Mass Spectrometric Detection and Analysis of Nitrogen Fixation in *Oscillatoria chalybea*

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By means of mass spectroscopic measurements in an artifical gas atmosphere containing the stable nitrogen isotope $^{15}N_2$ we were able to demonstrate nitrogen fixation capacity in the filamentous cyanobacterium *Oscillatoria chalybea*. Our technique proved to be well-suited also for investigations on the light-induced nitrogen fixation in the purple bacteria *Rhodobacter sphaeroides* and *Rhodobacter capsulatus*. *Oscillatoria chalybea* grown without combined nitrogen showed a substantial $^{15}N_2$-uptake which could clearly be correlated with nitrogen fixation. Nitrate grown cultures did not show this nitrogen uptake or only to a minimal extent. Addition of ammonium chloride resulted in a rapid deactivation of the nitrogenase system. Similar observations have been made with other so-called switch-off effectors like phenazine methosulfate. The structural integrity of the filaments appeared to be a prerequisite for nitrogen fixation also in this organism, as even mild mechanical homogenization strongly inhibited the N_2-uptake signals. Illumination of the assays under conditions where the photooxidition of water is not operational (Bader, K. P. (1994), Biochim. Biophys. Acta **1188,** 213–219) did not affect the nitrogen fixation in *Oscillatoria chalybea*. Illumination of cultures with concomitant release of oxygen from the water splitting reaction resulted in strong inhibition of $^{15}N_2$-uptake.

Introduction

All nitrogenase systems -known up to now- are more or less oxygen sensitive, and the question arises, how these 'incompatible' reactions of photosynthetic oxygen evolution and nitrogen fixation can be carried out within the same vegetative cell (for reviews see Hill, 1988 and Gallon, 1992). Among the strategies which had to be developed are temporal or spatial separation, respiratory activities or physiological mechanisms (Villbrandt *et al.*, 1990; Brass *et al.*, 1992). It was relatively easy to understand that the two processes can be separated by time, as photosynthesis is restricted to daytime, so that N_2-fixation can well proceed during the night at a low oxygen partial pressure. In this context the observation that cyanobacteria show substantial O_2-uptake reactions in the dark distinct from respiration might be of particular interest (Bader, 1994). A striking observation in context with spatial separation was that bundles of filaments are heterogeneous in the sense that only the outer cells were normally pigmented. Some observations and the interpretation that inner, lightly pigmented cells do not evolve oxygen, thus do not contribute substantially to a high oxygen partial pressure, do not fix CO_2 and are possible restricted to fix atmospheric nitrogen could not definitely be confirmed (Carpenter and Price, 1976; Carpenter *et al.*, 1990). However, also for another non-heterocystous cyanobacterium, *Lyngbya aestuarii* spatial separation between photosynthesis and nitrogen fixation has been reported. In this case the terminal regions of the filaments seem to play a prominent role in N_2-fixation (Pearl *et al.*, 1991).

In recent years we investigated the water oxidation reaction and the oxygen gas exchange in the filamentous non-heterocystous cyanobacterium *Oscillatoria chalybea*. This organism, like other cyanobacteria exhibits – beside normal respiratory activities – an additional oxygen uptake mechanism which leads to the formation of a peroxidic component. The resulting O_2/H_2O_2-cycle has been described in detail (Bader, 1994) and might be involved in the lowering of oxygen partial pressure what is required for efficient nitrogen

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fixation. Consequently, we wanted to answer the question whether *Oscillatoria chalybea* is capable of fixing molecular atmospheric nitrogen or not. Recent investigations suggested that acetylene reduction might not be necessarily linked to the nitrogen fixation reaction itself. This has been described for *Gloeothece* incubated under a 12 h light / 12 h dark cycle by Peschek *et al.*, 1991. Such reports prompted us to choose a mass spectrometric approach using the stable isotope $^{15}N_2$. By this method the proper substrate of nitrogenase, i.e. nitrogen gas should be detectable provided that *Oscillatoria chalybea* fixes nitrogen.

Materials and Methods

The filamentous cyanobacterium *Oscillatoria chalybea* was originally obtained from the Algal Collection in Göttingen (Germany) and cultivated on clay plates in large Petri-dishes using the medium D of Kratz and Myers (1955). The nitrogen source was either nitrate in the medium or N_2 from the atmosphere (nitrate omitted from the medium). Part of the "nitrogen free" grown cultures was cultivated microaerobically in the presence of N_2 + 1 % CO_2. The cultures were illuminated with approx. 12 $\mu E \times m^{-2} \times s^{-1}$ in a 14 h light / 10 h dark cycle. For most of the experiments pieces from the filamentous structures were cut with a size of approx. 10 cm^2 and transferred (as anaerobically as possible) to the measuring cell.

Mass spectrometry

The experiments were performed in a modified magnetic sector field mass spectrometer type "Delta" from Finnigan MAT (Bremen, Germany). The experimental set-up with the valve system has been described in detail by Bader *et al.* (1987). All experiments were carried out in a laboratory-made measuring cell in which a teflon membrane separates the reaction assay (liquid phase) from the ion source (gas phase). The gas tight lid of the cell is equipped with two valves which permit flushing with different gas mixtures. Calibration of the set-up and calculation of the isotope distribution was carried out as follows:

Various concentrations of [^{15}N]hydrazine were exposed to photooxidation by tobacco chloroplasts according to our earlier experiments (Renger *et al.*, 1990). The evolved $^{15}N_2$ was detected and recorded; the area under the respective curve was integrated and put into correlation to the applied [^{15}N]hydrazine concentration.

The mass spectrometric set-up which we use is a closed system with an unidirectional gas flow from the measuring cell towards the ion source. Accordingly, all signals are superimposed on a continously decreasing negative incline. This slope, however, is asymptote-like shaped and can easily be used as baseline for positive or negative changes in the respective recorder tracing. The resulting signals for $^{14}N_2$, $^{14}N^{15}N$ or $^{15}N_2$, ($^{16}O_2$, $^{16}O^{18}O$ or $^{18}O_2$) were detected in Faraday cups at a resistance of 1 giga-Ohm and recorded on a SE130–03 BBC Metrawatt three channel recorder. Fig. 1 shows the obtained results with *Rhodobacter sphaeroides*, an organism with well-known N_2-fixation capacity. Illumination of anaerobically grown bacteria in our mass spectrometric set-up results in a strong $^{15}N_2$- uptake detected at m/e =30. This uptake equalled 240 nmol $^{15}N_2 \times$mg dry weight$^{-1} \times h^{-1}$ (a value well known from the literature for nitrogen fixation in this organism), reacted almost immediately to the on- and off-set of light and was observed only with anaerobically grown cultures. For quantification, the differences in the deflection angles (light minus dark; I) and the integration of the surrounded area (II) were used and gave identical relative and absolute results. Illumination was per-

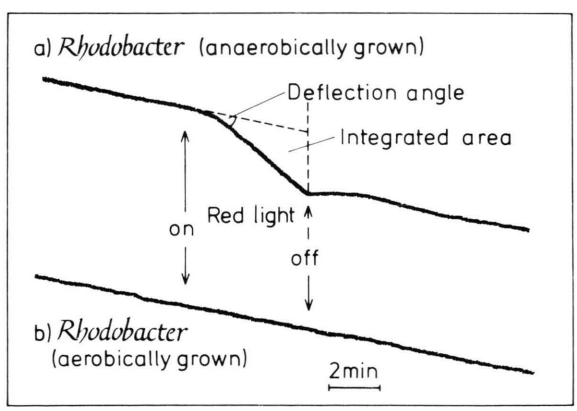


Fig. 1. Mass spectrometric detection of light-induced nitrogen uptake in *Rhodobacter sphaeroides*. The assay has been supplemented with the stable nitrogen isotope $^{15}N_2$. Quantitative determination of nitrogen fixation can be done by measurement of the deflection angle of the recorder tracing at *m/e* = 30 upon illumination or by integration of the respective signal area.

formed with a Leitz projector, Prado Universal. Nitrogen isotope $^{15}N_2$ was obtained from 'MSD Isotopes', Montreal, Canada.

Results and Discussion

The filamentous cyanobacterium *Oscillatoria chalybea* lives photoautotrophically on the surfaces of stones or porous supports and can easily be cultivated on clay plates in large Petri-dishes. As culture medium normally medium D of Kratz and Myers (1955) is used. When any nitrogen compound from this solution is omited, *Oscillatoria chalybea* still grows, although at a substantially decreased growth rate. The strong chlorosis which is observed after days or few weeks and the impaired growth can be mitigated by reduction of the light intensity or by the intercalation of dark intervals into the light periods. An even more significant amelioration of growth was observed following the reduction of the oxygen partial pressure in the gas atmosphere over the cultures. Under such conditions the growth rate could be more than doubled if calculated as increase in biomass production and dry weight (Table I). From these observations we concluded that *Oscillatoria chalybea* might be able to fix atmospheric nitrogen, although this had not been described up to now and the rate of fixation might be relatively small in comparison to well known nitrogen fixing organisms. Fig. 2 shows that in fact filaments from *Oscillatoria chalybea* exhibit a substantial $^{15}N_2$-uptake. For these experiments the assays have been extensively flushed with argon to remove the normal $^{14}N_2$-content from the atmosphere. Following the addition of appropriate amounts of $^{15}N_2$ to the gas atmophere the assays were equilibrated (to achieve an isotope equilibrium between gaseous and liquid phase) and the $^{15}N_2$-uptake was measured. Under these conditions *Oscillatoria chalybea* shows a nitrogen uptake rate of about 150–180 nmol $^{15}N_2 \times \mu g$ chlorophyll$^{-1} \times h^{-1}$. This uptake is clearly correlated with nitrogen fixation, as it is only observed with nitrogen-free grown cultures. Nitrate-grown *Oscillatoria* did not show any nitrogen uptake or only to a minimal extent. The same result of no significant N_2-fixation was observed with ammonium-grown cultures. Nitrogen-free grown cultures to which ammonium chloride (5 mM) was added lost part of their nitrogen fixing activity (50 %) within 2 hours and virtually all activity within 4 hours (Fig. 3). This observation of a somewhat moderate inhibition by ammonium ions might be seen in line with reports of a relative tolerance of the nitrogenase system to ammonium. The effect of a well-known switch-off effector namely phenazine methosulfate

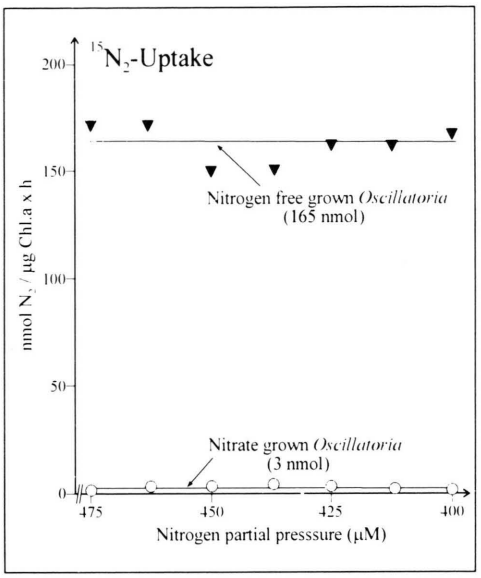


Fig. 2. Rates of $^{15}N_2$-uptake in nitrogen free grown- and in nitrate grown *Oscillatoria chalybea*. The rates of nitrogen fixation remain constant over the range of nitrogen partial pressure of the respective assays used for this experiment.

Table I. Effect of the gas atmosphere and the light intensity during the cultivation of nitrogen free grown *Oscillatoria chalybea* on growth and biomass production quantified as dry weight of the respective cultures after 8 weeks of growth.

Culture conditions	N-free; 24 $\mu E \times m^{-2} \times s^{-1}$; air	N-free; 12 $\mu E \times m^{-2} \times s^{-1}$; air	N-free; 12 $\mu E \times m^{-2} \times s^{-1}$; microaerobic atmosphere
Dry weight (mg/dish)	10.7 ± 4.3 mg	18 ± 1 mg	21.5 ± 1.5 mg

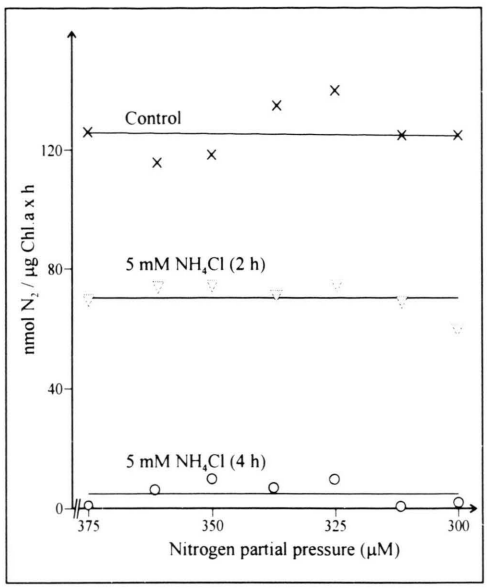


Fig. 3. Effect of ammonium chloride on the nitrogen fixation in *Oscillatoria chalybea*. Ammonium chloride has been added to give a final concentration of 5 mM and incubated with *Oscillatoria chalybea* cells for 2 and 4 h, respectively.

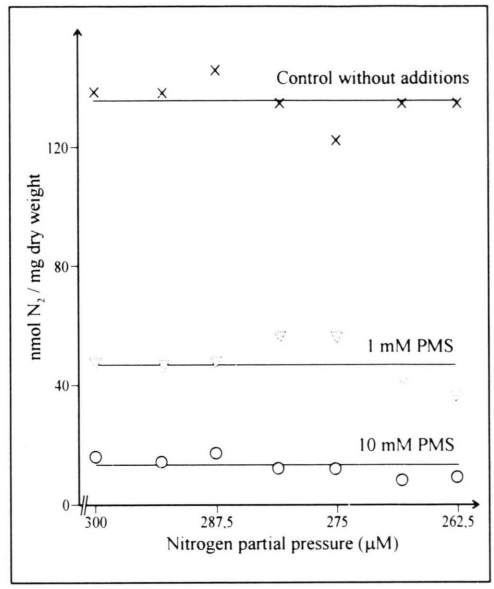


Fig. 4. Effect of phenazine methosulfate (PMS) on the nitrogen fixation in *Oscillatoria chalybea*. PMS has been added 30 min before the measurements.

on the nitrogen uptake in *Oscillatoria* is depicted in Fig. 4. Concentrations of 1 mM inhibit the N_2-fixation to about 40 % what is completely in line with values from the literature for other nitrogen fixing organisms. The pivotal feature of nitrogen fixation in non-heterocystous cyanobacteria is the question how the seemingly incompatible reactions of nitrogenase activity and photosynthetic oxygen evolution can be brought in line. This question and the possible strategies have been discussed (Gallon, 1992). However, cases with a more or less pronounced O_2-tolerance of the respective nitrogenase have also been reported. As a first approach in the case of *Oscillatoria chalybea* we investigated the significance of a structural integrity of the filaments for nitrogen fixation. Not least since the work by Carpenter and Price (1976) there has been debate that the formation of aggregates (trichomes) might be indispensable for certain organisms as it allows a spatial separation between 'inner' and 'outer' cells. Although *Oscillatoria* does not form real trichome-like structures but rather mattress-like aggregates on the surface of porous supports, even gentle homogenization (1 min) leads to a substantial attenuation of nitrogen consumption. Homogenization for several minutes abolishes any $^{15}N_2$-uptake (results not shown). As a working hypothesis one might assume that even gentle homogenization destroys (parts of) a protective layer or shell around the vegetative cells of *Oscillatoria chalybea* (*vide infra*).

One of the questions that remain open in context with nitrogenase and oxygenic photosynthesis is the one whether light as such is detrimental to nitrogen fixation or whether light induces an inhibition only because it leads to the photolysis of water and thus to an increase in the oxygen partial pressure. *Oscillatoria* is an organism well-suited for such investigations as this cyanobacterium does not oxidize water in an absolutely anaerobic environment. Consequently, no water is split and no oxygen evolved which might inhibit nitrogenase. Fig. 5 shows that under such conditions the nitrogen uptake is completely unaffected by light. Neither red light (55×10^{18} $Q\times m^{-2}\times s^{-1}$ = 3600 lux) nor white light (200×10^{18} $Q\times m^{-2}\times s^{-1}$ = 13100 lux) resulted in an inhibition of the nitrogen fixation capacity of *Oscillatoria chalybea* under conditions when no photolysis of water took place.

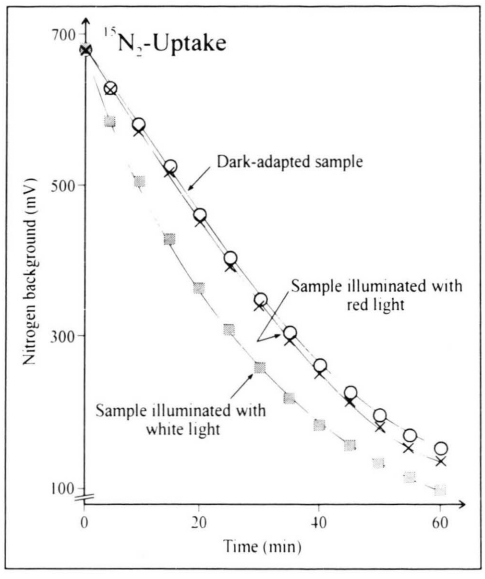
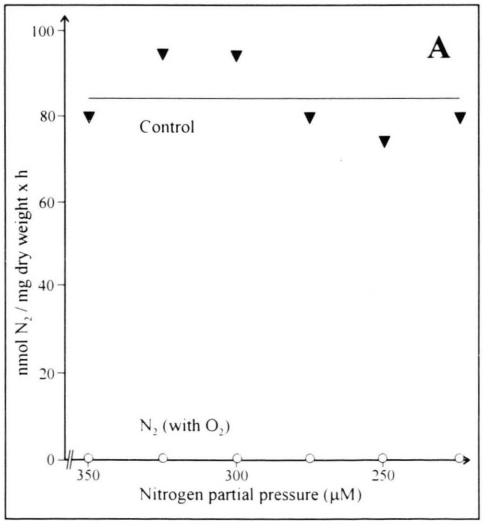


Fig. 5. Nitrogen fixation in filaments of *Oscillatoria chalybea*. The assays have been measured in darkness, red light (92 $\mu E \times m^{-2} \times s^{-1}$) or white light (333 $\mu E \times m^{-2} \times s^{-1}$), respectively. All measurements have been carried out under completely anaerobic conditions, when no photolysis of water takes place in this organism. A nitrogen background signal of 1000 mV corresponds to 250 µM N_2 under our conditions.

Consequently, the impaired N_2-uptake which can be observed upon illumination can only be derived from oxygen of the atmosphere and/or oxygen as the consequence of photosynthesis. Fig. 6 shows the effect of flushing of the assays with oxygen (≤ 100 mM) for some minutes. In this case nitrogen free grown *Oscillatoria chalybea* filaments looses virtually all nitrogen fixation capacity when cultures with low amounts of mucoids were used for the experiments (Fig. 6A). However, even under these conditions of a relatively high oxygen partial pressure *Oscillatoria* is largely able to protect against oxygen in case of high amounts of mucoid layers around the cells (Fig. 6B).

Thus, *Oscillatoria chalybea* appears to combine 2–3 different strategies in order to protect the nitrogenase system against oxygen. First, the high amount of polypeptidic slime around the cells hinders or even excludes a substantial diffusion of oxygen from the atmosphere into the cells. Consequently, the inside of the cells can be kept relatively anaerobic even in the oxygen containing atmosphere of today. (It should be kept in mind that filamentous cyanobacteria are supposed to have developed oxygenic photosynthesis about 3–4 billion years ago in a strongly reducing atmosphere.)



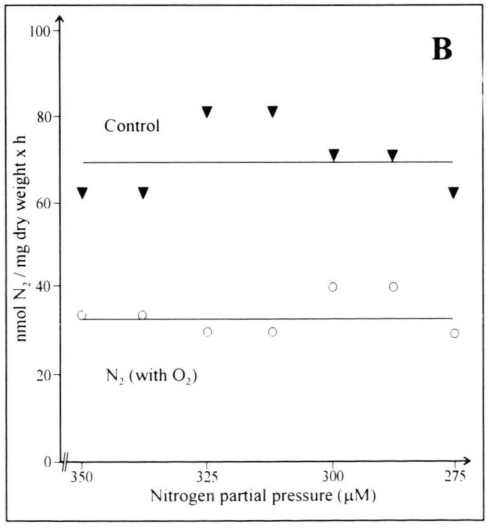


Fig. 6. Effect of oxygen on the nitrogen fixation capacity of *Oscillatoria chalybea*. The reaction assays have been flushed with nitrogen mixed with oxygen corresponding to ≤ 100 µM for 15 min. Filaments with low amounts of slime have been used in one case (A), whereas other experiments were carried out with cultures containing specifically high amounts of mucoids (B).

Second, the portion of atmospheric oxygen which penetrates the cells nevertheless together with the oxygen from photosynthesis can be removed by respiratory processes. Moreover, the O_2/H_2O_2-cycle which we describe since several years might also be efficient in lowering the internal oxygen partial pressure. On the other hand, this cycle might in addition provide the low concentrations of O_2 which are required for redox reactions within the cells (albeit the protective diffusion barrier of the mucoid layers). Photooxidations based on this cycle might well supply and regulate a low internal oxygen partial pressure independent from the surrounding atmosphere.

Acknowledgements

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