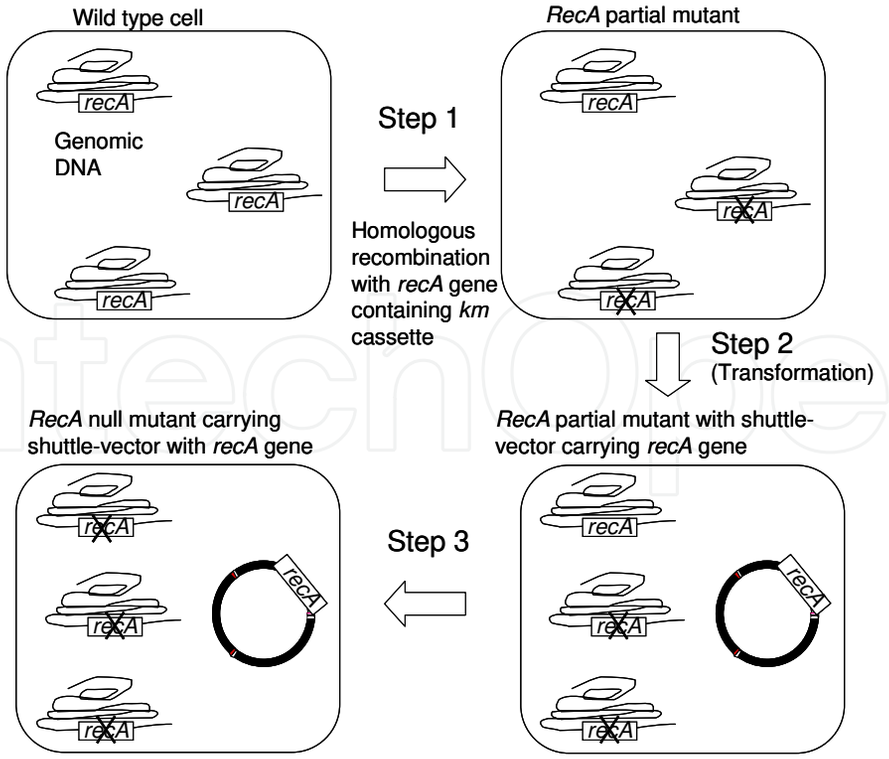

Figure 6. Procedure for generation of *rec*A null mutant cells carrying plasmids with *E. coli rec*A gene

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4. PHA production by recombinant cyanobacteria

4.1. Vector construct with *recA* complementation system for PHA production

PHAs are linear head to tail polyesters composed of 3-hydroxy fatty acid monomers (Figure 1), and there are at least 100 different 3-hydroxy alkanoic acids among the PHA constituents [25]. The first PHA discovered was poly(3-hydroxy-butyrate) (PHB). It is a highly crystalline thermoplastic sharing many properties with polypropylene, and the most abundant of the PHAs in nature. The PHB biosynthetic pathway consists of three enzymatic reactions catalyzed by three distinct enzymes (Figure 7A), 3-ketothiolase (PhaA), acetoacetyl-CoA reductase (PhaB), and PHA synthase (PhaC). These three enzymes are encoded by the genes of the *phbCAB* operon (Figure 7B). There are several well established PHA production systems using natural microorganisms such as *Wautersia eutropha*, *Methylobacterium*, and *Pseudmonas*, and also using recombinant bacteria such as *E. coli* [2, 26], and intracellular accumulation of PHA of over 90% of the cell dry weight has been reported. The use of agroindustrial by-products [27], forest biomass [28], and glycerol (by-product of bio-diesel production) [29] for the substrates of microbial PHA production has also been reported. The production of PHAs in transgenic plants carrying bacterial *phb* genes has also been investigated in *Arabidopsis thaliana*, *Gossypium hirsutum* (cotton), and *Zea mays* (corn) [30].

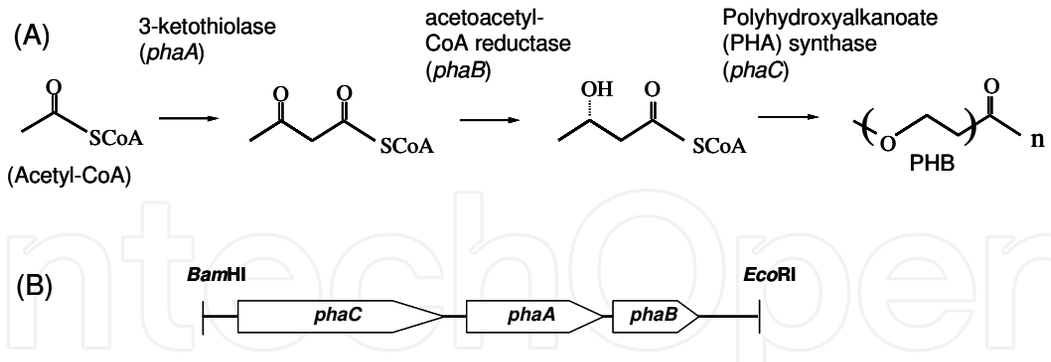

Figure 7. Biosynthetic pathway for PHA (A) and structure of pha genes (B)

There are several cyanobacterial strains which can naturally accumulate PHAs, but generally the PHA productivity in these strains are low [31, 32]. Several attempts have also been made to introduce PHA genes into non-PHA-producing cyanobacterial strains [33, 34].

We investigated the production of PHA by the recombinant cyanobacteria with the *recA* complementation antibiotics-free cyanobacterial expression system [35].

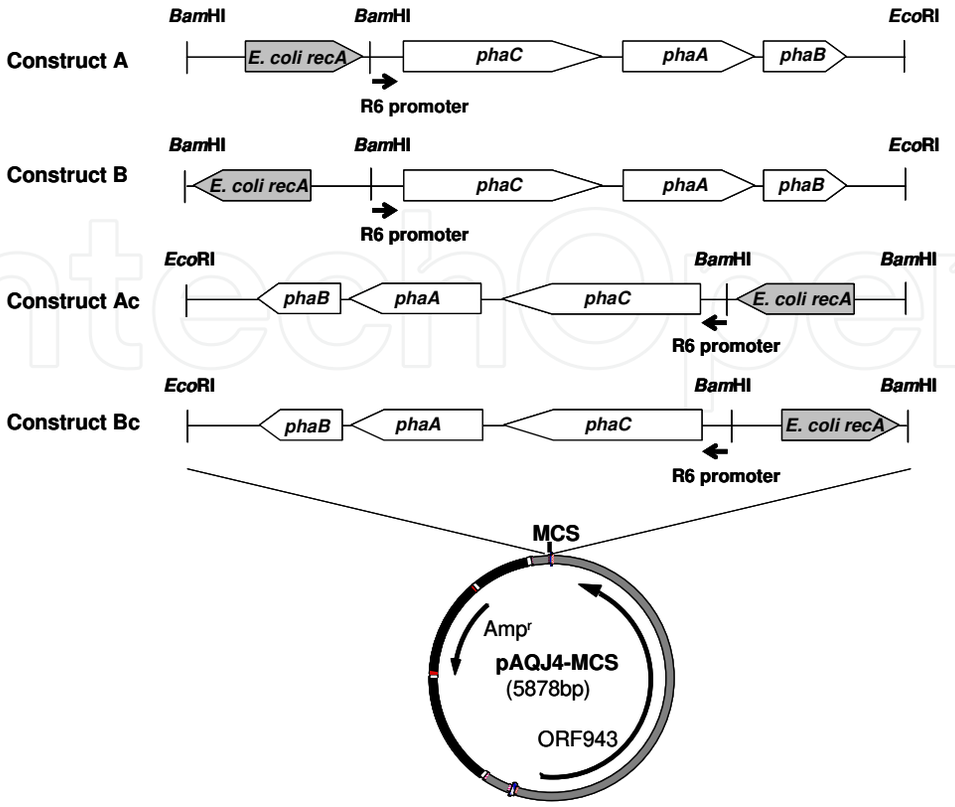

Figure 8. DNA constructs on pAQJ4 vector for PHA production

The *pha* genes and the *E. coli recA* gene were integrated on the shuttle vector pAQJ4, and the genomic *recA* genes of the transformant cyanobacteria were inactivated by an homologous recombination with the cyanobacterial *recA* gene containing a kanamycin resistance (*km*) cassette insertion. Figure 8 shows the *pha* and *E. coli recA* genes constructs on the pAQJ4 vector. For the *pha* genes, the *phaCAB* operon of *Wautersia eutropha* was used, and the *E. coli recA* gene (1.66 kb) with its upstream promoter region was connected to the *pha* genes in the same or opposite directions. For the expression of *pha* genes, the R6 promoter fragment (Figure 4), which lacks the CO_2-down-regulating element, was used. These two constructs were introduced into the pAQJ4-MCS (MCS: multiple cloning site) and pAQJ4-MCS(c) vectors (GenBank accession numbers AB480231 and AB480232, each contains the MCS in a different orientation), generating the four kinds of vector constructs A, B, A-complementary (Ac) and B-complementary (Bc) as shown in Figure 8. The partial *recA* mutant cells of *S.* PCC7002 was used for the transformation. The *recA* genes of the host cyanobacteria were partially inactivated by a homologous recombination with the *recA* gene containing a *km* cassette, and the partial *recA* mutant cells were transformed with the four kinds of *pha-recA* constructs (A, B, Ac, and Bc). The transformant cyanobacteria were obtained only for constructs B, and Ac, and these transformants were designated as Syn-pha/B and Syn-pha/Ac, respectively. The reason for the failure in the isolation of the transformants in the other constructs is not clear, but we speculate that the expression efficiency of *pha* genes might be too high in these constructs, and as a result the transformants could not gain enough energy and/or cellular metabolites for growth.

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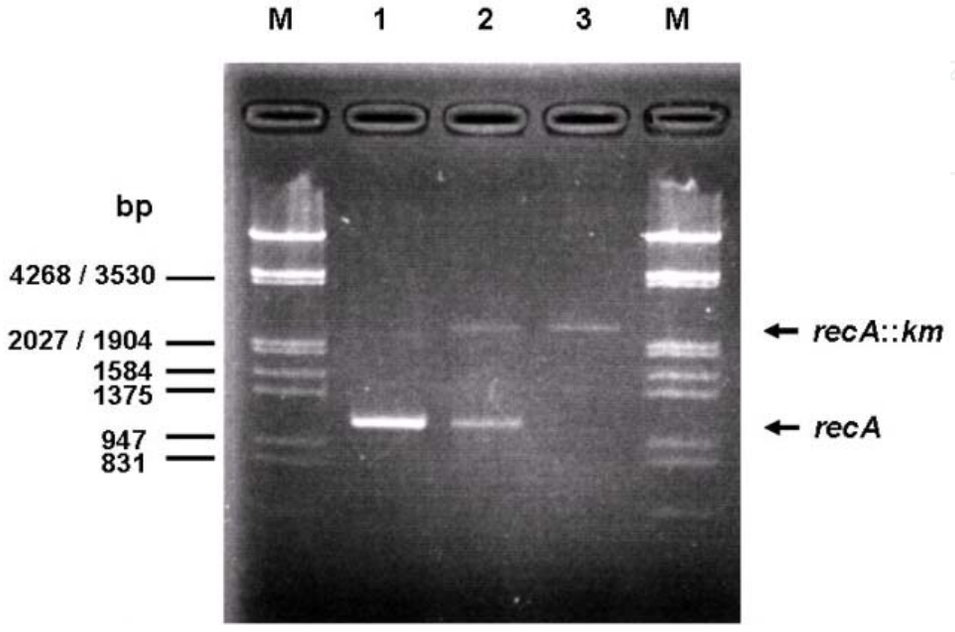

Figure 9. Integrity of the genomic *rec*A gene in transformant cyanobacteria. The genomic DNA was isolated from the wild type *S*. PCC7002, Syn-pha/B transformant, and Syn-pha/Ac transformant, and the *rec*A gene and *rec*A with a *km* cassette (*recA::km*) where amplified by PCR. The PCR products were analyzed with 1% agarose gel electrophoresis. M: Molecular weight marker, lane 1: wild type *S*. PCC7002, lane 2: transformant Syn-pha/B (*rec*A partial mutant), and lane 3: transformant Syn-pha/ac (*rec*A null mutant)

To obtain the *recA* null mutant of Syn-pha/B and Syn-pha/Ac transformants, the transformant cells were subcultured in the liquid medium with carbenicillin (4 μg / ml) and Km (200 μg / ml). Each liquid culture was allowed to grow into the late stationary phase prior to subculturing to enhance the efficiency of homologous recombination. After five times subculturing, the integrity of the genomic *recA* gene was examined with PCR (Figure 9). In the Syn-pha/Ac transformant cells, only the DNA fragment corresponding to the *recA* with *km* cassette was amplified, and no DNA fragment of wild type *recA* gene was detected on the agarose gel, indicating that the Syn-pha/Ac transformant was changed to a *recA* null mutant. On the other hand, in the Syn-pha/B transformant cells, both *recA* with *km* cassette and wild type *recA* fragments were amplified, thus the *recA* gene in cyanobacterial genome was not completely inactivated. The reason for the failure of *recA* null mutant segregation in the Syn-pha/B transformant is not clear, but a possible explanation is the insufficient complementation of RecA protein by the *E. coli recA* gene on the plasmid. The Syn-pha/Ac transformant was used for the following experiments for the *pha* gene stability and PHA production.

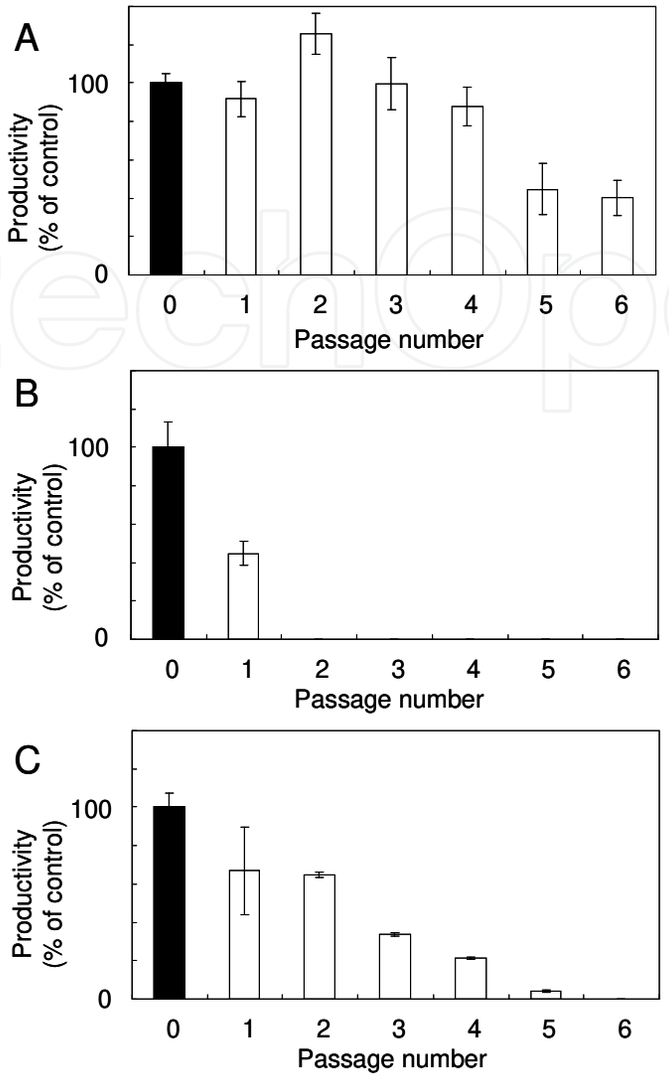

Figure 10. The stability of PHA productivity in the *recA* null mutant transformant (A), wild type (non-*recA*-mutant) transformant (B), and *recA* partial mutant transformant (C). All the transformants (*recA* null mutant, wild type, and *recA* partial mutant) carry the construct Ac plasmid of figure 8. The cells were subcultured in the antibiotics free medium for five times at one week intervals, and the PHA contents in the cells were determinated at the end of each culture. The PHA productivities were expressed as the percentage to the PHA content in the cells cultured with antibiotics (passage number 0, shown as black bars).

The stability of PHA productivity in the *recA* null mutant of Syn-pha/Ac transformant was examined in comparison to the wild type (non- *recA* -mutant) transformant cells carrying the construct Ac plasmid of Figure 8, and *recA* partial mutant of Syn-pha/Ac transformant. The cells were subcultured in the antibiotics free medium for five times at one week intervals, and the PHA contents in the cells were determined at the end of each culture. The cell densities at the end of cultures were 7.5×10^8 to 1×10^9 /ml, and the passage of culture was done by diluting the culture into a fresh medium at a dilution ratio of 1:1,000. Figure 10 shows the changes in the PHA productivities in the transformant cells of the *recA* null mutant (Figure 10A), wild type (non- *recA* -mutant) (Figure 10B), and *recA* partial mutant (Figure 10C). The PHA

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productivities were expressed as the percentage to the PHA content in the cells cultured with antibiotics (passage number 0, shown as black bars in Figure 10). The PHA productivities in the *recA* null mutant (Figure 10A) were kept at the approximately same level with that of passage number 0 at the passage numbers 1 through 4 in the antibiotics free medium, but suddenly decreased to 45% of the passage number 0 at the passage number 5. On the other hand, the PHA productivities in the wild type (non- *recA* -mutant) transformant significantly decreased at the passage number 1 (45% of the passage number 0), and no PHA production was detected at the end of passage number 2 (Figure 10B). Interestingly a partial positive effect for the PHA productivity was observed in the *recA* partial mutant (Figure 10C); the PHA productivity decreased gradually during the consecutive culture passages to a trace level of PHA production at the passage number 6. These results indicate that the *recA* complementation effectively acted as a selection pressure in the *recA* null mutant for the maintenance of the plasmid carrying the *pha* genes, at least for 3 to 4 passages at a dilution rate of 1:1,000. The cell number (and also culture scale) can be increased 10^9 times with three culture passages at a dilution rate of 1:1,000, and therefore this antibiotics-free PHA production system is applicable to the large scale PHA production. The reason for the sudden decrease in PHA productivity in the *recA* null mutant at the passage 5 is not clear, but this might not be caused by the loss of plasmid in the cyanobacterial cells because the cells of passage numbers 4 (high PHA productivity) and 5 (low productivity) did not show any difference in colony forming ability on the antibiotics (carbenicillin) plates. The decrease in the PHA productivity at the passage numbers 4 and 5 might, therefore, be attributed to the other reasons, such as the mutation in *pha* genes and/or the inhibition of the expression of *pha* genes on the plasmid.

4.2. PHA production by transformant cyanobacteria cells

Figure 11 shows the electron micrograph of the control wild type *S.* PCC7002 (A), and the PHA accumulating *recA* null mutant transformant (Syn-pha/Ac) (B) cells. The small PHA granules aligning along the thilacoid membrane were observed in the Syn-phaAc transformant cell. The molecular mass distribution of the PHA was estimated with the gel permeation chromatography (GPC). The molecular mass distribution of the PHA from the Syn-pha/Ac transformant was a little shifted to the higher side compared to that of the standard PHA from *W. eutropha* (Figure 12), but in the range previously reported for various microbial PHAs. The main component of the hydroxyalkanoic acid of the PHA from the Syn-phaAc transformant was hydroxybutyric acid (more than 98%), and a small amount of lactic acid (0.5 to 1.5 %), and a trace amount of hydroxyvaleric acid were also contained.

To obtain a higher PHA accumulation in the cyanobacterial cells, the nutrient condition of the culture was also examined. Since it is reported that the nitrogen and phosphorus supplies, much affect the PHA accumulation in microorganisms [36, 37], the Syn-phaAc transformant cells were cultured in the medium containing various concentrations of nitrogen ($NaNO_3$) and phosphorus (KH_2PO_4) sources, and the cell growth and PHA accumulation were compared (Figure 13). There was a clear negative relationship between cell growth and PHA accumulation, and nitrogen limitation seemed to be effective for the accumulation of PHA although the cell growth was significantly suppressed in the nitrogen limited medium. The maximum PHA

accumulation was 52% of cell dry weight, the highest among the ever reported PHA accumulation in cyanobacteria. Accordingly the two-staged culture system consisting of cell growth and PHA production phases should be applied to increase the total productivity (g per liter culture) of PHA. Asada et al. reported that acetyl-CoA flux is the limiting factor in PHA production by genetically engineered cyanobacterium [32], and the high PHA productivity in Syn-phaAc transformant cells suggests the abundant intracellular supply of acetyl-CoA in *S.* PCC7002.

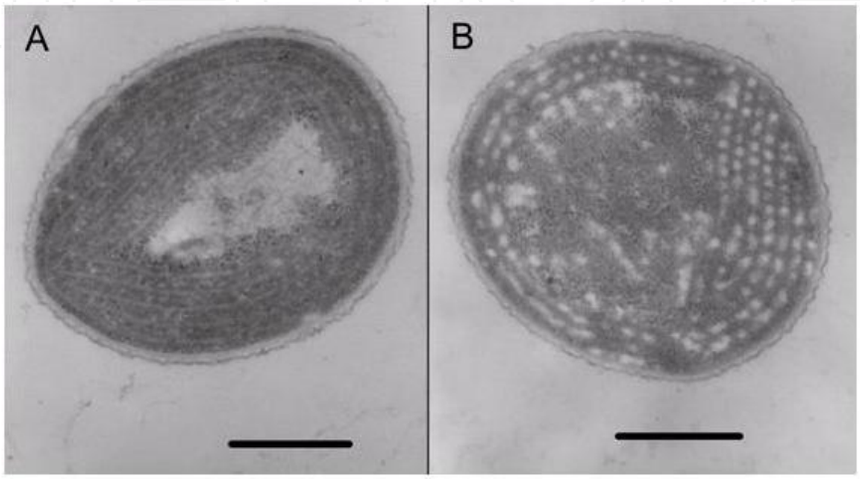

Figure 11. Electron micrograph of wild type *S.* PCC7002 (A) and PHA accumulating Syn-pha/Ac transformant cells (B) in the early exponential growth phase (OD550=2). The PHA content in the Syn-pha/Ac transformant cell is approximetly 10% of the dry weight. Scale bars represent 0.5 μm.

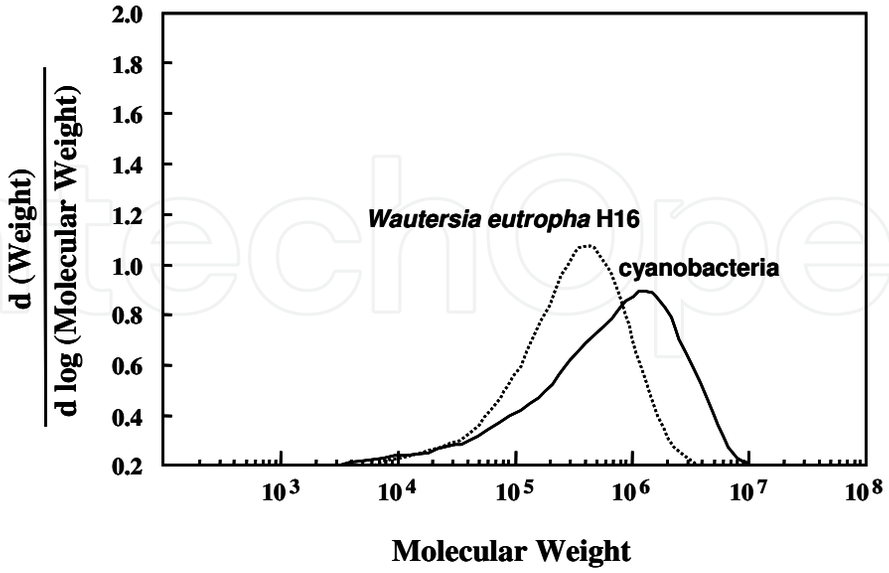

Figure 12. Molecular distribution of the PHA from *recA* null mutant Syn-pha/Ac transformant cells (solid line), and *Wautersia eutropha* H16 (broken line). Molecular weight of PHA samples was determined by gel permeation chromatography (GPC)

http://dx.doi.org/10.5772/54705

Our study is the first practical approach for the antibiotics-free maintenance of plasmid in cyanobacteria, and with this system the fixation and direct conversion of CO_2 into the useful bioplastics can be realized under low maintenance and low cost conditions.

The future research subjects to realize the on-site CO2 fixation and utilization system with recombinant cyanobacteria are the followings.

1. Although the promoter derived from the *rbc* gene was found to be quite effective both in cyanobacterial and bacterial cells, and the PHA production by the transformed cyanobacterial cells was also quite successful with this promoter, the use of a switchable promoter (ON/OFF type promoter) might further enhance the PHA production.

2. Method and system for the efficient harvesting of cyanobacteria at low energy and low cost should be developed.

3. Photosynthetic CO_2 assimilation only occurs during the day, and the productivity of biomaterials is much influenced by light condition. The use of cyanobacteria capable of growing photoautotrophic and also heterotrophic (with waste water) is one possible solution to this limitation.

4. Generally biomaterials, such as fuel and plastic, produced from CO_2 are low price. Simultaneous prodcuction of higher value products, such as fine chemicals, can lower the cost for the production of biomaterials from CO_2.

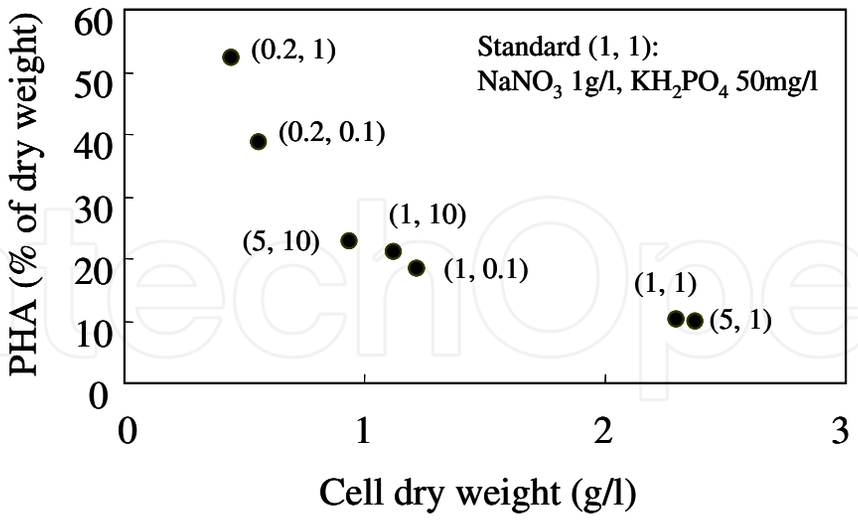

Figure 13. Effect of nitrogen and phosphorus concentrations in medium on cell growth and PHA accumulation in Transformant cyanovacteria. The cells (initial cell density; $5x10^6$ cells/ml) were cultured in the 50 ml medium containing various concentration of $NaNO_3$ and KH_2PO_4. The standard concentration of $NaNO_3$ and KH_2PO_4 are 1g/l and 50 mg/l, respectively. The standard concentrations of $NaNO_3$ and $KH2PO_4$ are shown as (1,1), and the ratio of each nutrient to the standard concentration is shown in the parenthesis.

5. Conclusion

The fixation and direct conversion of CO_2 into the useful biomaterials by the transgenic cyanobacteria are two processes of a promising technology for the coming low carbon economy. We have developed an efficient shuttle-vector between the marine cyanobacterium *Synechococcus sp.* PCC7002 and *E. coli*, and also a practical antibiotics-free cyanobacterial plasmid expression system by using the complementation of the cyanobacterial recA null mutation with the E. coli recA gene on the plasmid. Although considerable researches are still required to realize the practical on-site applications of the present system to the industrial emission sites, such as thermal power plants, this technology can be a promising option for the biological conversion of CO_2 into useful industrial materials.

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