Metabolic engineering of *Rhodopseudomonas palustris* for the obligate reduction of *n*-butyrate to *n*-butanol

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**Abstract**

**Background:** *Rhodopseudomonas palustris* is a versatile microbe that encounters an innate redox imbalance while growing photoheterotrophically with reduced substrates. The resulting excess in reducing equivalents, together with ATP from photosynthesis, could be utilized to drive a wide range of bioconversions. The objective of this study was to genetically modify *R. palustris* to provide a pathway to reduce *n*-butyrate into *n*-butanol for maintaining redox balance.

**Results:** Here, we constructed and expressed a plasmid-based pathway for *n*-butanol production from *Clostridium acetobutylicum* ATCC 824 in *R. palustris*. We maintained the environmental conditions in such a way that this pathway functioned as the obligate route to re-oxidize excess reducing equivalents, resulting in an innate selection pressure. The engineered strain of *R. palustris* grew under otherwise restrictive redox conditions and achieved concentrations of 1.5 mM *n*-butanol at a production rate of 0.03 g L⁻¹ day⁻¹ and a selectivity (i.e., products compared to the consumed substrate) of close to 40%. Since the theoretical maximum selectivity is 45%, the engineered strain converted close to its maximum selectivity.

**Conclusions:** The innate redox imbalance of *R. palustris* can be used to drive the reduction of *n*-butyrate into *n*-butanol after expression of a plasmid-based enzyme from a butanol-producing *Clostridium* strain.

**Keywords:** Metabolic engineering, Redox-driven obligate reduction, *n*-Butanol, *Rhodopseudomonas palustris*

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**Background**

*Rhodopseudomonas palustris*, which is a member of the purple non-sulfur bacteria group, is often noted for its metabolic versatility. One of these metabolic activities, which is H₂ production by photofermentation, utilizes organic carbon under anaerobic conditions with light and has become an attractive application for bioenergy production [1]. The ability to produce H₂ at high concentrations is driven by an excess of reducing equivalents that accumulate during photoheterotrophic growth [2]. Here, we aim to exploit the excess reducing equivalents generated during photoheterotrophic growth to drive an engineered reduction reaction for the obligate production of the liquid fuel *n*-butanol (hereafter butanol).

When growing on acetate, McKinlay and Harwood [1] reported that approximately half of all reducing equivalents that were generated were used for biosynthesis, while the other half required re-oxidation by some alternate pathway such as CO₂ fixation, H₂ evolution, or a combination of both. These two pathways were found to be complementary in this role, and when one route was made unavailable, the other facilitated the re-oxidation...
of all reducing equivalents with minimal changes occurring elsewhere in the central metabolism. However, when fed a more reduced substrate, such as \( n \)-butyrate (hereafter butyrate), it was observed that under non-H\(_2\) evolving conditions, \( R. \) \textit{palustris} could only grow if exogenous CO\(_2\) was supplemented into the medium \([2, 3]\). This finding demonstrates that under these conditions, \( R. \) \textit{palustris} generates more reducing equivalents than available electron sinks can accommodate, and ultimately suffers a lethal redox imbalance. Numerous studies have used these principles to improve \( H_2 \) yields from photofermentation using \( R. \) \textit{palustris} \([4–6]\).

Recently, Fixen et al. \([7]\) engineered a strain of \( R. \) \textit{palustris} to utilize excess reducing equivalents to reduce CO\(_2\) into CH\(_4\) by remodeling the nitrogenase enzyme active site and expressing the modified gene from the chromosome. A NifA* strain of \( R. \) \textit{palustris} was chosen as the host because it expresses the nitrogenase gene constitutively even in the presence of NH\(_3\). The result was a light-dependent \( R. \) \textit{palustris} strain that produced CH\(_4\) with ATP coming from cyclic photophosphorylation and electrons coming from either an organic substrate or thiosulfate. This reduction is, however, not obligatory and a large excess of \( H_2 \) was produced by the nitrogenase compared to CH\(_4\) \([7]\). Another attractive route to redirect the excess reducing equivalents and to maintain redox balance when growing on butyrate is to produce the fuel butanol through an obligate reduction reaction. The advantage of the obligate reduction is the lack of side products to achieve a high selectivity (i.e., products compared to the consumed substrate).

Butanol is an attractive biofuel molecule due to its higher energy density, lower volatility, and complete intersolubility with traditional fuels compared to ethanol \([8]\). Butanol is traditionally produced via fermentation of monosaccharides by certain strains of Clostridia. \( \textit{Clostridium acetobutylicum} \) ATCC 824, which has been widely studied for this activity, is generally seen as the model organism for solvent production and previously has been used as a source of genes for engineered butanol production \([9–11]\). In \( \textit{C. acetobutylicum} \), reduction of butyrate proceeds through a two-phase pathway. First, butyrate is activated to butyryl-CoA, which can occur by two different routes: (1) a two-step butyrate kinase/phosphotransbutyrylase reaction (Fig. 1); or (2) a one-step reaction with butyrate/acetoadetyl-CoA transferase transferring a CoA group from acetoadetyl-CoA to butyrate. Second, butyryl-CoA is reduced to butanol through the butyraldehyde intermediate \([12, 13]\). The AdhE2 enzyme from \( \textit{C. acetobutylicum} \) ATCC 824 (hereafter AdhE2\(_{824}\) and \( \text{adhE}_2\)\(_{824}\) for its gene) reduces butyryl-CoA to butyraldehyde and subsequently butanol (Fig. 1) \([14]\). Analysis of AdhE2\(_{824}\) has revealed that the protein is a bifunctional NADH-dependent fusion protein with the N-terminal domain retaining highly conserved sequences of the aldehyde dehydrogenase family and the C-terminal domain retaining conserved sequences of the iron-containing alcohol dehydrogenase family \([15]\). A second alcohol dehydrogenase enzyme BdhB has also been found to aid in the reduction of butyraldehyde to butanol in \( \textit{C. acetobutylicum} \), though its activity is not necessary for butanol production \([16]\).

In planning to introduce the butanol production pathway together with the existing catabolism of butyrate by \( R. \) \textit{palustris}, we established that before butyrate can be converted to two molecules of acetyl-CoA via anaerobic \( \beta \)-oxidation, butyryl-CoA is first formed through native butyrate kinase/phosphotransbutyrylase enzymes (Fig. 1). Under photoheterotrophic redox imbalance, the first oxidation reaction for the metabolism of butyrate (i.e., butyryl-CoA to crotonyl-CoA in Fig. 1) would likely be inhibited by the thermodynamic limitations of the reduced cofactor pool. Because of this, we anticipated the butyryl-CoA intermediate to accumulate and provide an ideal target for shunting excess reducing equivalents for the production of butanol by introducing only \( \text{adhE}_2\)\(_{824}\) (the bifunctional aldehyde/alcohol dehydrogenase gene) into \( R. \) \textit{palustris} (Fig. 1). After necessary optimizations, the production of butanol indicated that this assumption was correct.

A direct introduction of plasmid-based \( \text{adhE}_2\)\(_{824}\) did not result in the production of AdhE2\(_{824}\) (and butanol

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**Fig. 1** Butyrate metabolism and the proposed route for butanol production in \( R. \) \textit{palustris} by the activity of the butyryl-CoA to butanol AdhE2 enzyme. Dotted arrows represent the proposed pathway and solid arrows indicate native metabolism.
production) due to the major incompatibilities in codon utilization preference between the \textit{C. acetobutylicum} source and the \textit{R. palustris} host. Therefore, we pursued two alternate attempts to introduce the activity of \textit{AdhE2} by using: (1) a gene homologous to \textit{adhE2} \textit{824} from \textit{R. palustris} strain BisB18 (hereafter \textit{adhE} \textit{BisB18} and \textit{AdhEBisB18} for its protein); and (2) a codon-optimized \textit{adhE2} \textit{824} (hereafter \textit{adhE2} \textit{opti} and \textit{AdhE2opti} for its protein). In all our growth experiments, we omitted \textit{HCO}_3 − (CO_2) from, and added \textit{NH}_3 to, the growth medium to block the normal routes of shunting reducing equivalents, resulting in an obligate butyrate reduction. This strong selection pressure will only allow growth when butanol production occurs. In our hands, butanol production through the homologous \textit{AdhEBisB18} did not work, but \textit{AdhE2opti} enabled recovered growth through butanol production. Subsequently, we were able to increase the butanol selectivity and production rate in further experiments, accomplishing our aim of generating an engineered reduction reaction for the obligate production of the liquid fuel butanol in \textit{R. palustris}.

\textbf{Methods}

\textbf{Media and culture conditions}

\textit{Rhodopseudomonas palustris} CGA009 was cultured in anaerobic butyrate Rhodospirillaceae medium (RM) [17], consisting of 1.64 g \textit{NaHCO}_3, 0.5 g \textit{KH}_2\textit{PO}_4, 0.5 g \textit{K}_2\textit{HPO}_4, 0.2 g \textit{MgSO}_4 \cdot 7 H_2O, 0.4 g \textit{NaCl}, 0.05 g \textit{CaCl}_2 \cdot 2 H_2O, 0.01 g \textit{Fe-Citrate}, 1.0 g \textit{(NH}_4)_2\textit{SO}_4, 1.12 mL 10 M \textit{NaOH}, and 1 mL trace metals per liter. The trace-metal solution consisted of 0.25 mL concentrated \textit{HCl}, 70.0 mg \textit{ZnCl}_2, 100.0 mg \textit{MnCl}_2 \cdot 4 H_2O, 60.0 mg \textit{H}_3\textit{BO}_3, 200.0 mg \textit{CoCl}_2 \cdot 6 H_2O, 20.0 mg \textit{CuCl}_2 \cdot 2 H_2O, 20.0 mg \textit{NiCl}_2 \cdot 6 H_2O, and 40.0 mg \textit{Na}_2\textit{MoO}_4 \cdot 2 H_2O L \textsuperscript{−1}. After autoclaving, 1.0 g sterile butyric acid was added as the substrate. No toxicity or inhibition of \textit{R. palustris} growth was detected at this concentration (Additional file 1).

1 mL trace vitamins was added to the medium. The trace-vitamin solution consisted of 1.0 g \textit{p-aminobenzoic acid}, 1.0 g \textit{thiamine}, and 0.1 g \textit{biotin} L \textsuperscript{−1}. Sterile medium (50 mL) with a final pH of 7.0 was added to autoclaved serum bottles and sparged for 20 min with sterile-filtered 80:20 \textit{N}_2/\textit{CO}_2 gas. For the growth of engineered strains under restrictive butyrate-to-butanol conversion conditions, \textit{NaHCO}_3 was omitted from the medium, the addition of 10 M \textit{NaOH} was increased to 2.3 mL total, and serum bottles were sparged for 20 min with \textit{N}_2 instead of \textit{N}_2/\textit{CO}_2. In addition, 200 μg mL \textsuperscript{−1} of kanamycin sulfate was added for retention of the plasmid in all growth conditions with engineered strains. All serum bottle cultures were grown in an environmental growth chamber (GC8-2VH, EGC, Chagrin Falls, OH) at \textit{30 °C} with 80 μmol of photosynthetically active photons s \textsuperscript{−1} m \textsuperscript{−2} (photons between 400 and 700 nm) illumination from both fluorescent and incandescent lamps.

\textbf{DNA manipulation and cloning}

We used the green fluorescence protein \textit{GFPmut2} [18] as a reporter protein to screen for the activity of different expression vectors in \textit{R. palustris} under aerobic conditions. We tested two different plasmids as candidate expression vectors: (1) the endogenously derived \textit{pMG105} [19]; and (2) the broad-host \textit{pBBR1MCS-2} [20]. For detection by immunoreaction, we introduced a C-terminal 6X His tag prior to the termination site with PCR by the reverse primer (Table 1). \textit{GFPmut2} was inserted into the \textit{pMG105} and \textit{pBBR1MCS-2} vectors under the control of a range of promoters.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
\textbf{Primer} & \textbf{Length} & \textbf{Sequence (5′ → 3′)} & \textbf{Purpose} \\
\hline
\textit{pBBR1MCS-2} vector & & & \\
\hline
\textit{XhoI} \textit{GFPmut2} F1 & 60 & \textit{GGCGCGCTCGAGAGGAGGATCTATTCATGAGTAAAGGAGAAGAACTTTTCACTGGAGTTG} & Cloning \textit{GFPmut2} \\
\hline
\textit{XbaI} \textit{GFPmut2} R1 & 51 & \textit{GGCGCCCATCTAGACTATTGTATAGTTCATCCATGTCATGTAATCCCAGC} & Cloning \textit{adhE2} \textit{824} \\
\hline
\textit{BamHI} \textit{RBS AdhE2} \textit{824} F1 & 63 & \textit{GCCGCGGATCCAGGAGGATCTATTCATGAGTAAAGGAGAAGAACTTTTCACTGGAGTTG} & Cloning \textit{adhE2} \textit{824} \\
\hline
\textit{SacI} \textit{6X His AdhE2} \textit{824} R1 & 62 & \textit{GCCGCCGATCCAGGAGGATCTATTCATGAGTAAAGGAGAAGAACTTTTCACTGGAGTTG} & Cloning \textit{adhE2} \textit{824} \\
\hline
\textit{XhoI} \textit{RBS AdhEBisB18} F1 & 57 & \textit{GGCGCGGATCCAGGAGGATCTATTCATGAGTAAAGGAGAAGAACTTTTCACTGGAGTTG} & Cloning \textit{adhE} \textit{BisB18} \\
\hline
\textit{XbaI} \textit{6X His AdhEBisB18} R1 & 60 & \textit{GCCGCCGATCCAGGAGGATCTATTCATGAGTAAAGGAGAAGAACTTTTCACTGGAGTTG} & Cloning \textit{adhE} \textit{BisB18} \\
\hline
\textit{SacI} \textit{RBS AdhEBisB18} F1 & 52 & \textit{GCCGCCGATCCAGGAGGATCTATTCATGAGTAAAGGAGAAGAACTTTTCACTGGAGTTG} & Cloning \textit{adhE} \textit{BisB18} \\
\hline
\textit{pMG105P} vector & & & \\
\hline
\textit{XhoI} \textit{GFPmut2} F1 & 60 & \textit{GGCGCGCTCGAGAGGAGGATCTATTCATGAGTAAAGGAGAAGAACTTTTCACTGGAGTTG} & Cloning \textit{GFPmut2} \\
\hline
\textit{SalI} \textit{GFPmut2} R1 & 53 & \textit{GGCGGCCATCTAGACTATTGTATAGTTCATCCATGTCATGTAATCCCAGC} & Cloning \textit{adhE2} \textit{824} \\
\hline
\end{tabular}
\caption{Sequences of primers used for this study}
\end{table}

Restriction sites are underlined.
including the endogenous phosphoenolpyruvate carboxykinase (ppckA) from R. palustris, arabinose (ara), and lactose (lac) promoters. The adhE<sub>BisB18</sub> and adhE<sub>2824</sub> genes were amplified from R. palustris strain BisB18 and C. acetobutylicum ATCC 824, respectively, using Phusion HF polymerase (New England Biolabs, Ipswich, MA) and directionally ligated into the pBBR1MCS-2 expression vector using the XhoI/XbaI cloning sites. Restriction and ligation enzymes were purchased from Promega (Fitchburg, WI) and all constructs were verified with Sanger sequencing (Cornell University Genomics Facility, Ithaca, NY). Escherichia coli DH5α was used for routine cloning of constructs. Both E. coli and R. palustris were transformed by electroporation with a Bio-Rad Gene Pulser II Electroporator (Bio-Rad, Hercules, CA) with 1.8 kV, 200 Ω, 25 μF for E. coli, and 2.5 kV, 400 Ω, 25 μF for R. palustris. Electroporated cells were recovered in SOC for 1 and 3 h before plating on lysogeny broth (LB) agar plates with 50 and 200 μg mL<sup>−1</sup> kanamycin sulfate for isolation of individual E. coli and R. palustris transformants, respectively.

To successfully produce AdhE<sub>2824</sub> in R. palustris, codon optimization was pursued. Optimization design was performed in-house and largely utilized the one amino acid one codon approach, except in cases of multiple codon repeats and unfavorable energetics of mRNA secondary structure (Additional file 2). adhE<sub>2824</sub><sub>opt</sub> with a C-terminal 6X His tag was synthesized by GenScript (Piscataway, NJ) with flanking XhoI/XbaI restriction sites that were used for directional cloning into pBBR1MCS-2.

SDS-PAGE and western blot detection
For both SDS-PAGE and subsequent western blot detection, 50 μL of late-log E. coli and R. palustris cells were harvested by centrifugation (10 min, 12,000×g, 4 °C) and resuspended in 1 mL 1X Laemmli sample buffer [21] with a protease inhibitor (Protease inhibitor cocktail, Promega, Fitchburg, WI). The suspension was lysed by ultrasonication on ice using three 30 s on 1 min off pulses of 20 W (Branson Sonifier 150, Emerson, Danbury, CT). After sonication, debris was removed by centrifugation and the total soluble protein concentration was determined by BCA assay (Pierce BCA protein assay kit, Thermo Scientific, Rockford, IL). Forty-five μL of 3 mg mL<sup>−1</sup> soluble protein samples and 10 μL of Precision Plus Protein<sup>™</sup> standard (Bio-Rad, Hercules, CA) were loaded into pockets of a Mini-PROTEAN<sup>®</sup> TGX gel (Bio-Rad, Hercules, CA) and run at 100 V for 1.5 h before blotting onto PVDF membranes at 100 V for 1 h. For the initial western blot, immunoreactive bands were developed using manufacturer-suggested protocols for the 1 h Western<sup>™</sup> Standard Kit for rabbit (GenScript, Piscataway, NJ) and a 6X His primary antibody (Rabbit polyclonal, GeneTex, Irvine, CA). We used a similar protocol a second time to improve the sensitivity with these changes: we used 0.1 mL OD<sub>600</sub> 1.0 cells with 0.1 mL Laemmli sample buffer. The samples were boiled for 10 min before centrifugation. 15 μL of each sample was loaded into separate gel lanes. For development, an enhanced chemiluminescence protocol was used using the manufacturer-suggested protocol for promega ECL western blotting substrate (Fitchburg, WI). The film was allowed to incubate with the PVDF membrane for 30 min prior to development.

Bioreactor configuration and operation
Prior to operation, sealed glass bioreactors were autoclaved and filled with 350 mL sterile RM with 1 g L<sup>−1</sup> butyrate and without HCO<sub>3</sub><sup>−</sup>. The bioreactors were equipped with a recirculating water jacket that was operated at 30 °C. Magnetic stir plates (IKA Works Inc., Wilmington, NC) provided stirring of the reactors at 450 rpm. The bioreactors were continuously sparged with sterile-filtered N<sub>2</sub> gas at a flow rate of 0.1 L min<sup>−1</sup> to maintain an anaerobic environment. After sparging for 1 h to ensure anaerobic reactor conditions, 1 mL of actively growing R. palustris cells at an OD<sub>600</sub> of 0.1 was inoculated into the bioreactor. The preculture of R. palustris pBBR1MCS-2 lacp adhE<sub>2824</sub><sub>opt</sub> was grown in anaerobic RM without HCO<sub>3</sub><sup>−</sup>, while the preculture of R. palustris pBBR1MCS-2 lacp GFPmut2 (control) was grown in aerobic RM without HCO<sub>3</sub><sup>−</sup>. Gas entered the liquid phase of each bioreactor through a sparging stone to increase the gas/liquid interface for butanol stripping. Off-gas was connected to 2 Friedrich's condensers in parallel with recirculating water at 5 °C to condense the butanol that had been removed by gas stripping. Gas was released into the atmosphere after one passing through the condensers. The bioreactor volume was illuminated externally with a single 60-W incandescent lamp as used previously [22]. The bioreactors were operated in batch-mode and liquid samples were periodically taken for OD<sub>600</sub> and metabolite measurements.

Metabolite detection
Butyrate, butanol, and acetate were measured via HPLC (600 HPLC, Waters, Milford, MA) with a refractive index detector and an Aminex HPX-87H column (Bio-Rad, Hercules, CA). The column temperature was set to 60 °C, and a 5-mM sulfuric acid eluent at a flow rate of 0.6 mL min<sup>−1</sup> was used as the mobile phase. Butanol and butyraldehyde were measured with a gas chromatograph (HP 5890, Hewlett Packard, Palo Alto, CA) with a 7673 autoinjector and flame ionization detector. The gas chromatograph contained a custom-made packed bed glass column of 1.8 m × 2 mm id (Supelco, Sigma-Aldrich,
St. Louis, MO). The support matrix of the column was Chromosorb W/AW80 over 100 mesh; phases were preconditioned: phase A was 10% Carbowax-20M; phase B was 0.1% phosphoric acid. The inlet and detector temperatures were 220 and 240 °C, respectively. The column temperature profile was 100 °C for 2 min, a ramp of 40 °C min\(^{-1}\) to 180 °C with a 5-min hold. Butanol concentrations were verified by both HPLC and GC platforms.

**Results and discussion**

**GFP screen of expression vectors in *R. palustris* CGA009**

Different combinations of promoter and vector sequences were screened for highest levels of expression within *R. palustris*. The endogenous *ppckA* promoter of *R. palustris* is one of the few promoter sequences that had been previously validated to be active in *R. palustris* under gluconeogenic conditions [23]. However, we only observed a minimal increase in fluorescence for both the pMG105 and pBBR1MCS-2 *ppckA* GFPmut2 constructs compared to the empty vector controls (Fig. 2). While the pBBR1MCS-2 *ara* GFPmut2 construct yielded slightly higher levels compared to *ppckA*, the pBBR1MCS-2 *lac* GFPmut2 construct offered the best expression overall and was selected as the vector for all further expressions in *R. palustris* (Fig. 2). Expression from the *lac* promoter in *R. palustris* was not affected by the addition of the inducer IPTG and catabolite repression only showed a minor decrease in fluorescence (Additional file 3). This suggests that the *lac* regulatory response system utilized by *E. coli* is not functional in *R. palustris*. Although the pBBR1MCS-2 *lac* promoter system provided the best expression in *R. palustris* of all the combinations tested (Fig. 2), the fluorescent signal was still ~13 times lower than the signal from *E. coli* pBBR1MCS *lac* GFPmut2 (data not shown).

**Engineering adhE\(_{2824}\) in *R. palustris***

As determined by the lack of growth under restrictive conditions, the absence of butanol production (data not shown), and the lack of a signal in a western blot under permissive conditions (Fig. 3a, AdhE\(_{\text{BisB18}}\) Aerobic), direct introduction of *adhE\(_{2824}\)* in the pBBR1MCS-2 *lac* construct did not result in the production of AdhE\(_{2824}\) in *R. palustris*. This is likely due to the large GC discrepancy (30% vs. 65%) and codon utilization preference between *C. acetobutylicum* and *R. palustris* [24]. To address this, we looked for homologous proteins within *R. palustris*. Indeed, endogenous alcohol/aldehyde dehydrogenases with homology to AdhE\(_{2824}\) have been found to function in the production of butanol from butyrate when overexpressed. This was previously demonstrated with the fucO gene in *E. coli*, where overexpression allowed production of butanol from an engineered reverse β-oxidation pathway where no butanol production was observed.

![Figure 2](image_url)

**Fig. 2** Fluorescence screen of potential expression vectors in *R. palustris* CGA009. Fluorescence from each strain is expressed as relative fluorescence per 1 unit of OD\(_{600}\) in an exponentially growing culture. Technical triplicates with standard deviation and relevant \(p\) values are shown. Excitation/emission was measured at 480:510 nm. The control contained no cells. Different empty vectors and constructs with GFPmut2 are designated by vector/promoter heading.
previously [25]. In an attempt to enable butanol production in *R. palustris*, a BLAST search for an endogenous enzyme yielded the identification of RPC_4481 (AdhEBisB18) in *R. palustris* strain BisB18, which is an aldehyde/alcohol dehydrogenase with high homology to both domains of AdhE2824.

Before expressing *adhE* BisB18 as a candidate butanol-pathway gene, *R. palustris* strain BisB18 was screened for its ability to grow by producing butanol from butyrate under restrictive electron sink conditions (without HCO$_3^-$). Although no growth or butanol production occurred (data not shown), it is possible that wild-type production of this protein under these conditions is either inactive or insufficient for an observable effect, similar to the FucO protein in *E. coli* [25]. Because no experimental studies have previously characterized the activity of AdhE$_{BiB18}$, its highly conserved primary sequence was further investigated for its structural homology to the verified butanol-producing AdhE$_{2824}$.

Using Swiss-Pdbviewer/DeepView [26], three-dimensional structures of the individual protein domains for both AdhE$_{2824}$ and AdhE$_{BiB18}$ were generated. The predicted tertiary structure of both aldehyde/alcohol dehydrogenases appeared highly conserved with regard to overall shape, location, active site residues, channel volume, and surface charge (Additional file 4). This suggested that the two proteins likely recognized similar substrates. We, therefore, expressed *adhE* BisB18 in *R. palustris* using the pBBR1MCS-2 lacp construct. Transformants were inoculated into RM with butyrate and with and without HCO$_3^-$ to screen for viability and butanol production, respectively. Although transformants grew up with HCO$_3^-$, no growth was observed without HCO$_3^-$ condition (data not shown), suggesting that either AdhE$_{BiB18}$ was not successfully produced by *R. palustris* CGA009 or did not have butyryl-CoA-to-butanol conversion activity.

To investigate this further, a crude cell extract of the culture with HCO$_3^-$, along with *E. coli* harboring the same construct, was analyzed via western blotting. Although the strong signal at the expected size of 94.5 kD for AdhE$_{BiB18}$ for *E. coli* pBBR1MCS-2 lacp adhE$_{BiB18}$ demonstrated that the protein was produced (Fig. 3a, AdhEBisB18), the lack of a signal from *R. palustris* pBBR1MCS-2 lacp adhE$_{BiB18}$ suggested that the protein was either not produced or was produced at levels below detection (Fig. 3a, AdhEBisB18 Aerobic). Aerobic conditions were preferred since anaerobic cultures often had photosynthetic pigments overlapping the expected size for AdhE (Fig. 3a, AdhE$_{opti}$ Anaerobic). The lack of growth and butanol production under restrictive conditions, however, demonstrated conclusively that *R. palustris* pBBR1MCS-2 lacp adhE$_{BiB18}$ was not endowed with butyrate-to-butanol conversion functionality (data not shown).

**Engineering adhE$_{opti}$ in *R. palustris***

Another route to circumvent the large GC discrepancy and codon utilization preference between *C. acetobutylicum* and *R. palustris* was to perform codon optimization
on adhE2 opti to enable expression. The codon utilization table for *R. palustris* CGA009 was determined by analysis of all genomic coding sequences and was retrieved from the Kazusa database [27]. The adhE opti gene resulted in an increase of the average codon utilization frequency in *R. palustris* from 23% for the original adhE2 824 to 64% for the optimized adhE2 opti (Additional file 5). Codon optimization resulted in a shift of the GC content from 32.5% for adhE2 824 to 62% for adhE2 opti. In addition, the presence of rare codons (<10% utilization frequency), which previously composed 47.4% of all codons in adhE2 824, was entirely eliminated. One disadvantage to this approach of codon optimization is that rare codons are known to induce ribosomal pausing, which has been demonstrated important for proper folding of proteins. Thus, with the elimination of all rare codons from adhE2 opti, the elimination of codon-dependent ribosomal pausing could lead to improper folding and aggregation of the target protein [28]. For comparison with adhE2 opti, the codon utilization frequency of adhE2 opti 824 averaged 55% (Additional file 5), the gene possessed an overall 63% GC content, and the frequency of rare codons within the gene was 5%, demonstrating the utilization of rare codons for proper translation of proteins in endogenous alcohol/aldehyde genes (Additional file 6).

To test whether adhE2 opti would be expressed in *R. palustris* CGA009 and enable growth by conversion of butyrate to butanol, the gene was introduced using the pBBR1MCS-2 lacp construct. Transformants were then inoculated into RM with butyrate and with and without HCO3− for growth screening and western blot analysis. This engineered strain was able to grow without HCO3− for growth screening and Western blot analysis. This engineered strain was able to grow without HCO3− for growth screening and Western blot analysis. This engineered strain was able to grow without HCO3− (restrictive conditions) and it produced a measureable amount of butanol, demonstrating the successful production of AdhE2 opti with a functional activity. While a detectable signal was observed on a western blot for the *E. coli* cloning culture, no signal was initially detected from *R. palustris* pBBR1MCS-2 lacp adhE2 opti (Fig. 3a, AdhE2 opti Anaerobic and AdhE2 opti Aerobic). We performed a second western blot using a more sensitive X-ray film imaging method. For aerobically grown *R. palustris* pBBR1MCS-2 lacp adhE2 opti, we observed a single signal at approximately 94 kD, demonstrating successful expression (Fig. 3b, AdhE2 opti Aerobic). As stated above, GFPmut2 production from the pBBR1MCS lac promoter is much lower in *R. palustris* than in *E. coli*, so the lack of a signal in the majority of the samples is likely due to low protein production in *R. palustris*.

**Butanol production rates and selectivity for *R. palustris* pBBR1MCS-2 lacp adhE2 opti**

Because of the rescued growth and butanol production observed, *R. palustris* pBBR1MCS-2 lacp adhE2 opti was further investigated for its ability to grow under both permissive and restrictive conditions and these results were compared to *R. palustris* pBBR1MCS-2 lacp GFPmut2 as a control. *R. palustris* pBBR1MCS-2 lacp adhE2 opti was capable of growth without HCO3− (restrictive conditions) (Fig. 4b), while this growth did not occur for the control strain. The growth for *R. palustris* pBBR1MCS-2 lacp adhE2 opti occurred on a long time scale and reached a relatively low maximum cell density of ∼0.06 OD600 (Fig. 4b) at neutral pH levels (Fig. 4d). Following the onset of the stationary phase, consumption of butyrate and production of butanol still occurred (Fig. 4f, h), but the cultures only reached a maximum butanol concentration of <0.4 mM after extended incubation (Fig. 4h).

Although only low levels of butyrate were consumed (Fig. 4f), butanol production occurred with a 39.7 ± 7.6% selectivity based on moles of carbon. The theoretical maximum for butanol production is based on all electrons derived from complete oxidation of one molecule of butyrate reducing five molecules of butyrate to butanol (6 butyrate → 5 butanol + 4 CO2) for 83% conversion efficiency. However, based on previous empirical measurements of excess reducing equivalents of *R. palustris* growing with butyrate by McKinlay et al. [3], only an estimated 0.45 mol of butanol should be produced per mole of butyrate consumed for an effective 45% selectivity (6 butyrate → 2.7 butanol + biomass). Therefore, almost all reducing equivalents predicted to be in excess were being converted into butanol by *R. palustris* pBBR1MCS-2 lacp adhE2 opti (40% vs. 45%), which we had anticipated for an obligatory reaction.

To further investigate why *R. palustris* pBBR1MCS-2 lacp adhE2 opti showed low growth rates, *R. palustris* was screened for product (butanol) toxicity. However, no inhibition of growth was observed in *R. palustris* at butanol concentrations up to ∼30 mM (Additional file 7), removing our concerns about a concentration of ∼0.4 mM (Fig. 4h). In addition, we operated a bioreactor with product removal to investigate whether this would increase growth and butanol production rates by removing butanol from the substrate pool, thereby, improving the thermodynamics for butanol formation and eliminating any end-product inhibition. Sparging of N2 has been previously used to remove butanol from fermentation broths [29]. Here, N2 gas was continuously sparged into 350-mL anaerobic bioreactors to remove butanol produced by *R. palustris* pBBR1MCS-2 lacp adhE2 opti in batch-mode for 6 consecutive weeks. The *R. palustris* pBBR1MCS-2 lacp adhE2 opti culture grew for that entire period up to an OD600 of 0.12 (Additional file 8: Fig. S6A), and achieved an average maximum butanol production rate of 0.0017 g L−1 day−1 (Additional file 8: Fig. S6D) with a maximum butanol concentration of...
0.35 mM (Additional file 8: Fig. S6C) without depleting butyrate (Additional file 8: Fig. S6B). The lack of a considerable improvement compared to the earlier experiment showed that neither butanol toxicity nor thermodynamics nor end-product inhibition was a main reason for low growth rates. Finally, we found that the extraction system removed a majority of the butanol without removing any of the butyrate (Additional file 8: Fig. S6E, F).

The intermediate butyraldehyde within microbial cells is another metabolite that could have inhibited *R. palustris*, even though the maximum concentration in the supernatant was measured to be only 50 μM. However, due to the fusion nature of *AdhE2 opti*, we anticipate that butyraldehyde would rapidly be converted into butanol with a high flux rate, resulting in a low effective concentration [30]. Without butanol and butyraldehyde toxicity, we can only speculate about why growth and butanol production rates of our engineered strain are so low. Since *AdhE2 opti* is provided as the sole route to maintaining redox balance, all excess electrons must be funneled through this pathway. When the capacity of the *AdhE2 opti* pool is outweighed by the demand for regenerating oxidized reducing equivalents, this would introduce a bottleneck in the maximum rate of metabolism and growth. However, more work is required to be conclusive.

Some butanol production did also occur for *R. palustris* pBBR1MCS-2 lacp *adhE2 opti* with HCO$_3^-$ (permissive conditions) when exogenous CO$_2$ was available for maintaining redox balance (Fig. 4a, g) at a neutral pH level (Fig. 4c). Because the preculture had originally been maintained without HCO$_3^-$, a long lag phase and carryover of butanol producing activity was observed for *R. palustris* pBBR1MCS-2 lacp *adhE2 opti* (Fig. 4a), resulting in a maximum concentration of 0.37 mM with 11.5 ± 2.4% selectivity (Fig. 4e, g). Though the transient production of butanol was a surprise, this lower selectivity for the permissive condition was expected because more reducing equivalents could be used in central metabolism following the introduction of exogenous HCO$_3^-$, resulting in much higher growth (~10× OD at 200 h), the complete depletion of butyrate (Fig. 4e), no production of acetate, and ultimate consumption of all butanol produced (Fig. 4g). This contrasts with the
restrictive condition where all growth proceeds through the obligate engineered pathway, butyrate is not depleted, and all butanol produced remains in solution.

**Optimizing butanol production by pregrowing *R. palustris* pBBR1MCS-2 lacp adhE2opti**

In an attempt to improve the maximum butanol concentrations produced by the engineered strain, 50 mL aerobic cultures was grown to mid-log phase, washed 3 times with sterile RM, resuspended in 0.5 mL RM, and used as an inoculum into 20-mL anaerobic cultures under restrictive conditions. This procedure ensured that the butyrate-to-butanol conversion was not catalyst limited as the initial OD600 of these cultures was >1. By using a concentrated inoculum, *R. palustris* pBBR1MCS-2 lacp adhE2opti produced butanol concentrations of greater than 1.5 mM at volumetric butanol production rates of 0.034 g L⁻¹ day⁻¹ (Fig. 5d, f). All butanol was produced within 100 h of inoculation (Fig. 5d, f). Following 100 h, however, the *R. palustris* pBBR1MCS-2 lacp adhE2opti culture continued to grow and consume butyrate (Fig. 5a, c) with the main carbon product switching from butanol to acetate (Fig. 5b). Acetate had not been detected before in any of the previous anaerobic experiments and in terms of maintaining redox balance, acetate is an unfavorable redox product compared to butanol. This metabolism reduced the butanol selectivity (Fig. 5e). We do not know exactly why this switch occurred, but it was likely a result from having a high biomass culture that underwent a rapid switch from aerobic to anaerobic conditions.

**Conclusion**

The introduction of adhE2opti into *R. palustris* via a plasmid-based construct is an example of metabolically engineered fuel production that proceeds as an obligate strategy for growth. Although growth and production rates were low for this system, our selectivity of 39.7 ± 7.6% matched well with the empirical expectation of 45.0% under these conditions. Because the low growth rates and butanol production are likely constrained by
redox flux through the engineered pathway, rational design and directed evolution could be used to further improve the performance of this activity. If improved, the system could be easily integrated into a simple bioreactor design with highly efficient selectivity and recovery. While this proof-of-concept for harnessing an obligate reducing metabolism to drive engineered reactions used butanol as a target molecule, this redox-based driving force could be used to achieve other reactions for the production of desirable chemicals that require reduction reactions.

**Authors’ contributions**

DFRD designed and executed experiments, performed analysis, interpreted data, and drafted the manuscript. ECH executed experiments, performed analysis, and substrate oxidation state together determine the H₂ biofuel yield in photoheterotrophic bacteria. mBio. 2011;2(2):e00323. 4. Barbosa MJ, Rocha JMS, Trumper J, Wijffels RH. Acetate as a carbon source for hydrogen production by photosynthetic bacteria. J Biotechnol. 2001;85(1):25–33.

Additional files

| Additional file 1 | Growth with butyrate, containing Figure S1. |
| Additional file 2 | Sequence. |
| Additional file 3 | Fluorescence, containing Figure S2. |
| Additional file 4 | Structure, containing Figure S3. |
| Additional file 5 | Codons, containing Figure S4. |
| Additional file 6 | Codons and GC content, containing Table S1. |
| Additional file 7 | Growth with butanol, containing Figure S5. |
| Additional file 8 | Growth with product removal, containing Figure S6. |

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**Competing interests**

The authors declare that they have no competing interests.

**Availability of data and materials**

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

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