

Background

The pathway for the synthesis of PHB begins with the condensation of two molecules of acetyl-CoA to acetoacetyl-CoA by β -ketothiolase, which is encoded by the *phaA* gene. This product is then reduced to $D(-)$ -3-hydroxybutyryl-CoA by acetoacetyl-CoA reductase, a product of the *phaB* gene. PHA synthase, encoded by *phaC*, catalyzes the polymerization of PHB monomers through the use of two thiolate groups [1].

The catalytic activity of PHA synthase has been studied in detail and residues of interest have been identified and found to be highly conserved across PHA-producing microorganisms. These sites are: Ser-260, Cys-319, Gly-322, Asp-351, Trp-425, Asp-480, Gly-507, and His-508. Cys-319 and Gly-322 are part of the G-x-C-x-G-G motif that is required for the catalytic activity of the enzyme. When the highly conserved Cys-319 residue is mutated, PHA synthase activity is lost, suggesting that it is one of the thiolate groups.

In the proposed model for PHA synthase function, the enzyme forms a dimer with the first thiol group (Cys-319) on one subunit acting as the loading site, and the same thiol group on the other subunit serving as the elongation site. The first thiol group covalently binds to $D(-)$ -3-hydroxybutyryl-CoA, resulting in the release of coenzyme A. Similarly, the corresponding thiol group on the other subunit covalently binds to another molecule of $D(-)$ -3-hydroxybutyryl-CoA and cleaves the coenzyme A on that molecule. The subsequent $D(-)$ -3-hydroxybutyryl attached to the second thiol group is then subjected to nucleophilic attack. This activates the $D(-)$ -3-hydroxybutyryl and results in a transesterification reaction that attaches the $D(-)$ -3-hydroxybutyryl on the first thiol group to the end of the monomer bound to the second thiol group [2]. The elongation

process occurs several thousands of times to create polyesters of high molecular weights [1].

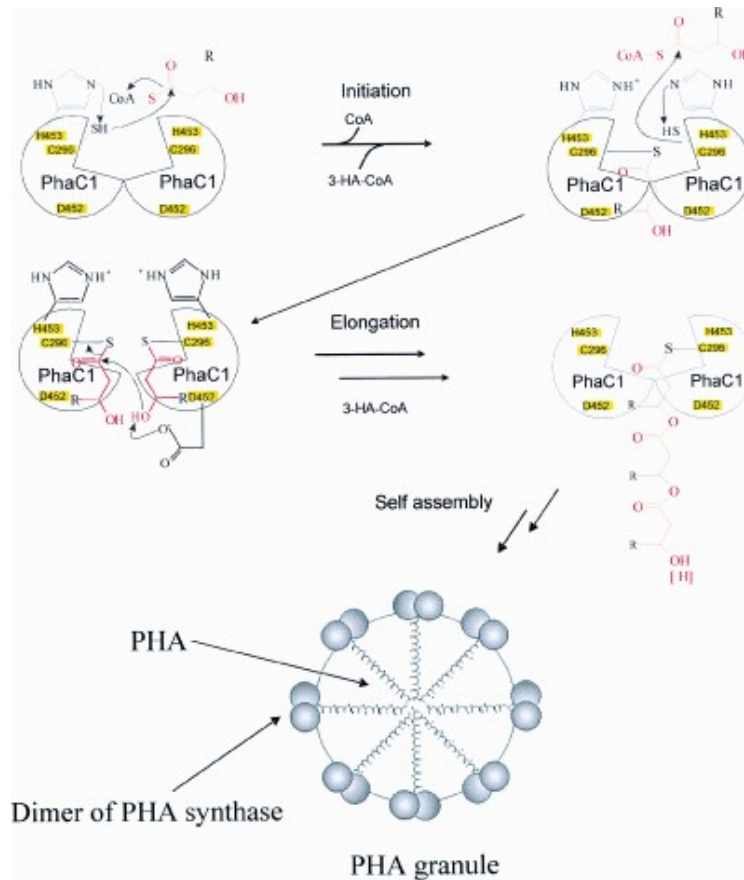


Figure 1: PHA synthase activity in *P. aeruginosa* class II PHA synthase [2]. Although this synthase is different from the class I synthase that is found in *C. necator*, the catalytic pathway for both synthases are comparable.

Although the above pathway results in the synthesis of a polyester of 3HB monomers, the formulation of a copolymer containing other monomers is largely accomplished through the use of precursor substrates as carbon sources. When *C. necator* was cultivated on 4-hydroxybutyric acid, a copolyester of 4HB and 3HB constituents were formed [3]. The 3HB monomers were also incorporated in the chain because of the catabolism of 4-hydroxybutyric acid to form intermediates that can be converted to 3-hydroxybutyryl-CoA. Moreover, Hein et al. formed a homopolymer of poly(4-hydroxybutyric acid) (poly(4HB)) through splicing together the *phaC* gene from

C. necator and the *cat2* succinic degradation gene from *C. kluyveri*. Insertion of this plasmid into *E. coli* cultivated in Luria-Bertani broth with glucose and 4-hydroxybutyric acid resulted in the formation of the poly(4HB) homopolyester. However, when cultivated in the absence of glucose, a copolyester of 3HB and 4HB monomers were synthesized instead. When grown in M9 mineral salts with glucose and 4-hydroxybutyric acid as carbon sources, poly(4HB) granules accounted for up to 80% of the cell's dry weight.

In addition, Valentin et al. [4] created a recombinant *E. coli* strain that produces the poly(3HB-co-4HB) when grown in medium containing glucose. The *E. coli* were transformed with two plasmids, one containing *phaCAB* and the other containing *cat2*, which consists of the *sucD*, *4hbD*, and *orfZ* genes. In their proposed pathway for copolyester formation, the succinic degradation genes are expressed in parallel with the PHA operon. *sucD* encodes succinic semialdehyde dehydrogenase which converts succinyl-CoA, an intermediate in the citric acid cycle, into succinic semialdehyde. 4-hydroxybutyrate dehydrogenase (encoded by *4hbD*) subsequently converts this product into 4-hydroxybutyrate. *orfZ* provides the final enzyme, 4-hydroxybutyrate-CoA:CoA transferase, which attaches coenzyme A to 4-hydroxybutyrate. This modification enables PHA synthase to bind to the molecule and catalyze the polymerization reaction of poly(3HB-co-4HB).

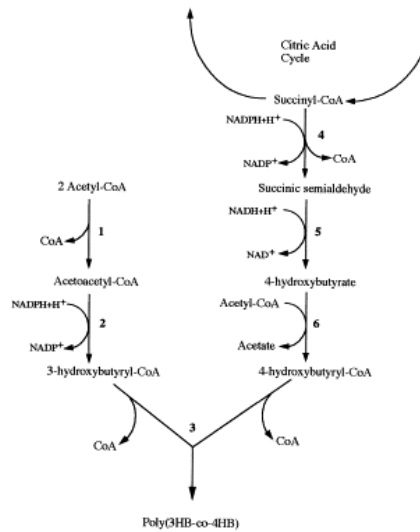


Figure 2: Proposed pathway for Poly(3HB-co-4HB) Production [4]

Transformation of *E. coli* with the plasmids containing *phaCAB* and *cat2* produced Poly(3HB-co-4HB) that was 50% of the cell dry weight and contained up to 2.8 mol% 4HB.

In a separate study by Hein et. al. [5], a recombinant strain of *E. coli* containing the PHA synthase gene, *phaC*, and *orfZ* (*cat2*) was used to produce P(4HB). These two genes were inserted onto the same plasmid and transformed into XL1-Blue *E. coli* cells. When grown in LB broth in the presence of glucose and 4-hydroxybutyric acid, P(4HB) was accumulated. When glucose was absent, P(4HB) was initially accumulated but after about a day, increasing amounts of 3HB was added to the polymer to approximately 70%, resulting in the accumulation of poly(3HB-co-4HB). This suggests that glucose limits the incorporation of 3HB into the polyester.

Methods (Detailed reactions for Wiki?)

The objective of this project was to engineer several vectors to enable the study of the metabolic pathway for the production of Poly(3HB-co-4HB). The first step of the project was to transfer the genomic phaCAB DNA into a plasmid. PCR TOPO Blunt-II and the pUC19 vector were used as cassettes to hold the phaCAB gene. These vectors were then used to design additional plasmids containing some combination of the phaCAB operon and the cat2 gene. The pASK-IBAC plasmid is used as the cassette containing most of the inserts because it contains a tetracycline promoter, which will suppress the expression of the insert unless tetracycline is added to the bacterial media. In addition, pASK-IBAC contains a strep tag that is useful in quantifying the expression of the genes contained in the insert. Vectors with and without this tag were designed. Ultimately, the design of these vectors will enable further research into methods for increasing the composition of 4HB monomers in Poly(3HB-co-4HB).

INSERT Method from Vinny: *3HB Genetic Pathway*

Creation of vectors

The cat2 gene had previously been inserted into a PSOS vector to form the PSOS-cat2 plasmid and the phaCAB plasmid (specifically the phaCAB in PCR Blunt II Topo #15) was obtained using the method described above. These two plasmids provided the template for the inserts used to create the following vectors. At first USER ligation was used to try to construct these vectors. However, after this approach failed to yield results, restriction enzyme digestion was used instead. This method requires PCR amplification of the insert, restriction digestion of the vector and insert, and then ligation of the fragments. (See appendix for design of the primers used in PCR amplification.)

Afterwards, these vectors will be used to modify the pathway for the synthesis of Poly(3HB-co-4HB).

Plasmid	Vector Name	Vector	PCR Reaction	Template for Insert	Restriction enzymes
1	pASKphaCAB-noTag	pASK	1	phaCAB in PCR Blunt II Topo # 15	XbaIFw, BamHI
2	pASKphaCAB-tag	pASK	2	phaCAB in PCR Blunt II Topo # 15	EcoRI, BamHI
3	pASKphaC-tag	pASK	3	phaCAB in PCR Blunt II Topo # 15	EcoRI, BamHI
4	pSOSCat2-phaC	pSOS	4	phaCAB in PCR Blunt II Topo # 15	BamHI, EcoRI
5	pASKphaAB-pLZCat2phaC	pASK	5	phaCAB in PCR Blunt II Topo # 15	XbaI, EcoRI,
			6	pSOSCat2-phaC	EcoRI, XhoI
6	pASKphaCAB-pLZCat2	pASK	7	phaCAB in PCR Blunt II Topo # 15	XbaI, EcoRI,
			8	pSOSCat2-phaC #9	EcoRI, BamHI

For PCR Reactions 3, 4:

Water	27.5
5x Phusion Buffer	10
2.5 mM dNTP (10 mM total)	4
DNA Template	3
Primer 1	2.5
Primer 2	2.5
Phusion Enzyme	.5
	50

Cycle	Step	Temperature	Time
1	x1	98°C	2 min
2	x30	98°C	30 s
		63°C	30 s
		72°C	1 min
3	x1	72°C	10 min
		4°C	∞

For PCR Reactions 1, 2, 5, 6, 7, 8:

H ₂ O	28.5
L Primer	2.5
R Primer	2.5
Template	2
5x Buffer	10
DNTP	4
Phusion	.5
	50 ul

Conditions for Reactions 1, 2, 5, and 6:

98°C	30s	x1
98°C	10s	x30
59°C	30s	
72°C	2 m	
72°C	10 m	x1
4°C	∞	

Conditions for Reaction 7:

98°C	30s	x1
98°C	10s	x30
66°C	30s	
72°C	2 m	
72°C	10 m	x1
4°C	∞	

Conditions for Reaction 8:

98°C	30s	x1
98°C	10s	x5
58°C	30s	
72°C	30s	
98°C	10s	x25
66°C	30s	
72°C	30s	
72°C	10 m	x1
4°C	∞	

Restriction enzyme digestion and ligation

For each plasmid, the vector and the insert were digested with restriction enzymes to form complementary sticky ends (see Table 2). Each restriction digestion consisted of 60-70 ng of DNA. Plasmids 1 and 2 required a two-segment ligation while plasmids 5 and 6 required a three segment ligation. The reactions were incubated at 37°C for 2 hrs and then heat shocked at 80°C for 5 min to degrade the enzyme. PCR purification was performed following digestion.

Plasmid 1:

	Insert (70)	PASK vector (60 ng)
H ₂ O	15	15
10x buffer	2	2
DNA	1	1
XbaI	1	1
BamHI	1	1
	20	20

Plasmid 2:

	Insert (70)	PASK vector (60 ng)
H ₂ O	15	15
10x buffer	2	2
DNA	1	1
EcoRI	1	1
BamHI	1	1
	20	20

Plasmid 3:

Plasmid 4:

	Insert (70)	PASK vector (60 ng)
H ₂ O	13	14
10x buffer	2	2
DNA	3	2
EcoRI	1	1
BamHI	1	1
	20	20

	Insert (70)	PASK vector (60 ng)
H ₂ O	13	15
10x buffer	2	2
DNA	3	1
EcoRI	1	1
BamHI	1	1
	20	20

Plasmid 5:

	Insert 5 (60)		Insert 6 (60)		PASK vector (60 ng)
H ₂ O	13	H ₂ O	15	H ₂ O	15
10x buffer	2	10x buffer	2	10x buffer	2
DNA	3	DNA	1	DNA	1
XbaI	1	EcoRI	1	XbaI	1
EcoRI	1	XhoI	1	XhoI	1
	20		20		20

Plasmid 6:

	Insert 7 (60)		Insert 8 (65)		PASK vector (60 ng)
H ₂ O	13	H ₂ O	11	H ₂ O	15
10x buffer	2	10x buffer	2	10x buffer	2
DNA	3	DNA	5	DNA	1
XbaI	1	EcoRI	1	XbaI	1
EcoRI	1	XhoI	1	XhoI	1
	20		20		20

Ligation was performed immediately following digestion using approximately twice as much volume of insert as vector for 10 ul total volume. For plasmids 5 and 6, which require three-segment ligation, a larger volume of the longer insert was used. No ligase and No Insert controls were used to verify the validity of the ligation reaction. All reactions were incubated at room temperature for one hour.

Ligation reaction for Plasmids 1-4:

Ligation reaction for Plasmids 5 and 6

	No ligase	No Insert	Exp
Insert	5.5	X	5.5
Vector	3	3	3
Ligase	X	.5	.5
Buffer	1	1	1
H ₂ O	.5	5.5	X
	10	10	10

	No ligase	No Insert	Exp
Insert	3	X	3
Insert	3.5		3.5
Vector	2	2	2
Ligase	X	.5	.5
Buffer	1	1	1
H ₂ O	.5	6.5	X
	10	10	10

Following ligation, plasmids were transformed into 50 ul of Library Efficiency DH5 α Competent Cells and plated on 2% agar plates containing the appropriate antibiotic and .5 ug/ml Nile Red.

Assembly PCR

Although clones containing pASKphaCAB-noTag and pASKphaCAB-tag were confirmed by restriction digestion to obtain the vector and the insert, plasmids pASKphaAB-pLZCat2phaC (plasmid 5) and pASKphaCAB-pLZCat2 (plasmid 6) proved much more difficult to construct. pASKphaCAB-pLZCat2 (plasmid 6) was deemed unnecessary because it contained essentially the same elements as pASKphaAB-pLZCat2phaC. Thus, attention was focused on the latter. Various methods were attempted to optimize the restriction digestion and ligation process. Prior to ligation, fragments were mixed in equimolar quantities to maximize efficiency. This method did not yield satisfactory results. Another method included separating the double digestion into two separate reactions. The first restriction digestion was incubated for 1.8 hrs

followed by heat shock at 80°C for 5 min and PCR purification. Then, the second restriction digestion was performed. Unfortunately, this method did not yield positive clones either.

A four fragment assembly PCR was then attempted: pASK vector, phaAB, terminator sequence, Cat2phaC. The terminator sequence halts transcription of the 8.15 kb insert after phaAB has been transcribed. This improves transcription efficiency of Cat2phaC. The plasmid was renamed pASKphaAB-Ter-pLZCat2phaC due to the addition of the terminator sequence.

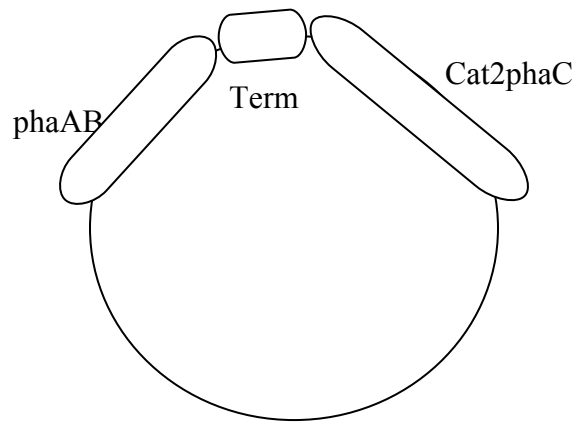


Figure 1. Assembly PCR of pASKphaAB-Ter-pLZCat2phaC with the terminator sequence included. The figure is not representative of the sizes of the fragments.

The primers were designed with overlapping sequences (see Appendix A). The optimized assembly PCR reaction was as follows:

	Term	pASK, AB, Cat2phaC
H ₂ O	26.5	28.5
L Primer	2.5	2.5
R Primer	2.5	2.5
Template	4	2
5x Buffer	10	10
dNTP	4	4
Phusion	.5	.5
		50 ul

Terminator			pASK, AB			Cat2phaC		
98°C	30s	x1	98°C	30s	x1	98°C	30s	x1
98°C	10s	x30	98°C	10s	x30	98°C	10s	x30
63°C	30s		66°C	30s		65°C	30s	
72°C	6 s		72°C	1m 45 s		72°C	2 m	
72°C	10 m	x1	72°C	10 m	x1	72°C	10 m	x1
4°C	∞		4°C	∞		4°C	∞	

PCR product was verified through gel electrophoresis on 1% agarose gels for all of the fragments except for the terminator sequence, which required a 2% gel.

Screening

Nile Red fluoresces at 598 nm when bound to PHA granules, and when viewed under UV light, can provide a visual indicator of the presence of PHA. The 4HB plastic-producing genes on pASKphaAB-pLZCat2phaC are controlled by the lacZ promoter and should thus produce 4HB in the absence of anhydrotetracycline. Plastic-producing colonies containing this plasmid and grown on Nile Red plates should fluoresce when exposed to UV light. pASKphaCAB-pLZCat2, pASKphaCAB-noTag and pASKphaCAB-tag require addition of anhydrotetracycline to express the plastic-producing genes. To determine the optimal level of anhydrotetracycline required to induce bioplastic production, colonies transformed with pASKphaCAB-noTag and pASKphaCAB-tag were plated on chloramphenicol, Nile Red plates with increasing concentrations of anhydrotetracycline: 0 ng/ml, 45 ng/ml, 100 ng/ml, and 200 ng/ml. The 100 ng/ml concentration of anhydrotetracycline appeared to be most effective, yielding two red colonies out of 32 total.

INSERT METHOD FROM VINNY: Polymer harvesting and NMR spectroscopy (last section)

Results

PCR Reactions for plasmids 1-6

The optimized PCR reactions yielded strong, single bands for most of the reactions except for reaction 7. The products were subjected to PCR purification before further use in restriction digestion.

Restriction Digestion to verify presence of insert

Eight colonies for both pASKphaCAB-noTag (plasmid 1) and pASKphaCAB-tag (plasmid 2) were randomly screened for the insert. A double digestion that cuts out the insert was performed and clones containing the insert should show two bands—one for the insert and one for the vector. For pASKphaCAB-noTag, the insert should be 3.87 kb and the vector 2.92 kb. For pASKphaCAB-tag, the insert is 3.85 kb and the vector is 3.0 kb.

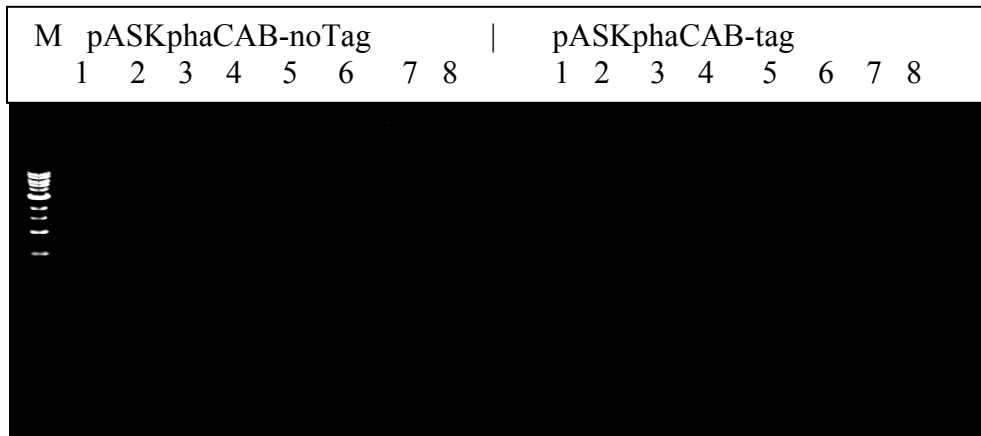


Figure 1. Gel picture of restriction digestion of random clones containing plasmid 1 and plasmid 2.

The gel illustrates that colonies 1 and 3 for pASKphaCAB-noTag and colonies 1, 4, and 8 for pASKphaCAB-tag contain the insert.

Plasmids 3 and 4 were also subsequently extracted and digested with BamH1 and EcoRI to confirm the presence of the insert. Inserts 3 and 4 should both be 1.77 kb in length. For insert 3, there should be two bands: one at 1.77 kb and another at 3.0 kb. The band at 3.0 kb is the pASK vector. Similarly for insert 4, there should also two bands: one at 1.77 kb for the insert and one at 3.99 kb for the pSOS vector.

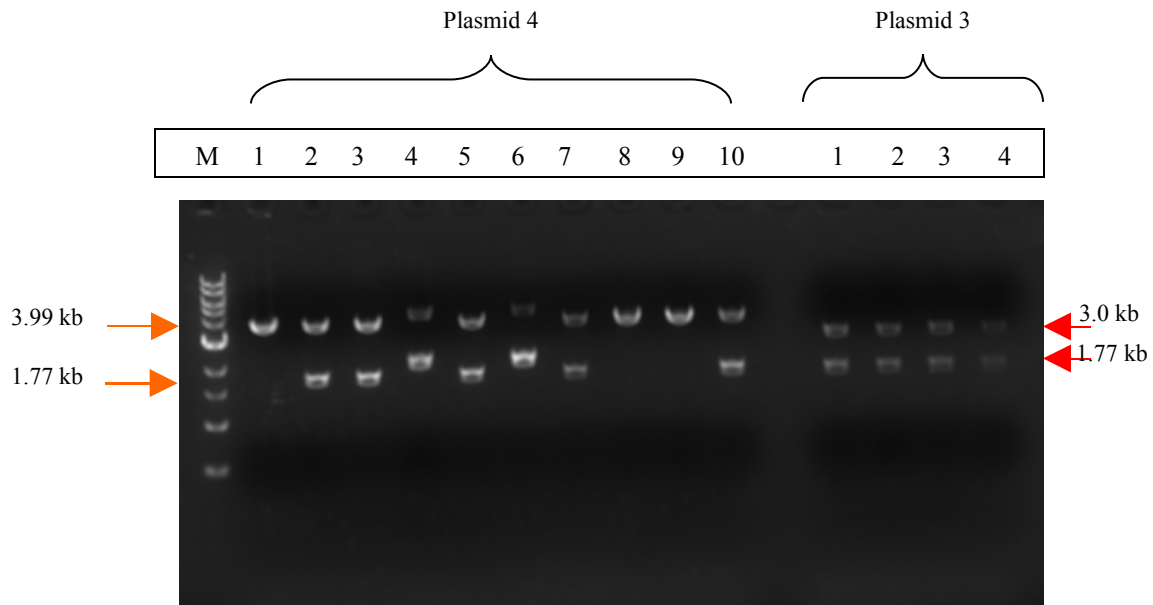


Figure 2. Gel picture of restriction digestion of random clones containing plasmid 4 and plasmid 3.

The gel picture demonstrates that for plasmid 4, colonies 2, 3, 5, 7, and 10 contain the insert. For plasmid 3, all four of the colonies contain the insert. These colonies display the required bands and suggest that the restriction enzyme digestion and ligation were successful.

Clones for plasmid 5, pASKphaAB-Cat2phaC, were screened for the insert using NdeI and NotI restriction digestion, which should cut the plasmid to form three fragments of 3.0 kb, 1.7 kb, and 3.6 kb. However, none of the selected clones were found to contain the insert.

Assembly PCR- Construction of pASKphaAB-Cat2phaC (plasmid 5)

Using new overlapping primers, the phaAB, cat2phaC, pASK, and terminator fragments were obtained through PCR.

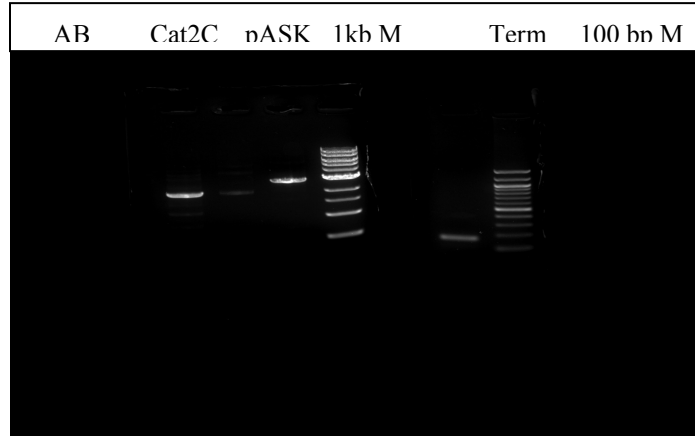


Figure 3. Gel picture of fragments used for assembly PCR.

The gel picture demonstrates that fragments phaAB, pASK, and the terminator were obtained. There does appear to be nonspecific binding for fragment phaAB and gel purification was used to extract the desired band. The PCR reaction for Cat2phaC was unsuccessful and a different template (pSOscat2#9-col5) was used instead.

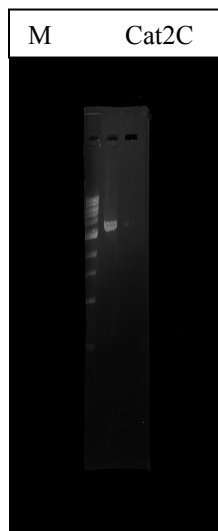


Figure 4. Gel picture of PCR reaction for Cat2phaC using template pSOscat2#9-col5.

The single band for cat2phaC is distinct and suggests that the product was obtained.

However, the attempt to assemble the disparate fragments through assembly PCR failed to yield any colonies with the insert present.

Sequencing Results

Clones for plasmids 1-5 were sent for sequencing. pASKphaCAB-noTag, pASKphaCAB-tag, and pASKphaC were verified to contain the insert. The clone for pSOSCat-phaC exhibited a frameshift mutation between cat2 and the lacZ promoter. This suggests that the original template used to create the plasmid, pSOScat2#9 also contained this mutation. The final clone sent for sequencing, pASKphaAB-pLZCat2phaC did not contain the insert.

Screening

The following is a picture of *E. coli* bacteria transformed with the PCR-Blunt II-phaCAB plasmid and grown on kanamycin plates containing Nile Red. When viewed under UV light, the reddish tint present on some colonies indicates the presence of PHA granules in the bacteria. This shows that the transformation was successful and that the genomic phaCAB gene was successfully inserted into a plasmid.

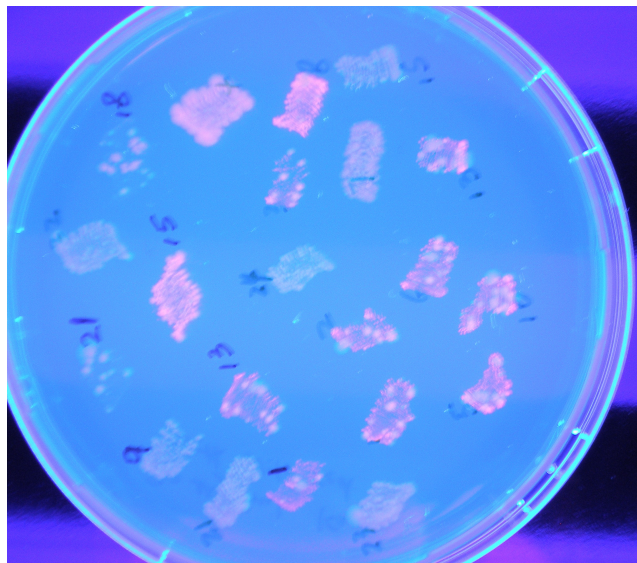


Figure 5. Colonies containing phaCAB insert glow red under UV light while those negative for the insert do not appear red.

NMR Results for harvesting plastic

The NMR results were obtained for commercial 3HB, a nonpolymer-producing control, and a polymer produced by pASKphaCAB-noTag bacteria. The NMR spectra for the control exhibits several peaks at 1, 6, and 7 ppm that are not present in the commercial and pASKphaCAB-noTag polymers. More significantly, a closer view of the 5 ppm and 2 ppm area for the commercial and pASKphaCAB-noTag polymers illustrate that the peaks are nearly identical. This suggests that the pASKphaCAB-noTag polymer is indeed 3HB.

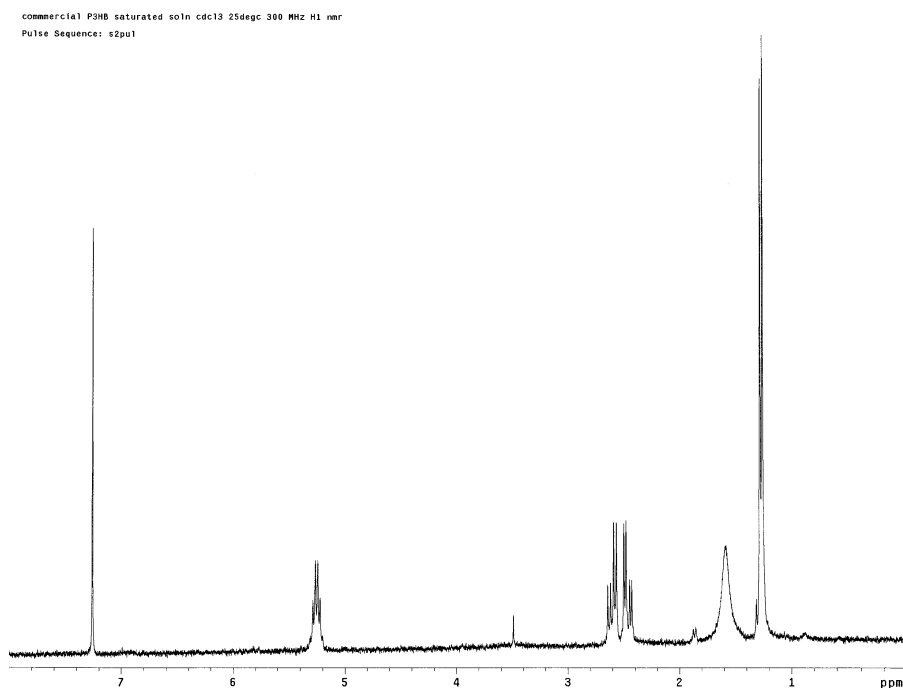


Figure 6. NMR for commercial 3HB.

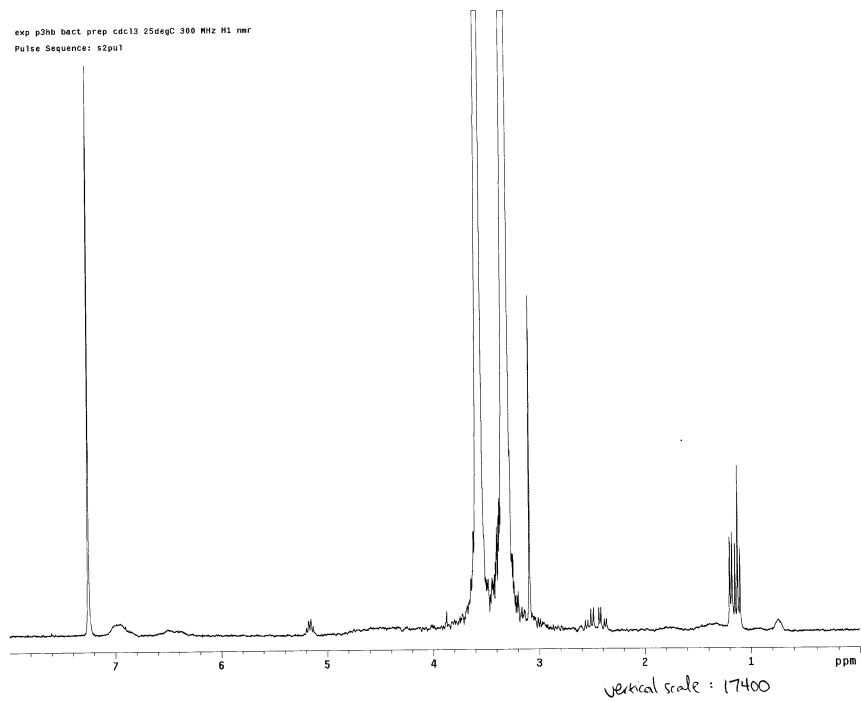


Figure 7. NMR for *pASKphaCAB-noTag* produced polymer.

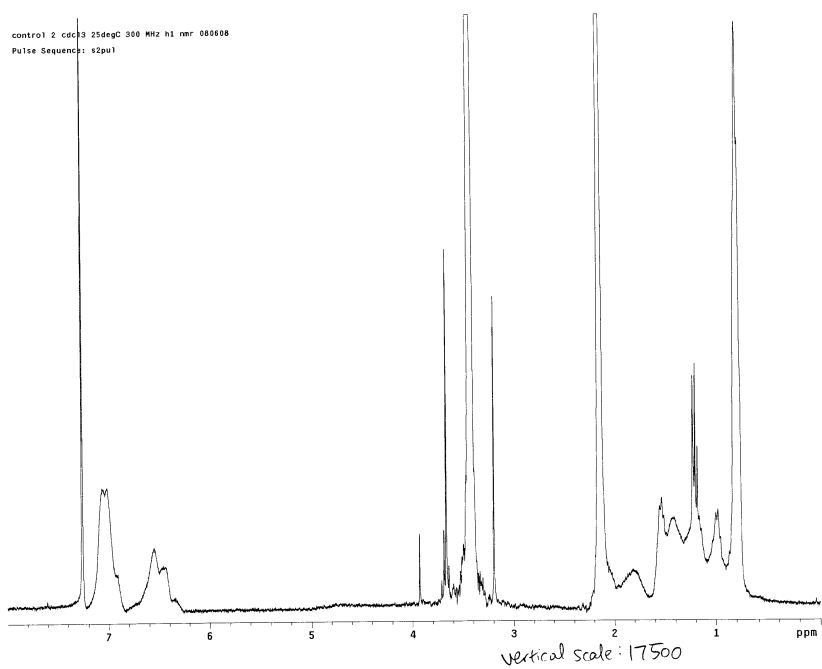


Figure 8. NMR for control.

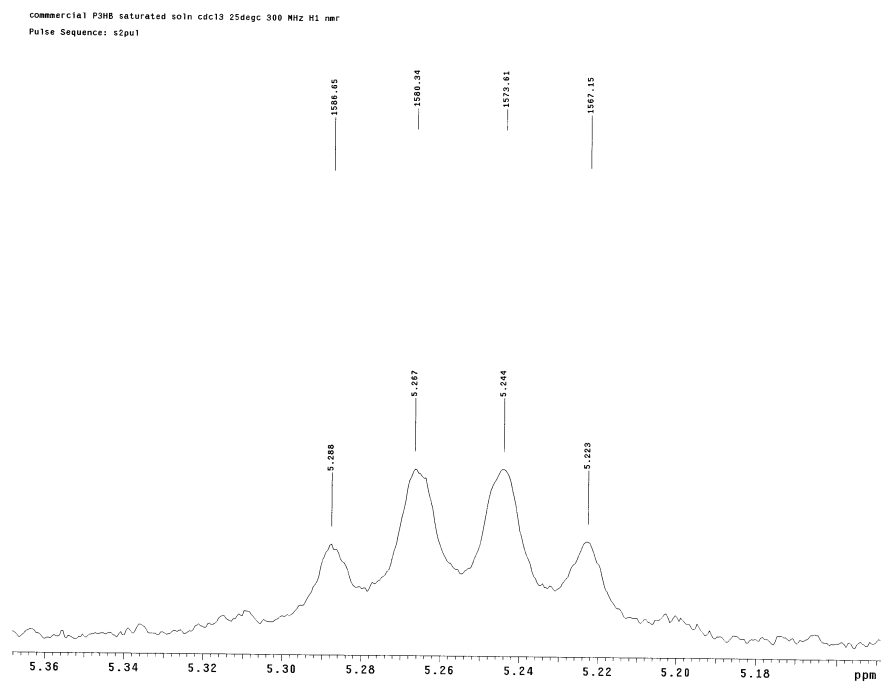


Figure 9. Close-up view of NMR of identifying region on commercial 3HB.

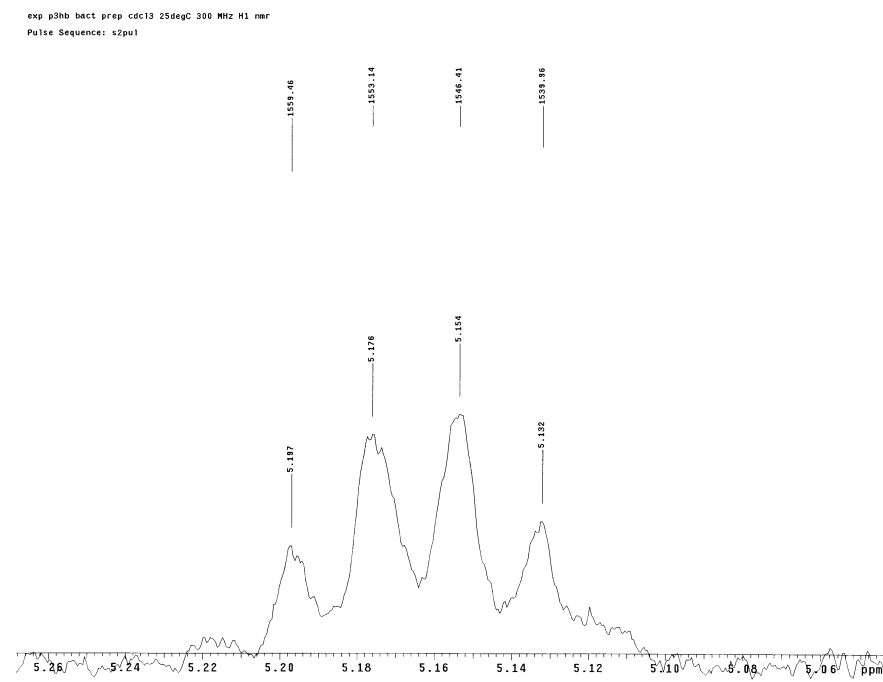


Figure 10. Close-up view of NMR of same identifying region on pASKphaCAB-noTag produced polymer.

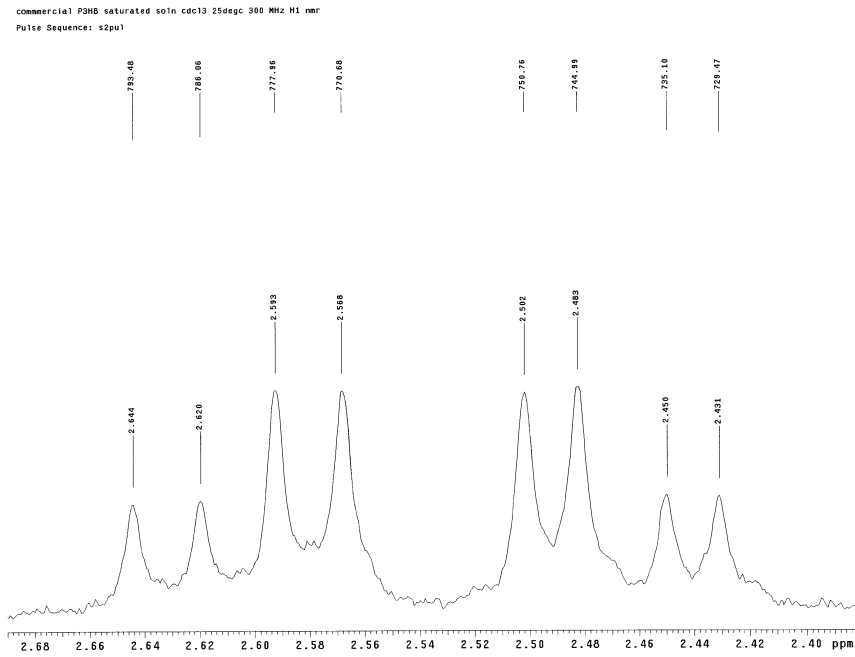


Figure 11. Close-up view of NMR of identifying region on commercial 3HB.

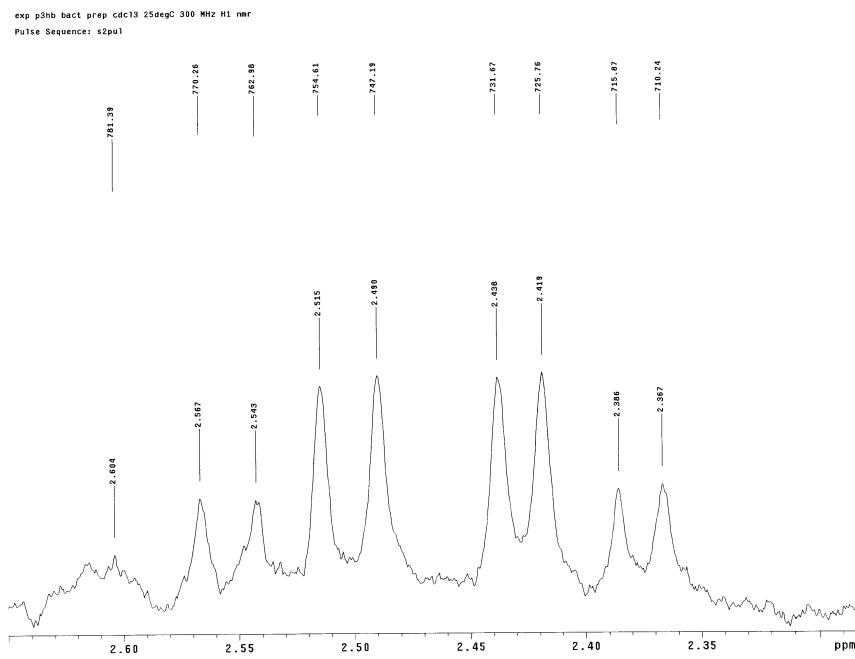


Figure 12. Close-up view of NMR of same identifying region on pASKphaCAB-noTag produced polymer

Discussion

Most of the plasmids have been constructed and verified except for a comprehensive plasmid containing the phaCAB operon and the cat2phaC gene. The development of this plasmid will enable further study on increasing the ratio of 4HB in poly(3HB-co-4HB). For the immediate future, it is necessary to repair the frameshift mutation in pSOScat2-phaC either through reconstructing the plasmid or by removing the lacZ promoter altogether. Better screening methods for the ratio between 3HB and 4HB in the copolymer should also be developed. One area that could be investigated is using lipases to degrade the plastic and then monitoring the lipase activity. This method distinguishes between 3HB and 4HB monomers because lipases only degrade 4HB monomers. However, this method has several problems. The plastic requires several days and the right conditions to accumulate in the bacteria. In addition, the bacteria have to be lysed and an effective assay for monitoring lipase activity found. Another method that could be employed after the plastic has been harvested is gas chromatography, or even melting point determination. Measuring the melting point of the copolymer is a relatively simple estimate of its composition as pure 3HB and poly(3HB-co-4HB) vary in T_m depending on the concentration of 4HB.

Achieving a higher composition of 4HB in the copolyester may also be feasible by using only the pSOScat2phaC plasmid after the frameshift mutation has been corrected. A study by Hein et. al. developed a recombinant strain of *E. coli* containing the PHA synthase gene, phaC, and orfZ (cat2) to produce P(4HB). These two genes were inserted onto the same plasmid and transformed into XL1-Blue *E. coli* cells. When grown in LB broth in the presence of glucose and 4-hydroxybutyric acid, P(4HB) was

accumulated. When glucose was absent, P(4HB) was initially accumulated but after about a day, increasing amounts of 3HB was added to the polymer to approximately 70%, suggesting that glucose limits the incorporation of 3HB into the polyester. A plausible experiment would be to grow a batch of pSOScat2phaC bacteria in the absence of glucose to look for the production of poly(3HB-co-4HB).

Other alternative methods for modifying the composition of poly(3HB-co-4HB) include mutagenesis of the PHA synthase enzyme, which is responsible for the polymerization reaction. Previous studies have used the in vivo evolutionary technique, which involves an error-prone PCR methodology to generate mutants with similar, lower, and much lower activity compared to the wild-type. These mutants are then identified and a second round of mutation is used to produce an enzyme with better characteristics than the wild-type. One of these mutants, E11-4 was generated after three rounds of mutations. In the first round, Proline was substituted for Serine at residue 80, rendering the enzyme more thermostable but reducing the activity. An additional mutation at Gly4Asp produced the E11-4 mutant which can accumulate higher levels of protein and P(3HB) production. Moreover, a separate mutant harboring a Phe420Ser change exhibited higher specificity toward 3HB-CoA. Knowing these important sites on PHA synthase for enzyme activity, site-directed mutagenesis could be used to target specific areas on the enzyme.

Past studies have also suggested that PHA synthase has equal affinity for 3HB and 4HB monomers. The in vitro polymerization of P(3HB-co-4HB) has been accomplished through the PHA synthase from *R. Eutropha* and an acyl-CoA synthetase, AlkK, from *Pseudomonas oleovorans*. The alkK gene product synthesizes 3-hydroxybutyryl-CoA (3HBCoA) and 4-hydroxybutyryl-CoA (4HBCoA) from the

hydroxyalkanoates and CoA. Despite previous studies that reveal that PHA synthase activity for 4HBCoA is only .03% that of 3HBCoA, this study demonstrates that the relative ratios of 3HB and 4HB added to the reaction mixture is approximately the ratio of the monomers in the finished product. This may be due to the low levels of CoA, which is known to inhibit PHA synthase [6]. A possible way to increase 4HB composition in poly(3HB-co-4HB) would thereby be to degrade CoA in the bacterial cells.

References

1. Steinbuchel, A., Hein, S. Biochemical and Molecular Basis of Microbial Synthesis of Polyhydroxyalkanoates in Microorganisms. *Advances in Biochemical Engineering/Biotechnology*. (2001), 82-119.
2. Rehm, B. Polyester synthases: natural catalysts for plastics. *J. Biochem.* (2003) 376, 15–33.
3. Steinbuchel, A, Lutke-Eversloh, T. Metabolic Engineering and Pathway Construction for Biotechnological Production of Relevant Polyhydroxyalkanoates in Microorganisms. *Biochemical Engineering Journal*. (2003) 16, 81-96.
4. Valentin, HE, Dennis, D. Production of poly(3-hydroxybutyrate-co-4-hydroxybutyrate) in recombinant *Escherichia coli* grown on glucose. *Journal of Biotechnology*. (1997) 58, 33-38.
5. Hein S., Soehling B, Gottschalk G., Steinbuechel A. Biosynthesis of poly(4-hydroxybutyric acid) by recombinant strains of *Escherichia coli*. *FEMS Microbiology Letters*. 1997. 153: 411-418.
6. Satoh, Y. Murakami, F. Tajima, K. Munekata M. Enzymatic synthesis of poly(3-hydroxybutyrate-co-4-hydroxybutyrate) with CoA recycling using polyhydroxyalkanoate synthase and acyl-CoA synthetase. *Journal of Bioscience and Bioengineering*. 2005. 508-511