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Elimination of Rubisco alters the regulation of nitrogenase activity and increases hydrogen production in *Rhodospirillum rubrum*

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Abstract

Nitrogenase not only reduces atmospheric nitrogen to ammonia, but also reduces protons to hydrogen (H₂). The nitrogenase system is the primary means of H₂ production under photosynthetic and nitrogen-limiting conditions in many photosynthetic bacteria, including *Rhodospirillum rubrum*. The efficiency of this biological H₂ production largely depends on the nitrogenase enzyme and the availability of ATP and electrons in the cell. Previous studies showed that blockage of the CO₂ fixation pathway in *R. rubrum* induced nitrogenase activity even in the presence of ammonium, presumably to remove excess reductant in the cell. We report here the re-characterization of *cbbM* mutants in *R. rubrum* to study the effect of Rubisco on H₂ production. Our newly constructed *cbbM* mutants grew poorly in malate medium under anaerobic conditions. However, the introduction of constitutively active NifA (NifA*), the transcriptional activator of the nitrogen fixation (*nif*) genes, allows *cbbM* mutants to dissipate the excess reductant through the nitrogenase system and improves their growth. Interestingly, we found that the deletion of *cbbM* alters the posttranslational regulation of nitrogenase activity, resulting in partially active nitrogenase in the presence of ammonium. The combination of mutations in *nifA*, *draT* and *cbbM* greatly increased H₂ production of *R. rubrum*, especially in the presence of excess of ammonium. Furthermore, these mutants are able to produce H₂ over a much longer time frame than the wild type, increasing the potential of these recombinant strains for the biological production of H₂.

Keywords

Rhodospirillum rubrum; nitrogenase; Rubisco; NifA; H₂ production

1. Introduction

Hydrogen gas (H₂) is seen as an energy carrier of the future by virtue of the fact that it is renewable and clean, since no carbon dioxide is produced and water is the only product of its combustion. Biological H₂ production has attracted attention in recent years because it is

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particularly environmentally friendly and can be produced from negative-value waste materials [1–3].

Purple non-sulfur photosynthetic bacteria have been studied extensively for biological H₂ production, since they have relative high substrate to product conversion yield and can produce highly pure H₂ without the contamination of oxygen. In addition, these bacteria can consume many different organic substrates, including those regarded as waste [1,4]. Several studies have focused on increasing the efficiency of H₂ production by optimization of growth conditions [5–9], co-culture cultivations [10–12], genetic engineering on H₂-producing or uptake enzymes [13–16] and the alteration of other regulatory mechanisms [17]. *Rhodospirillum rubrum* is one of the best model strains for the study of H₂ production, since it has several pathways to produce H₂, including nitrogenase [18], CO-induced Ni-Fe hydrogenase [19–21], pyruvate-formate hydrogenase [22–24] and other hydrogenases [25]. In *R. rubrum*, nitrogenase is the main route for H₂ production under photosynthetic and nitrogen-limiting conditions and it can efficiently produce nearly pure H₂ gas (>85% H₂ content) without O₂ as a byproduct [26].

The molybdenum nitrogenase is the main nitrogenase complex in *R. rubrum* and it consists of two proteins: dinitrogenase (also referred to as MoFe protein, coded by *nifDK* gene) and dinitrogenase reductase (also referred to as Fe protein, coded by *nifH* gene) [27]. In *R. rubrum*, the regulation of nitrogenase has been well characterized and it occurs at both the transcriptional and posttranslational levels [28–30]. NifA is a *nif*-specific transcriptional activator of the *nif* (nitrogen fixation) operon. Under nitrogen-limiting conditions, NifA is activated by GlnB-UMP, one of the modified P_{II} proteins [31]. Nitrogenase activity in *R. rubrum* is posttranslationally regulated by the ADP-ribosylation of dinitrogenase reductase in response to elevated levels of fixed nitrogen or the depletion of energy [32,33]. Two enzymes catalyze this reversible process: dinitrogenase reductase ADP-ribosyl transferase (referred to as DRAT, the gene product of *draT*) and dinitrogenase reductase activating glycohydrolase (referred to as DRAG, the gene product of *draG*). DRAT carries out the transfer of the ADP-ribose from NAD to one subunit of the dinitrogenase reductase homodimer, resulting in the inactivation of that enzyme. The ADP-ribose group attached to dinitrogenase reductase can be removed by DRAG, thus restoring nitrogenase activity [28–30]. In the past, we have identified mutations that circumvent both the NifA and the DRAT/DRAG regulatory systems. Mutants with specifically altered NifA (NifA-M173I or NifA-M173V, termed NifA*), express the *nif* genes even under nitrogen-excess conditions [34]. Mutations in *draT* eliminate the inactivating enzyme in the post-translational regulatory system [32]. Thus the nitrogenase in a *draT nifA** double mutant is no longer sensitive to negative stimuli such as NH₄⁺ or the depletion of energy and thus would be potentially useful for the production of biological H₂ [34].

The efficiency of H₂ production by nitrogenase largely depends on the availability of ATP and electrons in the cell. Previous studies indicated that blockage in the primary CO₂ fixation pathway, the Calvin-Benson-Bassham cycle (CBB, with Rubisco as the main enzyme), could increase the level of reductant in the cell, with significant effects on nitrogenase activity [35,36]. In some photosynthetic bacteria, such as *Rhodobacter sphaeroides* and *Rhodobacter capsulatus*, Rubisco-deficient strains are unable to grow photoheterotrophically in malate-ammonium medium, because too much reductant accumulates after blocking the CO₂ fixation pathway [37,38]. Spontaneous mutations at unknown loci in these Rubisco mutants led to the constitutive expression of nitrogenase, which utilized the reductant and restored normal growth [35,36,39]. However, *R. rubrum* was reported as an exception in that its Rubisco-deficient strain (I-19) could grow well under photoheterotrophic conditions [40]. The most surprising phenotype of strain I-19 was

that it expressed nitrogenase even in the presence of ammonia, suggesting that normal regulation of nitrogenase had been altered by the presence of the Rubisco deletion [35].

To further examine this intriguing possibility of a novel regulatory relationship between the fixation of CO₂ and of N₂ in *R. rubrum*, and the potential to increase H₂ production by blocking the CBB pathway, we analyzed a number of different strains altered in both processes and confirmed that there is a linkage between the two. As in other photosynthetic bacteria, *cbbM* mutants of *R. rubrum* grew poorly under photoheterotrophic conditions because of excess reductant in the cell. The introduction of specifically altered NifA*, which results in the constitutive expression of nitrogenase, helped *cbbM* mutants grow photoheterotrophically in malate medium. Additionally, we found that the previously reported *cbbM* mutant (strain I-19) has an unreported mutation in *nifA*, leading to constitutive nitrogenase expression and allowing its growth in malate-ammonium medium. We also found that the deletion of *cbbM* alters the posttranslational regulation of nitrogenase activity, resulting in partially active nitrogenase in the presence of ammonium. Finally, blockage of CBB pathway apparently diverts reductant to nitrogenase and increases H₂ production, because our *cbbM draT nifA** mutants produce about 2-fold more H₂ than does wild type under nitrogen-limiting conditions and 15-fold more under nitrogen-excess condition.

2. Materials and methods

2.1. Bacterial strains and growth conditions

R. rubrum UR2 is a spontaneous streptomycin resistant (Sm^r) strain derived from ATCC11170 and was used as the wild type [41]. *R. rubrum* was grown in yeast extract-supplemented malate-NH₄⁺ (SMN) rich medium aerobically [41,42] or grown in a nitrogen-limiting malate-glutamate minimal medium (MG, with glutamate as nitrogen source) or nitrogen-excess malate-NH₄⁺ minimal medium (MN) photoheterotrophically, as described previously [41,43,44]. *Escherichia coli* was grown in LC medium (similar to Luria-Bertani medium, but with 5 g/liter NaCl). Antibiotics were used at the following concentrations (mg l⁻¹): streptomycin (Sm), 100 (50 for strain I-19 [40]); kanamycin (Km), 12.5; nalidixic acid (Nx), 6; tetracycline (Tc), 1; gentamycin (Gm), 10; and chloramphenicol (Cm), 5 for *R. rubrum*; and ampicillin (Ap), 100; Km, 25; Gm, 5; Cm, 25; and Tc, 12.5 for *E. coli*.

2.2. Construction of *cbbM* mutants

To construct $\Delta cbbM$ mutants, two 1.2-kb fragments immediately 5' and 3' of *cbbM* respectively were amplified by PCR with the chromosomal DNA of *R. rubrum* strain UR2 (wild type) as a template. These fragments have *Hind*III/*Sac*I sites or *Sac*I/*Bam*HI sites at both ends and were cloned into the *Hind*III and *Bam*HI sites of pSUP202 [45]. *aacC1* (encoding Gm^r) from pUCGM [46] was inserted in both orientations into a *Sac*I site between these two fragments, yielding pUX2828 and pUX2829. These plasmids were conjugated into *R. rubrum* UR2 as described previously [32]. Sm^r Gm^r *R. rubrum* colonies were selected and replica-printed to identify Cm^s colonies resulting from a double-crossover recombination event (Cm^r is encoded by pSUP202). All mutations were verified by PCR. The $\Delta cbbM$ mutants were designated UR5251 ($\Delta cbbM1::aacC1$) with *cbbM* and *aacC1* transcribed in the same direction, and UR5252 ($\Delta cbbM2::aacC1$), in which they are transcribed in opposite directions.

To move the $\Delta cbbM::kan$ mutation from the previously reported *cbbM* mutant strain I-19 [40] into different backgrounds, total DNA isolated from strain I-19 was digested with *Xmn*I and *Bgl*II, and was then cloned into pSUP202 by selection for Km^r transformants. The pSUP202 derivative, termed pUX419, has a 2.7-kb *Xmn*I-*Bgl*II fragment carrying

$\Delta cbbM::kan$. This plasmid was conjugated into *R. rubrum* UR2 and $Sm^r Km^r Cm^s$ *R. rubrum* colonies resulting from a double-crossover recombination event were screened as described above. This $\Delta cbbM::kan$ mutant, which has the *cbbM::kan* mutation of strain I-19 in our wild-type (UR2) background, was designated UR865.

All other mutants mentioned in this work were constructed in the same way as described above.

2.3. Whole-cell nitrogenase activity assay

R. rubrum was grown in SMN (rich) medium, and then inoculated into MG or MN medium with a dilution of 1:60. Whole-cell nitrogenase activity was monitored by the acetylene reduction assay as described previously [33].

2.4. Quantitation of H₂ production

R. rubrum was inoculated into 50 ml of MG or MN medium in 100-ml bottles with a gas-tight rubber stopper. The bottle was degassed and filled with argon before illumination. After 48 h of growth, gas in each bottle was collected and measured as described previously [26]. The gas produced was approximately 85% H₂ as shown by gas chromatography using a thermal conductivity detector. H₂ production yield is expressed as mol H₂ produced per mol substrate (malic acid) used. Data points were obtained from the average of at least triplicate measurements from different individually grown cultures, and standard deviations were calculated.

2.5. Western immunoblotting of nitrogenase reductase

A trichloroacetic acid precipitation method was used for rapid protein extraction [47]. Proteins were separated on low-cross-linker SDS-PAGE, and immunoblotted with polyclonal antibody against *Azotobacter vinelandii* nitrogenase reductase as described previously [48].

3. Results and discussion

3.1. Growth of *cbbM* mutants in malate medium

To study the effect of Rubisco activity on the regulation of nitrogenase and its potential for H₂ production, two *cbbM* mutants, each with a gentamycin-cassette insertion replacing *cbbM*, were constructed using our wild-type (UR2), as described in Materials and methods. The mutants (UR5251 and UR5252) differed in the orientation of the *aacC1* insertion and were both analyzed in all the experiments described below. Because these mutants behaved identically, only the data for UR5251 are reported. While strain I-19, a $\Delta cbbM::kan$ mutant of *R. rubrum*, has been reported to grow well in malate medium [40], strain UR5251 ($\Delta cbbM::aacC1$) grew slowly in malate-glutamate (MG) medium (Fig. 1A), and very poorly in malate-ammonium (MN) medium (Fig. 1B). We were concerned that the growth differences between strains I-19 and UR5251 might be caused by differences in the nature of the *cbbM* mutation. To determine if this were indeed the case, we cloned the $\Delta cbbM::kan$ region from strain I-19, and moved it into our *R. rubrum* wild-type (UR2) background, creating UR865, as described in Materials and methods.

As shown in Fig. 1, UR865 has the same growth problems in MG and MN media under anaerobic conditions as those seen in UR5251. However, after a longer incubation period these *cbbM* mutants (UR5251 and UR865) eventually grew and the cell density reached an OD₆₀₀ of about 3 after 5 days of cultivation in MG medium (Fig. 1A). This eventual growth is not due to the selection for suppressor mutations, since 5-day-old cultures of UR5251 grew slowly after dilution into fresh MG medium (data not shown). In contrast to the poor

anaerobic growth of our newly construct *cbbM* mutants (UR5251 and UR865), strain I-19 not only grew well in both MG and MN media (Fig. 1), but also had moderate nitrogenase activity (Table 1) and H₂ production (data not shown) even in the presence of ammonia (MN medium). These results indicate that at least some of the growth properties seen in strain I-19 must be due to differences in its genetic background, and this is further investigated below.

Under aerobic growth conditions, our *cbbM* mutants (UR5251 and UR865) grew well in malate media (MN and MG) with final ODs similar to those seen from strain I-19 and wild type (UR2) (data not shown). These results suggest that oxygen could relieve the metabolic problems caused by the elimination of Rubisco, probably by removing excess reductant accumulated in the *cbbM* mutants. This was further confirmed by gas injection experiments. Three milliliters of O₂, CO₂, or N₂ were injected into the 12.5-ml headspace of anaerobic MG cultures, and the cultures were then grown under light for 3 days. Only O₂ dramatically improved the growth of *cbbM* mutants (data not shown). This is consistent with the hypothesis that O₂ respiration is dissipating excess reductant caused by the blockage of the main CO₂ fixation pathway in *cbbM* mutants [35,36,39].

3.2. Nitrogenase activity is critical for the anaerobic growth of the newly constructed *cbbM* mutants

It has been hypothesized that nitrogenase could remove excess reductant accumulated in Rubisco mutants of some photosynthetic organisms to support their photoheterotrophic growth [35,36,39], so we tested whether nitrogenase is important for anaerobic growth of these newly constructed *R. rubrum cbbM* mutants. We added a $\Delta cbbM$ mutation to a mutant background lacking the Mo-Fe nitrogenase system (UR206, a *nifH::kan* mutant) [49], creating UR2598. Similar to the $\Delta cbbM$ mutants (UR5251 and UR865), the *cbbM nifH* double mutant grew very poorly in MN medium under anaerobic conditions (Fig. 1B). However, this double mutant grew much more slowly in MG medium than did the *cbbM* single mutant when nitrogenase was expressed (Fig. 1A). This result supports the hypothesis that nitrogenase can remove excess reductant and improve the anaerobic growth of *cbbM* mutants [35,36,39]. The slow growth of the *cbbM nifH* mutant in MG medium might be due to the expression of an alternative nitrogenase system, which could slowly consume reductant accumulated in this mutant. We have shown previously that an alternate nitrogenase system in *R. rubrum* is expressed under nitrogen-deficient conditions when the Mo-Fe nitrogenase system is genetically eliminated [43]. This result shows that nitrogenase enhances the anaerobic growth of *cbbM* mutants.

3.3. Growth of *cbbM* mutants is greatly improved when the nitrogenase activity is not impaired by regulation

Previous studies showed that *R. capsulatus* and *R. sphaeroides* mutants lacking Rubisco enzymes were unable to grow photoheterotrophically in malate-ammonium medium [37,38]. Spontaneous suppressor mutants were obtained in both organisms and were shown to have functional nitrogenase in the presence of NH₄⁺, which causes the dissipation of excess reductant [35,36,39]. Since our newly constructed *R. rubrum cbbM* mutants (UR5251 and UR865) have the same anaerobic growth problem as Rubisco-deficient mutants in these related organisms, we tested whether mutations altering nitrogenase activity could also solve the growth problem in these *R. rubrum* Rubisco-deficient mutants.

In *R. rubrum*, the presence of fixed nitrogen blocks nitrogenase activity by effects at both transcriptional and posttranslational levels. Recently, we constructed several *draT nifA** double mutants that eliminate both levels of regulation, and these mutants are no longer sensitive to negative stimuli such as NH₄⁺ or the depletion of energy [34]. To test whether

the unregulated activity of nitrogenase could improve the growth of *R. rubrum cbbM* mutants, we constructed *cbbM nifA** double mutants (UR2561 and UR2562, which encode NifA-M173I and NifA-M173V, respectively), a *cbbM draT* double mutant (UR2560), and *cbbM draT nifA** triple mutants (UR2551 or UR2553, which encode NifA-M173I and NifA-M173V, respectively). Because these two *nifA** variants behaved identically, only the data for UR2553 and UR2562 are reported. As shown in Fig. 2, the *cbbM draT* double mutant behaved similarly to the *cbbM* mutants: All grew slowly in MG medium and grew very poorly in MN medium. However, the *cbbM nifA** double mutants and *cbbM draT nifA** triple mutants grew well in both MG and MN media. This indicates that the altered *nif* expression by NifA* provides better growth of the *cbbM* mutants. This is consistent with the hypothesis that the poor growth of *cbbM* mutants is caused by excess reductant in the cell, and that expression of nitrogenase could remove this excess reductant [35,36,39].

3.4. Identification of the additional mutation loci in strain I-19

The fact that our newly constructed *cbbM::aacC1* and *cbbM::kan* mutants (UR5251 and UR865) behaved very differently from a previously reported *cbbM::kan* mutant (strain I-19) led us suppose that there were differences in the genetic backgrounds of these strains. Given the above findings that the constitutive expression of nitrogenase by the genetically altered regulation of the nitrogenase system could improve the growth of *cbbM* mutants, we asked if strain I-19 had similar mutations, resulting in its better growth in both MG and MN media. We therefore cloned and sequenced genes from strain I-19 whose products are known to have roles in the regulation of *nif* expression or nitrogenase activity in *R. rubrum*: *nifR*, *ntrBC*, *glnB*, *glnJ* and *nifA*. All of these genes in I-19 had sequences identical to those in our wild type (UR2), with the striking exception of *nifA*, which had a mutation creating an M173V substitution. As we have shown previously and above, this substitution allows NifA to be active in the presence of ammonium [34]. This is a plausible basis for much of the improved growth of strain I-19 as compared to our *cbbM* mutants. However, we noticed that strain I-19 is not identical to our *cbbM nifA** (UR2562, which encodes the NifA-M173V variant), especially in that the cells of strain I-19 settled on the bottom of tubes when grown in MN or MG medium anaerobically. This suggested that strain I-19 might have alterations in its flagella, but sequence analysis of the *flhA flbD* region revealed no mutations.

Interestingly, we also noticed that the poor anaerobic growth of our *cbbM* mutant (UR5251) in MG correlated with a change in cell shape from a spiral to spindle shape, as observed by optical microscopy and electron microscopy (Fig. 3). The complementation of this *cbbM* mutant with a wild-type *cbbM* gene on a multicopy plasmid restored the cells to their normal shape (data not shown). However, the mechanism for this change in cell shape is unknown, and might be related to the excess reductant accumulated in the *cbbM* mutants.

All of these data indicate that the accumulation of excess reductant in Rubisco mutants in *R. rubrum* is unable to induce the derepression of nitrogenase in the nitrogen-excess condition. Similar to that seen in Rubisco mutants of *R. capsulatus* and *R. sphaeroides* [35,36,39], an additional mutation, such as in *nifA* in strain I-19, is necessary to allow the constitutive expression of nitrogenase to support their growth under nitrogen-excess conditions. It is likely that suppressor mutants obtained from Rubisco mutants of *R. capsulatus* and *R. sphaeroides* might also have mutations affecting NifA or other regulatory proteins involved in the regulation of nitrogenase. Suppressor mutations in *nifA* have also been obtained from a *glnB* mutant in *R. rubrum* when grown under nitrogen-fixing conditions [34], and in *Rhodopseudomonas palustris* when grown in the presence of electron-rich carbon and ammonium [50].

3.5. Posttranslational regulation of nitrogenase activity is altered in *cbbM* mutants

Nitrogenase activity was also monitored in *cbbM nifA**, *cbbM draT*, and *cbbM draT nifA** mutants (Table 1). We were unable to detect nitrogenase activity in *cbbM* and *cbbM draT* mutants when grown in MN medium, since they grew very poorly in this medium. However, these mutants grew slowly in MG with nitrogenase activity similar to that seen in wild type after normalization to cell density (although total nitrogenase activity in these mutants was much lower than that seen in wild type). Overall, all mutants showed similar nitrogenase activity when grown in MG medium, but significant differences were seen in MN-grown cultures. Because of the absence of both transcriptional and posttranslational regulation of nitrogenase, the *cbbM draT nifA** mutant (UR2553) showed high nitrogenase activity when grown in MN medium, in which condition the wild type (UR2) had little nitrogenase activity. It is somewhat surprising that the *cbbM nifA** mutant (UR2562) showed fairly high nitrogenase activity when grown in MN medium, while its parental strain (UR2503, with the *nifA** mutation only) displayed low nitrogenase activity in MN medium (Table 1), since dinitrogenase reductase was expected to be modified by DRAT in these mutants under nitrogen-excess conditions [34].

The *in vivo* modification of dinitrogenase reductase was also monitored in these strains when grown in MN medium and the data are shown in Fig. 4. The modification status of dinitrogenase reductase in *nifA** mutant (UR2503) and *draT nifA** mutant (UR2329) were identical to that reported previously [34]. The *nifA** mutant (UR2503) showed almost complete modification of dinitrogenase reductase under this condition. Since one subunit of the dinitrogenase reductase homodimer is modified by ADP-ribosylation through the DRAT/DRAG system, and the modified subunit (indicated as M) migrates more slowly than the unmodified subunit (indicated as U), UR2503 has both the modified and the unmodified band in a 1:1 ratio shown on the immunoblots (lane 1). As seen previously [34], there is a third band between the unmodified band and the modified band, which might be the alternative (Fe-only) dinitrogenase reductase or other unmodified form of dinitrogenase reductase. Because of the absence of DRAT, no modification of dinitrogenase reductase was seen in the *draT nifA** mutant (UR2329) under this condition. Consistent with the fairly high nitrogenase activity (Table 1), the *cbbM nifA** mutant (UR2562) showed only partially modified dinitrogenase reductase under nitrogen-excess conditions (lane 3). These results imply that the regulation of the DRAT/DRAG regulatory system is altered in *cbbM* mutants, allowing a substantial amount of dinitrogenase reductase to remain active even under nitrogen-excess conditions. It is unclear whether the *cbbM* mutation affects the signal transduction to the DRAT/DRAG regulatory system or the enzymes themselves. In either case, the effect could be on DRAT, on DRAG or on both. For example, DRAT might fail to modify dinitrogenase reductase, or DRAG might remain active and remove the ADP-ribose group from dinitrogenase reductase under NH_4^+ -excess conditions. Since NAD^+ is the donor of the ADP-ribose group attached to dinitrogenase reductase by DRAT [28], it is also plausible that the excess reductant in *cbbM* mutants could reduce the level of NAD^+ and thus affect DRAT activity. It has also been reported that DRAT prefers oxidized dinitrogenase reductase as a substrate, while DRAG prefers the reduced form of dinitrogenase reductase [51,52]. The reductant levels in the cell certainly could affect both DRAT and DRAG activities by shifting dinitrogenase reductase into different redox states.

3.6. Blocking the CBB pathway can increase H_2 production under nitrogen-excess conditions

Theoretically, the CO_2 and N_2 fixation pathways could compete for reductant, and the blockage of the main CO_2 fixation pathway in *cbbM* mutants could divert reductant to nitrogenase [35,36,39]. We were interested to see if blocking the CBB pathway in *cbbM* mutants could increase H_2 production in the *nifA** mutants. As shown in Fig. 5A, in MG

medium, a *cbbM* deletion led to a longer time frame of H₂ production for *R. rubrum* strains. Both *cbbM nifA** mutant (UR2562) and *cbbM draT nifA** mutant (UR2553) continuously produced H₂ for about 11 days, while the wild type (UR2) and the *nifA** mutant (UR2503) stop producing H₂ at the 4th day after inoculation, and the *draT nifA** mutant (UR2329) produced H₂ until the 9th day. This extended H₂ production might be due to the slower growth of these *cbbM* mutants in MG medium than other strains (Fig. 2A). However, unlike wild type, which stopped producing H₂ when cells stopped growth at the 4th day, the *cbbM draT nifA** mutant (UR2553) stopped growth at the 7th day, but kept producing H₂ for another 4 days. With the slower growth and longer term of H₂ production, this *cbbM draT nifA** mutant showed two-fold higher H₂ yield than that of wild type under nitrogen limiting (MG) conditions (Table 1). The *cbbM* deletion also increased the total H₂ production of *R. rubrum* strains, especially in the *cbbM draT nifA** mutant (UR2553), which showed 35% higher H₂ production than did the *draT nifA** strain (UR2329) in MG medium. A more significant increase in H₂ production was seen in MN-grown cultures: Both *cbbM nifA** (UR2562) and *cbbM draT nifA** (UR2553) produced about 2-fold more H₂ than did their respective *cbbM*⁺ controls. Overall, our triple mutant *cbbM draT nifA** (UR2553) produced about 2-fold and 15-fold more H₂ than did the wild type (UR2) in MG and MN media, respectively.

3.7. Implication of these altered *R. rubrum* strains for the H₂ production

Nitrogenase could potentially be used for H₂ production. However, the efficiency of H₂ production by nitrogenase is not very robust, and the limiting factor for H₂ production is not well defined. In vitro studies showed that the disassociation of oxidized dinitrogenase from the nitrogenase complex is the limiting step [53], but the availability of ATP and electrons in the cell could also affect H₂ production. We recently found that the nitrogenase system is very effective in competing with other ATP-utilizing systems for ATP, since moderate nitrogenase activity was seen in mutants that block purine synthesis, in which ATP levels are extremely low [54].

Recently, several studies have focused on the improvement of the efficiency of H₂ production in *R. rubrum* and other purple non-sulfur bacteria through genetic engineering, including heterologous expression of a hydrogenase system [25,55], elimination of an uptake hydrogenase system [26,56–58], alteration of the regulation of nitrogenase expression and activity [34,50], as well as modification of the electron transfer chains [59]. Overall, the regulation of nitrogenase activity by excess ammonium in the medium, greatly decreases ability of the nitrogenase system to produce H₂ [6,10,50,60]. As shown above, *R. rubrum* mutants with alteration in both NifA and the DRAT/DRAG regulatory system showed an order-of-magnitude increase of H₂ production under NH₄⁺-excess conditions versus wild type. Our new data also indicate that when cells are grown in malate-glutamate (MG) medium, there is enough reductant in the cell, and blocking the CBB pathway has a small effect on H₂ production. However, in the MN-grown cells, blocking the CBB pathway could increase H₂ production by as much as two-fold in some mutants.

4. Conclusions

In summary, blocking the CBB pathway causes growth problems for *R. rubrum* in malate medium under anaerobic conditions, especially in the presence of NH₄⁺. The presence of nitrogenase activity or exposure of the cells to O₂ improves growth, probably by removing the excess reductant accumulated in *cbbM* mutants. We also identified a mutation in a previously reported *cbbM* mutant (strain I-19) that allows NifA activity in the presence of ammonium. This explains why strain I-19 grows better than our newly constructed *cbbM* mutants in malate medium under anaerobic conditions. Surprisingly, a *cbbM* mutation alters the posttranslational regulation of nitrogenase activity by the DRAT/DRAG regulatory

system to allow some nitrogenase activity in the presence of NH_4^+ . In combination with mutants altering the regulation of nitrogenase activity, the *cbbM* mutation could provide excess reductant to nitrogenase, resulting in an order-of-magnitude increase of H_2 production under NH_4^+ -excess conditions.

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Fig. 1.

Growth curves of *R. rubrum* UR2 (wild type) (●), UR5251 ($\Delta cbbM::aacC1$) (○), UR865 ($\Delta cbbM::kan$) (Δ), I-19 ($\Delta cbbM::kan$) (▲) and UR2598 (*cbbM nifH*) (□) in malate-glutamate (MG) medium (panel A) and malate-ammonium (MN) medium (panel B) under anaerobic conditions. Each point represents the average of triplicate measurements from different individually grown cultures.

Fig. 2.

Growth curves of *R. rubrum* UR2 (wild type) (●), UR5251 ($\Delta cbbM::aacC1$) (○), UR2553 (*cbbM draT nifA**- encoding M173V) (△), UR2562 (*cbbM nifA**- encoding M173V) (▲), and UR2560 (*cbbM draT*) (□) in MG medium (panel A) and MN medium (panel B) under anaerobic conditions.

Fig. 3. Cell shape of MG-grown *R. rubrum* UR2 (wild type) and UR5251 ($\Delta cbbM::aacC1$) under optical microscopy (panel A) and electron microscopy (panel B). Samples were prepared as described previously [61].

Fig. 4.

Western blotting of dinitrogenase reductase in *nifA** (UR2503, lane 1), *draT nifA** (UR2329, lane 2) and *cbbM nifA** mutants (UR2562, lane 3) grown in MN medium. The ADP-ribosylated dinitrogenase reductase subunit (upper band, labeled M) migrates more slowly than does the unmodified subunit (lower band, labeled U). The faint middle bands routinely seen in immunoblots might be an alternative (Fe-only) dinitrogenase reductase or other unmodified form of dinitrogenase reductase as reported previously [34].

Fig. 5.

Total H₂ production of *R. rubrum* UR2 (wild type) (●), UR2503 (*nifA**) (□), UR2562 (*cbbM nifA**) (■), UR2329 (*draT nifA**) (△) and UR2553 (*cbbM draT nifA**) (▲) when grown in MG (panel A) or MN (panel B) medium under anaerobic conditions. Gas production for these strains reached a plateau and was not measured subsequently.

Table 1

Nitrogenase activity and H₂ production yield in *R. rubrum cbbM*, *draT* or *nifA** mutants.

Strains	Mutant description	Nitrogenase activity ^a		H ₂ production yield ^b (mol H ₂ /mol substrate)	
		MG Nitrogen-limiting	MN NH ₄ ⁺ -excess	MG Nitrogen-limiting	MN NH ₄ ⁺ -excess
UR2	Wild type	710	<10	3.79	0.18
UR2503	<i>nifA</i> *-encoding M173V	770 ^c	30 ^c	4.73	0.49
UR2562	<i>cbbM nifA</i> *-encoding M173V	690	440	5.34	1.04
UR2329	<i>draT nifA</i> *-encoding M173V	750 ^c	540 ^c	5.69	1.38
UR2553	<i>cbbM draT nifA</i> *-encoding M173V	720	990	7.89	2.74
UR2560	<i>cbbM draT</i>	680	<10	ND	ND
UR5251	<i>cbbM</i>	770	<10	ND	ND
I-19	<i>cbbM (nifA</i> *-encoding M173V)	570	340	ND	ND

^aEach unit of nitrogenase activity is expressed as nanomoles of ethylene produced per hour per milliliter of cells at an optical density at 600nm of 1. Each activity value is from at least three replicate assays from different individually grown cultures. The standard deviation is 5 to 10%.

^bH₂ production yield was expressed as mol H₂ produced per mol substrate (malic acid). Each value is from at least three replicate assays from different individually grown cultures. The standard deviation is 5 to 10%.

^cNitrogenase activity in UR2503 and UR2329 were similar to the previously published data [34].