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DNA Sequence Analysis of a Bioluminescent Marine Bacterium

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DNA SEQUENCE ANALYSIS OF A BIOLUMINESCENT MARINE

BACTERIUM: THE SEARCH FOR GENES INVOLVED

IN POLYHYDROXYALKANOIC ACID SYNTHESIS

AND ACCUMULATION

by
Benjamin Ryder

A Thesis Submitted in Partial Fulfillment
of the Requirements for the Master of Arts

in

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This thesis is dedicated to the memory of Sol Mitchell Ilowitz: friend, family and mentor.

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Abstract

Several putative genes have been previously identified by the DNA sequence analysis of a 2.9-kb *Bam*HI/*Sal*I subclone from a *Bam*HI genomic fragment from *Vibrio* species B-18. These genes included *phaA* and *phaC*, both of which are involved in the production of polyhydroxyalkanoic acid (PHA), a biodegradable storage polymer. In this study, the DNA sequence of the remainder of the 5.5-kb *Bam*HI genomic fragment was completed. Restriction enzymes were used to generate smaller subclones, which were sequenced and analyzed. Three open reading frames designated *orf4*, *orf5*, and *orf6* were found downstream from *phaC* arranged in a divergently transcribed operon. A BLAST search identified these putative genes as *napA*, *napB*, and *napC*, respectively. All three genes are involved in the reduction of nitrate into nitrite. The four closest matches in terms of amino acid sequence homology belonged to the genus *Vibrio*. It is possible that the activity of the *pha* and *nap* operons are linked as nitrogen limiting conditions often trigger accumulation of PHA.

Chapter One: Introduction

Polyhydroxyalkanoic acids (PHA) are plastics with properties similar to polypropylene, but are biodegradable and nontoxic. PHA synthases are anabolic enzymes that catalyze the conversion of hydroxyalkanoic acids (HA) into PHA (Figure 1). The substrate can either be short chain length (SCL) with 3-5 carbon atoms, or medium chain length (MCL) with 6-14 carbons atoms, or a mixture of both (hybrid). Bacteria use PHA synthase to make storage polyesters when carbon sources are plentiful, but other nutrients, like nitrogen or phosphorus, are limited (35). Although the genes for PHA synthase have been isolated from a wide variety of bacteria, large-scale production of PHA is not yet practical (reviewed in 1, 42, 48).

Figure 1. Synthesis of polyhydroxyalkanoic acid. A hydroxyacyl-CoA thioester is initially linked to another thioester or linked onto a growing chain of PHA by the enzyme PHA synthase, releasing coenzyme A (42).

The gene encoding PHA synthase is known as *phaC*. There are three major categories of PHA synthase are based on *phaC* gene structure and substrate specificity (Figure 2). Types I and II are both single proteins encoded by *phaC* genes that are

similar in size but distinct in nucleotide sequence. Type III consists of two subunits: one encoded by a third type of *phaC*, and the other encoded by *phaE* (42).

Type	Structure	Preferred substrates	Representative bacterium
II		HA _{MCL} -CoA	<i>P. oleovorans</i>
I		HA _{SCL} -CoA	<i>A. eutrophus</i>
III		HA _{SCL} -CoA	<i>C. vinosum</i>

Figure 2. Three types of polyhydroxyalkanoic acid synthases (42). This figure shows the genes needed to produce a functional enzyme, the preferred substrates of the enzyme, and the representative organisms containing these genes.

The substrates for PHA synthase can be produced by a PHA-specific NADH-dependent acetoacetyl-CoA reductase or they may be produced by other metabolic pathways, such as the fatty acid synthesis and beta-oxidation pathways (28, 29, 32, 40, 43) as seen in Figure 3. In some cases, additional PHA-specific enzymes, such as the transacylase encoded by *phaG* (9) and the hydratase encoded by *phaJ* (32, 46), may be used to convert intermediates into substrates for PHA synthase. Therefore, the composition of the PHA polymer depends upon both the substrate specificity of the PHA synthase as well as the availability of substrates in the cell.

Figure 3. Production of hydroxyacyl-CoA substrates for PHA synthase using the fatty acid synthesis and beta-oxidation pathways (43).

Type I PHA Synthase

Type I PHA synthases are encoded by the gene *phaC* and preferentially incorporate SCL hydroxyalkanoates into PHA. This enzyme is found in many bacteria and several attempts have been made to optimize PHA expression in heterologous host strains like *Escherichia coli* and *Klebsiella aerogenes* (6, 7, 13, 19, 20, 21, 44). Molecular studies of PHA synthase from *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*) have provided the foundation for all other studies concerning the molecular composition of type I PHA synthases (reviewed in 1, 42, 48). At least three *pha* genes are needed for the production of one of the most common forms of PHA, poly(3-

hydroxybutyric acid), in *E. coli*. The *phaA* gene encodes a 3-keto-thiolase that combines two acetyl-CoA molecules into acetoacetyl CoA. The *phaB* gene encodes a NADH-dependent acetoacetyl-CoA reductase that converts acetoacetyl-CoA into D-3-hydroxybutyryl-CoA, which is the substrate for PHA synthase. As mentioned previously, other metabolic pathways could provide the substrates for PHA synthase. Several studies have shown that the amount of PHA and its composition vary between organisms, even if they contained the same *phaC* gene (7, 44). In these studies, cloned *phaC* genes carried on plasmids were placed into heterologous hosts that do not normally accumulate PHA (*E. coli*, for example) or that have mutations in their existing *phaC* genes. Since the substrate specificity of the PHA synthase remains the same, these differences could be attributed to either differences in copy number or to differences in the metabolic “background” of the cells containing the cloned PHA synthase genes. In almost every case, the predominant monomers in PHA were four or five carbons in length.

A mutational analysis of the PHA synthase from *Aeromonas caviae* revealed that while the first 100 amino-acid residues had little effect on PHA accumulation, mutations within highly conserved regions severely reduced PHA accumulation indicating the possible location of an active site (16).

Type II PHA Synthase

Type II PHA synthases are encoded by the gene *phaC*, which is similar in size to type I (42). However, type II preferentially incorporates MCL hydroxyalkanoates into PHA. Most type II PHA synthases are found in the *Pseudomonas* species (25, 26, 31,

36). It is common for these organisms to have more than one *phaC* gene contained in the same operon (39, 42). Several experiments revealed the nature of the alpha/beta hydrolase fold found in PHA synthase. The alpha/beta hydrolase fold is a structure of eight beta sheets connected by alpha helices that is common to a large group of related enzymes (27). Mutational analyses revealed that this structure is crucial for substrate binding (36, 39, 40).

Type III PHA Synthase

The length of *phaC* is considerably shorter in type III PHA synthases than the other types, and the functional enzyme requires a second gene product, encoded by *phaE*. Type III PHA synthase has been intensively investigated in a wide variety of organisms and recent studies have examined the PHA synthase from phototrophic bacteria, including *Synechocystis* (12, 15), *Chlorogleocapsis fritschii* (12), and *Ectothiorhodospira shaposhnikovii* (47). In *Synechocystis*, both *phaC* and *phaE* must be expressed for PHA synthesis, but the proteins could not be found on the granule surface, unlike the situation with other organisms as seen in Figure 4. When *Ectothiorhodospira shaposhnikovii* was grown on nitrogen-limiting medium, cell growth continued in the presence of light but leveled off in the dark, while PHA accumulation was minimal in the light but dramatically increased in the dark, as did the activity of PHA synthase. When testing *in vitro* reaction rates, 1.5 M NaCl was found to decrease the lag time significantly. Size exclusion chromatography revealed that at low salinity (0-0.125 M NaCl), two PHA synthase complexes (473 kDa and 151 kDa respectively) were detected. The larger more abundant complex corresponded to a hexamer of three PhaC and three PhaE molecules,

while the smaller, less abundant complex corresponded to a dimer of one PhaC and one PhaE. At higher salinity (1.5 M NaCl) a large PHA synthase complex of 955 kDa predominated, corresponding to a dodecamer made of six PhaC and six PhaE. So as the salinity increased, PHA synthase hexamers combined to make more active dodecamers (47).

Regulation of PHA Synthase

A protein involved in the regulation of PHA synthase was recently discovered in *Bacillus megaterium* (24). This organism has an additional protein, encoded by the *phaR* gene, involved in PHA synthesis and accumulation. The PHA synthase was found to have 29.9%-32.7% identity with other type III PhaC, while PhaR had only 13%-14% identity with PhaE. The PhaR protein can be found in PHA inclusion bodies as demonstrated by SDS-PAGE and microscopy of cells containing the PhaR protein tagged with a fluorescent reporter. A strain of *B. megaterium* containing deletions of the phasin gene *phaP*, along with *phaB*, *phaC*, and *phaR* was transformed with plasmids containing the missing genes. Cells containing both *phaC* and *phaR* were able to accumulate PHA, while cells containing only *phaC* were not. This suggested PhaR was necessary for the proper functioning of PHA synthase, although it may not necessarily act as another subunit of the enzyme. Western blot analysis with antibodies against GFP found that GFP/PhaC fusion proteins were degraded in cells that did not accumulate PHA as compared to cells that did. This suggested that PhaC could have an inactive form when the cell is not making PHA and that PhaR regulates the activity of the synthase.

PHA Granule Proteins

In addition to proteins that help to produce substrates for PHA synthase, other proteins help to regulate PHA synthase expression. PHA is accumulated in cellular inclusions called granules (48). In these granules, a single phospholipid layer containing several proteins surrounds the PHA. These membrane proteins include PHA synthases, PHA depolymerases, and phasins to stabilize the granules (Figure 4). Additional other low molecular weight proteins are found on the surface (22, 30, 31). Altering the pathways of PHA synthesis alters the size and appearance of the granules, as does altering phasin genes.

Figure 4. PHA Granule Structure. PHA granules are enclosed in a unit membrane that contains PHA synthase (or PHA polymerase), PHA depolymerase, phasins, and other proteins (48).

The *phaP* gene encodes a phasin; in *Aeromonas hydrophila*, the *phaP* gene is located upstream from *phaC*. Cells overexpressing *phaP* accumulated the same amount of PHA as wild-type cells, but the granules were smaller and more numerous (Figure 5). Real-time quantitative PCR using recombinant strains showed that the amount of *phaC*

transcribed into RNA increased in cells where *phaP* was overexpressed. Also insertion of plasmids containing both *phaC* and *phaP* from *A. hydrophila* increased PHA accumulation in *E. coli*. This suggests that phasin influences both PHA synthase activity as well as the size and number of the granules within the cells (45).

Figure 5. Morphology of PHA Granules in *Aeromonas hydrophila*. Figure (a) shows granule formation in wildtype cells, while figure (b) shows granule formation in cells overexpressing the *phaP* gene (45). In both cases, cells accumulated a similar amount of PHA.

Another phasin was described in *Paracoccus denitrificans*. The GA16 protein is encoded by the *phaP* gene, located immediately upstream from the *phaC* gene. *E. coli* containing both *phaP* and *phaC* accumulated more PHA than cells that only contained *phaC*. Like the situation with *Aeromonas hydrophila*, cells overexpressing *phaP* contained granules that were smaller and more numerous. *P. denitrificans* also contains a *phaR* gene and there is evidence to suggest that PhaR regulates the production of GA16 (22).

The GA14 protein in *Rhodococcus ruber* was one of four proteins found in PHA granules and matched the predicted sequence of the protein encoded by a gene downstream of *phaC*. The molecular weight of the native protein was found to be around

53-kDa, suggesting a tetramer of four 15-kDa GA14 subunits. Western blotting analysis with rabbit antibodies to the GA14 protein showed that it was expressed in large amounts (45%) in normal cells able to make PHA synthase and low levels (4-21%) in mutants unable to make PHA synthase (30). The function of this protein is not yet known.

PhaF is a granule-associated protein that regulates PHA accumulation in *Pseudomonas oleovorans* Gpo1. This protein appears to repress *phaC1* gene expression when nitrogen is not limiting and it also appears to regulate the expression of another granule-associated protein encoded by *phaI*. The PhaF protein and PhaI share 58.6% identity. In addition to its regulatory functions, it is believed that PhaF and PhaI help to form granules as shown in Figure 6 (31). It is unknown if *phaI* regulates either of the PHA synthase genes found in this organism.

Figure 6. Model of PhaF and PhaI in PHA Granule Formation. The top of the figure shows the orientation of the *pha* genes. Black arrows indicate transcription was demonstrated by Northern blot analysis while

dashed arrows indicate where transcription was not demonstrated. The bottom half shows the expression of *phaC1*, *phaI*, and *phaF* genes in the presence of octanoic acid and in association with the PHA granule (31).

Previous Findings

The focus of this study is a bioluminescent bacterium that was isolated from the coastline of Virginia. A fatty acid profile analysis revealed that this organism shows the greatest homology with *Vibrio parahaemolyticus*, while a DNA-based identification showed that this organism aligns most closely with *Vibrio alginolyticus* (Figure 7).

Figure 7. Phylogenetic tree showing the results of a DNA-based identification system. C166122 is the code name for the bioluminescent marine bacterium isolated from Buckroe Beach, Virginia.

This organism, isolated from Buckroe Beach, has been designated as *Vibrio* species B-18. B-18 accumulates PHA composed of 3-hydroxybutyrate, 3-hydroxyhexanoate, and 3-hydroxyvalerate as seen in Table 1 (18). As such, this organism may contain a PHA synthase that is unique from the other four types.

Table 1. PHA accumulation in the bioluminescent *Vibrio* species B-18

Carbon Source	Yield	Polymer Composition (mol %)		
	% PHA/mg cells	% HB	% HV	% HC
Glucose	32.0	99.9	trace	trace
Gluconate	18.0	100.0		
Fructose	27.3	100.0		
Sucrose	4.3	100.0		
Butyric acid	2.5	100.0		
Valeric acid	2.6	100.0		
Hexanoic acid	3.2	83.4		16.6
Heptanoic acid	6.3	19.2	80.8	
Octanoic acid	11.0	87.2		12.8
Nonoic acid	47.1	90.4	9.6	
Decanoic acid	6.3	88.5		11.5

Cells were grown at 30°C, 2 days in minimal media supplemented with 0.1% nutrient broth, the appropriate antibiotic, and the carbon source listed above prior to centrifugation and lyophilization. PHA was isolated by methanolysis and the quantity and the composition of the PHA polymer was determined by gas chromatography as described previously (19, 38). Each number represents the average of at least two experiments. Trace levels are defined as 0.3% or less. Initials: HB, 3-hydroxybutyrate; HV, 3-hydroxyvalerate; HC, 3-hydroxyhexanoate.

Previous molecular genetic studies of the B-18 genome revealed an operon containing putative *phaA* and *phaC* genes. A 5-kb *Bam*HI fragment showed homology to both *phaA* and *phaC* genes and was cloned into a plasmid. A restriction map shows the relative location of *phaC* as determined by a Southern blot analysis as seen in Figure 8 (18).

Figure 8. Map showing the restriction sites within the cloned fragment. The gray striped areas indicate fragments that reacted with the PHA synthase gene probe. Fragments digested with either *SacI* or *SacII* were not exposed to the probe. *NotI*, *SmaI*, *SpeI*, and *XhoI* do not cut the fragment. The length of the fragments is not drawn to scale.

DNA sequence analysis of a 2.9-kb *BamHI/SalI* subclone revealed three open reading frames in the same orientation, presumably arranged as an operon as seen in Figure 9 (17). The first open reading frame is a partial gene with homology to *phaA*. The second open reading frame encodes a gene of unknown function, *orf2*. BLAST searches revealed homology with other genes, all of which are located upstream from the PHA synthase gene. The third gene in this fragment shows homology with *phaC*. Where

PHA synthase activity of these homologs was demonstrated, they have a similar PHA profile to that shown for B-18 (8, 35).

Figure 9. Map of the 2.9-kb *Bam*HI-*Sal*I fragment from the bioluminescent *Vibrio* species subjected to DNA sequence analysis. The large arrows indicate the orientation of the genes while the small vertical arrows indicate the distance between the genes.

The goal of this project is to complete the sequence of the entire *Bam*HI fragment in the hope of identifying open reading frames that could code for genes involved in PHA accumulation in this organism.

Chapter 2: Materials and Methods

Enzymatic manipulation of DNA. Restriction endonucleases and all other DNA modification enzymes were purchased from commercial sources (New England Biolabs) and all manipulations were performed using standard techniques (2) and manufacturer's recommendations. The details of the procedures are described below.

Plasmid Construction. The table below lists the plasmids used in this study. Both pPMSK4 and pPMSK4.6 were constructed prior to this study (18) and were used to create all of the plasmids in this study. Plasmid pPMSK4 contains the entire 5.3-kb *Bam*HI genomic fragment from B-18 cloned into pBluescriptSKII+ (Stratagene; La Jolla, CA) in the opposite orientation from that shown in the restriction map of Figure 8. This plasmid was digested with *Bam*HI and *Sal*I to create pPMSK4.6, which contains approximately 2.4-kb of the original insert that was not subjected to the DNA analysis shown in Figure 9.

Table 2. Plasmids Used in the Study

<u>Plasmid</u>	<u>Derivative</u>	<u>Restriction Enzyme</u>	<u>Insert Size (Kb)</u>
pPMSK4	original clone in pBluescriptSKII+	<i>Bam</i> HI	5.3
pPMSK4.6	pPMSK4 subclone	<i>Bam</i> HI, <i>Sal</i> I	2.4
BR1	pPMSK4.6	<i>Eag</i> I	4.4
BR3	pPMSK4	<i>Sac</i> II	7.1
BR4	pPMSK4.6	<i>Xba</i> I	3.9
BR6	pPMSK4.6	<i>Sac</i> II	5.0
BR7	pPMSK4.6	<i>Sac</i> II	3.7
BR9	pPMSK4	<i>Eco</i> RI	4.8
BR10	pPMSK4, pBluescriptSKII+	<i>Xba</i> I	4.7
BR11	pPMSK4, pBluescriptSKII+	<i>Eco</i> RI	1.5

Subclones BR1, BR3, BR4, BR6, BR7, and BR9 were constructed by digesting either pPMSK4 or pPMSK4.6 with the appropriate restriction enzyme overnight at 37 °C.

The resulting DNA fragments were separated by electrophoresis as described below. Fragments containing both vector DNA and a smaller piece of the cloned fragment were excised from the gels using the Qiaquick Gel Extraction Kit (Qiagen Corp.; Valencia, CA) and were recircularized using T4 DNA ligase at room temperature (approximately 25°C). In every case, the fragment contained sufficient vector DNA for replication in *E. coli*.

Subclones BR10 and BR11 were constructed by digesting pPMSK4 with either *Xba*I or *Eco*RI, respectively. After overnight digestion at 37°C, the fragments were separated by electrophoresis and the desired fragments were gel-purified as described above. The vector, pBluescriptSKII+, was digested with either *Xba*I or *Eco*RI and treated with calf intestine alkaline phosphatase to maintain the DNA in a linear state. The 4.7-kb *Xba*I fragment and the 1.5-kb *Eco*RI fragment, both of which were devoid of vector DNA, were ligated to the appropriate linear vector to create these subclones.

Agarose gel electrophoresis. DNA fragments were separated according to size by electrophoresis in 1% agarose-TAE gels that were run at 90 volts. Molecular weight markers were included to estimate fragment size. The gels were stained with ethidium bromide and the DNA fragments were visualized with an UV-transilluminator and photographed using 667 black and white Polaroid film.

Electroporation. During this study, it was discovered that the ingredients found in a typical ligation reaction greatly reduce the electroporation efficiency. To remedy this situation, it was necessary to passage the ligation mixture over the spin-columns found within the Qiaquick Gel Extraction Kit. Steps 6 through 13 of the gel extraction protocol

were followed. Electrocompetent *E. coli* XL1-Blue (Stratagene) were prepared as described (11). Aliquots of 150 µl were electroporated with the filtered ligation mixtures using 0.2 cm gap cuvettes and a MicroPulser electroporator (BioRad; Hercules, CA). After electroporation, the cells were incubated at 37°C for 45 minutes and plated on Luria-Bertani media containing 20 µg/ml X-Gal, 1 mM IPTG, 100 µg/ml ampicillin and 10 µg/ml tetracycline. These plates were then incubated overnight at 37 °C.

Screening clones. The cells that incorporated the plasmid became resistant to the antibiotics and formed colonies on the plates. Cells containing plasmids with insert DNA that disrupts the β-galactosidase gene (*lacZ*) on the plasmid are unable to utilize the lactose analog, X-Gal, and remain white on this medium. Cells that contain plasmids without any inserts have an intact *lacZ* gene and utilize X-Gal to form blue colonies. Bacteria from white colonies were cultured in tubes of Luria-Bertani broth containing the appropriate antibiotics at 37 °C. Plasmid DNA was purified from these cultures using the alkaline lysis procedure (2) and the pellets were resuspended in a final volume of 100 µl. To screen the clones, the purified DNA was digested with the appropriate restriction enzyme and subjected to agarose electrophoresis as described above. Fresh cultures of cells containing the desired plasmids were prepared in order to make freezer stocks, which were stored at minus 80°C.

DNA sequence analyses. Fresh cultures of cells containing the clones of interest were grown in Luria-Bertani broth containing the appropriate antibiotics at 37°C overnight. The plasmids were purified using the Qiafilter Plasmid Midi kit (Qiagen Corporation). Both the concentration and the purity of the plasmid DNA were

determined by spectrophotometry at 260 and 280 nm. The purified DNA was then ethanol precipitated. The resulting pellets were dried and sent to a commercial laboratory for DNA sequencing (MWG Biotech; High Point, NC). In all cases, the DNA sequencing reactions were performed using the T3 and T7 primer sites found on pBluescriptSKII+. The data was returned as a text file containing the DNA sequence and as the raw sequence data presented graphically as colored peaks (electropherograms or ABI files).

MacDNAsis was used to analyze the sequence data in the following manner. First, the DNA sequence was examined and any vector sequences were removed. DNA sequences from plasmids that were sequenced from both ends of the insert were first examined for overlapping sequences and any discrepancies were resolved by examining the ABI files. The data was combined to generate a consensus sequence for that plasmid. The consensus sequences from several plasmids were then compared to look for overlapping sequences and any discrepancies were resolved in a similar manner. The overlapping sequences were combined to create an overall consensus sequence for the entire fragment. This sequence was then examined using MacDNAsis for the presence of restriction sites and open reading frames. The restriction map based upon the sequence data was compared to the map that was determined empirically (Figure 8). The amino acid sequences encoded by open reading frames were compared using the Basic Local Alignment Search Tool (BLAST; National Center for Biotechnology Information) to determine the identity of putative genes and gene products encoded within the open reading frames. The polypeptide sequences from the five gene products from different

organisms showing the strongest identity to the open reading frames identified in this study were downloaded from Genbank and were aligned using the Higgins-Sharp (CLUSTAL4) multiple sequence alignment program found within MacDNAsis. This program aligns sequence information and generates phylogenetic trees based on the degree of sequence similarity.

Chapter 3: Results

Samples of each subclone were digested with the appropriate restriction enzyme to confirm that they contained inserts of the correct size, while the plasmids pPMSK4 and pPMSK4.6 were digested with *Sac*II (Table 2). Gel electrophoresis confirmed that all the important fragments were present (Figure 10).

Figure 10. Restriction digest and agarose gel electrophoresis of plasmid DNA. Lanes: 1, 2 Log DNA Ladder; 2, pPMSK4; 3, BR3; 4, BR9; 5, pPMSK4.6; 6, BR1; 7, BR4; 8, BR6; 9, BR7; 10, BR10; 11, BR11; 12, 2 Log DNA Ladder.

DNA sequencing revealed overlaps between the subclones in both the normal direction (Figure 11, rightward arrows) and the complementary direction (Figure 11,

leftward arrows) for the entire 5.5-kb region. This allowed for the construction of a consensus sequence with no gaps.

Figure 11. Connection map of subclones. Arrows indicate the direction of the reading frame and the length of each line indicates the length of the sequence. Plasmid pPM2.9 was sequenced in an earlier analysis (17).

An open frame reading analysis of the sequence in the normal direction revealed the previously discovered putative genes: a partial *phaA* (Figure 12, center row left side), *orf2* (Figure 12, bottom row left side), and *phaC* (Figure 12, bottom row right side).

Error!

```

File : Consensus 6
Mode : Normal      Range :      1 -      5549
Init : ATG
Term : TAA TAG TGA

```

Figure 12. Open frame reading analysis in the normal direction. Triangles represent methionine codons. Vertical lines represent stop codons.

An open frame reading analysis of the sequence in the complementary direction revealed three open reading frames, designated *orf4* (Figure 13, center row left side), *orf5* (Figure 13, center row right side), and *orf6* (Figure 13, bottom row).

```

File : Consensus 6
Mode : Complementary Range :      1 -      5549
Init : ATG
Term : TAA TAG TGA

```

Figure 13. Open reading frame analysis in the complementary direction. Triangles represent methionine codons. Vertical lines represent stop codons.

A BLAST search of the gene products encoded by *orf4*, *orf5*, and *orf6*, revealed that all three proteins align most closely with enzymes involved in nitrate reduction (like Nap or Nar as shown in Figure 21), not PHA synthesis. The gene products from four different organisms showing the strongest homology to the *orf4-6* gene products were selected for further study. In every case, the four strongest homologs were found in the same four *Vibrio* species (5, 14, 23, 34). Additionally, the identity of all of these homologs was determined via DNA sequence analyses of genomic DNA. Therefore, an additional protein identified through enzyme assays and other empirical studies was included in the analyses (3, 37, 41). A Higgins-Sharp alignment using these five sequences and the putative B-18 gene products identified in this study was performed and the results are shown in Figures 14, 15, and 16. It should be noted that the alignment of the *orf4* peptide to its homologs (Figure 14) revealed that the original *Bam*HI fragment (Figure 8) contained a partial gene for *orf4*. The lack of a suitable match to a Shine-Dalgarno sequence (ribosome binding site) upstream from the codon corresponding to the methionine residue at position 7 in the *orf4* peptide further supports this observation. Shine-Dalgarno sequences were found upstream from the hypothetical start codons (methionine codons) for both *orf5* and *orf6*.

Orf 4	1	10	20	30	40	50
A. eutrophus	1	DPNTKVMSLW	TMGMNQHTRG	VWMNNLVYNI	HLLTGKIATP	GNSPFSLTGQ
V. cholerae	1	DPNTKVMSLW	TMGFNQHTRG	TWANNMVYNI	HLLTGKIATP	GNSPFSLTGQ
V. fisheri	1	DPNTKVMSLW	TMGMNQHTRG	VWMQSLVYNI	HLLTGKIATP	GNSPFSLTGQ
V. parahaemo	1	DPNTKVMSLW	TMGMNQHTRG	VWMNNLVYNI	HLLTGKIATP	GNSPFSLTGQ
V. vulnificu	1	DPNTKVMSLW	TMGMNQHTRG	VWMNSLVYNI	HLLTGKIATP	GNSPFSLTGQ
Orf 4	51	60	70	80	90	100
A. eutrophus	51	PSACGTAREV	GTFAHRLPAD	MVVANPKHRK	IAENIWKLPE	GTIPPKPGFH
V. cholerae	51	PSACGTAREV	GTFSHRLPAD	MVVTNPKHRE	EAERIWKLP	GTIPDKPGYD
V. fisheri	51	PSACGTAREV	GTFAHRLPAD	MVVANPKHRA	IAEKVWKLPE	GTIPKPGFH
V. parahaemo	51	PSACGTAREV	GTFSHRLPAD	MVVANPKHRA	ISEKIWKLP	GTLNKPGKAH
V. vulnificu	51	PSACGTAREV	GTFAHRLPAD	MVVANPKHRQ	IAEKIWKLP	GTIPPKPGFH
Orf 4	101	110	120	130	140	150
A. eutrophus	101	AVLQDRMLHD	GVLNCYWVQC	NNMQAGPNI	NAERLPGYRN	PENFIVVSDP
V. cholerae	101	AVLQDRMLKD	GKLNAYWVQV	NNMQAAANL	MEEGLPGYRN	PANFIVVSDA
V. fisheri	101	AVQDRMLKD	GVLNCYWVQC	NNMQAGPNI	NEERLPGYRN	PENFIVVSDA
V. parahaemo	101	AVVQDRMLKD	GKINAYWVMC	NNMQAGPNI	NTERLPGYRN	PENFIVCSDP
V. vulnificu	101	AVLQDRMLND	GVLNCYWVQC	NNMQAGPNI	NTERLPGYRN	PENFIVVSDP
Orf 4	151	160	170	180	190	200
A. eutrophus	151	YPTATAQAAGD	LILPTAMWIE	KEGAYGNAER	RTQAWYQQVG	TIGDAKSDLW
V. cholerae	151	YPTVTALAAD	LVLPSAMWVE	KEGAYGNAER	RTQFWHLVD	APGEARSDLW
V. fisheri	151	YPTVTAQAAD	LVLPTAMWVE	KEGAYGNAER	RTQVWYQQVK	TVGESHSDSW
V. parahaemo	151	YPTATAQAAD	LILPTAMWVE	KEGAYGNAER	RTQAWYQQVQ	AKGEAKSDLW
V. vulnificu	151	YPTATAQAAD	LILPTAMWIE	KEGAYGNAER	RTQAWYQQVG	TVGDAKSDLW
Orf 4	201	210	220	230	240	250
A. eutrophus	201	QVMEFSKRFK	MEEVWPEELL	AKAPEYRGKT	MYDMLFKNGQ	VDQFPIEEAR
V. cholerae	201	QLVEFAKRFK	VEEVWPEELI	AKKPEYKGKT	LYDVLRYNGQ	VDFPKLDVN
V. fisheri	201	QVIEFSKRFK	VEDVWPEELL	AKAPQYRGKT	LYDVLFKNGQ	VDKFPLSEAR
V. parahaemo	201	QIMEFSKRFK	VEEVWGEELV	AKAPEYRGKT	MYDILFKNGQ	VDAPPLSEAR
V. vulnificu	201	QVMEFSKRFK	MEEVWPEELL	AKAPQYRGKT	MYDMLFKNGQ	VDKFPLEEAR
Orf 4	251	260	270	280	290	300
A. eutrophus	251	ELN--DDAHH	FGFYIQKGLF	EEYATFGRGH	GHDLPYDVY	HTVRGLRWPV
V. cholerae	251	AEYHNAEAKA	FGFYLQKGLF	EEYATFGRGH	GHDLPFDAY	HEARGLRWPV
V. fisheri	251	EL--NDDAHH	FGFYIQKGLF	EEYAEFGRGH	GHDLPYDVY	HQVRGLRWPV
V. parahaemo	251	EL--NDDAKA	QGFYVQKGLF	EEYASFGRGH	GHDLPYDITY	HTVRGLRWPV
V. vulnificu	251	ELN--DDSHH	FGFYVQKGLF	EEYATFGRGH	GHDLPYDVY	HTVRGLRWPV
Orf 4	301	310	320	330	340	350
A. eutrophus	301	VDGKETQWRF	KEGSDPYAKA	SGSWDFYGNA	DGKAKIISAP	YEAPPEVPNE
V. cholerae	301	VNGKETRWRY	REGSDPYVKA	GTGFQFYGNP	DGKAVIFALP	YEPPAESPPK
V. fisheri	301	VDGKETLWRF	KEGSDPYAKA	SGSWDFYGKP	DGKAWIISAP	YEAPPEMPNE
V. parahaemo	301	VDGKETQWRF	KEGSDPYAKK	SGSWDFYGNA	DGKAKIISAP	YEAPPEVPND
V. vulnificu	301	VDGKETLWRY	KEGSDPYAKK	SGSWDFYGKP	DGKALIISAP	YEAPPESPDA
Orf 4	351	360	370	380	390	400
A. eutrophus	351	EFDMWLCTGR	VLEHWHGTGM	TRRVPELYKA	VPDAVCYMHP	DDAKARNVRR
V. cholerae	351	EYPYWLVTGR	VLEHWHSGSM	TRRVPELYRS	FPNAVVFMMHP	EDAKALGLRR
V. fisheri	351	EYDLWLCTGR	VLEHWHGTGM	TRRVPELYKA	VPDALCFMHH	EDAQARGLRR
V. parahaemo	351	EFDMWLCTGR	VLEHWHGTGM	TRRVPELYKA	VPDALCYIHP	ADAKARNLRR
V. vulnificu	351	EFDLWLCTGR	VLEHWHGTGM	TRRVPELYKA	VPDAVCYMHP	EDAKARNVRR
Orf 4	401	410	420	430	440	450
A. eutrophus	401	GEEVLMANKR	GEVRVRVETR	GRNRPPKGLV	FVPFFDARIL	INKLILDATD
V. cholerae	401	GVEVEVVSRR	GRMRSRIETR	GRDAPPRGLV	FVPWFDSQL	INKVTLDATC
V. fisheri	401	GDEVLISNR	GEVRVRVETR	GRNKPPKGLV	FVPFFDARIL	VNKLILDATD
V. parahaemo	401	GDEVLISNKR	GEVRVRVETR	GRNRPPKGLV	FVPFFDARIL	INKLILDATD
V. vulnificu	401	GEEVVIANKR	GEVRVRVETR	GRNRPPKGLV	FVPFFDARIL	INKLILDATD
Orf 4	451	460	470	480	490	500
A. eutrophus	451	PLSKQTDFFKK	CPVKITKIA*			
V. cholerae	451	PISLQTDFFKK	CAVKIVKV--			
V. fisheri	451	PLSKQTDFFKK	CPVKITKVA-			
V. parahaemo	451	PLSKQTDFFKK	CPVKITKIA-			
V. vulnificu	451	PLSKQTDFFKK	CPVKITKV-A			
Orf 4	451	460	470	480	490	500
A. eutrophus	451	PLSKQTDFFKK	CPVKITKVA-			

Figure 14. Alignment of the *orf4* gene product to partial *napA* gene products from *Alcaligenes eutrophus* H-16 (37), *Vibrio cholerae* O395 (14), *Vibrio fischeri* ES114 (34), *Vibrio parahaemolyticus* RIMD 2210633 (23), and *Vibrio vulnificus* YJ016 (5).

		10	20	30	40	50
Orf 5	1	-----	-----	MKKLLVALLS	V-----GALL	TGVAQA-ELD
A. brasiliensis	1	M-----KT	RII-----FA	ALALAAAM--	-PLLVSGVFA	ADGAAPAKVP
V. cholerae	1	MHWAARQPRP	SIKPQGGEFT	MKKQLIALLS	VF-----TVL	AGIAHA-ELN
V. fischeri	1	-----	-----	MKKFIIALMS	AGLLFTGAVQ	AEAEQGSELH
V. parahaemo	1	-----	-----	MKKLLVALLS	V-----GALV	TGIAQA-ELD
V. vulnificu	1	M-----	-----	MKKLLMALLS	VSALSLGTLV	AGVAQA-ELD
		60	70	80	90	100
Orf 5	51	NPGGTGG LDS	LRGTAQLEDT	RPADDF-KDF	PKDQIVEDSF	VYQPPLIPHS
A. brasiliensis	51	S-----	--GHPITQE	IPADPMAKEI	TDDHKRARNY	ADQPPLIPHA
V. cholerae	51	NPGGIGGLES	LRGATEIEAT	RAADPM-KKY	PREQALESDF	VYQPPLIPHN
V. fischeri	51	NPGGIGGVES	LRGASELEAT	RPADDF-KRF	PRDHALDSY	VYQPPLVPHT
V. parahaemo	51	NPGGTGGLES	LRGMTQLEDT	RPADDF-KDF	PKDQIVEDSF	VYQPPLIPHS
V. vulnificu	51	NPGGIGGLES	LRGATNLEDT	RPADDF-KKF	PREQTLDSY	IYQPPLIPHN
		110	120	130	140	150
Orf 5	101	IRNYEVSLNA	NKCLACHSWK	NAKDAGATKI	SVTHYVNRED	AVLADMSPRR
A. brasiliensis	101	IRDYQIDLNI	NKCMTCHDRK	NTEGSQAPMI	SVTHFQDRDG	QTLGAVSPRR
V. cholerae	101	IRNYEVSLNA	NKCLACHSWK	NAKEMGATKI	SVTHFVNRED	AVLSDVSPRR
V. fischeri	101	IRNYEVSLNA	NKCLSCHSWK	NAKEMGATKI	SVTHYVNRED	AVLSDVSPRR
V. parahaemo	101	IRSYEVSLNA	NKCLACHSWK	NAKESGATKI	SVTHYVNRQD	AVLADVSPRR
V. vulnificu	101	IRGYEVTLNA	NKCLACHSWK	NAKEMGATKI	SVTHFVNRED	AVLADVSPRR
		160	170	180	190	200
Orf 5	151	YFCLQCHVPQ	TSATPLVENE	FKAVDSL---	----Q*....	
A. brasiliensis	151	YFCTQCHVPQ	TDAQPITGNR	FRDIDSILAG	GKEGAK....	
V. cholerae	151	YFCLQCHVLQ	ADAKPLVEND	FERVDSL---	----R-....	
V. fischeri	151	YFCLQCHVPQ	ADAKPLVENE	FERVDSL---	----R-....	
V. parahaemo	151	YFCLQCHVPQ	TNAKPLVENE	FQSVDSL---	----Q-....	
V. vulnificu	151	YFCLQCHVPQ	ANAKPLVEND	FERVDSL---	----RK....	

Figure 15. Alignment of the *orf5* gene products to *napB* gene product from *Azospirillum brasilense* (41), *Vibrio cholerae* O395 (14), *Vibrio fischeri* ES114 (34), *Vibrio parahaemolyticus* RIMD 2210633 (23), and *Vibrio vulnificus* YJ016 (5).

		10	20	30	40	50
Orf 6	1	MK-ILKAF--	--WKRLSRPS	KA-AAGVVLF	LGFAGGLLFW	GAFNTGMEAT
Pseudomonas	1	MIGMIKAVIL	WAWRILATPA	GTLGLGFLTL	GGFVGGVIFW	GAFNTALEVT
V. cholerae	1	MK-FLKAF--	--WLRLLSPS	KA-AVGVVLL	MGFVGGLLFW	GAFNTGMEAT
V. fischeri	1	MK-LLKAF--	--WKRFFSSPS	KA-AVGIVLF	MGFMGGLLFW	GAFNTGMEAT
V. parahaemo	1	MK-ILKAF--	--WKRLKNPS	KA-AAGVVLF	LGFAGGLLFW	GAFNTGMEAT
V. vulnificu	1	MK-LLKAF--	--WKRLSTPS	KA-AAGILLF	MGFAGGLLFW	GAFNTGMEAT
		60	70	80	90	100
Orf 6	51	NTEEFCSGCH	---APIVAEI	QETIHYSNRS	GVRAICSDCH	VPHEWTDKIV
Pseudomonas	51	NTEAFCTGCH	EMKTNVYEEL	TQTVHFSNRS	GVRASCPDCH	VPHQWTDKIA
V. cholerae	51	NSEEFCSGCH	---APIVAEI	QETIHYSNRS	GVRAICSDCH	VPHEWTDKIV
V. fischeri	51	NTEEFCSGCH	---APIVKEI	QETIHYSNRS	GVRAICSDCH	VPHNWTDKIV
V. parahaemo	51	NTEEFCSGCH	---APIVKEI	QETIHYSNRS	GVRAICSDCH	VPHEWTDKIV
V. vulnificu	51	NSEEFCSGCH	---APIVAEI	QETIHYSNRS	GVRAICSDCH	VPHEWTDKIV
		110	120	130	140	150
Orf 6	101	RKVQASKELF	AHYVTKTIDT	PEKFQARREH	LAEREWARMK	KNDSLECRNC
Pseudomonas	101	RKMQASKEVW	GK-IFGTIST	REKFLDKRLE	LAQHEWARLK	ANDSLECRNC
V. cholerae	101	RKVQASKELV	AHFI-GTIDT	PEKFQARRAH	LAEREWARLK	KNDSLECRNC
V. fischeri	101	RKVQASKELF	AHYVTKTIDT	EKFKERRAH	LAEREWHRMK	ENNSQECRNC
V. parahaemo	101	RKVQASKELF	AHYVLGTIDT	PEKFQARRGH	LAEREWARMK	KNDSLECRNC
V. vulnificu	101	RKVQASKELF	AHFI-GTIDT	PEKFQERRAH	LAEREWARLK	KNDSLECRNC
		160	170	180	190	200
Orf 6	151	HEFD FMDYSQ	QGKRAAEQHS	TALASGDKTC	VDCHKGIAHK	LPDMHGVE-G
Pseudomonas	151	HSSIAMD LTK	QTKRAADIHT	RYLLSGKATC	IDCHKGIAHE	LPNMDGVDPG
V. cholerae	151	HQFN YMDFSE	QSSRAAQHS	TALASGEKTC	VDCHKGIAHN	LPDMHGVE-G
V. fischeri	151	HEFDYMD FSE	QGPRSAKQHS	TALASGEKTC	VDCHKGIAHK	LPDMSGIE-G
V. parahaemo	151	HEFDYMDYSQ	QGSRAAAQHS	TALASGDKTC	VDCHKGIAHK	LPDMSGVE-G
V. vulnificu	151	HEFEYMD FSE	QGARS AKQHS	TALASGEKTC	VDCHKGIAHK	LPDMHGVE-G
		210	220	230	240	250
Orf 6	201	WQ-----	-----	-----	-----*	
Pseudomonas	201	WKVPAELMGK	AASHGLQD TD	QLAAYLAARE	GNIID.....	
V. cholerae	201	WQ-----	-----	-----	-----	
V. fischeri	201	W-----	-----	-----	-----	
V. parahaemo	201	WQ-----	-----	-----	-----	
V. vulnificu	201	WQ-----	-----	-----	-----	

Figure 16. Alignment of the *orf6* gene product to *napC* gene products from *Pseudomonas* species strain G-179 (3), *Vibrio cholerae* O395 (14), *Vibrio fischeri* ES114 (34), *Vibrio parahaemolyticus* RIMD 2210633 (23), and *Vibrio vulnificus* YJ016 (5).

Phylogenetic trees were constructed based upon the percentage of identical residues in the Higgins-Sharp alignments and the results are shown in Figures 17, 18, and 19.

Figure 17. Phylogenetic tree of *orf4* and its homologous gene products. The numbers indicate the percentage of identical residues between the gene products.

Figure 18. Phylogenetic tree of *orf5* and its homologous gene products. The numbers indicate the percentage of identical residues between the gene products.

Figure 19. Phylogenetic tree of *orf6* and its homologous gene products. The numbers indicate the percentage of identical residues between the gene products.

Chapter 4: Discussion

The putative gene products of *phaC*, *orf4*, *orf5*, and *orf6* all most closely aligned in the same four *Vibrio* species, based on the BLAST data. Not only did the putative gene products match closely in amino acid sequence (shown Figures 14-19), these genes were of similar lengths and orientations as seen in Table 3 and Figure 20. The alignments of the four sequences were similar as well. The most significant difference was the gap between *phaC* and *orf6*, which was substantially longer in *Vibrio vulnificus* (12,600 bp) and *Vibrio cholerae* (9,740 bp) and possibly contains enough space for additional genes.

Table 3. Alignment of *PhaC* with *Orf4*, *Orf5*, and *Orf6*

<u>Organism</u>	<u>Gene</u>	<u>Start</u>	<u>Stop</u>
<i>Vibrio parahaemolyticus</i> (34)	<i>phaC</i>	1277496	1275724
	<i>orf4</i>	1272881	1274263
	<i>orf5</i>	1274345	1274800
	<i>orf6</i>	1274829	1275404
<i>Vibrio</i> species B-18 (17)	<i>phaC</i>	818	2590
	<i>orf4</i>	5530	4142
	<i>orf5</i>	4066	3607
	<i>orf6</i>	3579	3004
<i>Vibrio vulnificus</i> (5)	<i>phaC</i>	1330390	1328621
	<i>orf4</i>	1313482	1314864
	<i>orf5</i>	1314945	1315412
	<i>orf6</i>	1315449	1316021
<i>Vibrio cholerae</i> (14)	<i>phaC</i>	688177	686405
	<i>orf4</i>	674168	675550
	<i>orf5</i>	675620	676075
	<i>orf6</i>	676093	676665

Once sequenced, the *Bam*HI fragment revealed three open reading frames running in the complementary direction to *phaC* (Figure 20). Based on the BLAST search results,

orf4 most closely matched *napA*, *orf5* with *napB*, and *orf6* with *napC*. In addition the greatest homology in these sequences was found in organisms in the same genus, *Vibrio*. This suggests a common evolutionary history between them.

Figure 20. Alignment map of the *Bam*HI fragment from *Vibrio* species B-18 compared to the three other *Vibrio* species that showed the greatest homology. The large arrows indicate the orientation of the genes while the small vertical arrows indicate the distance between the genes. This figure is not drawn to scale.

The genes in the *napA-C* locus code for a periplasmic nitrate reductase involved in reducing nitrate (NO_3^-) to nitrite (NO_2^-) in bacteria (33, Figure 21). The *napA* gene codes for a large 90 kDa subunit, while the *napB* gene codes for a small subunit ranging in size between 13-19 kDa (10). The *napC* gene codes for a membrane-bound cytochrome *c* that assists in electron transfer to the soluble NapA/B complex. These genes have several possible functions including accepting electrons in anaerobic conditions, denitrification, or scavenging for nitrate when it is in limited quantities (4, 10,

37). The last possibility suggests a linkage between the *nap* and *pha* loci as PHA production is often induced under conditions where nitrogen is limiting.

Figure 21. Bacterial enzymes involved in modifying nitrogen-containing compounds (33).

Conclusion and Future Directions

There is the possibility that the *nap* locus is involved in processing intermediates in the nitrogen cycle, which in turn can affect the biosynthesis of PHA synthase, so these putative genes downstream of *phaC* may be indirectly involved in the regulation of PHA synthase expression and activity. Studies that explore the expression of *phaC* in the presence or absence of nitrate and nitrite could be helpful in discerning their roles. Another approach would involve creating mutations in the *nap* locus to compare the expression of *phaC* with and without the *nap* genes.

The identity of the open reading frames will only be confirmed by transcriptional and translational studies, but given the strong homology with previously discovered sequences in closely related organisms provides strong evidence for their putative identity. Since none of the three closest matches for sequence homology have had their identity determined empirically, a comparison with a more distant match was necessary

for each open reading frame in this analysis. A logical next step involves expression studies to ascertain the true identity and role of these genes.

In conclusion, while these new sequenced open reading frames are not directly involved in the synthesis of PHA synthase, they may have a strong indirect role in the expression of this gene.

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