Gas Exchange in the Filamentous Cyanobacterium *Nostoc punctiforme* Strain ATCC 29133 and Its Hydrogenase-Deficient Mutant Strain NHM5

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*Nostoc punctiforme* ATCC 29133 is a nitrogen-fixing, heterocystous cyanobacterium of symbiotic origin. During nitrogen fixation, it produces molecular hydrogen (H₂), which is recaptured by an uptake hydrogenase. Gas exchange in cultures of *N. punctiforme* ATCC 29133 and its hydrogenase-free mutant strain NHM5 was studied. Exchange of O₂, CO₂, N₂, and H₂ was followed simultaneously with a mass spectrometer in cultures grown under nitrogen-fixing conditions. Isotopic tracing was used to separate evolution and uptake of CO₂ and O₂. The amount of H₂ produced per molecule of N₂ fixed was found to vary with light conditions, high light giving a greater increase in H₂ production than N₂ fixation. The ratio under low light and high light was approximately 1.4 and 6.1 molecules of H₂ produced per molecule of N₂ fixed, respectively. Incubation under high light for a longer time, until the culture was depleted of CO₂, caused a decrease in the nitrogen fixation rate. At the same time, hydrogen production in the hydrogenase-deficient strain was increased from an initial rate of approximately 6 μmol (mg of chlorophyll a)⁻¹ h⁻¹ to 9 μmol (mg of chlorophyll a)⁻¹ h⁻¹ after about 50 min. A light-stimulated hydrogen-deuterium exchange activity stemming from the nitrogenase was observed in the two strains. The present findings are important for understanding this nitrogenase-based system, aiming at photobiological hydrogen production, as we have identified the conditions under which the energy flow through the nitrogenase can be directed towards hydrogen production rather than nitrogen fixation.

Cyanobacteria are an ancient and widespread group of photosynthetic organisms, found in many different environments on Earth. Some of them have the ability to fix nitrogen from the atmosphere and thus are able to live in nitrogen-poor media with sunlight as their source of energy and water as the source of reductant.

The enzyme complex that performs nitrogen fixation, the nitrogenase, is oxygen sensitive, and nitrogen-fixing cyanobacteria have therefore evolved a variety of strategies to combine nitrogen fixation with oxygen-evolving photosynthesis. Some separate the two processes temporally, and others separate them spatially. The primary spatial strategy, deployed by certain filamentous cyanobacteria, is to develop specialized cells called heterocysts, in which there is no photosystem II activity, and thus no O₂ evolution, an enhanced level of respiration scavenging O₂, and a protecting cell envelope limiting O₂ penetration (22). This allows nitrogen fixation to take place in a microaerobic environment in these cells. The heterocysts are supported with carbohydrates from the surrounding vegetative cells, and export combined nitrogen in return. The activation of nitrogen fixation is determined by the nitrogen status of the filament. When no source of fixed nitrogen (e.g., ammonium, nitrate, or urea) is available, some cells will develop into heterocysts and start expressing the nitrogenase. This highly regulated process has been most extensively studied in *Anabaena* and *Nostoc* species, including *Nostoc punctiforme* (for reviews, see references 10, 19, and 33).

*Nostoc punctiforme* ATCC 29133 is a filamentous cyanobacterium capable of nitrogen fixation. It was originally isolated from a symbiotic association with the cycad *Macrozamia* sp. (25). In symbiosis, the cyanobacterium grows inside specialized roots of the plant, where it performs nitrogen fixation for its host, and in return can use carbohydrates from the plant. The heterocyst frequency in the symbiotic filament is elevated, reaching as high as 50% heterocysts in the older parts of the root, compared to the heterocyst frequency in the free-living state which is around 5% (4).

In *N. punctiforme* ATCC 29133, there is only one type of nitrogenase, the MoFe–nitrogenase complex, encoded by the structural genes *nifHDK* (20). Electrons derived from degradation of carbohydrates through the oxidative pentose phosphate pathway (29) are used to reduce N₂ to ammonia (34). The electrons are delivered to nitrogenase via NADPH, ferredoxin–NADP-reductase, and a heterocyst-specific ferredoxin (or flavodoxin) or via NAD(P)H and thylakoid electron carriers, through photosystem I, which reduces ferredoxin in the light (26).

The nitrogenase itself is remarkably conserved between different species, and the reaction takes place according to the formula

\[
2N_2 + 8H^+ + 8e^- \rightarrow 2NH_4^+ + 2H_2 \]

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N\textsubscript{2} + (6 + 2n)\textit{H}^{+} + (6 + 2n)e^{-} + p(6 + 2n)\textit{ATP} \rightarrow 2\textit{NH}_{3} + n\textit{H}_{2} + p(6 + 2n)\textit{ADP} + p(6 + 2n)\textit{P}_{i}

where \( n \geq 1 \) and \( p \geq 2 \), \( n \) representing the number of hydrogen molecules coupled to reduction of one molecule of nitrogen and \( p \) representing the number of ATP molecules hydrolyzed per electron transferred (24).

The ATP consumed by the process is generated in the heterocysts by cyclic electron flow around photosystem I and/or by oxidative phosphorylation (34). Molecular hydrogen is formed as a by-product. The formation of hydrogen is obligatory for the reaction, and at least one fourth of the electrons are consumed by that process. In vivo, the ratio of nitrogen fixed to hydrogen evolved is in fact variable, as expressed in the formula above, and typically more than one molecule of \( \text{H}_{2} \) will be produced for each molecule of \( \text{N}_{2} \) fixed. Theoretically, if no \( \text{N}_{2} \) or other substrate is available, e.g., if the cells are incubated under an argon atmosphere, all electrons available to the nitrogenase would be used for hydrogen production (6).

Several parameters influence the activity of the nitrogenase. The requirement for ATP makes light conditions a determining factor, particularly in the absence of an external supply of sugars that can be used as an energy source (17). Also, carbohydrates must be imported to the heterocysts from the vegetative cells to provide reducing power for the nitrogenase itself. The ATP-dependent mechanism. The hypothesis for this inactivation is that an abundance of ammonia will direct the use of reducing power for the nitrogenase itself and for respiration, which protects the nitrogenase from inactivation by oxygen. It has not been shown what compound(s) is imported, although the available evidence indicates that sucrose is a likely candidate (34). A supply of carbon skeletons is also necessary for biosynthesis to incorporate ammonia. The presence of ammonium will have a negative effect on the synthesis of new nitrogenase (9) and will, under certain physiological conditions, induce a modification of the iron protein, NifH, which reversibly inactivates the nitrogenase by an oxygen-dependent mechanism. The hypothesis for this inactivation is that an abundance of ammonia will direct the use of available carbohydrates to ammonia assimilation, thereby creating a decreased supply of reductant for respiration, resulting in an increased level of oxygen in the heterocyst, which in turn induces the modification of NifH (7). Other conditions, such as carbon starvation, can also lead to this modification (8).

In all nitrogen-fixing cyanobacteria examined so far, molecular hydrogen produced by the nitrogenase is reoxidized by an uptake hydrogenase (30). This enzyme belongs to the class of membrane-bound NiFe hydrogenases (32), as identified by sequence homology. It consists of two subunits, the small and large subunit being encoded by the structural genes \( hup\textit{S} \) and \( hup\textit{L} \), respectively. The large subunit contains the active center of the enzyme, splitting \( \text{H}_{2} \) into protons and electrons. The electrons are presumably passed on through FeS clusters in the small subunit to an acceptor in the respiratory electron transport chain. The hydrogen uptake activity is oxygen dependent (3), and the recycling of hydrogen through the hydrogenase may therefore contribute to preventing inactivation of the nitrogenase by oxygen as well as to generating ATP and serving as a reductant for nitrogen fixation through the photosynthetic electron transport chain (28).

A mutant of \( \textit{N. punctiforme} \) ATCC 29133 lacking the uptake hydrogenase has been constructed. This hydrogenase-free mutant strain, \( \text{NHM5} \), was shown to evolve \( \text{H}_2 \) when grown under nitrogen-fixing conditions (15). In order to further characterize this strain, and specifically to investigate the properties relevant for hydrogen production, we studied gas exchange in both \( \textit{N. punctiforme} \) ATCC 29133 and \( \text{NHM5} \) grown under nitrogen-fixing conditions. A mass spectrometer was used to detect exchange of \( \text{O}_2 \), \( \text{CO}_2 \), \( \text{N}_2 \), and \( \text{H}_2 \) in the cultures. Isotopic labeling with \( ^{18}\text{O} \) and \( ^{13}\text{C} \) was used to separate evolution and uptake of \( \text{O}_2 \) and \( \text{CO}_2 \). Hydrogen-deuterium (H-D) exchange activities were measured as a means of studying nitrogenase and hydrogenase activities.

Previous studies of gas exchange in cyanobacteria with mass spectrometry include experiments in which \( ^{15}\text{N}_2 \), \( \text{N}_2 \), \( ^{18}\text{O}_2 \), \( \text{O}_2 \), and \( \text{CO}_2 \) (5, 23) were used to demonstrate gas exchange activities. This study, however, is to our knowledge the first case in which nitrogen uptake and oxygen, carbon dioxide, and hydrogen exchange have been measured simultaneously in single experiments and in which it was also possible to study hydrogen production under air due to the lack of an uptake hydrogenase. This has given more detailed insight into the potential of a system for nitrogenase-based hydrogen production by cyanobacteria.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** Nitrogen-fixing axenic cultures of \( \textit{N. punctiforme} \) ATCC 29133 (PCC 73102) (25) and the hydrogenase-free mutant strain \( \text{NHM5} \) (15) were grown in BG\textit{11} medium in glass bottles bubbled with air in continuous light as described previously (14).

**Mass spectrometric measurements of gas exchange.** Cultures were harvested, centrifuged, resuspended in 35 mM HEPES-NaOH buffer, pH 7.5, and homogenized with a potter in order to break up large aggregates. The cell suspension was placed in the measuring chamber (1.5 ml) of a mass spectrometer, model MM 8-80 (VG Instruments, Cheshire, United Kingdom). The bottom of the chamber (Hansatech electrode type) was sealed with a polypropylene membrane, allowing dissolved gases to be directly introduced through a vacuum line into the ion source of the mass spectrometer. The chamber was thermostated at 25°C, and the cell suspension was stirred continuously with a magnetic stirrer. Light was supplied to the suspension by a fiber optic illuminator (Schott, Main, Germany). Experiments were performed at either 20 \( \mu \text{mol} \) of photons m\(^{-2}\) s\(^{-1}\) incident light (low light, corresponding to the culture conditions) or at 1.000 \( \mu \text{mol}\) of photons m\(^{-2}\) s\(^{-1}\) (high light, fully saturating).

The spectrometer sequentially scans the abundance of the different gases (\( \text{H}_2 \), \( \text{N}_2 \), \( \text{O}_2 \), \( ^{18}\text{O}_2 \), \( \text{Ar} \), \( \text{CO}_2 \), and \( ^{13}\text{CO}_2 \)) by automatically adjusting the magnet current to the corresponding mass peaks (\( m/e = 2, 28, 32, 36, 40, 44, \) and 45, respectively). \( ^{18}\text{O}_2 \) tracing was used to separate photosystem II activity (which produces \( \text{O}_2 \) from \( \text{H}_2\text{O}, \) i.e., essentially \( ^{16}\text{O}_2 \) from \( \text{O}_2 \) uptake phenomena (which consume the \( ^{16}\text{O}-\text{labeled mix} \) in the light. In a similar manner, \( ^{13}\text{CO}_2 \) tracing was used to separate \( \text{CO}_2 \) production (from stored carbohydrates, essentially \( ^{12}\text{CO}_2 \) from labeled \( \text{CO}_2 \) uptake by photosynthesis). \( \text{D}_2 \) (deuterium) tracing was used to assay the H-D exchange activity in the cells, which concerns both nitrogenase and hydrogenase. (For details of the calculation principles see the Appendix and references 5 and 12.) Measuring one mass peak typically took 0.5 s. Once corrected from the background value (due to the residual concentration of the gas in the vacuum line), the amperometric signal collected by the spectrometer is directly proportional to the gas concentration in the chamber, the proportionality coefficient varying from one mass to the other according to the ionization properties of the corresponding gas. Gas concentrations were calculated with reference to biologically neutral \( \text{Ar} \), which is present at around 1% in air, in order to reduce the measurement noise. This was especially necessary for calculating \( \text{N}_2 \) exchange rates; indeed, the relative variations in \( \text{N}_2 \) concentrations were low, as \( \text{N}_2 \) is very abundant in air and \( \text{N}_2 \) uptake rates are low.

Finally, in order to calculate the biologically relevant rates of gas exchange in the vessel, the time derivation of gas concentrations had to be corrected from the slow but significant rate of gas consumption by the mass spectrometer, which is superimposed on production or uptake rates. The mass spectrometer consumption of gases was assayed in cell-free buffer; it showed first-order kinetics with time constants at around 0.09 min\(^{-1}\) for \( \text{H}_2 \), 0.02 min\(^{-1}\) for \( \text{N}_2 \), and 0.015 min\(^{-1}\) for
RESULTS

Three sets of measurements were made: (i) simultaneous measurements of nitrogen uptake, hydrogen evolution, and CO2 and O2 exchange in the wild-type *N. punctiforme* ATCC 29133 and in the hydrogenase-free mutant NHM5, (ii) levels of N2, H2, O2, and CO2 were recorded in cultures of both strains under conditions in which CO2 was allowed to become depleted, and (iii) H-D exchange activities and their light dependency in the two strains.

Nitrogen uptake, hydrogen evolution, and CO2 and O2 exchange. The cultures were incubated with continuous stirring in a chamber connected via a gas-permeable membrane to a mass spectrometer. During the measurements, light was shifted from initial darkness to low light (20 μE s−1 m−2), followed by a period of high light (1,000 μE s−1 m−2), and then darkness again.

The rates of N2 uptake were similar between the two strains (Fig. 1A and Table 1). In accordance with previous measurements (15), the wild-type strain did not evolve hydrogen in the light (Fig. 1A), whereas the mutant did (Fig. 1D). An interesting effect seen in Fig. 1D is that when the light was low, the ratio of hydrogen evolved to nitrogen taken up (nitrogen fixation) was approximately 1.4, which is close to the maximum theoretical efficiency of the nitrogenase (one H2 produced for each N2 fixed) (Table 1). However, at higher light intensity, the relative amount of evolved hydrogen increased, resulting in a ratio of hydrogen produced to nitrogen fixed of about 6.1. It is also worth noting that there was enough hydrogenase activity in the wild type to recapture all of the produced hydrogen under high light, so that there was no net hydrogen evolution even under these conditions. As can be seen in Fig. 1D, it was possible to observe some uptake of hydrogen in the mutant strain in the dark period after exposure to high light.

In the same measurements, the exchange of O2 and of CO2 was recorded in both the wild type and the mutant. 18O2 and 13CO2 tracing was used to allow separation of evolution and uptake of each gas. The two strains showed similar characteristics (Fig. 1 and Table 1). In the light, as expected, a net production of O2 from photosystem II and an uptake of CO2 could be observed. Considering the uptake and evolution of O2 and CO2, as detected with labeled 18O2 and 13CO2, respectively, a slight inhibition of CO2 production could be observed in the light, and there was a rapid uptake of CO2 at the onset of light. For O2, in low light there was a slight inhibition of respiratory uptake, and in high light, the O2 uptake was significantly increased.

Gas exchange under CO2 limitation. A second set of measurements were made without any isotopic tracing. In these experiments, the cultures were left for a longer time under high light conditions, allowing the culture to consume nearly all the available CO2. Levels of CO2, O2, N2, and H2 were recorded. In the wild type, as CO2 became limiting, the rate of O2 evolution decreased (Fig. 2A and Table 2). For N2 uptake (Fig. 2B), there was also a decrease as the CO2 level became low. The rate, however, at the end of the experiment, 1.2 μmol (mg of chlorophyll a)−1 h−1, was still higher than the rate of N2 uptake in the dark, 0.7 μmol (mg of chlorophyll a)−1 h−1. A slight but significant hydrogen evolution by the wild type could also be seen in this experiment (Fig. 2B and Table 2).

For the mutant strain (Fig. 2C and D and Table 2), the characteristics of O2, CO2, and N2 exchange were very similar to those of the wild type. The H2 evolution showed some interesting features. The rate of H2 evolution was inhibited, like the rate of N2 uptake, when CO2 became limiting (Fig. 2D and Table 2, mid-light phase). However, after a short interval, the activity was stimulated again and even increased compared to the beginning of the light period, 8.9 μmol (mg of chlorophyll a)−1 h−1, although the rate of N2 uptake stayed low.

H-D exchange. In the last set of measurements, deuterium (D2) was used to study H-D exchange in the cultures. In both the wild type and mutant, there was a significant activation of H-D exchange by light (Fig. 3). Since the two strains behaved very similarly, it can be concluded that the effect was due mainly to the hydrogenase activity of the nitrogenase and that the activity under these conditions is light dependent.

DISCUSSION

Nitrogen uptake, hydrogen evolution, and CO2 and O2 exchange. The observed decrease in the ratio of N2 fixed to H2 evolved after the switch from low light to high light in our experiment could occur because nitrogenase is capable of evolving hydrogen without fixing nitrogen. The mechanism for substrate reduction by nitrogenase has not been fully elucidated, and it may be that the stoichiometric ambiguity of the ratio of H2 to N2 is the result of the nitrogenase having a branched pathway for the two products (24). Under high light, it is possible that photosystem I activity in the heterocysts will not only produce ATP by cyclic electron flow but will also reduce ferredoxin, with carbohydrates-derived NAD(P)H as an electron source (34), making the availability of ATP and the reducing power higher than the capacity of the nitrogenase to fix N2, and more hydrogen is produced instead.

The observed minor hydrogen uptake activity observed in the dark in the mutant strain (Fig. 1D) could be explained by released H2 equilibrating between the medium and the vegetative cells in the culture; however, the observed uptake is slow for such a phenomenon.

In the 18O2 tracing experiment, there was a low level of oxygen consumption under low light, which increased under high light (Table 1). Under low light, the plastoquinone pool is efficiently oxidized by photosystem I and electrons are passed on to the Calvin cycle. This is probably why respiratory O2 uptake fell below its value in the dark. Under high light, however, reduced plastoquinone will accumulate as a result of limited access to the photosystem I substrate, the activity of photosystem II exceeding the capacity of CO2 fixation by the Calvin cycle. This will favor respiration as well as Mehler reactions (direct photoreduction of O2) (2), resulting in the observed increase in oxygen uptake. Thus, both O2 uptake and H2 evolution seem to operate as means to dispose of the excess reducing power produced in high light.
By tracing CO$_2$ with $^{13}$CO$_2$, we observed respiratory production of CO$_2$ as well as photosynthetic uptake during the light periods. The uptake occurred very rapidly at the onset of light, most likely due to the activity of CO$_2$ concentrating mechanisms, the interruption of which accounts for the intense CO$_2$ release observed when the light was switched off. In line with this observation, the genes for CO$_2$-concentrating enzymes are present in the *N. punctiforme* genome (20). After the initial phase, the steady-state CO$_2$ uptake rate in the light corresponds to fixation by the Calvin cycle and is fairly similar between the wild-type and mutant strains, suggesting that hydrogenase impairment does not significantly affect photosynthetic carbon uptake capacity.

FIG. 1. Mass spectrometric recording of N$_2$ uptake, H$_2$ evolution, and O$_2$ and CO$_2$ exchange in cultures of *N. punctiforme* ATCC 29133 and the hydrogenase-free mutant strain NHM5 for close to 30 min. CO$_2$ and O$_2$ exchange was measured by isotopic tracing with $^{13}$CO$_2$ and $^{18}$O$_2$. (A and D) Mass spectrometric measurement of N$_2$ uptake and H$_2$ evolution in a culture of *N. punctiforme* ATCC 29133 (A) and NHM5 (D). (B and C) CO$_2$ and O$_2$ exchange recorded simultaneously with N$_2$ and H$_2$ in panel A. (E and F) CO$_2$ and O$_2$ exchange recorded simultaneously with N$_2$ and H$_2$ in panel D. Note that the recording was started after the recording of N$_2$ and H$_2$ in panel D. In the CO$_2$ and O$_2$ graphs, the three different lines show evolution (E), uptake (U), and net exchange (N). Bars under the graphs denote light conditions; black bar, darkness; grey bar, low light, 20 μmol of photons m$^{-2}$ s$^{-1}$; white bar, high light, 1,000 μmol of photons m$^{-2}$ s$^{-1}$. 
Gas exchange under CO₂ limitation. The total electron flow through the nitrogenase (calculated as the sum of 6 e⁻/N₂ and 2 e⁻/H₂; see Table 2) in this experiment was higher at the beginning of the light period than at the end, indicating an overall reduction in the efficiency of the nitrogenase, affecting nitrogen fixation more than hydrogen production. The observed decrease in nitrogen fixation activity after a longer period under high light and under CO₂ limitation could have more than one explanation: When CO₂ was depleted in our experiment, there may not have been enough carbohydrates available to the heterocysts to use as a reductant and as carbon skeletons for biosynthesis. This would affect the nitrogen fixation process.

### Table 1. N₂ uptake, H₂ evolution, and CO₂ and O₂ exchange in wild-type strain ATCC 29133 and mutant NHM5

<table>
<thead>
<tr>
<th>Strain</th>
<th>Light conditions</th>
<th>N₂ uptake</th>
<th>H₂ evolution</th>
<th>CO₂ uptake</th>
<th>CO₂ net exchange</th>
<th>O₂ evolution</th>
<th>O₂ uptake</th>
<th>O₂ net exchange</th>
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<td>−0.1</td>
<td>2.2</td>
<td>0.3</td>
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<td>0.8</td>
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<td>1.4</td>
<td>−1.0</td>
<td>0.5</td>
<td>40.2</td>
<td>−26.3</td>
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<tr>
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<td>−7.3</td>
<td>−5.7</td>
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<td>0.4</td>
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<td>−40.3</td>
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*The data here correspond to the experiment shown in Fig. 1 and have been normalized for chlorophyll content.

*b Light conditions were cycled during the experiment from darkness to low light (20 μE s⁻¹ m⁻²) to high light (1,000 μE s⁻¹ m⁻²) and back to darkness.

**FIG. 2.** Mass spectrometric recording of O₂, CO₂, N₂, and H₂ for close to 120 min. (A) O₂ and CO₂ in a culture of *N. punctiforme* ATCC 29133; (B) N₂ and H₂ measured simultaneously with the O₂ and CO₂ in panel A, (C) O₂ and CO₂ in a culture of NHM5. (D) N₂ and H₂ measured simultaneously with the O₂ and CO₂ in panel C. Bars under the graphs denote light conditions: black bar, darkness; white bar, high light (1,000 μmol of photons m⁻² s⁻¹).
tion rate in different ways. First, the use of available carbon would be directed to biosynthesis, leading to a lack of reductant for the nitrogenase. A lack of reductant would also affect respiration, increasing the level of oxygen in the heterocysts, which could in turn lead to inactivation of nitrogenase by a reversible modification (8, 34). This seems like a plausible explanation given the observed CO₂ depletion (Fig. 2A and C). However, if a lack of carbon and the downstream effects of such a deficiency were the reason for the observed decrease in the level of nitrogen fixation, it is difficult to explain the observed increase in the rate of H₂ production from the nitrogenase in the mutant strain (Fig. 2D), since a supply of carbohydrates to be used as a reductant would still be needed for any proton reduction to take place. Still, there may have been some inactivation of the nitrogenase, causing the observed decrease in nitrogen fixation activity, while at the same time there were sufficient carbohydrates available to supply the reducing power for the remaining nitrogen fixation and hydrogen evolution activities. Second, ammonium would accumulate and could inhibit the synthesis of new nitrogenase (9). However, if the reason for the decreased nitrogen fixation was a decreasing amount of nitrogenase, there would not have been an increase in H₂ production at the same time.

Another possible explanation, apart from inhibition of the nitrogenase due to lack of carbon and subsequent accumulation of ammonium, is that the level of oxygen in the closed reaction chamber became high after a period of incubation in

<table>
<thead>
<tr>
<th>Strain</th>
<th>Light conditions</th>
<th>Rate (µmol [mg of chlorophyll a]⁻¹ h⁻¹)</th>
<th>N₂</th>
<th>H₂</th>
<th>CO₂</th>
<th>O₂</th>
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<tr>
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<tr>
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<td>Mid-light phase</td>
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* The data here correspond to the experiment shown in Fig. 2 and have been normalized for chlorophyll content.

* Light conditions were cycled during the experiment from darkness to high light (1,000 µE s⁻¹ m⁻²) and back to darkness.
However, as we have seen in this study, there are conditions that energy will normally be used for hydrogen production. For energy in the form of ATP and the fact that only a part of cells have to be developed. There is also the obligatory need which in the heterocystous cyanobacteria means that special proteins as well. A microaerobic environment is necessary, amounts and requires a rather large investment in accessory cells (11). The nitrogenase itself must be synthesized in large slow and the process of nitrogen is deemed as being low, since the turnover rate is, in e.g., the late part of the light period in Fig. 2D amounts to 9 μmol (mg of chlorophyll) a^{-1} h^{-1}. These rates from different experiments with cyanobacterial strains are comparable to, e.g., the system of hydrogen production from green algae under sulfur deprivation, in which the production rates lie in the range of up to 17 μmol (mg of chlorophyll a)^{-1} h^{-1} (21). However, cyanobacteria can produce hydrogen during growth under air, while green algae need anaerobic conditions. Furthermore, the growth of cyanobacteria is entirely autotrophic, whereas green algae, although able to grow autotrophically, do not produce H2 unless acetate is provided to drive respiration, making the culture anaerobic. These points make the cyanobacterial system easier to implement than the green algal system. Based on previous studies (15, 16, 31) and our experiments (e.g., Fig. 2), it may be concluded that favorable conditions for improved hydrogen production from a culture of N. punctiforme should be where the cell growth is limited by a low supply of CO2 but light is abundant, such as growth in a bioreactor in a sunny area under air.

In this study, we continued the characterization of a hydrogen-producing cyanobacterial strain. Conditions under which the energy flow through the nitrogenase can be directed towards hydrogen production rather than nitrogen fixation were identified. Based on the findings presented here, further studies on how to improve the system for hydrogen production can be designed.

**APPENDIX**

Calculations of hydrogenase activity from H-D exchange rates: modeling of the H-D exchange reaction catalyzed by hydrogenase or nitrogenase in the presence of various relative concentrations of D2 and of H2-consuming or H2-producing processes. As a first approximation for modeling H-D exchange, we will assume that the cleavage is heterolytic and that the D\(^{+}\) concentration in water is negligible, so that each D2 exchange event leads to 1 H-D and each exchange of an H-D molecule leads with a 0.5 probability to either 1 H\(_2\) or 1 H-D, the H\(_2\) interaction with the enzyme being neutral in this respect. Hydrogen production or uptake can be superimposed on the H-D exchange activity and is equal to the net rate of change in hydrogen concentration (all isotopic species joined).
We will also assume that the probability that one hydrogen species interacts with an active site (either for H-D exchange or for uptake) is equal to its proportion in the mix and that hydrogen production, apart from the H-D exchange, yields only H₂ coming from water protons. In the following equations, the total concentration of hydrogen species is called \( \Sigma = [D₂] + [H₂] + [H_D] \); \( d\Sigma /dt \) is then the net production or uptake of hydrogen (<0 in the case of uptake, >0 in the case of production); \( V_{exch} \) is the turnover rate of hydrogen species at the active site leading to the H-D exchange reaction (i.e., the H-D exchange activity of the enzyme); and \( \tau \) is the isotopic ratio of hydrogen:

\[
\tau = \frac{[D₂] + 1/2[HD]}{\Sigma}
\]

A simple model for H-D exchange combined with hydrogen production or uptake can then be drawn:

\[
\begin{align*}
\frac{d[D₂]}{dt} &= -V_{exch} [D₂] - 0 + \frac{\Sigma}{\Sigma} [D₂] \\
\frac{d[HD]}{dt} &= V_{exch} \left( \frac{[HD]}{\Sigma} \right) - 0 + \frac{\Sigma}{\Sigma} [HD] \\
\frac{d[H₂]}{dt} &= \frac{1}{2} V_{exch} \left( \frac{[HD]}{\Sigma} \right) + \frac{\Sigma}{\Sigma} [H₂]
\end{align*}
\]

The hydrogenase activity can then be deduced from \( D₂, H-D, \) and \( H₂ \) concentration changes by simple rearrangement of these equations.

Then, in the case of a simple H-D exchange \( (d\Sigma/dt = 0) \), in which only the first column of the equations table is considered, we combine the equations on lines 2 and 3:

\[
2 \frac{d[H₂]}{dt} + \frac{d[HD]}{dt} = V_{exch} \left( \frac{[HD]}{\Sigma} \right) + \frac{\Sigma}{\Sigma} \frac{d[HD]}{dt} = V_{exch} \cdot \tau
\]

which gives

\[
V_{exch} = \frac{1}{\tau} \left( \frac{2d[H₂]}{dt} + \frac{d[HD]}{dt} \right)
\]

When \( H₂ \) production occurs simultaneously with H-D exchange \( (d\Sigma/dt > 0) \), we take columns 1 and 2 into account, it yields

\[
V_{exch} = \frac{1}{\tau} \left( \frac{d[H₂]}{dt} + \frac{d[HD]}{dt} - \frac{d\Sigma}{dt} \right)
\]

When uptake occurs \( (d\Sigma/dt < 0) \), columns 1 and 3 apply, and calculation gives

\[
V_{exch} = \frac{1}{\tau} \left( \frac{2d[H₂]}{dt} + \frac{d[HD]}{dt} - 2(1 - \tau) \frac{d\Sigma}{dt} \right)
\]

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