Engineering nonphotosynthetic carbon fixation for production of bioplastics by methanogenic archaea

Kershanthen Thevasundaram, Joseph J. Gallagher, Freeman Cherngb, and Michelle C. Y. Chang

The escalating impact of greenhouse gas emissions on the environment has driven efforts to develop new approaches to capture, sequester, and utilize CO₂ (1–5). One strategy to recycle CO₂ is to use it as a feedstock for chemical synthesis in place of petroleum sources, thereby sequestering CO₂ while reducing its production. Biological carbon fixation is particularly well suited to this goal, as diverse organisms have evolved to trap ambient CO₂ as a carbon source for biosynthesis and growth. Indeed, photosynthetic carbon fixation serves to fix and capture 120 Gt each year and plays a key role in the global carbon cycle (6). Consequently photosynthetic autotrophs have been developed as a metabolic engineering hosts for chemical synthesis (7–11) but can present challenges for industrial fermentation because of the requirement for light as an energy source and the need to manage light penetration, low thermodynamic efficiency, and solar intermittency (12). As an alternative, engineering non-photosynthetic carbon fixation pathways with downstream biosynthetic pathways from heterotrophs to produce heat, fuels, chemicals, pharmaceuticals, and others in heterotrophic hosts (Scheme 1) (15–17). Their central metabolism is the most energy-efficient carbon fixation pathway in nature, as measured by the adenosine triphosphate (ATP) requirement per mole of fixed CO₂ (18). Methanogens have also been utilized in anaerobic digesters to valorize waste biomass streams such as landfill waste and cattle manure to produce biomethane, an easily separated metabolic by-product, which could be used as a source of heating fuel, chemical precursors, and energy (19). Although the use of acetyl-CoA is relatively limited in methanogens, it serves a central building block for producing a broad range of products such as food, feed, chemicals, fuels, pharmaceuticals, and others in heterotrophic hosts (Scheme 1) (20–22). It may, therefore, be possible to interface upstream chemoautotrophic metabolism for carbon fixation with downstream biosynthetic pathways from heterotrophs to ultimately synthesize a range of targets from CO₂ and H₂ or soluble carbon and energy sources such as formate. However, the evolutionary distance between archaea and other metabolic engineering; K.T., J.J.G., and F.C. performed research; K.T., J.J.G., and M.C.Y.C. analyzed data; and K.T., J.J.G., and M.C.Y.C. wrote the paper. The authors declare no competing interest.

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engineering its availability and turnover was essential for reaching high product yields. These results demonstrate the potential of this species as a platform for the production of value-added chemicals from CO₂ and H₂. By using H₂ or other chemical energy sources rather than light as the energy input, biosynthesis in chemoautotrophs can be driven electrochemically using inorganic catalysts and renewable sources of energy, including but not limited to solar power (30, 31). In conclusion, combining these complementary processes into a hybrid, versatile platform could provide a route to sustainable chemical synthesis from CO₂.

Results

Design and Analysis of a Synthetic Pathway for Monomer Production. We initiated these studies by designing a single, three-gene, NADH-dependent pathway for conversion of acetyl-CoA into S-3HB. The pathway consists of the well-characterized enzymes PhaA (Ralstonia eutropha), Hbd (Clostridium acetobutylicum), and TesB (Escherichia coli) (32–34) (Fig. 1A). The Hbd ketoreductase was selected based on its use of NADH rather than NADPH, which is attributed to its use in energy metabolism and also results in its formation of the 35 stereoisomer. A library of polycistronic and monocistronic constructs was assembled and screened for mRNA and protein expression (SI Appendix, Table S1 and Fig. S1). Engineered strains expressing polycistronic constructs containing the phaA-hbd-tesB pathway had detectable mRNA for pathway genes in many cases (SI Appendix, Fig. S2). However, protein levels from pathway genes were undetectable, suggesting a possible bottleneck in mRNA translation. When testing monocistronic constructs, we achieved robust protein expression of all enzymes in the pathway using the PmcrB promoter from Methanosarcina barkeri (SI Appendix, Fig. S2).

To better understand the expression profile, we carried out long-read sequencing of 5’ untranslated regions of native monocistronic transcripts from M. maripaludis. Analysis of these sequences revealed a median 5’ untranslated region length of 20 nucleotides with a bias toward two guanine bases 8 nucleotides upstream from the start codon and a weak bias toward a 5’ – GAGGT – 3’ sequence starting at the +8 position (SI Appendix, Fig. S3). This finding suggests that M. maripaludis may utilize a consensus ribosome-binding site sequence that is recognized by the 3’ end of the 16S rRNA sequence found in M. maripaludis, resembling a bacteria-like ribosome binding sequence because of its position upstream of the Hbd promoter from M. maripaludis (35, 36). The PmcrB promoter used for our monocistronic constructs does contain a 5’ – GAGGA – 3’ sequence from 8 to 12 nucleotides away from the start codon, which may explain the successful protein expression with this promoter (37). Interestingly, the 5’ region upstream of flaB1 or hbrC1 did not yield detectable protein expression when included as promoter elements in our constructs and did not include this consensus in the 8 to 12 nucleotides upstream of the start codon (5’- AATAC – 3’, flaB1; 5’ - GAGGT – 3’, hbrC1).

Despite expression of all pathway enzymes, S-3HB titers were below the limit of detection when engineered strains were grown in rich, undefined McCas medium. To troubleshoot the lack of small-molecule production, we generated strains expressing each of the pathway enzymes alone and tested for enzyme activity in cell lysates supplemented with substrates and cofactors (Fig. 1B). This result suggests that the production problem was not due to an intrinsic lack of enzyme activity but another limiting factor.
Identifying Metabolic Bottlenecks for Small Molecule Production. Since methanogenesis has evolved to use coenzyme F420 and 2[4Fe-4S] Fd as cofactors rather than NADH (SI Appendix, Figs. S4 and S5) (15), we postulated that intracellular NADH levels could be low in methanogens. Low intracellular NADH levels could limit the flux through the ketoreduction step catalyzed by Hbd and, consequently, the production of S-3HB. To estimate the overall usage of NADPH, we used a genome-scale metabolic model of *M. maripaludis* (imMR539) (38) to perform flux balance analysis (39), assuming standard nutrient consumption rates in defined mineral medium with supplemented acetate. From this model, NAD(P)H appears to be predominantly associated with amino acid and coenzyme biosynthesis pathways. Despite the variety of reactions that are putatively associated with NAD(P)H, our flux balance analysis results also suggested that the steady-state turnover of these cofactors is less than 1.5% of the turnover of Fd and coenzyme F420 (SI Appendix, Fig. S5). This suggests that the majority of redox cofactor-dependent activity in *M. maripaludis* is confined to a small number of Fd- and coenzyme F420-dependent reactions, most of which appear to be in central metabolism.

To test this hypothesis, we measured the NAD(H) levels in *M. maripaludis* grown in McCas medium and compared these with NAD(H) levels in *E. coli* grown anaerobically in Luria-Bertani medium. Remarkably, we found that the total NAD(H) levels in *M. maripaludis* were 12.7 ± 2.4% of the NAD(H) levels in *E. coli*. It is interesting to note that the NAD+ to NADH ratio in *M. maripaludis* is higher (SI Appendix, Fig. S5), although the role of this ratio in methanogens has not been clearly elucidated compared with *E. coli*, where it is known to regulate transcription, allosterically modulate enzyme activity, and control metabolic flux (40). Measuring expression levels of *de novo* NAD biosynthesis pathway genes from *M. maripaludis* by qPCR showed that they were expressed at less than 30% of the levels for a housekeeping gene control (SI Appendix, Fig. S6), suggesting that NAD biosynthesis rates may also be low. Overall, this suggests that low NAD(H) levels may represent a metabolic bottleneck for our biosynthetic pathway and more generally for other NADH-dependent pathways expressed in this species as well.

Increasing NAD(H) Availability via Nitrogen Metabolism and Alanine Dehydrogenase. We next sought to gain more insight into NAD(H) use in this species by performing affinity chromatography using adenosine monophosphate–agarose on crude lysates to enrich for proteins that bind to adenosine-containing cofactors like NAD. Using shotgun proteomics on this enriched protein sample, we found alanine dehydrogenase (ald) to be the most abundant protein (Dataset S1). Ald catalyzes NAD+-dependent oxidative deamination of L-alanine to liberate ammonium and pyruvate and had previously been identified to be essential for growth of *M. maripaludis* in mineral medium with L-alanine as the sole nitrogen source (McNA-Ala) (41). Heterologous expression, purification, and biochemical characterization showed that the *K_m* for Ald with respect to NAD+ (0.16 ± 0.02 mM) was not particularly low compared with other NAD-dependent Alds (~0.01 to 0.2 mM) (42, 43) (SI Appendix, Fig. S7). Based on these observations, we hypothesized that growth of *M. maripaludis* in McNA-Ala medium may drive greater in vivo NAD+ turnover than growth in McCas medium, leading to higher titers of S-3HB. To test this hypothesis, *phaA*–*hbd*–*tesB*-containing strains of *M. maripaludis* were grown in McNA-Ala medium as well as a defined medium with 10 mM ammonium chloride as the sole nitrogen source (McNA) and McCas medium. Through these production experiments, we found that growth in McNA-Ala medium led to S-3HB titers of 16.3 ± 2.7 mg/L, the highest titer of the three media conditions (Fig. 2D).

Next, transcriptomic studies were performed to elucidate the effects of medium choice on production titers (Fig. 2E and SI Appendix, Fig. S8). During growth in either minimal media condition (McNA and McNA-Ala), most genes involved in the modified Wood–Ljungdahl and methanogenesis pathways were up-regulated compared with the rich McCas medium. Since
amino acids are not supplied in minimal media (with the exception of Ala as a nitrogen source in McNA-Ala), acetyl-CoA formation is necessary for cell growth. Conversely, acetyl-CoA formation may be more limited in McCas medium since most amino acids, vitamins, and cofactors can be taken up from the medium. This suggests that acetyl-CoA production could be a metabolic bottleneck in a rich medium such as McCas but can be increased in a minimal medium in which greater biosynthetic activity is required. Isotopic labeling studies with $^{13}$C-alanine were carried out to assess its role in increasing 3-HB production, which could be related to altering carbon flux and redox state or by direct usage of Ala-derived pyruvate as a carbon source (SI Appendix, Fig. S9).

Interestingly, Ald (MMP1513) was up-regulated in both minimal media conditions, not just in McNA-Ala, in which Ala is provided as the sole nitrogen source. We interpret these data to mean that M. maripaludis must use Ald to produce Ala, an essential amino acid, during growth in McNA. Conversely, the reverse reaction is necessary for growth when Ala is the sole nitrogen source, suggesting that NAD$^+$ regeneration is a necessary feature of growth in McNA-Ala medium, in particular. Consistent with this interpretation, NADH levels and the NADH to NAD$^+$ ratio were observed to be higher in McNA-Ala medium than during growth in McCas or McNA media (Fig. 2B and C). In addition, the highest expression of the sodium-alanine symporter gene (ageS) was observed in McNA-Ala growth conditions, suggesting that it may be up-regulated to import Ala (SI Appendix, Fig. S7). Follow-up comparative proteomic studies revealed that Ald protein levels were higher in McNA-Ala growth than in McNA (SI Appendix, Fig. S10). These observations led us to the model in which increased intracellular Ala flux and up-regulation of Ald could provide a thermodynamic driving force for the oxidative deamination and NADH regeneration activity of ald during growth in McNA-Ala medium, leading to improved NADH to NAD$^+$ ratios. Overall, our data suggest that the choice of medium in our experiments had a profound influence on acetyl-CoA formation and NADH-dependent metabolism, particularly through the activity of Ald.

**Engineering NAD(H) Pools in M. maripaludis.** In order to increase NAD(H) pools, we used two different approaches to engineering M. maripaludis biosynthesis (Fig. 3A). The first approach was to tune native NAD biosynthesis, which avoids heterologous expression issues but is subject to native regulation and feedback regulation that could set a ceiling on the increases available. The second approach was to design a synthetic salvage pathway that could allow for potentially higher NAD levels overall. We first systematically overexpressed each gene in the de novo NAD biosynthesis pathway native to M. maripaludis. This strategy revealed that overexpression of the first three steps in the pathway, aspartate dehydrogenase (MMP0737), quinolinate synthase (nadA), and quinolinate phosphoribosyltransferase (nadC), led to an approximately two-fold increase in total NAD(H) levels during growth in McCas medium (Fig. 3B), with similar results in McNA and McNA-Ala media (SI Appendix, Fig. S11). The effect of overexpression of these genes may relate to increasing flux to the first dedicated step of NAD biosynthesis to produce iminoaspartate. Since iminoaspartate is an unstable intermediate, overexpression of the following two steps may help drive the overall pathway equilibrium forward away from decomposition. McNA medium was used for all subsequent production experiments because of its defined components and relative simplicity.

In addition to exploring de novo NAD biosynthesis, we also tested the ability of a hybrid cofactor salvage pathway to
increase NAD(H) pools. This approach allowed us to titrate the amount of supplemented NAD precursor and perhaps better optimize conditions. We chose nicotinamide as a precursor because it has no charge under physiological conditions and is known to be transported across the membrane (47). Unlike many bacterial and eukaryotic species, no nicotinamide salvage transporter or by passive diffusion through the membrane, as it is a small and neutral molecule at the pH of the medium. Comparing the two approaches for increased NADH pools, the greatest improvement in NAD(H) levels came from overexpression of the synthetic pathway to produce PHB. Our original synthetic pathway to produce PHB. Our original synthetic

Engineering a Synthetic Pathway for PHB and R-3HB Monomers. Strains with engineered NAD(H) were further modified with a synthetic pathway to produce PHB. Our original synthetic pathway to produce PHB. Our original synthetic pathway to produce PHB. Our original synthetic pathway to produce PHB. Our original synthetic pathway to produce PHB. Our original synthetic pathway to produce PHB. Our original synthetic pathway to produce PHB. Our original synthetic pathway to produce PHB. Our original synthetic pathway to produce PHB. Our original synthetic pathway to produce PHB. Our original synthetic pathw...
pathway was modified by replacing Hbd with an NADH-dependent PhaB from \textit{Halomonas bluephagenesis}. This enzyme preserves the use of NADH rather than NAPD and thus produces the \( R \)-3-hydroxybutyryl-CoA, the stereoisomer required for incorporation into the growing PHB polymer by PhaC (49). We then constructed a third biosynthetic pathway that contains the PHB polymerase (PhaC) from \textit{Cupriavidus necator} (50) in combination with PhaA from \textit{C. acetobutylicum} and PhaB from \textit{H. bluephagenesis}. Initial measurements showed that PHB product titers (67 ± 2 mg/L) were similar to those of \( S \)-3HB (75 ± 4 mg/L) and \( R \)-3HB (90 ± 7 mg/L). Adding the NadMV salvage pathway and supplementing with 50 mM nicotinamide to the PhaABC pathway then led to a 48 ± 4% increase in PHB titers (Fig. 4). Characterization of the PHB molecular weight by gel permeation chromatography showed that the average molecular weight was 5.6 × 10^6 g/mol (SI Appendix, Fig. S13).

Given that endogenous reactions to recycle NAD\(^+\) back to NADH are not expected to occur widely in \textit{M. maripaludis}, a formate dehydrogenase (Fdh1) from \textit{Candida boidinii} (51) was then introduced for this purpose. Fdh1 is a single subunit enzyme that has been shown to improve NADH yield in engineered heterotrophic hosts by increasing NAD\(^+\) turnover by its reduction with formate (52). Since formate is a known substrate for growth for \textit{M. maripaludis}, formate should be transportable using native mechanisms (53). As an added benefit, the CO\(_2\) produced by formate oxidation can be used as a carbon source for biosynthesis so that it is not released from media during the fermentation process. Initial characterization showed that \textit{M. maripaludis} strains containing Fdh1 had 2.0–6.0-fold higher formate-dependent NAD\(^+\) turnover rates compared with wild type (Fig. 3D). Next, NadMV and Fdh1 were combined with both the PhaAB-tesB and PhaABC pathways, which showed a further improvement of 25 to 43% to final titers of 158 ± 6 mg/L (\( R \)-3HB) and 171 ± 4 mg/L, respectively (Fig. 4). These yields correspond to 26.3 ± 1.6% and 24.0 ± 1.9%, respectively, of dry cell weight, which shows that a significant amount of \textit{M. maripaludis} carbon flux could be diverted to our synthetic pathway. The combination of higher NAD(H) pools from NadMV and improved in vivo NAD\(^+\) turnover had a synergistic benefit, leading to higher product titers than strains expressing either NadMV or Fdh1 alone. Both of these strains were observed to have higher productivity in a formate- and nicotinamide-dependent manner, indicating that the improvements in product titer were directly related to the introduced genetic modifications (SI Appendix, Fig. S14). The difference in productivity between NadMV-Fdh1–containing strains and unoptimized strains broadened over the course of production, suggesting that our improvements to the NAD(H) pool may lead to more robust production compared with unoptimized strains over time.

### Discussion

Carbon fixation or primary production plays an essential part of the global carbon cycle, providing the chemical basis for life on Earth by transforming inorganic to organic carbon. Despite the enormous scale, biological carbon fixation remains an environmental process and relatively untapped for industrial chemical production. Although less well-characterized than photosynthesis, non-phototrophic CO\(_2\) assimilation is thought to contribute 5 to 22% of ocean primary production and occurs without light input (54). As such, chemoaautotrophs allow sustainable synthesis using CO\(_2\) as the carbon building block with a broader range of sustainable energy inputs, such as electrocatalytically or photocatalytically generated H\(_2\). Given the low efficiency of photosynthetic carbon fixation (~8 to 9%) (55), it is also possible that coupling sustainable H\(_2\) or formate production to engineered chemoaototrophic hosts could match the yields and efficiencies of photosynthetic organisms.

In this work, we focused on developing methods to interface the carbon-fixation abilities of methanogenic archaea with downstream biosynthetic pathways derived from heterotrophs. \textit{M. maripaludis} uses the modified Wood–Ljungdahl pathway for CO\(_2\) assimilation, where two equivalents of CO\(_2\) are used to generate one acetyl-CoA in the most energy-efficient carbon-fixation pathway known in nature in terms of ATP required per mole of fixed CO\(_2\). One challenge in utilizing methanogenic archaea as a metabolic engineering host platform is that they have evolved to use orthogonal redox cofactors, compared with typical downstream biosynthetic pathways that utilize NAD(P)(H). Toward this end, we utilized 3HB and PHB production as a model heterotrophic pathway to examine its potential as a host for metabolic engineering; as there are many other systems to benchmark its production. Heterotrophs fed sugar-beet molasses, sucrose, cooking oils, glucose, and other carbon sources, of course, achieve the highest yields of more than 50% of cell dry weight in pilot and larger-scale production (56, 57). The native bacterial PHB producer, \textit{Cupriavidus necator}, which can grow heterotrophically or chemoaototrophically via the Calvin–Benson–Bassham cycle, can produce similar yields of 80% cell dry weight of PHB using CO\(_2\) and H\(_2\) (58).

### Table

<table>
<thead>
<tr>
<th>Strain</th>
<th>Pathway 1</th>
<th>Pathway 2</th>
<th>NADH regeneration</th>
<th>Nicotinamide</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>phaAB-hbd-tesB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>phaAB-tesB</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>3</td>
<td>phaAB-tesB</td>
<td>nadMV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>phaAB-tesB</td>
<td>Fdh1</td>
<td></td>
<td>50 mM</td>
</tr>
<tr>
<td>5</td>
<td>phaABC</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>6</td>
<td>phaABC</td>
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<td>8</td>
<td>phaABC</td>
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<tr>
<td>9</td>
<td>phaABC</td>
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![Fig. 4](https://www.pnas.org)
However, other native chemoautotrophic and phototrophic producers of PHB that utilize the Wood–Ljundahl pathway produce ~20 to 30% cell dry weight under optimized scale-up conditions. There are also other chemoautotrophs, such as acetogens, that can be engineered to produce 3HB and PHB, as they contain Rnf complexes that allow for equilibration between Fd and NAD pools (59). In comparison, our engineered strain of *M. maripaludis* can achieve similar yields of PHB (25%) as these native producers under unoptimized, laboratory-scale growth, suggesting that it could serve as a reasonable host for metabolic engineering, given its other attributes, such as the high ATP utilization efficiency for carbon fixation, the production of methane as a valuable by-product, and the potential to use formate as a carbon and electron source to avoid gas mass-transfer issues with H2. At this time, *M. maripaludis* is still in the relatively nascent stages of development as an industrial host and further work to identify sequence determinants for tunable gene expression, the use of an expanding genetic toolkit for genomic engineering, and the development of more robust metabolic models will assist with the continuing domestication of this host for metabolic engineering.

A long-term goal is to work toward domesticating new hosts for metabolic engineering, as the broad range of chemical phenotypes in nature can serve as advantages in accelerating the formation of a fermentation-based industry for chemical production. Given the scale of the challenge, many different solutions are needed to aggregate reduce environmental impact and increase efficiency of energy and resource utilization. Compared with canonical fermentation approaches using photoautotrophs or heterotrophs, a hybrid fermentation approach that uses complementary microbial and electrochemical catalysts could have lower land requirements and provide soluble carbon and energy sources in situ for fermentation. In this approach, purified CO2, water, and renewable electricity could be provided as inputs, and value-added chemicals derived from acetyl-CoA could be produced, such as alcohols, amino acids, and isoprenoids. In addition, methane could be harnessed using similar processes as those typically used in anaerobic digesters to generate a renewable fuel and energy source. Through a range of physiological studies, we were able to design a highly expressed engineered pathway in *M. maripaludis*. With further transcriptomic and proteomic studies in addition to other experiments, we showed that NADH availability was a limiting factor for small-molecule production. Using a combination of a synthetic nicotinamide salvage pathway and a formate dehydrogenase to recycle the NADH consumed in our pathway, we were able to achieve titers of PHB and its monomer of up to 171 ± 4 mg/L and 24.0 ± 1.9% of cell biomass, which is two orders of magnitude more than previous efforts in its use as a host (23). Taken together, we hope that insights presented in this work provide a foundation for more extensive metabolic engineering efforts in *M. maripaludis* and other archaea, allowing us to better tap the diverse chemical abilities found in nature.

**Materials and Methods**

**Materials.** Reagents were purchased from commercial sources as described in SI Appendix and used without further purification. Ultra-high-purity gases purchased from Praxair were used for all anaerobic manipulations. Purified CO2, water, and renewable electricity were provided for all anaerobic manipulations. Distilled water was deionized using a STAK-PAK palladium catalyst and desiccant system in a Coy Laboratory Products unheated fan box. *M. maripaludis* cultures were propagated in 18 x 150 mm Balch tubes with butyl rubber stoppers and aluminum crimp seals using defined mineral media ([McNA (48) or McNA-Ala] or complex, undefined medium [McCas (37)]). In unoptimized conditions, cultures were grown at 37 °C without shaking. Under optimized conditions, 10-mL cultures were grown in 250-mL anaerobic glass bottles at 30 °C with shaking at 250 rpm in defined media. Bottles were sparged with H2/CO2 (80%/20%) to 275 kPa every 24 h for 14 d. Stock cultures were stored at room temperature in the dark after growth and were propagated by diluting 1:100 into fresh McCas, McNA, or McNA-Ala medium at least every 2 wk. The stock cultures were used until changes in growth patterns were observed. Glycerol stocks were kept at −80 °C for long-term culture storage.

**Measurement of Intracellular NADH Pools.** For quantification of the NAD+ and NADH pools, overnight cultures of *M. maripaludis* or anaerobically grown *E. coli* were used. The assay was performed using an NAD+/NADH Glo Assay Kit (Promega) according to the manufacturer’s protocol for measuring the NAD+ and NADH pools individually with minor modifications. Cells were harvested by centrifugation for 1 min at 13,000g at room temperature. For *E. coli*, 500 μL of pelleted culture was used per sample. For *M. maripaludis*, 5 mL of pelleted culture was used per sample. After resuspending in at least 500 μL of lysin buffer, the samples were mixed with 0.1-mm glass disruption beads (300 μL) in 2-mL O-ring screw-cap tubes and lysed using a BioSpec Products Mini-BeadBeater by beating for 45 s at 4 °C. For cell dry weight measurements, 20 mL of culture from each sample was centrifuged and collected into a tared 2-mL tube by two rounds of centrifugation for 1 min at 13,000g at room temperature. As much residual medium as possible was removed before samples were vacuum concentrated for 30 min at room temperature. The change in mass of tubes was determined using a microbalance.

**3-HB Quantification Using High-Performance Liquid Chromatography-Tandem Mass Spectrometry.** Production cultures were sampled 7 d postinoculation and culture samples were stored at −80 °C until analysis. Inoculated samples were screened for stable genetic expression after 2 d. Cell culture samples (1 mL) were thawed, mixed with 0.1-mm glass disruption beads (300 μL) in 2-mL O-ring screw-cap tubes, and then lysed using a BioSpec Products Mini-BeadBeater by beating for 45 s at 4 °C. Cell lysates were centrifuged for 10 min at 20,000g at 4 °C to pellet cell debris. Cleared cell lysates (50 μL) were diluted into 50 μM adipic acid (150 μL), which served as the internal standard. Samples were filtered through a 96-well MultiScreenHTS plate (Millipore-Sigma) and analyzed on an Agilent 1290 high-performance liquid chromatography (HPLC)-6460 triple quadrupole mass spectrometer equipped with an autosampler. Samples were chromatographed on a Rezex-ROA Organic Acid H+ column (150 x 4.6 mm, 8 μM; Phenomenex) fitted with a CarboH+ Security Guard cartridge (Phenomenex) at 55 °C with an isocratic gradient of 0.5% (volume per volume) formic acid as the mobile phase (0.3 mL/min) for 10 min. 3-HB was quantified by mass spectrometry (MS) on an Agilent 6460 triple quadrupole mass spectrometer with an electrospray ionization source, operating in negative ion multiple reaction monitoring transition mode with the fragmentor voltage set at 70 V. Between 5 and 8 min, the following transitions and collision energies were monitored: *m/z* 145.1 → 83.1, 10 V (adipic acid, internal standard); *m/z* 103.1 → 59.2, 5 V (3-hydroxybutyric acid). Samples were quantified relative to a standard curve of 7.8125, 15.625, 31.25, 62.5, 125, 250, 500, and 1,000 mg/L 3-HB prepared in McCas medium.

**PHB/3HB Quantification Using High-Performance Liquid Chromatography-Ultraviolet.** Production cultures were sampled 14 d postinoculation and culture samples were stored at −80 °C until analysis. Inoculated samples were screened for stable genetic expression after 2 d. Cell culture samples (1 mL) were thawed, and centrifuged for 10 min at 20,000g at 4 °C to pellet cells. Cell pellets were acid digested using concentrated sulfuric acid (500 μL) at 90 °C for 30 min. They were then neutralized with 1 M KOH (500 μL) and filtered through a 96-well MultiScreenHTS plate. The filtered sample (100 μL) was diluted into 50 μM adipic acid (100 μL), which served as the internal standard. Samples were analyzed using an Agilent 1260 HPLC-multi wavelength detector equipped with an autosampler. Samples were chromatographed on an Aminex HXP-87H ion-exclusion organic acid analysis column (300 x 7.8 mm, 9 μM; Bio-Rad) fitted with an Aminex XBP-85X ion-exclusion guard column (Bio-Rad) at room temperature with an isocratic gradient of 0.014N H2SO4 as the mobile phase (0.6
ml/min) for 30 min. Absorbance of crotonic acid and adipic acid was measured at 235 nm and 425 nm, respectively. Samples were quantified relative to a standard curve of 31.25, 62.5, 125, 250, 500, and 1,000 mg/L crotonic acid prepared in MilliQ water. For cell dry weight measurements, 2 mL of culture from each sample was centrifuged and was collected into a tared 2-mL tube by two rounds of centrifugation for 1 min at 13,000 g at room temperature. As much residual medium as possible was removed before samples were vacuum concentrated for 30 min at room temperature. The change in mass of tubes was determined using a microbalance.

Data Availability. All studies data are included in the article and/or supporting information, including sequences and source data for all figures.

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Author affiliations: Department of Molecular & Cell Biology, University of California, Berkeley, CA 94720-3200; Department of Chemistry, University of California, Berkeley, CA 94720-4760; Department of Chemical & Biomolecular Engineering, University of California, Berkeley, CA 94720-1462.