BIOLOGICAL HYDROGEN FROM FUEL GASES

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ABSTRACT

Upon adding CO to the gas phase of the photosynthetic bacterium Rubrivivax gelatinosus CBS, a CO oxidation: H₂ production water-gas shift pathway is induced. Based on chloramphenicol inhibition studies, CO induces the de novo synthesis of new proteins in the CO shift pathway. When various amounts of CO were added during sub-culturing, a minimal level of 10% (v/v) is required for the maximal induction of the CO shift activity. Daily feeding of 20% CO also prolongs the CO shift activity at a higher and constant rate versus a culture fed with CO only during inoculation. After screening a library of transposon mutants, we identified two lines deficient in CO shift activity. Biochemical analysis indicated that mutant GV1214 lacks carbon monoxide dehydrogenase (CODH) activity and mutant GV 1762 lacks hydrogenase activity. Sequence analysis suggests that the mutations occurred in a CODH gene and hydrogenase large subunit gene, respectively. The physiological and molecular biology studies provide insight into the biological CO shift pathway and have significance in producing additional H₂ from gasified waste biomass.

INTRODUCTION

The use of H₂ as a clean fuel not only addresses environmental concerns, it also helps the nation in coping with energy independence and security. The development of an economic means of H₂ production is essential for H₂ economy. Microbial production of H₂ has gained substantial interests in recent years due to the wide variety of substrates microbes can use to generate H₂. These substrates can either be waste biomass or commercial waste streams containing organic compounds. Microbial H₂ production therefore has the dual benefits of generating a clean fuel while reducing waste. The enzymatic reaction responsible for H₂ production from the reduction of protons is catalyzed by hydrogenase, the existence of which is widespread among the domains of Bacteria and Archaea (Vignais et al., 2001).

The use of waste biomass as feedstock for H₂ production is an attractive choice. However, due to its heterogeneous nature, biomass utilization by microbes is extremely slow. Instead, biomass can be gasified via a rather rapid thermochemical process to generate a homogeneous synthesis gas (syngas) mixture containing CO and H₂. A small number of microbes contain the CO oxidation: H₂ production water-gas shift pathway in which CO is converted to H₂ according to the equation CO + H₂O ↔ CO₂ + H₂ (Davidova et al., 1994; Martin et al., 1983; Svetlitchnyi et al., 2001; Uffen, 1981). Syngas therefore can be fed to a microbial culture to yield additional H₂.
Upon adding CO to the culture gas phase, *Rubrivivax gelatinosus* CBS synthesizes a CO oxidation system with the concomitant production of H\textsubscript{2} (Uffen 1976; Maness *et al*., 2002). Although a phototroph, this purple non-sulfur bacterium contains a CO shift pathway that is highly active in darkness. While CBS contains a H\textsubscript{2}-oxidizing system, this pathway does not function in darkness, so the H\textsubscript{2} produced remains in the culture gas phase (Maness and Weaver, 1994). The hydrogenase from *Rx. gelatinosus* is highly O\textsubscript{2} tolerant, compared to most evolving hydrogenases reported in literature (Maness *et al*., 2002). Another feature of the CO shift reaction lies in its high equilibrium constant, near 78,000 at 30 °C, making this process ideal for scale-up H\textsubscript{2} production without the worry of product feedback inhibition.

On the molecular level, the biological water-gas shift reaction catalyzed by *Rx. gelatinosus* CBS is modeled after the analogous system in *Rhodospirillum rubrum*, where H\textsubscript{2} is produced by an intricate enzymatic system initiated by the oxidation of CO by a carbon monoxide dehydrogenase (CODH). This enzyme transfers electrons (via a ferridoxin-like iron sulfur protein) to a heteromultimeric hydrogenase, which then reduces two protons to form H\textsubscript{2} (Uffen, 1981; Bonam *et al*., 1989).

To fully utilize the CO shift reaction as a renewable energy resource, it is necessary to understand the mechanism of how *Rx. gelatinosus* CBS produces H\textsubscript{2} from CO. In *Rs. rubrum*, CO indirectly induces the CODH and hydrogenase via a CO-activated transcription factor (Shelver *et al*., 1995). Carbon monoxide is also a potent inhibitor to many hydrogenases (Adams *et al*.; 1981; Houchins and Burris, 1981; Peters *et al*., 1998), including the CO-linked hydrogenase from *Rx. gelatinosus* CBS when the bulk of the CODH is removed (Maness *et al*., 2002). Carbon monoxide therefore plays multiple roles in the microbial CO oxidation system serving as a substrate, an inducer and an inhibitor. In order to obtain optimal activity, we must understand how CO regulates the overall pathway. This report documents the effect and concentrations of CO on the maximal induction, synthesis, and maintenance of enzymatic activities in the CO shift pathway.

Another approach to gain insight into the CO shift is to clone and characterize genes involved in the pathway. For example, gene discovery will help in the understanding of the rate-limiting step during H\textsubscript{2} production, as well as how CO shift enzymes are produced and degraded. The genetic system of the CO shift pathway in *Rs. rubrum* has been studied (Kerby *et al*. 1992; Fox *et al*., 1996; He *et al*., 1999), yet very little is known about the genes responsible for the CO shift reaction in *Rx. gelatinosus*. We report in this paper the discovery of two CBS mutants unable to shift CO to H\textsubscript{2}, and the successful determination of the partial sequences of four putative CO shift genes.

**MATERIALS AND METHODS**

**Organism and growth condition.** *Rubrivivax gelatinosus* CBS was isolated from garden soil that has been exposed to elevated levels of CO (Maness and Weaver, 1994). Medium preparations, photosynthetic growth conditions and cell dry weight determinations were as described (Maness and Weaver, 2001) with sodium malate (32 mM) serving as the carbon source supplemented with yeast extract (0.05%, w/v) (designated as BG medium). Approximately 20% (v/v) CO was added to the culture gas phase during inoculation to induce the CO shift activity.

**CO shift rate measurement.** A log-phase photosynthetic culture of *Rx. gelatinosus* CBS having an optical density (OD) at 660nm of 0.7-0.8 was diluted to near 0.1 OD in BG medium (final volume 50 ml) in the presence of 1 mM dithiothreitol inside a 158-ml glass bottle. The
diluted suspension was pre-incubated in 20% CO by shaking at 250 rpm for two hours in the dark at 30 °C, followed by gassing out with CO (20%) to initiate the kinetic measurement of CO uptake and H$_2$ production in darkness. Hydrogen and CO were separated with a molecular sieve 5A column (2 mm x 2 m) using Ar as the carrier gas (40cc/min), and detected with a gas chromatograph (Hewlett Packard 5890, series II) equipped with a thermal conductivity detector. Data were analyzed with a HP3365 ChemStation software.

**Hydrogenase and CODH assays.** Hydrogenase activity was determined by the evolution of H$_2$ from the electron mediator methyl viologen (MV, 2 mM) reduced by sodium dithionite (5 mM) according to Maness et al. (2002). Carbon monoxide dehydrogenase activity was measured based on the reduction of MV from CO oxidation monitored at 578 nm (Bonam et al., 1984) in a Cary 5E spectrophotometer (Varian).

**Transposon insertional mutagenesis and screening of CO shift mutant.** Rubrivivax gelatinosus CBS was mutagenized using the EZ::TN™ <R6K$^\gamma$ori/Kan-2> Tnp Transposome™ Kit (Epicentre) following the manufacturer’s protocol. Cells were grown in 15-ml culture tubes under photo-heterotrophic conditions to an OD660 of 0.5, and washed four times with sterile, ice cold 10% (v/v) glycerol. After each wash, the cells were resuspended in one-half the starting volume. After the final wash, cells were resuspended in 0.5 ml, and electroporated immediately or frozen at – 80°C. Electroporation was conducted inside 0.2 mm gap cuvettes using a Bio-Rad Gene pulser® set at 2.0 kV (voltage), 200 Ω (resistance) and 25 μF (capacitance). Approximately 50 μl of cells were mixed with 1 μl of the transposome prior to electroporation. After electroporation cells were immediately added to 15 ml of BG media and incubated in the dark for 6 hours. After outgrowth, transformants were recovered by plating on BG agar plates containing 15 μg/ml kanamycin. Transposition was confirmed via Southern analysis.

To identify CO-shift mutants, individual transformed colonies were added to 10 ml anaerobic vials containing 5 ml BG media supplemented with 15 μg/ml kanamycin. These cultures were grown photosynthetically overnight to an OD ~1.0, followed by inducing with 1.0 ml of pure CO and shaking at 250 rpm for two hours prior to being placed in the dark. Hydrogen and CO contents in the gas phase were analyzed using a Hewlett Packard 5890 series II gas chromatograph as noted above.

The aforementioned transposome contains both a kanamycin resistance marker as well as the R6K$^\gamma$ origin of replication, which facilitates the rescue cloning of mutated genes. DNA was prepared from CBS mutants using the Qiagen genomic DNA extraction kit (Qiagen). Approximately 1 μg of DNA was digested to completion with Sac I (New England Biolabs), and self-ligated in a 1 ml reaction using the DNA Ligation Kit manufactured by Stratagene. Ligations were concentrated and de-salted using a Centricon-30 concentrator (Millipore). *E. coli* cells expressing the *pir* gene were electroporated with 25 ng of ligated DNA and selected on Luria-Bertani agar plates containing 50 μg/ml kanamycin. DNA was isolated from two colonies per mutant using the QIAprep Spin Miniprep Kit (Qiagen) following the manufacturer’s protocol. DNA flanking the transposon was sequenced at the Davis Sequencing automated DNA sequencing facility in Davis, California using the dye terminator cycle sequencing method with PE Biosystem ABI Prism DNA sequencers.
RESULTS

The induction of CO shift activity by CO

To determine if CO induces the CO-shift activity in *Rx. gelatinosus* CBS, we study the effect of chloramphenicol on cultures previously incubated in the absence of CO. Chloramphenicol inhibits new protein synthesis while exerting no effect on existing hydrogenase activity (Bonam *et al.*, 1989). Figure 1 shows that when CO was added to a CBS culture previously not induced for CO-shift activity, CO consumption and H₂ production were detected within two hours following CO addition (square). This experiment was conducted in light to allow for protein synthesis within a reasonable time period, which explains the low levels of net H₂ due to its simultaneous consumption via a light-dependent H₂ uptake pathway. When added along with CO, however, chloramphenicol (50 µg/ml) immediately stopped the appearance of the CO-shift activity for up to a seven-hour period (triangle), suggesting that CO acts as an inducer for the *de novo* synthesis of both CODH and hydrogenase of the CO-shift pathway. Consequently, in order to maintain CO shift activity, CO would have to be present to ensure that new enzymes are constantly being synthesized.

![Figure 1](image-url)

**Fig. 1.** Effects of CO and a protein synthesis inhibitor chloramphenicol (Chloram) on inducing CO shift activity.

Optimal amounts of CO for maximal induction

The induction data gathered above suggest that an optimal or minimal level of CO may be required for maximal induction of the CO shift activity. To verify, we cultured CBS in various amounts of CO in BG medium (see Methods). After 16 hours of incubation in light, the cultures were then diluted to 0.1 OD and assayed for CO uptake coupled to H₂ production in 20% CO. Data from Fig. 2 indicate that between 0 and 10% CO added during inoculation, levels of the newly induced CO shift activity correlates positively with incremental amounts of CO in the gas phase. Beyond 10% CO, the CO shift rate is maximally induced. Similar induced rate was also detected when 100% CO was fed to a culture to induce CO shift pathway (data not shown).
Effect of CO on the longevity of the CO shift activity

Since CO facilitates the production of new CO shift enzymes, its constant presence is likely required to maintain steady-state levels of CO shift proteins. To verify, we prepared two identical CBS cultures in BG medium and fed 20% CO during inoculation. To the control culture, no CO was fed once it was consumed within 10 hours, and to a parallel culture CO (20%) was fed daily. Aliquots of cells were withdrawn from both cultures and rates of H₂ production from CO were measured daily using diluted suspension saturated with CO (20%) (Methods) so that mass transfer is not limited during assays. Results from Fig. 3 indicate that over a period of five days, the culture receiving CO daily was able to maintain a more constant CO shift rate, between day two and day five, 56% higher than that of the control without daily CO feeding. Both cultures exhibited similar growth rates during the time course of the experiment.

Cloning the CO shift genes

To identify the genes involved in the CO shift pathway in *Rx. gelatinosus* CBS, we created a library of transposon mutants, which were then screened for their CO shift activity. Mutants unable to either consume CO or produce H₂ would suggest that gene(s) responsible for that function has been interrupted with the transposon insertion. After screening approximately 2000 mutants, we identified two mutants unable to consume CO or produce H₂ (Table 1). Enzymatic assays of partial reaction indicate that mutant GV1214 lacks the CODH activity (unable to reduce MV from CO oxidation), yet it still retains partial hydrogenase activity. To the contrary, mutant GV1762 retains 32% of the CODH activity, yet its hydrogenase activity is abolished completely. The mutant genes were rescue cloned, sequenced, and compared to known sequences in GenBank. GV1214 is interrupted in an ortholog of the *Rs. rubrum* cooS gene, which encoded a CODH protein. The predicted amino acid sequence of the putative CBS CODH is 56% identical and 70% similar to that of the *Rs. rubrum* protein. The predicted amino acid sequence flanking the putative CBS CODH is homologous to the *Rs. rubrum* CooF protein (58% similar and 66% identical), which is involved in the transfer of electrons from CO oxidation to the hydrogenase. Mutant 1762 is interrupted in a gene resembling the *Rs. rubrum*
hydrogenase large subunit CooH (68% identical and 89% similar). Although incomplete at this time, the DNA sequence 5’ of the predicted cooH gene resembles cooU, which codes for a protein as part of the hydrogenase complex.

Fig. 3. Effect of CO feedings on rates and longevity of the CO shift reaction

Table 1. Enzymatic activities and homology comparison of putative CO shift genes of *Rx. gelatinosus* CBS.

<table>
<thead>
<tr>
<th></th>
<th>CODH*</th>
<th>Hydrogenase*</th>
<th>CO to H₂*</th>
<th>Homology with <em>Rs. rubrum</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>CBS Parent</td>
<td>14.8</td>
<td>0.36</td>
<td>0.87</td>
<td></td>
</tr>
<tr>
<td>GV 1214</td>
<td>0</td>
<td>0.03</td>
<td>0.00</td>
<td>CODH, ~56% identical</td>
</tr>
<tr>
<td>GV 1762</td>
<td>4.75</td>
<td>0.00</td>
<td>0.00</td>
<td>Hydrogenase, ~68% identical</td>
</tr>
</tbody>
</table>

* unit: mmol/min/g cell dry weight

Photo-oxidative killing of a carotenoidless mutant

During the mutagenesis process, we unexpectedly obtained several mutants unable to produce carotenoid, the photoprotective pigment responsible for scavenging oxygen radicals. Without the carotenoid pigment, these colonies appear blue-green on nutrient agar plates. One of these cell lines was designated G1, which due to its lack of carotenoid, will die when exposed to both high light and O₂ simultaneously. Data from Fig. 4 show that when a G1 culture was bubbled with air, the bulk of the cells were killed completely within one minute of illumination with a source of white light having an intensity of 630 μmol/sec/m². Little impact was observed with a parallel control containing CBS wild-type cells.
DISCUSSION

Similar to the CO oxidation system in *Rs. rubrum* (Bonam *et al.*, 1989; Shelver *et al* 1995; Maness and Weaver, 2001), CO also induces the CO shift pathway in *Rx. gelatinosus* (Fig. 1). Carbon monoxide has been demonstrated to bind to a transcriptional regulator to activate the expression of the genes in the CO shift pathway (Shelver *et al*., 1995; He *et al*., 1999). Data from Fig. 2 suggest that at least 10% CO has to be present at all times to ensure the maximal induction of the CO shift pathway. Ten percent CO in the gas phase yields a dissolved CO concentration of 72 µM in solution under a 0.82 atmospheric pressure, implying the sensitivity of the CO sensing mechanism in this organism. Since long-term goal of this project is to produce H₂ using syngas or refinery gas as feedstock, which may contain variable amounts of CO depending on the process for its generation, it is important to know the minimal level of CO required for the maximal induction of the CO shift pathway.

With CO serving as an inducer of the CO shift reaction, its constant presence is thereby necessary in order to maintain this reaction both at a higher rate and for a longer duration. Results from Fig. 3 strongly support this assumption. Each data point in Fig. 3 represents an average of 56 measurements, while the data from day two represent the average of 84 measurements. It is not clear why the culture fed with additional CO at day two exhibited a CO shift rate 20% less than that of the control. Nonetheless, the culture receiving daily feeding was able to maintain a higher and constant rate for the duration of the experiment comparing to its control.

The collection of carotenoidless pigmented mutants provides us with another means of generating CO shift mutant for the elucidation of the CO shift genes. Based on the susceptibility of the pigmented mutant to photo-oxidative killing (Fig. 4), CO shift mutant can be selected from a population of cells cultured in CO as the sole carbon substrate. Those growing cells with an intact CO shift pathway will be selectively killed in light and O₂, leaving behind non-growing yet viable cells with an altered CO shift pathway. Using a similar strategy, we have successfully

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**Fig. 4.** Photo-oxidative killing of *Rx. gelatinosus* CBS and its carotenoidless mutant G1.
isolated a mutant deficient in uptake hydrogenase activity from the carotenoidless *Rs. rubrum* G9 mutant by subjecting G9 to photo-oxidative killing after culturing it under an H₂-CO₂ atmosphere as the sole electron donor and carbon substrate to fully induce its H₂-uptake pathway (Maness and Weaver, 2001).

By identifying putative CO shift genes (*cooS, cooF, cooH, and cooU*), we have begun to dissect, on the molecular level, how *Rx. gelatinosus* CBS responds to CO and produces H₂. Since bacterial genes of related functions are normally clustered within the chromosome, we can use our current sequence information to clone nearby genes and predict, by sequence homology, if they are involved in producing H₂. Once obtained, these genes can be manipulated to optimize the production of H₂. For example, targeted mutagenesis may enable the overproduction of CO shift enzymes as well as increasing their overall catalytic efficiency.

**ACKNOWLEDGEMENTS**

This work was supported by the U.S. Department of Energy Hydrogen Program.

**REFERENCES**


