SUPPLEMENTARY INFORMATION

Chemistry with an artificial primer of polyhydroxybutyrate synthase suggests a mechanism for chain termination

*Rachael M. Buckley*, *JoAnne Stubbe*
Construction of pRBphaCc-C406A by the primer overlap extension method.

Briefly, phaCc was amplified from pRBphaCc in two fragments: fragment 1 contained the 5’ region of phaCc from the start codon through the codon corresponding to C406, and was amplified using the primers phaCfw and C406Arev; fragment 2 contained the 3’ region from the codon corresponding to C406 to the stop codon, and was amplified using the primers C406Afw and phaCrev. Primer phaCfw inserted an NheI site onto the 5’ end of fragment 1, and C406Arev introduced the mutation. Primer phaCrev introduced a BamHI site onto the 3’ end of fragment 2 and C406Afw introduced the mutation. Fragments 1 and 2 were then combined and used as a template for a third PCR reaction using the primers phaCfw and phaCrev to “stitch” the two fragments together. The amplified product was digested with NheI and BamHI and ligated into pET28a digested with the same enzymes to generate pRBphaCc-C406A.

Table S1. Primers used in this study.

| Name         | Sequence<sup>a</sup>                                                                 |
|--------------|-------------------------------------------------------------------------------------|
| phaCΔNfw     | 5’ - GATATAGCTAGCGTCAAGCCCTCTCGGCGAAT - 3’                                        |
| phaCΔNrev    | 5’ - GATATAGGATCCTCAGGTTGACTTACCAGCAC - 3’                                        |
| phaCfw       | 5’ - GATATAGCTAGCATGGCCACGGCGAA - 3’                                              |
| phaCrev      | 5’ - GATATAGGATCCTCAGGTTGACTTACCAGCAC - 3’                                        |
| phaC406Afw   | 5’ - GCCGTAACCCGGTAGGCTACCCATCGGCGGCA - 3’                                        |
| phaC406Arev  | 5’ - AGCAGGGGTGCCGGCGATGCCGCTACCGTGTTGACGC - 3’                                  |
| phaC406Sfw   | 5’ - CCGTGGCTACTCCATCGGCGGACCC - 3’                                               |
| phaC406Srev  | 5’ - GGTTGCGCCCGATGGAGTGACCTACGGAC - 3’                                           |
| phaCD562Nfw  | 5’ - GTGCAGTCTCTGACTACCCAAACCACATCGCG - 3’                                       |
| phaCD562Nrev | 5’ - CGCAGTGTGGTGTCTCTGAGGACTGCAC - 3’                                            |
| phaCH590Qfw  | 5’ - CCATGGCCGCTGTCAGGACTCGCGCGGCGGCGATCAAACC - 3’                               |
| phaCH590Qrev | 5’ - GGTTGATACCCGGCGATGTAACCGGAACCACCACGCCATGGA - 3’                            |

<sup>a</sup>Restriction sites and mutated codons are underlined.
Table S2. Plasmids used in this study.

| Plasmid                | Description                                                                 | Reference |
|------------------------|-----------------------------------------------------------------------------|-----------|
| pRBphaCc               | *phaCc* inserted into pET28a between NheI and BamHI restriction sites; encodes N-terminal His$_6$-tag and the linker SSGLVPRGSHMAS containing a thrombin site (underlined). | This work |
| pRBphaCc-C406A         | Same as pRBphaCc, with point mutation C406A                                 | This work |
| pRBphaCc-C406S         | Same as pRBphaCc, with point mutation C406S                                 | This work |
| pRBphaCc-D562N         | Same as pRBphaCc, with point mutation D562N                                 | This work |
| pRBphaCc-H590Q         | Same as pRBphaCc, with point mutation H590Q                                 | This work |
| pRBphaCcΔN             | *phaCc*,ΔN inserted into pET28a between NheI and BamHI restriction sites; encodes N-terminal His$_6$-tag and the linker SSGLVPRGSHMAS containing a thrombin site (underlined). | This work |
**Figure S1.** Sequence alignment of PhaC<sub>Re</sub> and PhaC<sub>Cc</sub>. Black dots indicate conserved active site residues: Cys (C319 in PhaC<sub>Re</sub>, C406 in PhaC<sub>Cc</sub>); Asp (D480 in PhaC<sub>Re</sub>, D562 in PhaC<sub>Cc</sub>); and His (H508 in PhaC<sub>Re</sub>, H590 in PhaC<sub>Cc</sub>).
Purification of PhaCs.

Culture medium was supplemented with 50 µg/mL kanamycin (km). Single colonies were grown to saturation at 37 °C in 5 mL LB, then used to inoculate 2 L LB cultures in 6 L shake flasks. The 2 L cultures were grown at 37 °C with shaking at 220 rpm until they reached OD_{600} = 0.6, then rapidly cooled on ice to ~18 °C, and induced with 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). The cultures were grown an additional 4 h at 18 °C with shaking at 220 rpm, then the cells were harvested by centrifugation at 3,500 x g for 10 min. Cell pellets were flash frozen in liquid nitrogen and stored at -80 °C until use. A typical yield was 2 g wet cell paste per L.

All purification steps were carried out at 4 °C. Cells were resuspended in 3-5 mL/g wet cell paste in lysis buffer containing: 50 mM NaH_{2}PO_{4}, 300 mM NaCl, 10 mM imidazole, pH 8.0. The cells were lysed by two passes through a French pressure cell at 14,000 psi. The insoluble cell debris was removed by centrifugation at 17,000 x g for 20 min. The soluble fraction was equilibrated with 1 mL of Ni-NTA resin (Qiagen) per 5 mL of soluble extract for 30 min with gentle stirring. The slurry was loaded onto a column and allowed to settle, and the flow-through was collected. The column was washed with 20 column volumes (cv) lysis buffer, followed by 10 cv lysis buffer with 50 mM imidazole. Protein was batch eluted with lysis buffer containing 250 mM imidazole.
Table S3. Purification of PhaC<sub>Cc</sub>.

| Purification step         | Volume (mL) | Protein (mg) | Activity (U<sup>a</sup>/mg) | Total units | % yield<sup>b</sup> |
|---------------------------|-------------|--------------|------------------------------|-------------|---------------------|
| Crude soluble extract     | 11          | 180          | 4.9                          | 880         | 100                 |
| Crude insoluble extract   | 30          | 18           | 14                           | 250         | n/a                 |
| Ni-NTA flowthrough        | 10          | 120          | 1.4                          | 170         | 20                  |
| 10 mM imidazole wash      | 40          | 28           | 0.9                          | 25          | 2.8                 |
| 50 mM imidazole wash      | 24          | 12           | 8.5                          | 100         | 11                  |
| Pure sample               | 1.0         | 11           | 50                           | 550         | 63                  |

<sup>a</sup>One unit of activity is one µmol CoA released per min.

<sup>b</sup>% yield is calculated based on total units in crude soluble extract.

Table S4. Purification of ΔNPhaC<sub>Cc</sub>.

| Purification step         | Volume (mL) | Protein (mg) | Activity (U<sup>a</sup>/mg) | Total units | % yield<sup>b</sup> |
|---------------------------|-------------|--------------|------------------------------|-------------|---------------------|
| Crude soluble extract     | 8           | 87           | 0.3                          | 24          | 100                 |
| Crude insoluble extract   | 6.5         | 93           | 0.09                         | 8.1         | n/a                 |
| Ni-NTA flowthrough        | 14          | 63           | 0.1                          | 6.3         | 26                  |
| 10 mM imidazole wash      | 42.5        | 6.7          | 0.04                         | 0.3         | 1.2                 |
| 50 mM imidazole wash      | 25          | 5.6          | 0.09                         | 0.5         | 2.1                 |
| Pure sample               | 0.75        | 14           | 1                            | 14          | 60                  |

<sup>a</sup>One unit of activity is one µmol CoA released per min.

<sup>b</sup>% yield is calculated based on total units in crude soluble extract.
Figure S2. Typical 10% SDS-PAGE purification gel of PhaCc and ΔNPhaCc. A) Purification gel of ΔNPhaCc. Lane 1, $M_w$ standards. Lane 2, soluble cell-free extract. Lane 3, insoluble cell-free extract. Lane 4, Ni-NTA agarose flowthrough. Lane 5, 10 mM imidazole wash. Lane 6, 50 mM imidazole wash. Lane 7, final purified ΔNPhaCc. B) Purification gel of PhaCc. Lane 1, $M_w$ standards. Lane 2, whole cell pre-induction sample. Lane 3, whole cell post-induction sample. Lane 4, soluble cell-free extract. Lane 5, insoluble cell-free extract. Lane 6, Ni-NTA agarose flowthrough. Lane 7, 10 mM imidazole wash. Lane 8, 50 mM imidazole wash. Lane 9, final purified PhaCc.
Figure S3. Reactions of ΔNPhaC<sub>Cc</sub>, wt PhaC<sub>Cc</sub> and active site mutants of PhaC<sub>Cc</sub> with HBCoA at 30 °C. A) CoA release from ΔNPhaC<sub>Cc</sub>. The inset shows an expansion of the first five minutes to highlight the lag phase. B) CoA release from wt PhaC<sub>Cc</sub>. The inset shows an expansion of the region between 0-4 min. C) Initial rate of CoA release catalyzed by PhaC<sub>Cc</sub> as a function of [HBCoA]. Data are fit to the Hill Equation. D) CoA release from mutant PhaCs PhaC-D562N (●, blue), PhaC-C406S (□, red), PhaC-C406A (▲) and PhaC-H590Q (★). All error bars represent the standard deviation from the mean of three experiments.
Figure S4. CoA release from the reaction of PhaC<sub>Cc</sub> with sTCoA at 30 °C. Error bars represent standard deviation from the mean of three experiments. Equivalents of CoA released are given per PhaC monomer. CoA release from 30 s to 5 min occurs at 0.004 s<sup>-1</sup>.

Figure S5. Chemical competence of sT-PhaC<sub>Cc</sub>. A) GPC trace from the reaction of PhaC<sub>Cc</sub> with [1-<sup>14</sup>C]-(R/S)-HBCoA (specific activity = 175 cpm/nmol). Polymer elutes with a retention time of 12.9 min. Small molecules, which have not been identified, elute with retention times of 21-23 min. B) GPC trace from a reaction containing [<sup>3</sup>H]-sT-PhaC<sub>Cc</sub> and HBCoA. Polymer elutes as a
low, broad peak from 11-19 min, and small molecules, which have not been identified, elute after 21 min.

**Figure S6.** Effect of priming with sTCoA on the activity of PhaC<sub>Cc</sub>. A) The reactions contained 50 nM PhaC<sub>Cc</sub> reacted with either 1 mM HBCoA (●, blue) or PhaC<sub>Cc</sub> reacted first with 500 μM sTCoA then diluted to a final concentration of 50 nM PhaC<sub>Cc</sub> and 0.5 μM sTCoA (□, red). B) Expansion of the early time points. The first 2 min are fit to a linear function to calculate specific activity. The specific activity of the unprimed synthase was 42 U/mg, whereas the specific activity of the primed synthase was 72 U/mg.

**Quantitation of [<sup>3</sup>H]-sT-PhaC by Sephadex G50 chromatography.**

In a final volume of 75 μL, the first reaction contained: 50 μM PhaC<sub>Cc</sub> and 500 μM [<sup>3</sup>H]-sTCoA (specific activity 1900 cpm/nmol) in 20 mM Hepes pH 7.5, 20 mM NaCl. A second reaction contained in a final volume of 50 μL: 50 μM PhaC<sub>Cc</sub> and 500 μM [<sup>3</sup>H]-sTCoA (specific activity 1300 cpm/nmol) in 20 mM Hepes pH 7.5, 20 mM NaCl. Both reactions were initiated by addition of enzyme and immediately loaded onto 10 mL Sephadex G50 columns (7 mm x 300
mm) at 4 °C. The first reaction was eluted with 20 mM Hepes pH 7.5, 200 mM NaCl at 0.6 mL/min. The second reaction was eluted with 20 mM KH$_2$PO$_4$ pH 4.5, 2 M urea at 0.3 mL/min. The eluent was collected in fractions and analyzed by $A_{260}$, $A_{280}$, and scintillation counting.

**Figure S7.** Changes as a function of time in the amounts of the peaks at 15 min (green, closed triangles), 31 min (cyan, open triangles), 37 min (blue, closed circles), 52 min (green, open squares), sT$_4$CoA (dark blue, open diamonds), and the species from 56-65 min (red, closed squares). The peak with a retention time of 15 min had radioactivity but no $A_{260}$, and increased in the first min to ~30 nmol then did not increase further. The peak with a retention time of 31 min had $A_{260}$ and no associated radioactivity; its levels fluctuated throughout the reaction. Finally, the peak with a retention time of 37 min was associated with radioactivity but had no $A_{260}$, and was present in the control without enzyme; its levels fluctuated somewhat during the reaction as well. These three species were not identified. At the 5 min time point, approximately 18% of the total radioactivity eluted with retention times of 52-60 min with no apparent $A_{260}$. The identity of these species remains unknown, but they are likely acids, again formed from chain extension with small amounts of contaminating HBCoA and subsequent hydrolysis.
MALDI-TOF identification of selected small molecule products of the reaction of PhaC<sub>Cc</sub> with sTCoA.

In a final volume of 50 µL, the reaction contained: 50 µM PhaC<sub>Cc</sub> and 500 µM sTCoA in 20 mM Hepes pH 7.5, 20 mM NaCl. The sample was then treated and analyzed by HPLC as described in the Materials and Methods of the main text. The peaks from the HPLC were collected, flash frozen in liquid nitrogen and lyophilized. The lyophilized samples were re-dissolved in 100 µL ddH<sub>2</sub>O and submitted for MALDI-TOF analysis at the MIT Biopolymers Facility. Ten µL of each sample was desalted using a Zip-tip (C18, Millipore, Billerica, MA) and eluted with 50% acetonitrile, 0.1% trifluoroacetic acid (TFA) and then combined 1:1 with matrix (α-cyano-4-hydroxycynammic acid) and analyzed.

**Figure S8.** MALDI-TOF analysis of the products of reaction of sTCoA and PhaC<sub>Cc</sub>. A) Negative mode MALDI-TOF spectrum of the peak with an elution time of ~54 min. sT<sub>4</sub>CoA has a predicted M<sub>w</sub> of 1095.9 Da. The sample contained a peak with a m/z of 1095.3, consistent with the predicted m/z of the [M-H]<sup>-</sup> species of sT<sub>4</sub>CoA (1094.9). B) Positive mode MALDI-TOF of
sDCoA (retention time 43 min) in positive mode. The calculated $M_w$ of is 923.71 Da, and the major peak has a m/z of 924.75, consistent with [M+H]$^+$. 

Analysis of the products of reaction of PhaEC$_{Av}$ and sTCoA.

PhaEC$_{Av}$ was expressed and purified as previously described.$^1$ The reaction was carried out at 30 °C and contained 50 μM PhaEC$_{Av}$ and 500 μM sTCoA in a final volume of 110 μL. After 10 min and 60 min reaction, 50 μL aliquots were withdrawn and quenched in 20 μL ice cold 10% TCA. The samples were centrifuged at 4 °C for 5 min at 20,000 x g to pellet precipitated protein. The supernatant was removed and the pellet was washed with 50 μL ice cold H$_2$O. The wash and supernatant were combined and adjusted to pH ~5 by careful addition of 1 M NaOH with rapid mixing to avoid high local concentrations of base. One hundred μL each sample was injected onto a nucleotide/nucleoside column (Alltech, 7 μm, 4.6 mm x 250 mm) attached to a Waters HPLC equipped with a diode array detector. Samples were eluted with 20 mM KP$_i$, pH 4.7 (mobile phase A) and methanol (mobile phase B) using a linear gradient from 5% to 70% mobile phase B and from 0-60 min.
Figure S9. Small molecule products from the reaction of PhaEC$_{Av}$ with sTCoA. The reaction was carried out for 10 min (red) and 60 min (blue) at 30 °C. The samples were analyzed as described in the Experimental Procedures section for the analysis of the reaction of PhaC$_{Cc}$ with sTCoA. A) A$_{260}$ trace, showing a peak at 31 min (also observed in the reaction with sTCoA and PhaC$_{Cc}$), the increase in the peak at 43 min (sDCoA) and a decrease in the peak at 47 min (sTCoA). B) Expansion of the region around the peak corresponding to sDCoA.

REFERENCES

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