The conversion of CO\textsubscript{2} to value-added products allows both capture and recycling of greenhouse gas emissions. While plants and other photosynthetic organisms play a key role in closing the global carbon cycle, their dependence on light to drive carbon fixation can be limiting for industrial chemical synthesis. Methanogenic archaea provide an alternative platform for an autotrophic microbial species capable of non-photosynthetic CO\textsubscript{2} fixation, providing a potential route to engineered microbial fermentation to synthesize chemicals from CO\textsubscript{2} without the need for light irradiation. One major challenge in this goal is to connect upstream carbon-fixation pathways with downstream biosynthetic pathways, given the distinct differences in metabolism between archaea and typical heterotrophs. We engineered the model methanogen, *Methanococcus maripaludis*, to divert acetyl-coenzyme A toward biosynthesis of value-added chemicals, including the bioplastic polyhydroxybutyrate (PHB). A number of studies implicated limitations in the redox pool, with NAD(P)(H) pools in *M. maripaludis* measured to be <15% of that of *Escherichia coli*, likely since methanogenic archaea utilize F\textsubscript{420} and ferredoxins instead. Multiple engineering strategies were used to precisely target and increase the cofactor pool, including heterologous expression of a synthetic nicotinamide salvage pathway as well as an NAD\textsuperscript{+}-dependent formate dehydrogenase from *Candida boidinii*. Engineered strains of *M. maripaludis* with improved NADH pools produced up to 171 ± 4 mg/L PHB and 24.0 ± 1.9% of dry cell weight. The metabolic engineering strategies presented in this study broaden the utility of *M. maripaludis* for sustainable chemical synthesis using CO\textsubscript{2} and may be transferable to related archaean species.

Carbon fixation | metabolic engineering | archaea | bioplastics | redox cofactors

The escalating impact of greenhouse gas emissions on the environment has driven efforts to develop new approaches to capture, sequester, and utilize CO\textsubscript{2} (1–5). One strategy to recycle CO\textsubscript{2} is to use it as a feedstock for chemical synthesis in place of petroleum sources, thereby sequestering CO\textsubscript{2} while reducing its production. Biological carbon fixation is particularly well suited to this goal, as diverse organisms have evolved to trap ambient CO\textsubscript{2} 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 where chemoautotrophic organisms use hydrogen (H\textsubscript{2}) and other compounds as sources of energy and electrons rather than light offers new possibilities (13, 14).

Among the chemoautotrophs, methanogens stand out for their favorable metabolic characteristics. Methanogens are a group of obligately anaerobic archaean that use a modified Wood–Ljungdahl pathway to fix CO\textsubscript{2} for cell growth via acetyl-coenzyme A (acetyl-CoA) as an intermediate (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 CO\textsubscript{2} (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 CO\textsubscript{2} and H\textsubscript{2} or soluble carbon and energy sources such as formate. However, the evolutionary distance between archaea and other
Chemolithotrophic metabolic engineering. The methanogen
well-characterized pathways from typical heterotrophic organisms.
The relatively orthogonal metabolic processes of archaea with the more
CoA as an intermediate for biosynthesis and methane. However, their biosyn-
production. In nature, NAD(P)H is used as the predominant
reduction reactions (27). However, methanogenic archaea have
time to use ferredoxins (Fds) and coenzyme F420 instead for
redox systems between the upstream
methanogenic archaea have been
determined empirically and gene regulatory processes have not
orthogonal pathway between the upstream
methanogens (5
34) (Fig. 1
A). The Hbd
ketoeductase 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
polymeric and monocistronic constructs was assembled and
screened for mRNA and protein expression (SI Appendix, Fig. S1).
Engineered strains expressing polymeric 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 Promob
promoter from Methanocarcina 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’ – GGGGG – 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 start codon
(35, 36). The Promob promoter used for our monocistronic con-
structs do contain a 5’ – GGGGG – 3’ sequence from 8 to 12
nucleotides away from the start codon, which may explain the
successful protein expression with this promoter (37). Interest-
ingly, the 5’ region upstream of flaB1 or hbdC1 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’, hbdC1).
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 cofac-
tors (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 F₄₃₀ 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 keto-reduction 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* (iMR539) (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 F₄₃₀ (SI Appendix, Fig. S5). This suggests that the majority of reductase cofactor-dependent activity in *M. maripaludis* is confined to a small number of Fd- and coenzyme F₄₃₀-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 up-regulated compared with the rich McCas medium. Since NAD(H) levels may represent a metabolic bottleneck for our biosynthetic pathway and more generally for other NAD(H)-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 *K₅₅* 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-Ala) (41). 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-Ala) (41). 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-Ala) (41).
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). The increased labeling in the product (4%) was small, suggesting that the major source of increase was not related to use of Ala as a direct carbon source for biosynthesis of acetyl-CoA. However, it is possible that the pyruvate produced during oxidative deamination of Ala may be used as a precursor for the reverse tricarboxylic acid cycle and gluconeogenesis, producing amino acids, nucleotides, and glycogen (44–46).

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 \textit{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. 2 B and C). In addition, the highest expression of the sodium-alanine symporter gene (\textit{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 \textit{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 \textit{M. maripaludis}**. In order to increase NAD(H) pools, we used two different approaches to engineering \textit{M. maripaludis} biosynthesis (Fig. 3.A). 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 \textit{M. maripaludis}. This strategy revealed that overexpression of the first three steps in the pathway, aspartate dehydrogenase (MMP0737), quinolinate synthase (\textit{nadA}), and quinolinate phosphoribosyltransferase (\textit{nadC}), led to an approximately two-fold increase in total NAD(H) levels during growth in McCas medium (Fig. 3 B), 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
A

De novo NAD biosynthesis pathway

L-Aspartate → Iminoaspartate → Quinolinic acid → Nicotinamide ∣

NADM

B

NAD salvage pathway

Nicotinamide → NAD

C

NAD(H) levels in M. maripaludis

D

Engineering a Synthetic Pathway for PHB and R-3HB Monomers

E

NAD recycling rates in M. maripaludis

Fig. 3. Engineered M. maripaludis shows improved NAD+ recycling rates and NAD(H) levels. (A) The NAD pool was manipulated by overexpression of individual genes from the de novo NAD+ biosynthetic pathway or by insertion of a synthetic nadMV salvage pathway. NMIN, nicotinamide mononucleotide; NaMN, nicotinamide in vivo.

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 pathway appears to exist in this methanogen and, therefore, a new pathway was designed consisting of nicotinamide phosphoribosyltransferase (NadV) from Haemophilus ducreyi (48), which converts nicotinamide into nicotinamide mononucleotide, and a second copy of the native nicotinamide mononucleotide adenylyltransferase (NadM) from M. maripaludis (Fig. 3A). Combining these two genes allowed for the constitution of a nicotinamide salvage pathway in M. maripaludis. Using this pathway, intracellular NAD(H) levels could be increased by 3.4 ± 0.6-fold upon medium supplementation of 50 mM nicotinamide in McCas medium (Fig. 3B) with similar changes seen in McNA and McNA-Ala media—dependence growth (SI Appendix, Fig. S12). Although there is no known transporter of nicotinamide in this species, it may be possible for nicotinamide to traverse the cell membrane through a nonspecific 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 NadMV.

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 was modified by replacing Hbd with an NADH-dependent PhaB from *Halomonas bluephagenosis*. This enzyme preserves the use of NADH rather than NADPH but produces the R-3-hydroxybutyryl-CoA, the stereoiomer 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 *Cupriavidus necator* (50) in combination with PhaA from *C. acetobutylicum* and PhaB from *H. bluephagenosis*. Initial measurements showed that PHB product titer (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 NADH back to NADH are not expected to occur widely in *M. maripaludis*, a formate dehydrogenase (Fdh1) from *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 NADH turnover by its reduction with formate (52). Since formate is a known substrate for growth for *M. maripaludis*, formate should be transportable using native mechanisms (53). As an added benefit, the CO₂ 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 *M. maripaludis* strains containing Fdh1 had 2.0- ± 0.6-fold higher formate-dependent NADH turnover rates compared with wild type (Fig. 3D). Next, NadMV and Fdh1 were combined with the 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 *M. maripaludis* carbon flux could be diverted to our synthetic pathway. The combination of higher NAD(H) pools from NadMV and improved in vivo NADH turnover had a synergistic benefit, leading to higher product titer 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 CO₂ assimilation is thought to contribute 5 to 22% of ocean primary production and occurs without light input (54). As such, chemoaototropic hosts allow sustainable synthesis using CO₂ as the carbon building block with a broader range of sustainable energy inputs, such as electrocatalytically or photocatalytically generated H₂. Given the low efficiency of photosynthetic carbon fixation (≤8 to 9%) (55), it is also possible that coupling sustainable H₂ or formate production to engineered chemoautotrophic 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. *M. maripaludis* uses the modified Wood–Ljungdahl pathway for CO₂ assimilation, where two equivalents of CO₂ 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 CO₂. 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, *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 CO₂ and H₂ (58).
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 conventional 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. Distilled water was deionized to a resistivity of 18.2 MΩ·cm using a Millipore Milli-Q UF Plus system.

M. maripaludis Cell Culture. M. maripaludis S2 (BAA-2049) was purchased from the American Type Culture Collection. All culture manipulations were performed inside a Vacuum Atmospheres Nexus One glovebox with an atmosphere of 90% nitrogen and 10% H2. Oxygen levels and humidity levels within the box were controlled 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 Bala tubes with butyl rubber stoppers and aluminum crimp seals using defined mineral media (M6NA (48) or Mn6NA-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, Mn6NA, 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 lysis 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 Carbo-H+ 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–Ultrascan. 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 HPX-87H ion-exclusion organic acid analysis column (300 x 7.8 mm, 9 μm; Bio-Rad) fitted with an Aminex XPE-85X ion-exclusion guard column (Bio-Rad) at room temperature with an isocratic gradient of 0.014N H2SO4 at a flow rate of 0.6 mL/min for 10 min. The change in mass of tubes was determined using a microbalance.
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 Milli-Q 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 13,000g 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.

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

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