Expression of alternative nitrogenases in *Rhodopseudomonas palustris* is enhanced using an optimised genetic toolset for rapid, markerless modifications

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Abstract

The phototrophic bacterium *Rhodopseudomonas palustris* is emerging as a promising biotechnological chassis organism, due to its resilience to a range of harsh conditions, a wide metabolic repertoire, and the ability to quickly regenerate ATP using light. However, realisation of this promise is impeded by a lack of efficient, rapid methods for genetic modification. Here, we present optimised tools for generating chromosomal insertions and deletions employing electroporation as a means of transformation. Generation of markerless strains can be completed in 12 days, approximately half the time for previous conjugation-based methods. This system was used for over-expression of alternative nitrogenase isozymes with the aim of improving biohydrogen productivity. Insertion of the *pucBa* promoter upstream of *vnf* and *anf* nitrogenase operons drove robust over-expression up to 4000-fold higher than wild-type. Transcript quantification was facilitated by an optimised high-quality RNA extraction protocol employing lysis using detergent and heat. Over-expression resulted in increased nitrogenase protein levels, extending to superior hydrogen productivity in bioreactor studies under non-growing conditions, where promoter-modified strains better utilised the favourable energy state created by reduced competition from cell division. Robust heterologous expression driven by the *pucBa* promoter is thus attractive for energy-

intensive biosyntheses suited to the capabilities of *R. palustris*. Development of this genetic modification toolset will accelerate the advancement of *R. palustris* as a biotechnological chassis organism, and insights into the effects of nitrogenase over-expression will guide future efforts in engineering strains for improved hydrogen production.

Keywords

Biohydrogen; *Rhodopseudomonas palustris*; genetic toolset; alternative nitrogenases; gene overexpression; insertional mutagenesis

FOR TABLE OF CONTENTS USE ONLY

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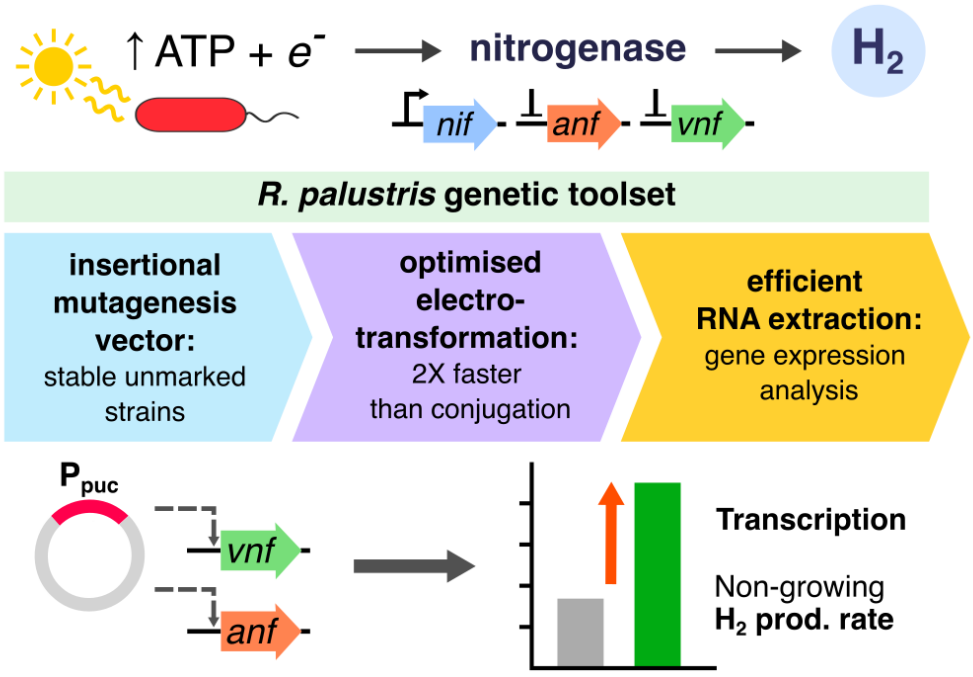

The phototrophic purple non-sulfur bacterium (PNSB), *Rhodopseudomonas palustris*, is a model organism used to study anoxygenic photosynthesis[1] and extracellular electron transport[2]. Due to its extraordinary metabolic versatility, capacity for carbon fixation and resilience against toxic heavy metals and aromatic or chlorinated compounds[3], *R. palustris* is also emerging as a potential biotechnology platform for bioremediation[4], production of bioplastics[5], and possibly bioenergy generation using microbial fuel cells[6]. The metabolic potential of *R. palustris* has thus far mostly been applied to biohydrogen production[7,8], since this native pathway shows potential for the generation of a clean energy source from a multitude of waste materials (reviewed by Adessi et al.[9]). During anaerobic photoheterotrophic growth, light-derived ATP synthesis drives energy-intensive H_2 production, with reducing power derived from heterotrophic metabolism[10]. The ready supply of ATP and electrons creates a conducive cellular energy state which has also been co-opted for the production of a wide variety of products, including methane[11] and butanol[12]. The ability of *R. palustris* to produce abundant ATP under anaerobic phototrophic conditions also renders it a useful platform for production of redox- or oxygen-sensitive compounds and intermediates. Concomitantly, the challenges of adequate oxygenation of the aerobic organisms typically employed for demanding biosyntheses are avoided.

Hydrogen production in *R. palustris* is the obligate consequence of fixation of atmospheric nitrogen to ammonia, mediated by nitrogenase complexes. Due to the high energetic cost in terms of ATP and electrons for this reaction, expression and activity of this pathway is under strict control. Tight transcriptional repression and inactivation of nitrogenases by post-translational modification occurs while fixed nitrogen remains available to the cell[13]. Despite possessing three nitrogenase isozymes, only the molybdenum-containing nitrogenase Nif is expressed under most nitrogen-fixing conditions. The alternative nitrogenases, Vnf and Anf (enzymes with vanadium and iron-only co-factors respectively), are expressed only under extreme nitrogen starvation[14]. Altering expression of these complexes shows promise for enhancing hydrogen productivity since the reaction stoichiometries of Vnf and Anf favour a 2- and 4-fold respectively higher rate of hydrogen production per unit of ammonia than Nif[15]. In the

absence of N_2, energy efficiency is enhanced since ATP and electrons are directed solely to proton reduction, and thus stoichiometric yield of H_2 is increased 4-fold[8]. Under these conditions, a further doubling in hydrogen production rates was observed in strains of the PNSB *Rhodobacter capsulatus* expressing Anf instead of Nif[16,17]. Additionally, since nitrogenases exhibit notoriously low catalytic rates of approximately 6 reactions per second[18], maximising gene expression and thus the total pool of nitrogenase could potentially boost hydrogen production[19,20].

The potential of *R. palustris* as a biotechnology platform has been hampered by a lack of standardised, efficient engineering methods to rapidly generate both unmarked gene deletions and insertions, which are necessary for repeated genetic manipulation. Knockouts have been widely constructed using a variety of systems, but inserted genes have been mostly limited to transient heterologous expression on non-native plasmids with non-optimised wide host range promoters and requiring antibiotics for maintenance[21,22]. Native, stably maintained plasmids have been isolated from *R. palustris* and developed as expression vectors[23]. However, these have shown poor expression performance in reporter assays when compared to non-native plasmids[12]. Long-term, stable expression would be better achieved by chromosomal integration with cassettes under the control of strong promoters, along with the ability to manipulate different genes repeatedly within the organism without the persistence of selection markers.

Genomic insertions have only sporadically been reported[24–26]; thus we aimed to develop a rapid, reliable and versatile recombination-based genetic modification technique tailored to *R. palustris*, with the aim of metabolic engineering of the hydrogen synthesis pathway to enhance productivity. In order to circumvent innate control over alternative nitrogenase expression, we replaced native promoters upstream of the *vnf* and *anf* gene operons to generate over-expressing strains. Transcript quantification showed robust expression of up to 4000-fold over wild-type levels under nitrogen-fixing conditions, which resulted in higher levels of nitrogenase protein, and translated to improved hydrogen productivity in non-growing bioreactor studies.

The techniques developed allow for robust and rapid genetic modification of *R. palustris*, both in terms of

insertions and deletions. Under ideal conditions, unmarked modified strains can be generated in 12 days, allowing for multiple loci to be modified in quick succession. In addition, a simple and effective method for reliable extraction of high-quality RNA is presented, facilitating expedient expression analysis in a difficult-to-lyse organism. Together, these tools advance the utility of *R. palustris* as a biotechnological chassis organism, allowing users to exploit the unique capabilities of this organism for innovative applications.

Results and discussion

Generation of plasmids and identification of promoters for over-expression of genes encoding nitrogenase subunits

In order to facilitate rapid engineering of strains suitable for biotechnology applications, we set out to develop a versatile genetic modification toolset capable of both deletion of chromosomal regions and insertion of foreign DNA into the *R. palustris* genome. The suicide vector pK18*mobSacB* was chosen[27], as it has been widely used for genetic modification in a variety of organisms, including *R. palustris*[21,25,28]. This system exploits a two-step homologous recombination system. The plasmid vector is inserted into the genome under selection pressure incurred by encoded kanamycin resistance. Subsequent excision of the plasmid backbone is then selected by exposing recombinant strains to sucrose, which is lethal via the formation of levansucrase by *sacB*, resulting in stable markerless strains. The absence of selection markers in the final strain facilitates repeated modifications in this strain utilising the same method.

In order to over-express alternative *vnf* and *anf* nitrogenases, a suitably strong promoter was required which is highly active under photoheterotrophic conditions. Transcriptomic studies of *R. palustris* strain CGA009 cultured under anaerobic phototrophic conditions showed *pucBa* to be strongly expressed under both high and low light[29]. This gene encodes the light harvesting antenna protein LH2 peptide A, and is expressed only under low oxygen tension via action of the PpsR repressor mechanism[30]. The *pucBa* promoter is therefore potentially ideal for inducible expression under photosynthetic conditions conducive to high cellular energy availability, with repression under aerobic conditions. This promoter has also

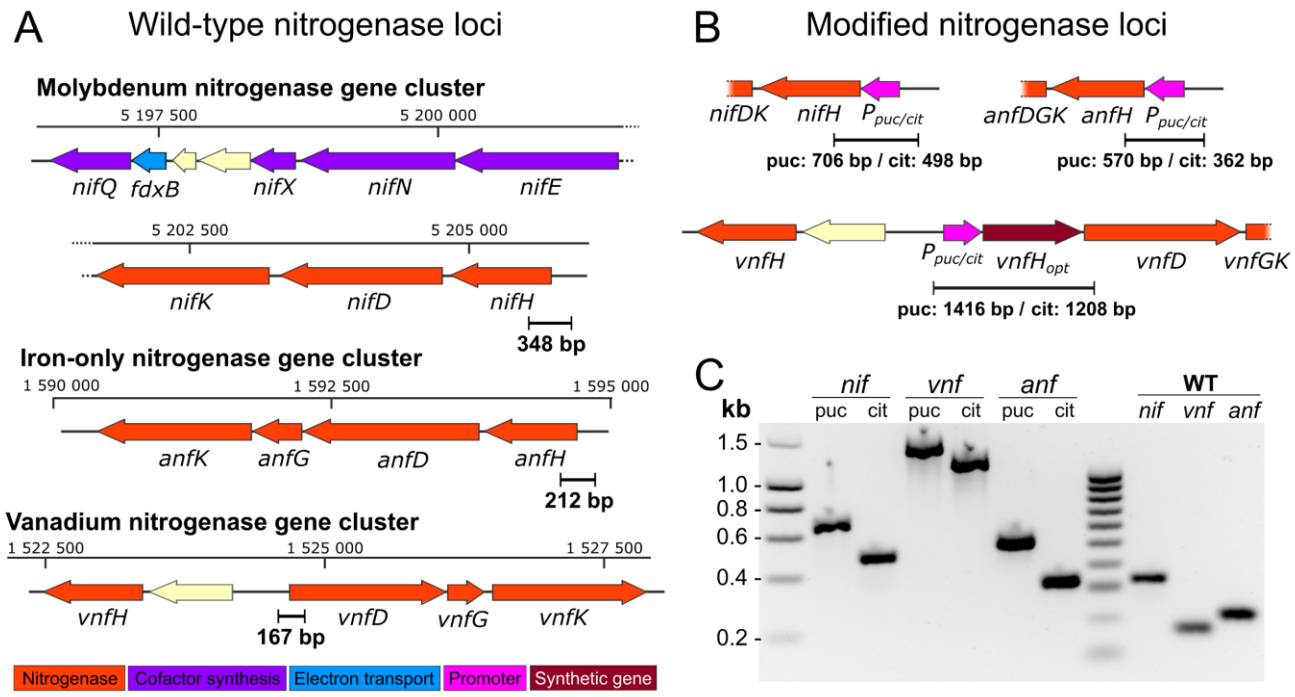

Figure 1 Organisation of nitrogenase genes in *R. palustris* (**A**) and summary of promoter insertion modifications (**B**). Expected PCR product sizes are indicated for genotyping primer pairs spanning the insertion region. Promoters were inserted upstream of gene clusters. A synthetic codon-optimised *vnfH* gene (*vnfH*$_{opt}$) was co-inserted with the promoter to create a unified vanadium nitrogenase operon. Gel electrophoresis of PCR products confirmed genotypes of six modified strains (**C**) which were subsequently verified by sequencing. Gene functions annotated as per Oda et al. (15); cream-coloured genes denote undefined functions.

successfully been used in the PNSB *Rhodobacter sphaeroides* for tuneable expression of membrane proteins by control of culture oxygenation[31] and in vectors designed for heterologous expression[32]. For comparison, the *cisY* promoter was chosen as a constitutively-expressed control, since citrate synthase forms part of the TCA cycle which should be active under most environmental conditions[33].

The Nif nitrogenase enzyme complex consists of two subunits: iron-cofactor dinitrogenase reductase (NifH; Fe protein), which facilitates the maturation and ATP-mediated transfer of electrons to the molybdenum-containing catalytic component dinitrogenase (an $\alpha_2\beta_2$ tetramer of NifDK; MoFe protein)[34]. These genes are clustered on the CGA009 chromosome (Figure 1A) along with additional genes responsible for the complex process of cofactor synthesis and assembly of the catalytically-active holoenzyme[35]. The alternative nitrogenases are similarly composed, with the exception of additional AnfG

and VnfG subunits in the respective $\alpha_2\beta_2\gamma_2$ dinitrogenase complexes.

We thus designed plasmids to insert *pucBa* (*puc*) and *cisY* (*cit*) promoters directly upstream of these three putative nitrogenase operons in order to drive over-expression of all requisite nitrogenase H, D, G and K components (Figure 1B). These insertions preserved the native intergenic sequences to avoid disrupting terminators and potential downstream control regions for the preceding genes; elements which have not been well characterised in *R. palustris*. The native vanadium nitrogenase genes do not form a single cluster, since *vnfH* is transcribed from the reverse strand (Figure 1A). To consolidate the operon, we generated a second synthetic version (*vnfH*$_{opt}$) which was codon-optimised to limit potential off-target recombination with the native gene during co-insertion with the promoter. These constructs were designed for insertion upstream of the native *vnfD* ribosome binding site to facilitate translation from the polycistronic transcript.

Optimisation of a rapid electroporation-based genetic modification protocol

The pK18*mobSacB* plasmid was originally designed to be mobilised into target cells by conjugation. However, this process is slow and labour-intensive since specific *E. coli* donor strains must be generated for each construct and subsequently eliminated after transfer[36,37]. Published protocols require approximately 24 days for *R. palustris* unmarked strain construction[38] (Figure 2A). Therefore, we trialled transformation by electroporation, which is more rapid and flexible.

Initial attempts with published electroporation parameters for *R. palustris*[23,39] yielded very low transformation efficiency of around 200 colonies per µg of plasmid DNA. Replicative plasmids display much higher transformation efficiencies than vectors such as pK18*mobSacB* which require chromosomal insertion for persistence; therefore, parameters need to be specifically optimised for insertional

mutagenesis, in particular by allowing sufficient recovery time for integration to occur[40]. In the PNSB *Rhodobacter sphaeroides*, increasing recovery time after transformation and before exposure to antibiotics has also been linked to markedly higher efficiency[41]. We thus attempted to identify the optimal

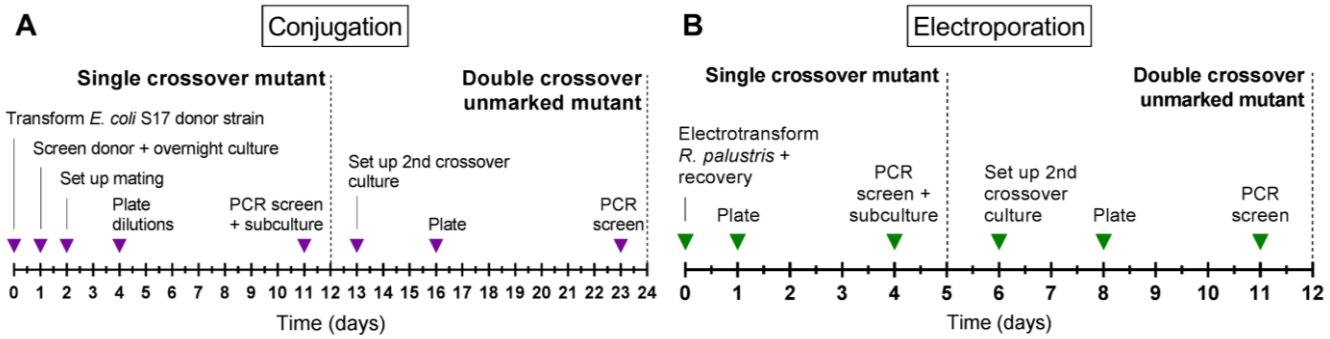

Figure 2 Timelines for transformation and generation of unmarked strains in *R. palustris* by conjugation (**A**) and electroporation (protocol used in this study; **B**). Times indicated represent the minimum possible for each protocol under ideal conditions. Conjugation timeline constructed from protocol by Giraud et al. (38).

electroporation parameters in terms of field strength (voltage), pulse duration (resistance), and optimal post-incubation conditions including growth medium and recovery time. Electroporation of cells at 2.0 kV and 800 Ω, immediate recovery in cold VNG medium and subsequent incubation in antibiotic-free medium at 30°C for 18 hours before plating on selective media, resulted in the highest transformation efficiency of 1.79 – 3.57 x 10^3 colonies per µg of plasmid DNA. Despite larger insert sizes of around 1.5 kb for the *vnf* constructs, these plasmids were integrated with similar efficiency to promoter-only insertions. Furthermore, use of frozen electrocompetent cells did not result in an observable decrease in transformation efficiency, which greatly enhances the convenience of the electro-transformation method.

Since *R. palustris* is a relatively slow-growing species with a doubling time of 8 – 11 hours in mineral medium[42], obtaining transformants during selection steps has been one of the most time-consuming and thus major rate-limiting steps in published methods (Figure 2A)[38]. In order to reduce the time required for generation of visible colonies on agar plates, we tested a range of complex culture media, including Van Niel's yeast agar[43], yeast peptone (YP), tryptone soy (TS) and nutrient agar (NA). In addition, defined mineral medium (PM), used in the majority of *R. palustris* genetic modification studies, was tested for comparison[42,44]. Culturing transformants on Van Niel's yeast agar (VN) at 30°C resulted in the fastest growth amongst the media tested, with colonies appearing approximately 3 days after electroporation, consistent with this medium being specifically developed for PNSB[43,45]. Equivalent growth on YP, TS,

and NA required approximately 4 – 5 days while colonies did not appear on minimal medium or PM plates for at least one week, consistent with previously reported times[38]. Further refinement of conditions by the addition of glycerol to VN medium as a carbon source (referred to as VNG medium) and incubation at 35°C, resulted in even more rapid colony formation after only 2 – 3 days.

R. palustris displays high innate resistance to antibiotics due to the presence of drug efflux pumps[3], which may contribute to the substantial frequency of false positives observed during screening. Use of kanamycin concentrations up to 400 µg.mL^{-1} has been reported[46,47], but the effect of high concentrations on the growth rate of transformants has not been definitively determined. In addition, long-term cultivation in the presence of kanamycin leads to spontaneous mutations in the 16S rRNA of many bacterial species, resulting in antibiotic resistance and further increasing the frequency of false positives over time[48]. In order to decrease the frequency of false positives seen in initial experiments using 100 µg.mL^{-1} kanamycin, transformants were subsequently routinely cultured on agar plates containing kanamycin at a concentration of 200 µg.mL^{-1} and screened as promptly as possible. These measures resulted in on average 70% of colonies successfully showing a single cross-over, with a range of 50 – 83%, perhaps reflecting variation in recombination efficiency for different constructs and loci (Table S1).

Single cross-over strains were then cultured on sucrose-containing plates to generate unmarked strains. Screening by PCR showed ~50% of colonies were wild-type revertants as expected for the pK*mobSacB* vector system[27], and all six *R. palustris* insertion strains with either *puc* or *cit* promoters placed to drive expression of each putative nitrogenase operon were successfully generated (Figure 1C), and confirmed by sequencing of the loci. In order to test the efficacy of the procedure for generating deletions rather than insertions, a pK18*mobSacB* vector targeting *glnK2* was used. Deletion of *glnK2*, a component of the nitrogenase post-translational control mechanism, has previously been performed using an insertional mutagenesis technique[13]. Similar success rates using our optimised technique demonstrated that deletions can be achieved with comparable efficiency to insertions (Table S1). Together, these results confirm the versatility of the electro-transformation method for precise multi-unit modifications in *R.*

palustris. Combining the advantages of electro-transformation and faster selection steps using VNG agar, markerless strains were generated in 12 days, approximately half the time required in previous conjugation-based protocols (Figure 2B).

Development of an optimised RNA extraction method for analysis of overexpressed genes

Confirmation of increased transcription of target genes is typically achieved by reverse-transcription followed by quantitative PCR (RT-qPCR). RNA-based expression analysis requires the purification of high-quality RNA. However, utilising widely-used kit-based methods for *R. palustris*[15,47] resulted in poor yields, degraded RNA and extensive gDNA contamination (Figure S1). We thus tested RNA extraction methods *de novo* with the aim of addressing these problems, while simplifying the methodology as far as possible.

TRI reagent (acidic guanidinium thiocyanate-phenol) has an extensive track record for reliable isolation of intact RNA[49], further improved by the more recent development of direct column-based purification. We therefore determined the efficacy of this method in combination with detergent and enzymatic pre-treatment methods to improve cell lysis and RNA extraction efficiency (Figure 3). Lysozyme digestion offered reasonably improved yield over samples with no additional treatment (Figure 3A, lane 1). Lysis in 1% sodium dodecyl sulfate (SDS) resulted in both the best yield and RNA integrity for the single treatments investigated, as determined by electrophoresis (Figure 3A, lane 4). The favourable denaturing environment in the presence of SDS offers additional protection of RNA integrity from the activity of RNases. Although the combination of lysozyme and SDS offered the most efficient RNA extraction (Figure 3A, lane 2), we focused further optimisation on the SDS pre-treatment to simplify and expedite the extraction protocol with high sample throughput in mind.

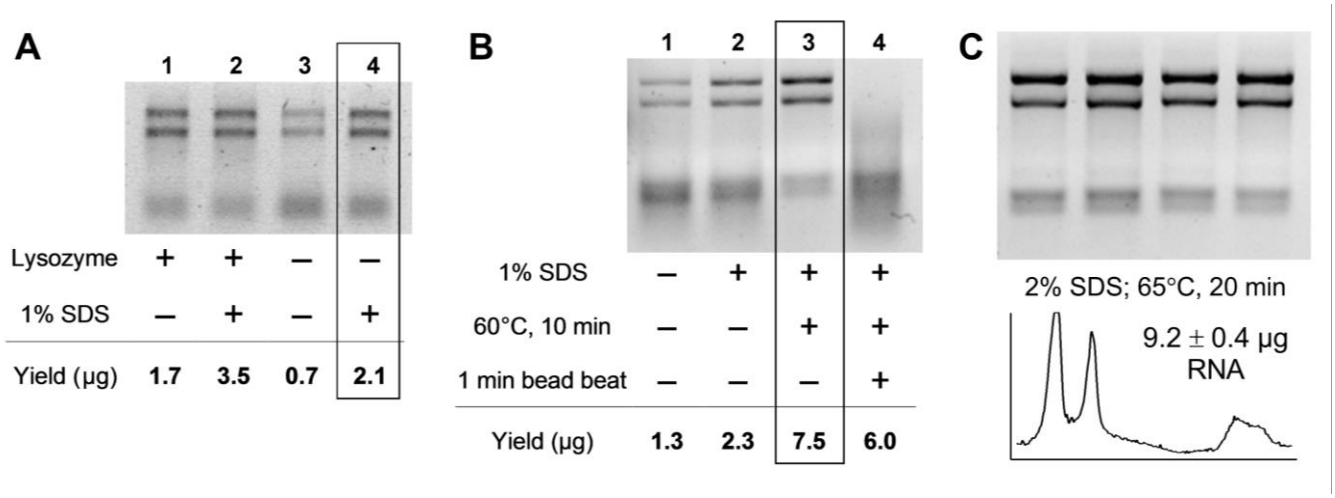

Figure 3 Evaluation of cell lysis pre-treatments for RNA extraction from frozen pellets of 1 mL *R. palustris* cultures. Lysozyme digestion (**A**) was performed by addition of 250 U of Ready-Lyse lysozyme (Epicentre) with incubation at room temperature for 15 min. SDS and lysozyme treatments were aided by the presence of EDTA in the TE resuspension buffer (pH 8). For bead disruption (**B**), RNase-free 0.1 - 0.2 mm acid-washed glass beads (Sigma) were added after thorough mixing with TRI reagent (as per methods section). Pre-chilled samples were treated for 1 min in a Biospec mini-beadbeater and immediately cooled on ice. 150 ng RNA was loaded in each lane of 1% agarose TBE bleach gels for evaluation of RNA integrity. Optimal RNA quality and yield was seen using 2% SDS and 20 minutes of heat treatment (**C;** lanes represent biological replicates). Densitometry analysis of the agarose gel allows comparison to Agilent Bioanalyzer electropherograms used to compute RNA integrity numbers (RIN).

In subsequent experiments RNA yield was further increased by the addition of a heat treatment step. Incubation at ~65°C has been reported to enhance lysis efficiency[50], which corresponds with results seen here (Figure 3B, lane 3). Marked improvement in RNA quality was also observed, probably due to the synergistic disruptive action of heat and detergent which also accelerates the inactivation of RNases. Conventionally, mechanical disruption with silica beads is used to lyse resilient bacteria to improve yields and this method has been widely applied to isolate RNA from *R. palustris*[15,51,52]. However, when used in combination with SDS and heat treatment, even 1 minute of bead disruption resulted in severe RNA degradation (Figure 3B, lane 4). A higher 2% SDS concentration with a longer 20 minute incubation at 65°C ultimately offered the highest reproducible yields of up to 10 µg per 1 mL culture sample (OD_{660} ~1) with excellent RNA integrity as evidenced by sharp rRNA bands and densitometry peaks (Figure 3C). Comparison of gel densitometry to reference electropherograms[50,53] indicated all samples had RNA integrity number (RIN) values of at least 8. This reflects excellent RNA integrity suitable for expression

studies.

Despite the high resistance to cell disruption, relatively gentle lysis of *R. palustris* with heat and detergent seems to give much higher quality RNA than bead beating. In addition, RT-qPCR controls showed little evidence of gDNA carryover with only two integrated DNase treatment steps, in contrast to extensive digestion required for bead beating protocols[52]. SDS and heat thus offer an effective method for extraction of high-quality RNA, with the added benefit of a simple, rapid protocol (Supplementary Protocol 3).

Quantifying gene expression of recombinant strains

Using the optimised RNA extraction method we analysed transcript levels of multiple genes in each putative operon under nitrogen-fixing conditions in the six promoter-substitution strains by RT-qPCR (Figure 4). Transcript levels for all genes investigated in each putative operon showed significant overexpression in both *puc*- and *cit*-promoter strains, with the single exception of *nifH*, the first gene in the operon, which did not differ from wild-type levels (Figure 4; $p = 0.054 – 0.1$). Since the canonical molybdenum nitrogenase is well-known to be highly expressed under nitrogen fixing conditions, it would seem that the activities of these promoters offer only equivalent expression potential to the native *nif* promoter, albeit with the distinction of being active under non nitrogen-fixing conditions as well. Interestingly, however, downstream genes in the operon were over-expressed despite the lack of change in transcript level of *nifH*. These downstream genes include the non-nitrogenase subunit genes *nifE* and *nifQ*, which encode assembly and helper proteins, confirming that these accessory genes are co-transcribed.

In the case of the alternative *vnf* and *anf* nitrogenase promoter-substitution strains, significantly higher relative expression was observed for all genes. As expected, promoter insertion was much more effective here since these nitrogenases are normally repressed due to their nature as fall-back isozymes, with the repression confirmed by very low transcript abundance in WT samples (Figure 4). Up-regulation of between 1000 to 4000-fold compared to wild-type was achieved for *vnf* and *anf* subunit genes under

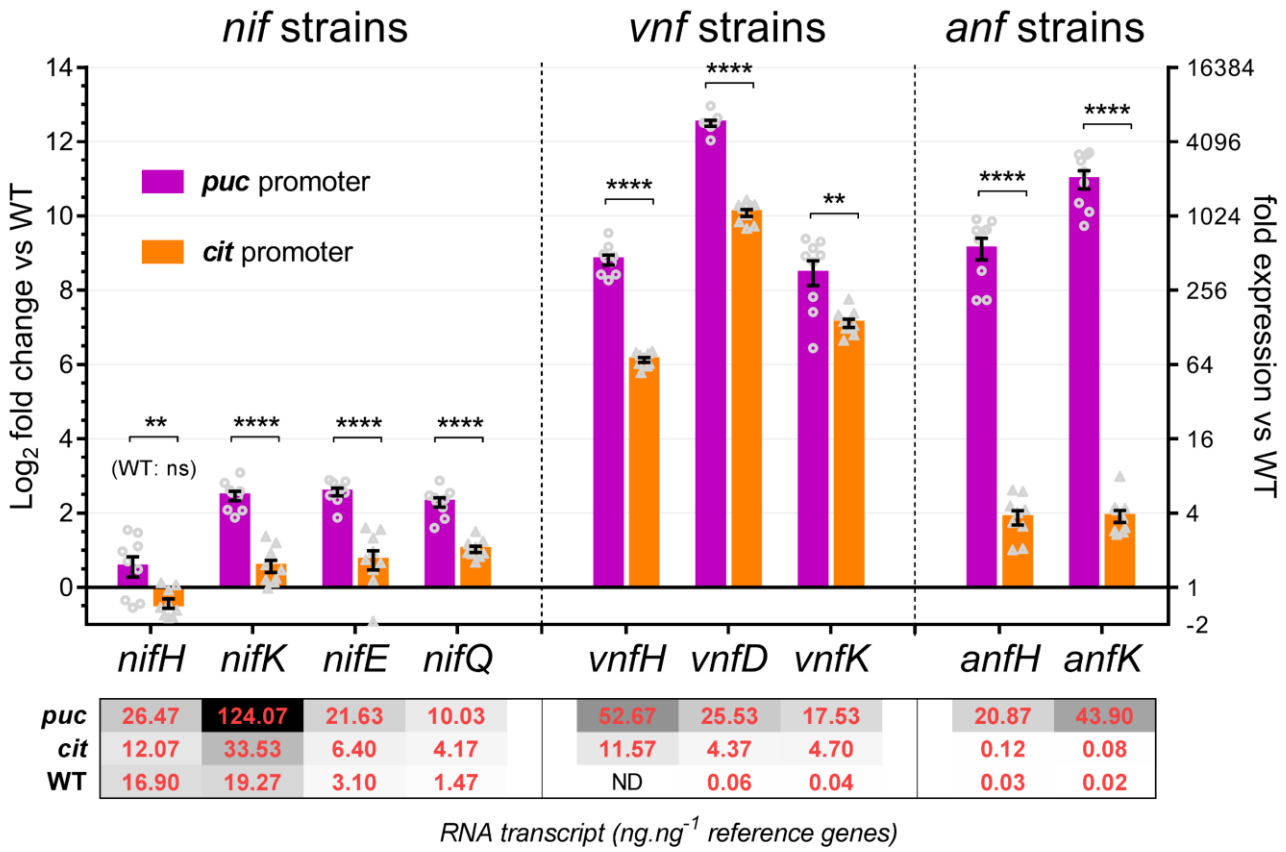

Figure 4 Quantification of gene expression by RT-qPCR from genetically modified nitrogenase operons under control of pucBa (P_{puc}) or cisY (P_{cit}) promoters. Cultures were grown under nitrogen-fixing conditions for 48 h. Transcript levels were normalised to reference genes and wild-type samples to give relative log_2 expression (left axis) and -fold difference (right axis). Heatmap below graph shows differences in normalised transcript abundance between strains (ND: not detected). Data reflects average ± SEM from 3 biological with 3 technical triplicates each; datapoints shown as grey symbols. Statistical significance from two-tailed t-tests with Holm-Sidak correction (α = 0.05) represented by: ****: $p < 0.0001$; **: $p < 0.01$; ns: not significant.

control of the *puc* promoter, indicating excellent activity under photoheterotrophic conditions. Expression of native *pucBa* transcripts in wild-type samples was 10-fold higher than those for *nifH* while *cisY* transcripts were 23-fold lower, consistent with the nature of citrate synthase as a constitutively expressed but low-abundance protein. This highlights the usefulness of the *puc* promoter as a robust driver of gene expression with wider potential applications.

Nitrogenase protein expression

Detection of total nitrogenase protein in the modified strains was performed by western blot analysis (Figure 5) using antibodies against a peptide epitope conserved in all dinitrogenase reductase subunits (NifH, VnfH, AnfH) which further verified successful over-expression of the alternative nitrogenases. Nitrogenase protein abundance was increased in all strains relative to wild-type except for the *nif:cit* strain, following the same trend observed for transcript quantification. A modest increase in nitrogenase in the *nif:puc* strain suggests that the *puc* promoter performs at least equivalently to the native *nif* promoter. In *Rhodopseudomonas capsulatus*, nitrogenase comprises up to 40% of intracellular protein under nitrogen-fixing conditions[54], and if this is similar in *R. palustris*, the result with the *puc* promoter demonstrates its potential for high-level expression. In the *nif:cit* strains, decreased nitrogenase protein is consistent with the lack of increase in transcript levels, indicating inferior performance of the *cit* promoter compared to the native promoter.

The *anf* and *vnf* strains possess intact *nif* genes, and under nitrogen-fixing conditions high-level expression of the main molybdenum nitrogenase would occur in addition to that of the modified loci. Assuming similar translation efficiency between *nif* and alternative nitrogenases, over-expressing *vnf* and *anf* transcripts to levels similar to that of *nif* should lead to a doubling in total nitrogenase protein. However, protein expression was raised only modestly with a maximum of approximately 30% in the *anf:puc* strain. Thus, there is a possibility that the translation machinery is saturated under these

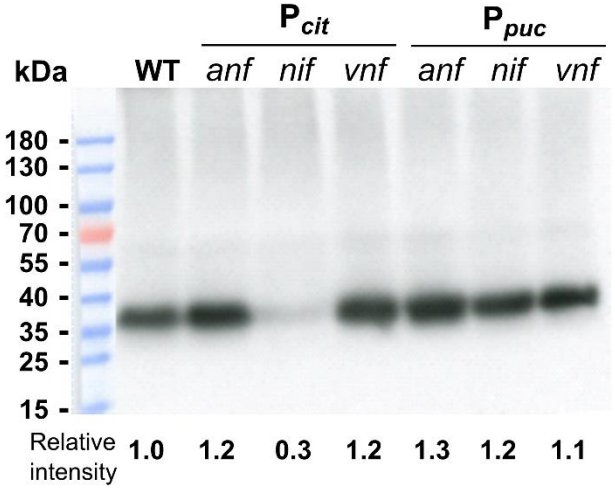

Figure 5 Western blot for the detection of total nitrogenase via a conserved peptide epitope in all dinitrogenase reductase subunits (NifH, VnfH, AnfH). 40 µg protein loaded in each sample lane, extracted from each promoter-substitution strain in which nif (Mo), vnf (V) and anf (Fe) nitrogenase genes are expressed under control of either *pucBa* or *cisY* promoters (P_{puc} and P_{cit}). All strains grown under nitrogen-fixing conditions. Band intensity relative to wild-type determined by densitometry. Bands correspond to expected size of dinitrogenase reductase subunits.

conditions, leading to competitive bottlenecks in expression of the alternative isozymes and a practical limit on the total nitrogenase present within the cell. In addition, post-transcriptional control mechanisms may reduce translational efficiency despite high transcript levels. However, these promoters may be useful for heterologous expression of other proteins under phototrophic conditions where nitrogenases are not expressed, allowing higher translation capacity.

In addition, a two amino acid difference in the VnfH antibody epitope target may affect the detection efficiency for the vanadium nitrogenase, which may lead to an underestimation of the total nitrogenase content in the *vnf* samples. Nevertheless, protein expression levels further validate the over-expression of genes using the *puc* (and in some cases *cit*) promoters; and the optimised genetic modification method as a viable metabolic engineering strategy.

Hydrogen production by modified strains

Along with modest improvement of nitrogenase protein expression, subtle differences in the relative populations of expressed isozymes may yield distinct changes in hydrogen output due to the higher hydrogen production stoichiometries of the iron and vanadium nitrogenases[14,15,17]. To test this, growth and hydrogen production studies were conducted with the promoter-modified strains in 0.5 L test-scale bioreactors under nitrogen-fixing conditions. Initial growth of all modified strains was slightly slower than wild-type, perhaps reflecting a small metabolic burden on cells due to the modification, although the difference in growth rates was not significant (Figure 6A). The specific hydrogen production rate was in turn also lower, most notably in the case of the *nif* strains (Figure 6B) during the early phase of growth, when cultures are at low density and light availability per cell is highest. These strains only attained a maximal production rate of ~10 $mL.g^{-1}.h^{-1}$ at 90 hours, around half of the wild-type maximum rate, which suggests that substitution of the native *nif* promoter does not enhance hydrogen production performance

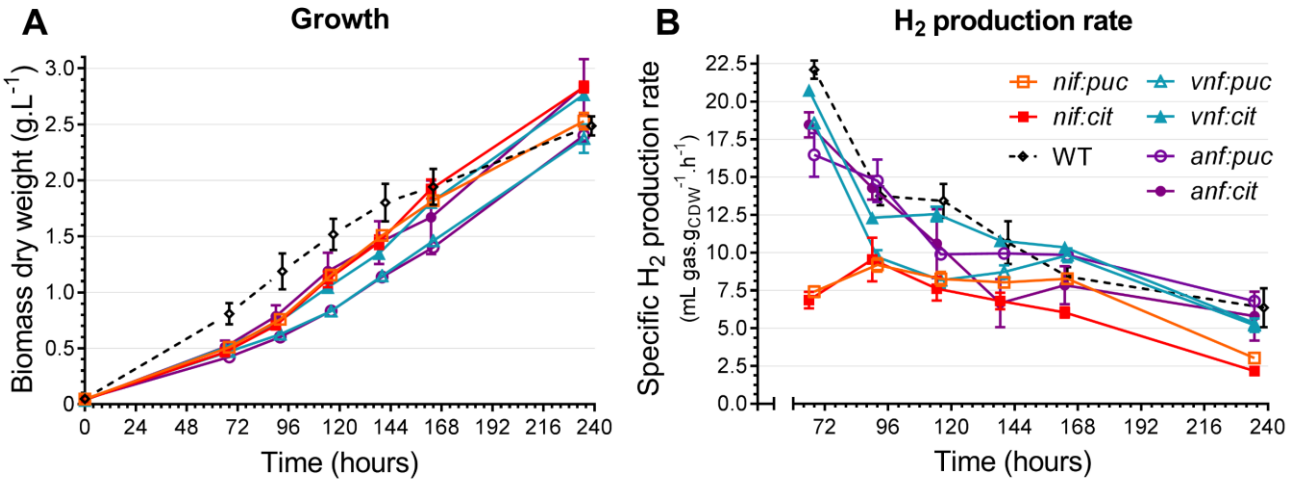

Figure 6 Growth and hydrogen production by nitrogenase promoter-modified strains under nitrogen-fixing conditions in 0.5 L test bioreactors. Cell growth was tracked by converting OD_{660} to cell dry weight (**A**). Hydrogen production was normalised to biomass in the reactor to give specific production rate (**B**). Data reflects averages and error bars represent SEM for 3 to 6 biological replicates.

by Mo nitrogenase. Notwithstanding equivalent transcription in the *nif:puc* strain and wild-type, the replacement of native regulatory elements in the 5' UTR may disrupt correct post-transcriptional processing, translation or the intricate enzyme assembly and maturation process[55], leading to decreased concentration of functional enzyme.

Despite the presence of unmodified *nif* loci in *vnf* and *anf* strains, a slight decrease in hydrogen productivity was observed relative to wild-type levels, especially in terms of the peak in production rate in the early culture phase (Figure 6B). In these strains, expressing an additional nitrogenase isozyme from a second locus, only increased or at the very least equivalent hydrogen productivity would be likely compared to wild-type unless the over-expression places a burden on the cell. Again, high transcript and protein levels from expression of two nitrogenase genes may reduce expression of other proteins required for optimal hydrogen production, including those involved in correct nitrogenase assembly. Alternatively, lower production rates may reflect innate limitation in terms of either ATP or electron availability for reduction of protons to H_2. This may result from either non-optimal light availability within the relatively large 80 mm diameter bioreactor with a low surface-to-volume ratio, or saturation of the pathway supplying electrons to nitrogenase, since optimal nitrogenase activity is reliant on associated ATP and

electron supply mechanisms operating at similar efficiency.

Alongside biomass synthesis, the Calvin Cycle CO_2 fixation pathway is the main metabolic competitor for reducing equivalents[56], and inactivation of this pathway resulted in 1.5 to 2.5 times higher specific H_2 production rates[57]. It is thus also possible that the promoter-substitution strains would show superior hydrogen production compared to wild-type under non-growing conditions with reduced competition from biomass accumulation[58]. This hypothesis was subsequently tested using *puc* promoter strains in the same test-scale bioreactor system. Late-log phase cells were resuspended in medium free of nitrogenous substrates to curtail further cell division, and at low optical density to allow light penetration deep into the bioreactor (Figure 7). Specific gas production rate in all strains decreased from an initial peak of around 16 $mL.g^{-1}.h^{-1}$ after 24 hours; values comparable to those in growing cultures (Figure 6B). However, both the *vnf:puc* and *anf:puc* strains maintained higher production rates compared to wild-type at subsequent timepoints from a minimum of 1.2-fold at 48 h (*vnf:puc*) to greater than 5-fold after 212 h (Figure 7). Non-growing conditions thus seem to unlock the additional hydrogen production capacity of the promoter-modified strains due to reduced competition for available energy and reductant flux. This observation is interesting in context of the evidence that expression of alternative nitrogenases is stimulated by nitrogen starvation[15], as employed in this experiment to inhibit cell growth. The improved productivity of the modified strains therefore occurred despite some potential degree of alternative nitrogenase expression

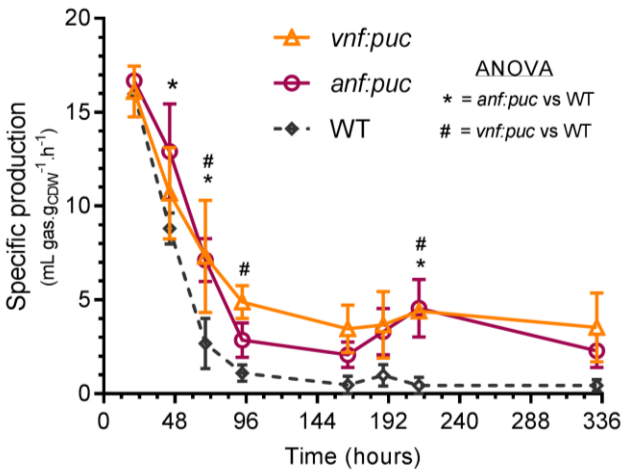

Figure 7 Non-growing hydrogen production by nitrogenase promoter-modified strains of *R. palustris* under nitrogen-fixing conditions in 0.5 L test bioreactors. Biomass was resuspended in minimal medium free of nitrogenous substrates at OD_{660} ~ 0.5. Hydrogen production normalised to biomass in the reactor is reported as specific production rate.

Data reflects averages and error bars represent SEM for 3 to 4 biological replicates. Statistical significance tested using two-way ANOVA with Holm-Sidak multiple comparisons tests ($p < 0.05$).

in the wild-type controls, supporting the effectivity of the over-expression technique. These higher production rates further continued for the entire 2 weeks of batch culture, suggesting that improved productivity may extend to long-term non-growing cultivation, as we have previously demonstrated with immobilised *R. palustris* in continuous mode[59]. Such a system, employing promoter-modified strains and nitrogen-free substrates, may be an achievable way to make further use of the favourable energy status present in non-growing cells for improved hydrogen production[58]. Deletion of *nifHDK* from the *vnf:puc* and *anf:puc* strains in order to restrict expression solely to alternative nitrogenases would allow full realisation of the productive potential of these strains.

While enhancing production rates is a key goal for the feasibility of biohydrogen, improving overall bioprocess compatibility is equally important for implementation. Production of hydrogen via nitrogenases expressed from native promoters will be strongly repressed by nitrogenous compounds such as ammonia and urea[60], which are commonly found in potential waste feedstocks. The NifA* strain, which expresses higher levels of constitutively-active nitrogenase due to a 16 amino acid deletion in the master transcriptional activator NifA[56], shows highly reduced sensitivity to the presence of ammonia[61]. The maintenance of nitrogenase activity was hypothesised to be due to the lack of expression of post-translational inactivation system GlnK2-DraT2 since the NtrBC control regulon is not activated under nitrogen-replete conditions[13]. This hypothesis should similarly apply to the promoter-modified strains under nitrogen replete conditions. However, no hydrogen was produced by these strains grown with 5 mM urea, which is converted to ammonia intracellularly, suggesting that over-expression is not sufficient to overwhelm basal activity of the switch-off system (data not shown). Additional factors thus seem to be involved with maintenance of nitrogenase activity in the NifA* strain, warranting further exploration of nitrogenase control mechanisms. Further engineering of the *vnf:puc* and *anf:puc* strains by knocking out the GlnK2-DraT2 control system may result in efficient hydrogen-producing strains insensitive to nitrogenous feedstocks.

Nevertheless, the *pucBa* promoter shows great promise for high-level heterologous expression in *R.*

palustris, applied to synthesis of products with fewer demands and in the absence of sophisticated native control mechanisms. Further investigation into the function of the promoter sequence, identification of core elements and clarification of post-transcriptional control mechanisms would allow full development of an effective heterologous expression system.

Exacting maximal hydrogen productivity from *R. palustris* will clearly require comprehensive metabolic engineering to address pathway bottlenecks. The optimised tools presented here expedite the repeated genetic modifications required for further genetic dissection, while also advancing the potential of *R. palustris* as a biotechnology platform for additional high-value products suited to its metabolic capabilities.

Methods

Media and culture conditions

Rhodopseudomonas palustris strain CGA009 was routinely cultured using Van Niel's Yeast medium for fast growth and recovery during genetic manipulations, comprising 10 g yeast extract, 0.5 g $MgSO_4$, 1 g K_2HPO_4 (with 15 g agar for solid media) per litre; supplemented with 50 mM sterile glycerol after autoclaving (VNG medium).

Growth and expression studies were conducted in modified Rhodospirillacea minimal medium as previously described[59]. Medium was supplemented with 50 mM glycerol, 1 $mL.L^{-1}$ trace element solution[62] and either 10 mM glutamate (monosodium) or 0.3% w/v additional yeast extract as the nitrogen source. These conditions allow for expression and function of the native Nif nitrogenase (nitrogen-fixing conditions). For non-nitrogen fixing conditions, 5 mM urea was used a nitrogen source, which avoids culture pH disturbances caused by more commonly-used ammonium salts. Vanadium for Vnf nitrogenases was supplied as $NaVO_3$ at a final concentration of 165 nM, equimolar to molybdenum. Cultures were incubated at 35°C, previously verified as the optimum temperature for strain CGA009[63], under illumination from 100W incandescent light bulbs for anaerobic liquid cultures and in the dark for aerobic agar plates. Irradiance intensity was calibrated in the wavelength range 500 – 1100 nm using a compact spectrometer and cosine correcting probe (RGB photonics Qmini VIS-NIR). Cultures were

verified as axenic using VNG agar plates to confirm absence of contaminating organisms before each experiment.

Screening of growth media for transformants was performed using YP agar (0.3% yeast extract, 0.3% peptone, 1.5% agar), tryptone soy agar (TS; BD Bacto), and nutrient agar (NA; Merck). Defined mineral medium (PM) was used as described by Rey et al[42].

E. coli NEB5α (New England Biolabs) was grown in LB medium at 37°C, with 50 μg.mL^{-1} kanamycin as required.

Plasmid construction

The genome sequence of *R. palustris* CGA009[3] was consulted via the Ensembl database (bacteria.ensembl.org). Candidate promoters were identified as the upstream intergenic sequences of *pucBa* (LH2 beta chain A; RPA2654; 350 bp) and *cisY* (Citrate synthase; RPA2907; 150 bp), referred to as the *puc* and *cit* promoters. Promoters and ~1 kb regions flanking the desired insertion site directly upstream of the target gene start codon (or ribosome binding site –21 bp from the start codon for *vnf* modifications) were amplified by PCR using Phusion high-fidelity polymerase (Thermo Scientific) with supplied GC buffer and 3% DMSO to optimise performance on GC-rich *R. palustris* gDNA template. Primers (Table S3) were designed using the NEBuilder tool (nebuilder.neb.com; New England Biolabs) to generate PCR fragments suitable for Gibson assembly[64] with minimum 30 bp overlaps. Promoter sequences were assembled between the 5' and 3' flanking regions in order to precisely target the insertion. In the case of *vnf* promoter-substitution strains, a maximum diversity codon-optimised sequence of the *vnfH* gene was generated using the Kazusa codon-usage database (kazusa.or.jp/codon/) to reduce homology to the native *vnfH* gene (*vnfH*$_{opt}$; sequence provided in Supplementary Information). This fragment was synthesised by GeneArt (Thermo Scientific) and PCR amplified to be co-assembled downstream of the promoter in order to create a unified single operon. PCR products were purified by gel electrophoresis and extraction using the PureLink Quick Gel Extraction Kit (Invitrogen). Promoter and flanking fragments were then assembled into *Bam*HI/*Xba*I-digested

pK18*mobSacB*[27] with the NEBuilder High-Fidelity DNA Assembly Cloning Kit (New England Biolabs) with a 90-minute incubation, followed by cloning into *E. coli* NEB5α and blue-white screening for successful constructs. Component PCR fragments and final assembled plasmids were verified by Sanger sequencing before use (DNA Sequencing Facility, University of Cambridge).

Optimised *R. palustris* transformation and markerless strain generation

Electrocompetent cells were prepared from 50 mL log-phase cultures (OD_{660}: 0.3 – 0.5) grown in VNG medium. Cultures were chilled on ice for 10 min and centrifuged (5000 *g*, 4°C, 10 min), followed by 3 washes with 50 mL ice-cold 10% glycerol and final resuspension in 500 µL 10% glycerol. Cells were either used immediately or quick frozen on dry ice and stored at –80°C. No discernible difference in transformation efficiency was observed for frozen cells compared to those prepared from fresh cultures. Approximately 500 ng plasmid DNA was mixed with 100 µL electrocompetent cells in a pre-chilled 1 mm electroporation cuvette (VWR), electroporated (2.0 kV, 800 Ω, 25 µF), followed by immediate addition of 1 mL ice-cold VNG medium and incubation on ice for 5 min. Cells were allowed to recover during overnight 18-hour incubation at 30°C under incandescent light (80 – 100 $W.m^{-2}$) and subsequently plated on VNG agar with 200 $\mu g.mL^{-1}$ kanamycin. Protocols for electrocompetent cell preparation and electro-transformation are supplied in the supplementary information (Supplementary protocols 1 and 2).

Kanamycin-resistant transformants were promptly genotyped by colony PCR using GoTaq G2 DNA polymerase (Promega) and primers spanning and within the insertion site to confirm the presence of a single homologous recombination event. Screening of colony PCRs was rapidly performed by capillary electrophoresis using a QIAxcel Advanced system with DNA high-resolution kit (Qiagen). Confirmed single-recombination clones were cultured in 15 mL VNG medium for 2 days and plated on 10% sucrose VNG agar to select for double recombination events. Verified sucrose-resistant colonies were screened for kanamycin sensitivity and genotypes of resultant unmarked knock-in strains were verified by PCR using primers flanking and within the insertion region (Table S4). Sequencing of the amplicon confirmed correct insertion of the promoter construct.

Electroporation procedure refinement

Initial tests of electroporation parameters affecting transformation efficiency in *R. palustris* were performed using a pK18*mobSacB* plasmid targeting a deletion in RPA1309. This gene is a transposase pseudogene which is thought to be frameshifted and incomplete, and thus inactivation is unlikely to have a deleterious effect on cell viability. 60 µL mid-log electrocompetent cells (prepared as above from culture OD_{660}: 0.3) were transformed with 250 ng of plasmid DNA in 1 mm electroporation cuvettes. Following electroporation (1.5 – 2.5 kV, 200 – 1000 Ω, 25 µF), 1 mL of either ice-cold or prewarmed SOC medium was added and samples were allowed to recover for 90 min at 37°C, before plating on YP agar plates with 100 $\mu g.mL^{-1}$ kanamycin and incubation at 30°C. Protocol refinements leading to the highest transformation efficiencies are reflected in the optimised procedure above.

Optimised RNA isolation

Each strain was inoculated in triplicate from a 3-day preculture into 50 mL of Minimal medium supplemented with 0.3% yeast extract and 50 mM glycerol in completely-filled 25 cm^2 transparent polystyrene cell culture flasks (solid cap; Nunc, Thermo Scientific) at a starting OD_{660} of 0.025. Cultures were grown anaerobically at 35°C with shaking under 80 – 100 $W.m^{-2}$ illumination (500 – 1100 nm) from incandescent lightbulbs for 48 hours (OD_{660} < 3). RNA was isolated in duplicate from 1 mL aliquots of each culture, with the remainder kept for protein extraction. Samples were chilled on ice and cells harvested by centrifugation (14000 *g*, 4°C, 5 min). The pellets were snap frozen in liquid nitrogen and stored at –80°C until further processing. Cell pellets were lysed by resuspension in 55 µL 2% SDS in RNase-free Tris-EDTA (TE) buffer (pH 8; Invitrogen) with incubation at 65°C for 20 minutes followed by thorough mixing with 640 µL TRI Reagent (Sigma-Aldrich). After 15 minutes of agitation, samples were centrifuged to pellet cell debris (20000 *g*, 10 min.). RNA was purified from the supernatant using the Direct-Zol RNA miniprep kit (Zymo Research) with on-column DNase digestion for 30 minutes, and 2-minute incubation prior to final elution. This method allows isolation of RNA directly from TRI reagent, obviating the chloroform phase separation normally required and thereby minimising both complexity and

the number of sample handling steps involving hazardous TRI reagent. The optimised protocol for RNA extraction is supplied in the Supplementary Information (Supplementary protocol 3).

RNA quantity and purity were determined by UV spectrophotometry (NanoDrop 1000, Thermo Scientific). RNA integrity was rapidly assessed using 1% agarose TBE bleach gels[65] before further use and storage at –80°C. 5 µL containing 150 ng of each RNA sample were pre-incubated with 5 µL 2X formamide RNA loading buffer at 65°C for 5 minutes to denature RNA secondary structure followed by snap cooling on ice prior to electrophoresis. Densitometry of gel images was performed using the GelQuant program[66].

Procedures tested during optimisation and not forming part of the final isolation procedure are described in the legend of Figure 3.

Gene expression analysis by RT-qPCR

A total of 300 ng of RNA from each sample was used for cDNA synthesis using SuperScript IV VILO Mastermix with EZDNase (Invitrogen) containing both oligo d(T) and random hexamer primers. This input quantity was optimal to ensure linearity of the RT step[67]. Reverse transcription was performed at 65°C to minimise secondary structure interference due to the GC-rich template. Linearity of RT-qPCR was verified using a titration of a pool of input RNAs, which was also used to rule out gDNA contribution (RT–).

Quantitative PCR (qPCR) primers (Table S5) were designed using OligoArchitect (Sigma-Aldrich) with a target T_m of 66°C, strictly avoiding primer and cDNA secondary structure or dimer formation where possible. Product sizes were limited to 80 – 120 bp to enable fast reaction cycling, and primers were confirmed to be target-specific using PrimerBlast (NCBI). Efficiency of all primer sets was determined by titration over 4 orders of magnitude from 10 pg to 10 ng (MIQE guidelines[68]) using isolated gDNA from the *vnf:puc* strain (ChargeSwitch gDNA bacterial kit, Life Technologies) which contained all the target sequences. Multiple primer sets were tested for each gene and best-performing sets were selected. qPCR was performed using PowerUp SYBR green mastermix in 384-well plates on a QuantStudio 5 real-time PCR system (both Applied Biosystems). Triplicate 10 µL reactions contained 400 nM primers, 0.75

ng cDNA and 5% v/v 1,2-propanediol (0.68 M) to improve amplification of templates with problematic secondary structure due to the high GC content[69]. The 2-step PCR fast-cycling program consisted of 95°C, 2 min; and 40 cycles of 95°C, 1 s; 60°C, 30 s. C_q values were converted to gDNA ng equivalents using a reference titration of gDNA amplified alongside the cDNAs, and normalised to average quantification values of *rpoD* and *dnaA* reference genes[29]. Quality Control: The existence of a single end product was verified by melt-curve analysis. Genomic contribution to RT-qPCR was ruled out by processing a pool of input RNAs without reverse transcriptase (RT–), as well as ensuring negligible amplification from an untranscribed inter-operon genomic area (CAE27092–93) in the RT-qPCR samples. No substantial readings were obtained in reverse transcription or PCR samples performed with water alone (RT0 and NTC).

Western blot

Cells were harvested from the remaining 50 mL cultures by centrifugation (4°C, 5000 g, 10 min) and resuspended in 3 mL hypotonic STE buffer (20 mM NaCl, 50 mM Tris-HCl, 5 mM ETDA) and 1 mL lysis buffer (10% glycerol, 1% Triton X-100, 1 mM PMSF, 10 µg.ml^{-1} RNase/DNase I, 10 mM MgCl2). Samples were lysed by sonication on ice for 2 min (5 s on, 15 s off). After centrifugation at 14000 g for 2 min to pellet debris, protein concentration was measured using Pierce 660 nm protein assay reagent (Thermo Scientific). Samples were diluted to 1 µg.µl^{-1} protein in 1X Laemmli sample buffer (with 50 mM DTT) and incubated at 40°C for 30 min. For each sample 40 µg protein was loaded into wells of 4–15% Mini-PROTEAN TGX gels (Bio-Rad) and run at 200 V. Transfer onto PVDF membranes was performed using the iBlot dry blotting system (Invitrogen) at 20V for 7 min. NifH was detected with chicken polyclonal anti-NifH (Agrisera AS01021A; 1:7500 dilution) and secondary goat anti-chicken horseradish peroxidase-conjugated IgG (ImmunoReagents Inc., 1:7500 dilution) with Pierce ECL Western Blotting Substrate (Thermo Scientific). Images were captured using a ChemiDoc system (Bio-Rad) and densitometry was performed using Image Studio lite (Li-Cor Biosciences).

Photobioreactor configuration and conditions

Bioreactors were comprised of autoclave-sterilised 500 mL borosilicate glass reagent bottles (Simax) and polypropylene caps modified with gastight stainless-steel liquid and gas sampling tubes (Figure S2). 500 mL minimal medium, with 50 mM glycerol and 10 mM glutamate, was inoculated from a 4-day preculture to a starting OD_{660} of 0.05 – 0.075 (~1% inoculum). For non-growing experiments, pre-cultures were grown to OD_{660} of ~1 before cells were harvested by centrifugation in 250 mL bottles (Nalgene, Thermofisher) at 3500 *g* for 15 min. Cell pellets were washed twice with 200 mL aliquots of nitrogen-free minimal medium (standard formula omitting yeast extract and glutamate), followed by centrifugation and final resuspension in nitrogen-free medium at OD_{660} ~ 0.5. Anaerobic and dinitrogen-free conditions were created by sparging sterile argon gas though cultures for 10 minutes. Magnetic stirring provided agitation at ± 200 RPM with 50 mm PTFE-coated stirrer bars. Bioreactor temperature was maintained at 35°C (± 0.2) by immersion in a water-filled glass tank equipped with a heating circulator and cooling loop. Irradiance intensity was calibrated to 200 $W.m^{-2}$ (± 20) at the internal surface of the bioreactors. Hydrogen gas production was quantified by displacement of water in inverted 1 L measuring cylinders via low hydrogen-permeability tubing (Tygon E-3603, Saint-Gobain) and one-way valves to preclude culture contamination. Cell growth was determined by monitoring optical density (OD_{660}) and converting to dry cell weight concentration using a standard curve. Data was analysed using GraphPad Prism, and statistical methods used to determine significance are described in the relevant figure legends.

Supporting Information

Details of plasmids and primer sequences used, rates of false-positive transformants after selection on kanamycin, results of RNA isolation using previous methods and image of photobioreactor configuration.

Supplementary protocols: *R. palustris* electrocompetent cell preparation (protocol 1), *R. palustris* electro-transformation procedure (protocol 2), Optimised *R. palustris* RNA isolation procedure (protocol 3).

Author Contributions

J-P du Toit: Conceptualisation, Methodology, Investigation, Formal Analysis, Visualisation, Writing - Original Draft, Writing - Review & Editing. **D.J. Lea-Smith:** Conceptualisation, Methodology, Writing -

Review & Editing. **A. Git:** Methodology, Investigation (RT-qPCR), Writing - Review & Editing. **J.R.D. Hervey:** Methodology. **C.J. Howe:** Conceptualisation, Supervision, Writing - Review & Editing, Funding Acquisition. **R.W.M. Pott:** Conceptualisation, Supervision, Writing - Review & Editing, Funding Acquisition.

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Declarations of interest:

The authors declare that no conflicts of interest exist.

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