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Effect of oxygen concentration on growth and hemoglobin production in *Frankia*[1]

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Abstract

Frankia strain ArI3 was grown in large culture tubes that were vigorously bubbled with 1, 20, or 40% oxygen. In 20 and 40% oxygen, ammonium-fed (+N) cultures reached maximum biomass (protein) at 4 days and remained in the stationary phase of growth until 7 days. In 1% oxygen, +N cultures grew more slowly reaching maximum biomass at 7 days and remaining in the stationary phase until 10 days. Among treatments, the highest hemoglobin concentrations (hemoglobin/protein ratios) were produced in 1% oxygen, consistent with a possible role of hemoglobin in delivering oxygen to respiratory enzymes. Within each treatment, the highest hemoglobin concentration was found at the end of the stationary growth phase, consistent with a possible role of hemoglobin in removing toxic nitric oxide. The nitrogen-fixing (–N) cultures grew in media lacking ammonium. These cultures grew much more slowly and remained in the growth phase throughout the 12 day experimental period in all three oxygen concentrations. The strongest growth occurred in 20% oxygen accompanied by modest hemoglobin production. In 40% oxygen, hemoglobin concentrations were very low. Thus hemoglobin is unlikely to have a role in directly protecting nitrogenase from oxygen.

Keywords: Culture growth, *Frankia*, hemoglobin, hypoxia, oxygen, nitrogen oxide, truncated hemoglobin

1. Introduction

Frankia are actinobacteria capable of fixing nitrogen in culture and in symbiosis with eudicotyledenous, woody plants belonging to eight plant families (Baker and Schwintzer, 1990). Production of hemoglobin is widespread in *Frankia* and may occur in all *Frankia* strains. All six *Frankia* strains examined to date either produce hemoglobin or have genes for hemoglobin. Hemoglobin is produced in the following five strains that were selected to represent a wide range of the genetic diversity previously identified in the genus *Frankia* (Beckwith et al., 2002): CcI3, (originally isolated from *Casuarina cunninghamiana*, river she-oak), EAN1pec (*Elaeagnus angustifolia*, Russian olive), ArI3 (*Alnus rubra*, red alder), EUN1f (*Elaeagnus umbellate*, autumn olive), and Cc.1.17 (*Colletia cruciata*, anchor plant).

In addition, hemoglobin genes have been found in each of the fully sequenced genomes of strain ACN14a, originally isolated from *Alnus*, as well as in strains CcI3 and EAN1pec (Niemann et al., 2005). Finally, hemoglobin genes have also been found in strains CN3 and EuI1c (Niemann et al., 2005).

When grown in culture, *Frankia* strain CcI3 produces hemoglobin that has typical optical absorption spectra for the ferrous (Hb), carboxyferrous (HbCO), and oxyferrous (HbO_2) forms of hemoglobin (Tjepkema et al., 2002). This hemoglobin has a molecular mass determined by gel filtration of 14.1 kDA and extremely rapid O_2 binding kinetics. The oxygen association rate constant is 206 μM^{-1} s^{-1}, the oxygen dissociation rate constant is 56 s^{-1}, and the equilibrium oxygen binding constant is 274 nM (Tjepkema et al., 2002). A similar hemoglobin is produced by cultures of *Frankia* strain EAN1pec (Beckwith et al., 2002).

The relatively small molecular mass of *Frankia* hemoglobin suggests that it is a truncated hemoglobin (trHb). Truncated hemoglobins share little amino acid sequence similarity with classical hemoglobins and may be of very ancient origin (Wittenberg et al., 2002).

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Besides having a shorter amino acid sequence than the classical hemoglobins, their tertiary structure has a novel two over two α-helical sandwich fold rather than the three over three fold of the classical hemoglobins (Pesce et al., 2000).

Truncated hemoglobins have been found in a variety of bacteria, several protozoans, and also in plants (Watts et al., 2001; Wittenberg et al., 2002). Three major groups, Groups I, II, and III, of truncated hemoglobins have been proposed and some bacterial species have genes for two or even all three of these groups (Wittenberg et al., 2002). *Mycobacterium bovis* (bovine tubercle bacillus) produces two hemoglobins, trHbO (Group II) and trHbN (Group I), and these have different functions: trHbO enhances oxygen uptake under hypoxic conditions (Pathania et al., 2002), and trHbN provides protection from NO (Ouellet et al., 2002). Presence of truncated hemoglobins in *Frankia* is confirmed by genetic analysis. The genomes of all three *Frankia* strains that have been fully sequenced contain genes for two truncated hemoglobins, namely trHbO and trHbN (Niemann et al., 2005).

All five *Frankia* strains that have been examined for production of hemoglobin produce hemoglobin in cultures supplied with ammonium (+N; nitrogen fixation absent or minimal) and in nitrogen-fixing cultures (–N, cultures without combined nitrogen in the medium) (Beckwith et al., 2002). The effect of oxygen (2 and 20%) on hemoglobin production has only been examined in strain EAN1pec. Here the concentration of oxygen had little effect in cultures supplied with ammonium but in nitrogen-fixing cultures hemoglobin production was enhanced by 2% oxygen (Beckwith et al., 2002).

The rapid oxygen binding kinetics of *Frankia* hemoglobin suggest that it may have a role in supplying oxygen to respiratory enzymes under hypoxic conditions as has been found for trHbO in *Mycobacterium bovis* (Pathania et al., 2002). It may also protect nitrogenase from oxygen under hyperoxic conditions by removing oxygen from the vicinity of nitrogenase. In *Mycobacterium bovis* trHbN is expressed during the stationary phase of culture growth (Couture et al., 1999) and functions to detoxify NO (Ouellet et al., 2002). A similar function is possible in *Frankia*.

In this study we examined the effect of hypoxic (1%), ambient (20%) and hyperoxic (40%) oxygen concentrations on culture growth and hemoglobin production in +N cultures (ammonium present) and also nitrogen-fixing cultures (–N, ammonium absent).

We did this to determine whether hemoglobin production: 1) increases under hypoxic conditions indicating a likely role in respiration, 2) increases under hyperoxic conditions indicating a possible role in protecting nitrogenase, and/or 3) increases in the stationary phase of culture growth indicating a possible role in removal of NO as has been found in *Mycobacterium bovis*.

2. Materials and Methods

Culture of Frankia

Frankia strain ArI3 was grown in medium containing ammonium (P+N) or lacking ammonium (P–N). Cultures grown on P+N obtained their nitrogen from the medium and those grown on P–N obtained it by nitrogen fixation. P+N medium is a modification of the defined propionate minimal medium originally described by Baker and O'Keefe (1984). The P+N medium contained (per liter): 574 mg KH_2PO_4, 549 mg K_2HPO_4, 100 mg $MgSO_4.7H_2O$, 10 mg $CaCl_2.2H_2O$, 50 mg NH_4Cl, 7.3 mg Fe-EDTA (ferric-sodium salt), 1.2 g Na propionate, and 1 ml microelements as described by Beckwith et al. (2002). The pH of the medium was adjusted to 6.8. For –N cultures the NH_4Cl was omitted and 240 µg biotin was added because it stimulates growth in strain ArI3 in –N medium when propionate is used as the sole C source (Murry et al., 1984b).

The cultures were grown at 28°C in 35×195 mm culture tubes containing 100 ml of medium. The medium was bubbled with humidified gas mixtures containing 1, 20, or 40% O_2 and 0.2% CO_2 delivered to the bottoms of the tubes through 130 mm long, 50 µl glass micropipets at a flow rate of 110 ml/min/tube. The gas mixtures were made by combining gases using flow meters and N_2, O_2, and CO_2 from gas tanks and air from the laboratory as needed, humidified by bubbling through distilled water via air stones, and then passed through in-line PTFE filters (0.2 µ, 50 mm diameter).

The inoculum was prepared from actively growing cultures grown in 100 ml P+N medium in 250 ml Erlenmeyer flasks without shaking. These cultures formed only occasional vesicles. The hyphae were collected by centrifugation and then homogenized in a Wheaton Potter-Elvehjem tissue grinder to produce small, even sized fragments. The protein concentration of the inoculum was determined by the BCA method (see below) and an aliquot of inoculum equal to 0.09 mg protein was added to each culture tube. To ensure uniformity among aliquots, the inoculum was continuously stirred with a stir bar.

When the culture tubes were first inoculated, the *Frankia* were barely visible to the naked eye but the colonies became clearly visible after a few days of growth although they remained small. Rapid bubbling kept the colonies in slow, continuous motion, and thus minimized depletion of nutrients in the vicinity of the growing hyphal tips. It also prevented the formation of large masses of hyphae with much reduced, and unknown oxygen concentrations at their centers.

Determination of hemoglobin and protein

Frankia cells were harvested by vacuum filtration using

0.4 μm filters (Osmonics, Minnetonka, MN). To minimize the amount of culture medium adhering to the cells, filtration was continued until the hyphal mass was tightly pressed against the filter. The *Frankia* cells were weighed and placed in a 15-ml glass centrifuge tube together with 1.7 ml of extraction buffer (Beckwith et al., 2002) and 3 g of 0.1 mm glass beads (Biospec Products, Bartlesville, OK). The tube was bubbled with CO for 30 s, closed with a stopper, and the cells were disrupted by shaking for 5 min on a mixer mill (Retsch MM2, Brinkmann Instruments, Westbury, NY) equipped with a custom-built tube holder. The tube was centrifuged for 10 min at 5700 × g, the supernatant transferred to a cuvette, the absorption spectrum recorded from 390–450 nm, and the concentration of hemoglobin determined as described by Beckwith et al. (2002). A baseline was drawn between 405 and 440 nm, and the difference (ΔA) was measured between this baseline and the HbCO absorption peak at 420–421 nm. To calculate the concentration, a ΔE (mM) of 180 was used (Tjepkema and Asa, 1987), where ΔE is the difference in the extinction coefficient of pure hemoglobin. Lastly, the protein concentration of the supernatant was determined by the BCA protein assay (Pierce, Rockford, IL).

The hemoglobin within the cultures was expressed as the ratio of hemoglobin/protein to allow direct comparison of cultures grown in different oxygen concentrations regardless of their biomass.

Effect of oxygen concentration in +N and –N cultures

In the first experiment, cultures were grown in the presence of combined nitrogen (+N) in 1, 20, or 40% oxygen in P+N medium and harvested at 4, 7, or 10 d after inoculation. Six tubes were harvested for each treatment at each harvest date. The tubes were assigned at random to the treatments and harvest dates.

In the second experiment, cultures were grown in the absence of combined nitrogen (–N) in P–N medium. In this experiment the hyphae used to prepare the inoculum were washed twice with distilled water prior to homogenization to minimize carry-over of combined nitrogen into the nitrogen-free medium. The cultures were harvested at 6, 9, and 12 d to allow for the time needed by *Frankia* to form vesicles and the slower growth of nitrogen-fixing cultures. Other procedures were the same as for the +N experiment.

3. Results

Effect of oxygen concentration in +N cultures

Cultures grown at ambient (20%) oxygen reached their maximum protein content at 4 d when they contained 1.54 mg protein/culture (Fig. 1). At 7 d protein had declined slightly, and at 10 d it had declined strongly. Thus these cultures reached the beginning of the stationary phase of growth at approximately 4 d and maintained the stationary phase until approximately 7 d. By 10 d they were clearly in the decline phase. Cultures in the hyperoxic treatment (40% oxygen) closely followed the pattern of those grown in ambient oxygen. In contrast, cultures in the hypoxic treatment (1% oxygen) grew substantially more slowly. At 4 d they contained only 37 % as much protein as those in the ambient oxygen treatment. They continued to grow until 7 d when they attained their maximum protein content, and showed only a slight decline at 10 d. Thus these cultures remained in the growth phase until approximately 7 d and were in the stationary phase from 7 d until at least 10 d, when the experiment ended.

Cultures grown in ambient oxygen contained the maximum amount of hemoglobin/ unit of protein at 7 d and lower amounts at 4 and 10 d (Fig. 2). Again cultures grown in 40% oxygen closely followed the pattern of those grown in ambient oxygen. In contrast, cultures grown in 1% oxygen contained substantially more hemoglobin at all three harvest dates than the cultures grown at 20 and 40% oxygen. Moreover, the 1% cultures reached their maximum hemoglobin/protein ratio at 10 instead of 7d. In all three treatments, the maximum hemoglobin/protein ratio occurred towards the end of the stationary phase of growth.

Effect of oxygen in nitrogen-fixing (–N) cultures

Nitrogen-fixing cultures grew much more slowly than those supplied with combined nitrogen. They made their best growth at ambient (20%) oxygen where their protein content increased linearly from 6 to 12 d (r^2=0.82, P=<0.0001) and contained 0.412 mg protein at the end of 12 d (Fig. 3). Cultures in the hyperoxic treatment (40% oxygen) also grew linearly (r^2=0.74, P=<0.0001) but more slowly than those grown at ambient oxygen and contained only 0.140 mg protein/culture at the end of 12 d. Cultures in the hypoxic (1% oxygen) treatment also grew very slowly and reached a final protein content of 0.221 mg at the end of 12 d. The cultures in all three treatments remained in the growth phase of the growth curve throughout the experiment.

Moderate hemoglobin/protein ratios were found in cultures grown in 1 and 20% oxygen while only low ratios were found in 40% oxygen (Fig. 4). The highest hemoglobin/protein ratios occurred in 1% oxygen after 6 d of growth and then declined until 12 d when they were similar to those of the cultures grown in ambient oxygen. In contrast, the cultures grown in 40% oxygen had very low hemoglobin/protein ratios throughout the experiment. These ratios were lower than any others in either the +N or –N cultures.

We considered reporting hemoglobin relative to fresh mass instead of relative to protein. We did not do this, however, to avoid errors caused by varying amounts of culture media being included in the fresh mass and accumulation of dead cells as cultures age. As expected, the

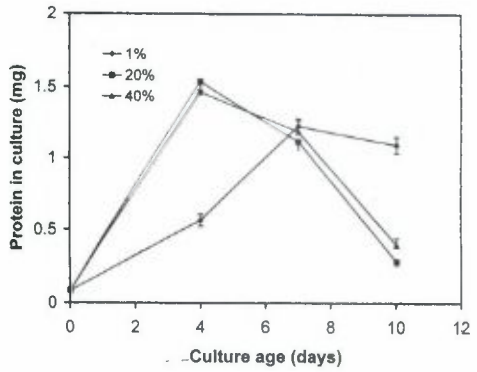

Figure 1. Effect of oxygen concentration on growth (mg protein) in ammonium-fed (+N) cultures of *Frankia* ArI3. Cultures were vigorously bubbled with gas mixtures containing 1, 20, or 40% oxygen. Values are mean ± SE, n=6.

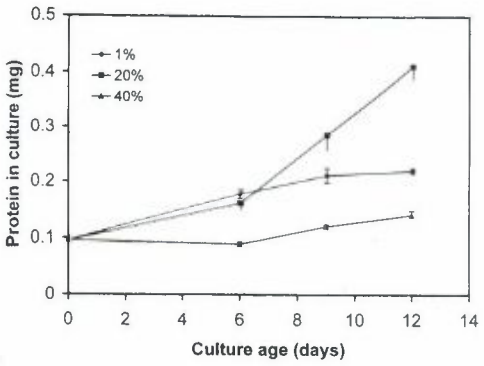

Figure 3. Effect of oxygen concentration on growth (mg protein) in nitrogen-fixing (–N) cultures of *Frankia* ArI3. Cultures were vigorously bubbled with gas mixtures containing 1, 20, or 40% oxygen. Values are mean ± SE, n=6.

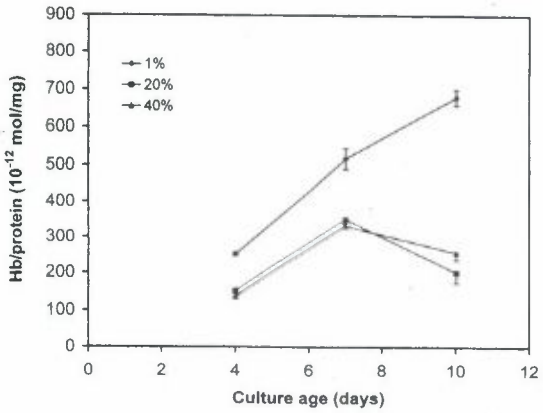

Figure 2. Effect of oxygen concentration and age of the culture on hemoglobin production (10^{-12} mol Hb/ mg protein) in ammonium-fed (+N) cultures of *Frankia* ArI3. Cultures were vigorously bubbled with gas mixtures containing 1, 20, or 40% oxygen. Values are mean ± SE, n=6.

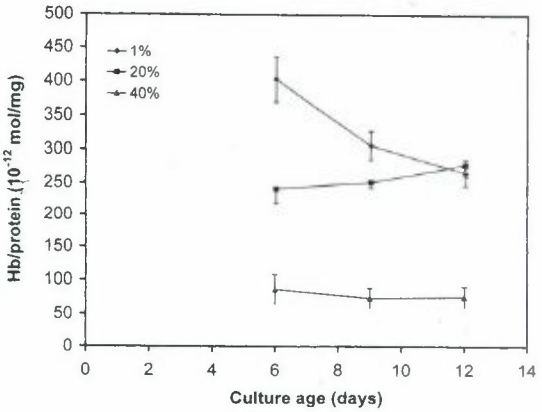

Figure 4. Effect of oxygen concentration and age of the culture on hemoglobin production (10^{-12} mol Hb/ mg protein) in nitrogen-fixing (–N) cultures of *Frankia* ArI3. Cultures were vigorously bubbled with gas mixtures containing 1, 20, or 40% oxygen. Values are mean ± SE, n=6.

amount of fresh mass relative to protein increased with culture age in the +N cultures. Comparing mg fresh mass per mg protein at 4 and 10 d for the three oxygen treatments, the value increased 2.3 to 2.6 fold over time. This is consistent with accumulation of dead cells as the cultures aged. The amount of hemoglobin relative to fresh mass varied with the culture conditions and culture age. The highest values, 2.3–3.1 × 10^{-6} moles Hb kg^{-1} wet mass were found in +N cultures grown at 1% oxygen and the lowest values, 0.22–0.23 × 10^{-6} moles Hb kg^{-1} fresh mass, were found in –N cultures grown at 40% oxygen.

4. Discussion

Effect of oxygen concentration in +N cultures

The amount of oxygen in the medium strongly affected both growth of strain ArI3 and the amount of hemoglobin produced in +N cultures. Growth was clearly limited by 1% oxygen and hemoglobin production was strongly enhanced. The reduction in growth is probably caused, at least in part, by oxygen limitation of respiration.

In contrast, strain ArI3 readily tolerated hyperoxic (40% oxygen) conditions. Both its growth rate and the amount of hemoglobin produced were unaffected by increasing the oxygen concentration from 20 to 40%. Given this tolerance to high oxygen concentrations, it is unlikely that oxygen concentrations up to 40% ever limit growth of strain ArI3 in the presence of combined nitrogen.

The observed increase in hemoglobin production in 1% oxygen is consistent with a possible role in delivering oxygen to respiratory enzymes within the hyphae. In +N culture, strain ArI3 produces very few vesicles and nitrogen fixation cannot be detected (Murry et al., 1984b) thus essentially all oxygen uptake occurs in the hyphae. This is in contrast to cultures grown under nitrogen-fixing conditions (–N cultures) where oxygen uptake occurs in

both the hyphae and vesicles (Murry et al., 1984a). It is likely that *Frankia* hemoglobin delivers oxygen to respiratory enzymes because its rapid oxygen binding kinetics make it well suited for facilitation of oxygen diffusion over short distances (Tjepkema et al., 2002; Beckwith et al., 2002). Consistent with this, trHbO supplies oxygen to respiratory enzymes in *Mycobacterium bovis* under hypoxic conditions (Pathania et al., 2002).

The observed increase in hemoglobin in the late stationary phase of growth in all three oxygen concentrations is consistent with a possible role of the hemoglobin in removal of toxic concentrations of NO (Ouellet et al., 2002). Toxic concentrations of NO are most likely to form in the stationary phase of culture growth when cultures are at their maximum density. Consistent with this, trHbN is expressed in *Mycobacterium bovis* during the stationary phase of culture growth (Couture et al., 1999).

Effect of oxygen in nitrogen-fixing (–N) cultures

In the –N cultures, growth could not begin until the *Frankia* had produced mature vesicles that were able to fix nitrogen. Initially the cultures contained essentially no vesicles because when strain ArI3 is grown on +N medium, such as that used to produce the inoculum, it forms only extremely small numbers of vesicles and nitrogenase activity is not detectable (Murry et al., 1984b). The length of time needed for vesicles to differentiate and mature depends on the oxygen concentration in the medium (Harris and Silvester, 1992). For example, in strain CcI3, nitrogenase activity first appears after 4 d in 5% oxygen, 5 d in 21 % oxygen and 7 d in 40% oxygen. Vesicles formed in lower oxygen concentrations have thinner envelopes (Parsons et al., 1987) and consequently are able to mature more rapidly than those formed in higher oxygen concentrations (Harris and Silvester, 1992). Vesicle formation may follow a similar time course in strain ArI3. In 1 and 20% oxygen, there was a modest increase in protein at 6 d over that present in the inoculum but in 40% oxygen there was no increase until 9 d.

At all three oxygen concentrations, growth was much slower in the nitrogen-fixing, –N cultures than growth in the ammonium-fed, +N cultures. The high metabolic costs of nitrogen fixation were probably a major cause of the slower growth rates.

As in the case of the +N cultures, the amount of oxygen in the medium strongly affected both growth of strain ArI3 and the amount of hemoglobin produced in nitrogen-fixing (–N) cultures. The very limited growth in 1% oxygen was unexpected because at this oxygen concentration, only thin vesicle envelopes are needed to protect nitrogenase from oxygen. However, this oxygen concentration is very limiting to growth in general as seen in the +N cultures, and the added metabolic demands of nitrogen fixation may further limit growth. In the 20% oxygen concentration, growth was steady throughout the 12 d period and showed no sign of approaching the stationary phase. Consistent with this, the hemoglobin/protein ratio was moderate and varied little. In the 40% oxygen concentration, growth was very slow but steady during the 12 d period. Hemoglobin/protein ratios were very low throughout and substantially lower than in any of the other treatments. The cause of these very low hemoglobin/protein ratios is not known but these low ratios make it unlikely that hemoglobin has a role in directly protecting nitrogenase from oxygen.

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