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Transcriptional Regulation of δ-Aminolevulinic Acid Dehydratase Synthesis by Oxygen in *Bradyrhizobium japonicum* and Evidence for Developmental Control of the *hemB* Gene

SARITA CHAUHAN AND MARK R. O'BRIAN*

Department of Biochemistry and Center for Advanced Molecular Biology and Immunology, State University of New York, Buffalo, New York 14214

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An increased demand for cytochromes is associated with symbiotic development and microaerobic metabolism in the bacterium *Bradyrhizobium japonicum*, and evidence suggests that *hemB*, rather than *hemA*, is the first essential bacterial heme synthesis gene in symbiosis with soybean. Steady-state levels of mRNA and protein encoded by *hemB* were strongly and rapidly induced by O_2 deprivation as determined by RNase protection and immunoblot analyses, but *hemH* message was not induced. Oxygen limitation resulted in a greater-than-10-fold increase in the rate of *hemB* mRNA synthesis as determined by transcriptional runoff experiments, whereas *hemH* transcription was unaffected by the O_2 status. Thus, *hemB* is a regulated gene in *B. japonicum* and is transcriptionally controlled by O_2. Unlike the expression in parent strain I110, *hemB* expression was not affected by O_2 in the *fixJ* strain 7360, and O_2-limited cultures of the mutant contained quantities of *hemB* mRNA and protein that were comparable to uninduced levels found in aerobic cells. In addition, spectroscopic analysis of cell extracts showed that increases in *b*- and *c*-type cytochromes and the disappearance of cytochrome aa_3 in response to microaerobic growth in wild-type cells were not observed in the *fixJ* mutant. FixJ is a key transcriptional regulator that mediates O_2-dependent differentiation in rhizobia, and therefore *hemB* expression is under developmental control. Furthermore, the data suggest a global control of cytochrome expression and heme biosynthesis in response to the cellular O_2 status.

Bacteria of the genera *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium*, collectively referred to as rhizobia, interact with certain leguminous plants, which culminates in the formation of symbiotic root nodules (reviewed in references 12 and 25). The nodule is comprised of highly differentiated plant and bacterial cells, and the prokaryote is found within cells of the eukaryotic host. The symbiotic bacteria, or bacteroids, convert atmospheric N_2 to ammonia, which the plant can use to fulfill its nutritional nitrogen requirement, and the host provides the endosymbiont with carbon sources. Bacterial nitrogen fixation is energy intensive, requiring alterations in heme protein expression by both plant and bacterial cells during nodule development to accommodate this demand. We are interested in the mechanism and regulation of heme biosynthesis, particularly in *Bradyrhizobium japonicum*-soybean symbiosis. Leghemoglobin is a plant protein strongly induced in nodules to facilitate O_2 diffusion to respiring bacteroids and to maintain a low free-O_2 tension. Accordingly, soybean genes encoding heme synthesis enzymes are strongly induced and maintained at elevated levels within nodules (2, 16, 21, 23, 33), and these observations run counter to the long-held view that the host lacks the synthetic capacity for heme formation in the symbiotic organ (reviewed in reference 26). The universal first committed heme precursor δ-aminolevulinic acid (ALA) is synthesized from glutamate by soybean in nodules, and biochemical and genetic evidence indicate that the so-called C_5 pathway (7) catalyzes that synthesis (16, 32, 33). Induction of soybean ALA synthesis in nodules is due, at least in part, to activation of *gsa1*, a gene normally expressed strongly only in photosynthetic tissue in nonsymbiotic plants (16). Rhizobial differentiation involves an overall increase in cytochrome heme protein expression and de novo synthesis of certain cytochromes found specifically in nodules or in microaerobically grown cultured cells (2, 3, 5, 19). At least some of these cytochromes are required for respiration under hypoxic conditions, such as the cytochrome cbb_3 oxidase, which has a high affinity for O_2 (22, 29, 30). The *fixNOQP* operon encodes the oxidase subunits and is induced in O_2-limited cells via the two-component regulatory system FixL-FixJ (10, 11, 18). FixL and FixJ are primary components of a regulatory cascade initiated by O_2 limitation, and although the specific features of these cascades are not identical in all rhizobial species, the FixL-FixJ system is necessary for bacterial development and microaerobic metabolism in all species examined thus far (6, 11).

Analysis of rhizobial heme synthesis mutants reveals unexpected and interesting features of the pathway that give clues to mechanisms and control. *B. japonicum* ALA synthase, the *hemA* product that catalyzes the first step in heme synthesis, is not required for heme formation in symbiotic bacteroids, but the *hemB* and *hemH* genes are essential (8, 9, 13). Furthermore, the *hemA* gene of other rhizobial species is essential for symbiosis with their respective hosts. These observations can be reconciled by evidence that suggests that the *B. japonicum hemA* mutant is rescued symbiotically by provision of soybean-derived ALA and that ALA uptake is deficient in species that require the *hemA* gene for symbiosis (24, 31). These findings have turned our focus to ALA dehydratase and the *hemB* gene that encodes it, because the model predicts that the second step of the pathway is the first essential bacterial step for *B. japonicum* heme synthesis in nodules. Herein, we show that *hemB* is a regulated gene in *B. japonicum* and that its control by O_2 is part of a regulatory system necessary for cellular differentiation and development.

* Corresponding author. Mailing address: Department of Biochemistry, 140 Farber Hall, State University of New York at Buffalo, Buffalo, N.Y. 14214. Phone: (716) 829-3200. Fax: (716) 829-2661. E-mail: mrobrian@acsu.buffalo.edu.

MATERIALS AND METHODS

Bacterial strains, media, and growth. *B. japonicum* I110 is the wild-type strain used in this study. *B. japonicum* 7360 is a *fixJ* mutant (1) and was provided by H. Hennecke. These strains were routinely grown in GSY medium as described previously (15) and were harvested at an optical density at 540 nm of 0.4 to 0.6. For growth under restricted aeration, flasks containing inoculated media were sealed with a rubber stopper and flushed with N_2; then, O_2 was injected to the desired concentration. For cytochrome analysis, 1-liter cultures were grown in 4-liter flasks, and for protein analysis, 15-ml cultures were grown in 125-ml flasks. Plasmids used in the present study were maintained and propagated in *Escherichia coli* DH5α or XL1-Blue.

Isolation of *B. japonicum* RNA. Total RNA was prepared from cultured cells as follows. Cells grown to an optical density at 540 nm of 0.4 to 0.6 were harvested by centrifugation at 4°C, and cell pellets from 12.5 ml of culture were resuspended in 600 μl of lysis mixture containing 10 mM NaCl, 10 mM Tris (pH 8), 5% sodium dodecyl sulfate, and 200 μg of proteinase K per ml and were incubated at 37°C for 5 min. A total of 300 μl of 5 M NaCl was added to the lysed cells, and the mixture was kept on ice for 10 min, followed by centrifugation at 4°C. RNA in the supernatant was precipitated by the addition of 3 volumes of ethanol at −70°C for 1 h, followed by centrifugation. The crude RNA pellet was resuspended in 1 mM EDTA and was acid phenol-chloroform extracted, followed by ethanol precipitation. This preparation was treated with DNase I in the presence of human placental RNase inhibitor to eliminate any contaminating DNA. In our hands, the DNase I treatment was necessary to avoid false-positive signals in the RNase protection analysis resulting from RNA-DNA hybrids.

Analysis of steady-state levels of *hemB* and *hemH* mRNA. Analysis of RNA levels of specific genes was carried out by a quantitative RNase protection assay. pRPhemB1, containing a portion of the *hemB* gene, was derived by deletion of a 0.7-kb *Sph*I-*Kpn*I fragment from pZEN (8). pRPhemH2 contains a portion of the *hemH* gene and was constructed by deletion of a 0.3-kb *Sph*I-*Pst*I fragment of pKShemH18 (14). Each plasmid was linearized with *Stu*I and used for in vitro transcription from the vector-borne T7 promoter of nontemplate strand DNA for synthesis of antisense riboprobes 489 and 188 bases in size, for the *hemB* and *hemH* genes, respectively. The transcription reaction was carried out with the MAXIscript in vitro transcription kit (Ambion Inc.) according to the manufacturer's instructions. The riboprobes were purified by electrophoresis on a 5% denaturing polyacrylamide gel, followed by elution of the excised fragments containing the RNA for 1 to 3 h at 37°C. RNase protection analysis to quantify *hemB* and *hemH* mRNA was performed with the Hybspeed RPA kit (Ambion Inc.) according to the manufacturer's instructions. A total of 1 to 4 μg of total RNA was used in each reaction. The protected regions of the *hemB* and *hemH* fragments were 478 and 92 bp, respectively and were analyzed by polyacrylamide gel electrophoresis and autoradiography as described previously (4). The bands on autoradiograms were quantified by using a Bio-Rad model GS-700 imaging densitometer in the transmittance mode and the Molecular Analyst software package, version 1.4.1. We analyzed several exposures of a single blot to be certain that the data were examined within the linear range of the densitometer. The lower limit of detection was estimated by scanning various amounts of radiolabeled *hemH* or *hemB* riboprobes as controls.

Analysis of *hemB* and *hemH* mRNA levels in aerobic and O_2-limited cells. *B. japonicum* cells were grown aerobically to mid-log phase in liquid medium. To remove O_2, the cultures were either flushed with N_2 or aliquoted into flasks with no headspace, without shaking. The flasks were sealed with rubber stoppers and paraffin wax and were incubated at 29°C for various periods. RNA was harvested from the cells, and *hemB* and *hemH* messages in them were analyzed by a quantitative RNase protection assay as described above.

Transcriptional runoff assays. Cells grown aerobically were split into two cultures and treated aerobically or anaerobically, as described above, for 20 min before being harvested by centrifugation. Forty milliliters of cells was used per runoff reaction. Cell pellets were resuspended in a solution containing 5 ml of 5 mM Tris (pH 8), 2.5 mM $MgCl_2$, and 150 mM KCl per 40 ml of the original culture and were permeabilized with 1% toluene on ice for 5 min, with gentle mixing every minute. Toluene was removed by one wash with the above-described buffer and resuspended in 1 ml of the same buffer per 40 ml of the original culture. The cells were divided into 1-ml aliquots and centrifuged, and pellets were frozen in a dry ice-methanol bath and stored at −80°C until further use. To initiate transcription, pellets were thawed on ice and were then resuspended in 60 μl of the transcription cocktail. The reactions proceeded for 20 min at 30°C. The transcription cocktail contained the following: 10 μl of 10× transcription buffer from the Ambion MAXIscript kit, 16.6 mM dithiothreitol, 0.83 mM (each) ATP, CTP, and GTP, 62.5 U of human placental RNasin, 250 μCi of [α-^{32}P]-UTP (800 Ci/mmol), and 33 μg of rifampin per ml. Total RNA was harvested as described above and was passed through a Sephadex G-50 column to remove unincorporated nucleotides. A total of 10^7 cpm of each RNA preparation was hybridized with a nitrocellulose membrane filter containing 400 ng each of *hemB* or *hemH* and pBluescript SK DNA slot blotted onto it as described previously (4). Autoradiograms were analyzed, and bands were quantified as described above for the RNase protection analysis.

Analysis of ALA dehydratase protein. Strains I110 and 7360 were grown in the presence of various concentrations of oxygen. Total cellular proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to Immobilon membranes (Millipore), and probed with antibodies raised against *B. japonicum* ALA dehydratase as described previously (9). Cross-reactive material that bound to nitrocellulose filters was analyzed with peroxidase-conjugated goat anti-rabbit immunoglobulin G and visualized by chemiluminescence by using the Renaissance kit (DuPont-NEN) according to the manufacturer's instructions. Bands on autoradiograms were analyzed by imaging densitometry within the linear range as described above for the RNase protection analysis.

Cytochrome analysis. *B. japonicum* wild-type strain I110 and the mutant strain 7360 were grown in the presence of air (20% O_2) or 2% oxygen. For the latter, flasks were flushed with N_2 gas, followed by injection of the appropriate volume of O_2. Cell extracts were prepared as described previously (15), and bacterial cytochromes were analyzed by absorption difference spectroscopy of dithionite-reduced minus ferricyanide-oxidized samples with an SLM-Aminco DW2000 scanning spectrophotometer. The spectral features were analyzed as described previously (15).

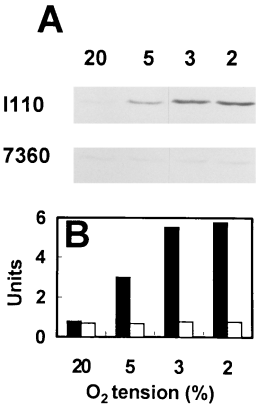

FIG. 1. Effects of O_2 tension on ALA dehydratase protein levels in *B. japonicum* parent strain I110 and *fixJ* mutant strain 7360. Cells were grown under 20, 5, 3, or 2% O_2 and were analyzed by Western blot analysis (A) with anti-ALA dehydratase antibodies. Forty micrograms of protein was loaded per lane. Quantification of the autoradiograms in the figure is shown in the bar graph (B). Black bars, strain I110; white bars, strain 7360. In two experiments, ALA dehydratase levels in strain I110 at 2% O_2 were 7.5 ± 0.2-fold (mean ± standard deviation) greater than those in aerobic cells of that strain.

RESULTS

O_2-dependent expression of *hemB*. *B. japonicum* I110 cells were grown aerobically under various O_2 tensions, and ALA dehydratase was measured by Western blot analysis of whole-cell protein with anti-ALA dehydratase antibodies (Fig. 1). ALA dehydratase levels were sevenfold greater in cells grown under 2% O_2 tension than in cells grown in air (20% O_2), indicating that O_2 affects *hemB* expression. The kinetics of O_2-dependent ALA dehydratase protein induction were evaluated by growing cells in air and then removing O_2 from the culture flask headspace. A sixfold increase in the protein was observed by 1 h after O_2 was removed and was maintained at an elevated level for at least 4 h (Fig. 2). To determine whether the control of *hemB* by O_2 is at the protein or message level, *hemB* mRNA was measured in cells subjected to O_2 limitation by an RNase protection assay (Fig. 3). Removal of O_2 from aerobically grown cells of parent strain I110 resulted in a greater-than-150-fold increase in the *hemB* message level compared with that for aerobic cells. This observation contrasts with that for the *hemH* message level, which was not induced by O_2 deprivation but was moderately repressed. The enzyme activity of ferrochelatase, the *hemH* product, was unaffected by O_2 (data not shown); thus, the basis for the three- to fourfold decline in *hemH* mRNA was not investigated further. The induction of *hemB* mRNA was observed by 15 min after O_2 removal (Fig. 3) and preceded that of its translation product, ALA dehydratase. The data show that *hemB* is a regulated

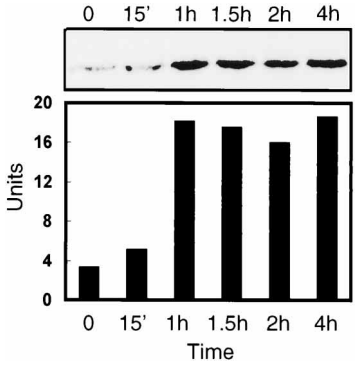

FIG. 2. Kinetics of ALA dehydratase induction in O_2-limited cells. O_2 was removed from aerobic cultures, and then cells were harvested at various times and analyzed for ALA dehydratase by Western blot analysis with anti-ALA dehydratase antibodies. Forty micrograms of cell protein was loaded in each lane. Quantitation of the blot is shown in the bar graph. In two experiments, ALA dehydratase levels were induced 6.1 ± 0.3-fold at the 1-h time point and 6.3 ± 0.1-fold at the 4-h time point.

gene in *B. japonicum* and is controlled at the level of mRNA accumulation. *hemB* mRNA was elevated to a much greater extent than protein, and thus, full expression of the gene is not proportional to the message level. It is plausible that another step in gene expression, such as translation, becomes limiting once the transcript level is induced.

The *hemB* gene is transcriptionally controlled by O_2. The rate of *hemB* mRNA synthesis was more than 10-fold greater in cells subjected to O_2 limitation than in aerobic cells, as determined by transcriptional runoff assays (Fig. 4). The elevated transcription rate of *hemB* was observed in cells subjected to O_2 deprivation for 20 min prior to being harvested for the runoff assay; thus, the response to O_2 limitation is rapid. In contrast to *hemB*, oxygen had little effect on the rate of *hemH* transcription (Fig. 4), consistent with the RNase protection analysis of steady-state message levels (Fig. 3). We conclude that O_2 regulates the *hemB* gene at the level of message synthesis.

The *hemB* gene is controlled by FixJ. Oxygen is a key developmental regulator of rhizobial differentiation during nodule development (35), and the FixL-FixJ system mediates this response by allowing cells to sense O_2 and to initiate a regulatory cascade for symbiosis and microaerobic metabolism in response to O_2 limitation (10, 11, 18). Deoxygenated FixL phosphorylates FixJ, which is then functional as a transcriptional activator of genes, at least some of which encode transcriptional regulators (reviewed in references 6 and 11). Because alterations in the cytochrome profile are necessary for bacteroid development and for the microaerobic respiration of free-living cells, we wanted to determine whether O_2-dependent *hemB* expression involves FixL-FixJ. Specifically, we measured and compared the expression of *hemB* mRNA and protein in cultured cells of the parent strain I110 and the *fixJ* mutant strain 7360. RNase protection analysis revealed no induction of *hemB* message in response to O_2 limitation in mutant strain 7360, in contrast to the strong induction observed in the parent strain, I110 (Fig. 3). In addition, the levels of immunologically detectable ALA dehydratase in the *fixJ* strain were independent of the O_2 tension in which the cells were grown, and the amounts found in O_2-restricted cells of the mutant were similar to those found in aerobic cells (Fig. 1). These findings show that FixJ is required for activation in response to microaerobiosis, supporting the argument that the gene is under developmental control.

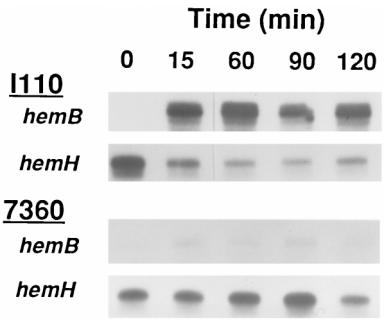

FIG. 3. Effects of O_2 on the expression of *hemB* and *hemH* mRNA in *B. japonicum* parent strain I110 and *fixJ* mutant strain 7360. O_2 was removed from aerobically grown cells, which were harvested afterwards at the indicated times for RNA analysis. *hemB* and *hemH* mRNA were analyzed by a quantitative RNase protection assay with antisense riboprobes. One microgram of total RNA was used for each time point.

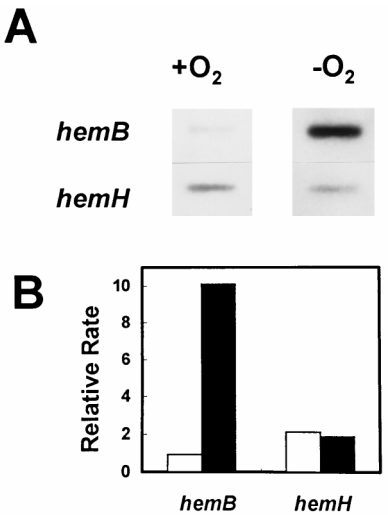

FIG. 4. The effects of O_2 on transcription rates of *hemB* and *hemH* in *B. japonicum*. Aerobic cells and those in which O_2 was removed for 20 min were analyzed by a transcriptional runoff assay to determine the rate of message synthesis. Total RNA from each cell type, radiolabeled with ^{32}P in vivo for 20 min before extraction, was used to hybridize a filter containing *hemB* and *hemH* DNA. The autoradiograms (A) were quantitated as shown in the bar graph (B).

FixJ is involved in expression of the microaerobic cytochrome profile. *B. japonicum* cells respond to microaerobiosis in culture and symbiosis with increases in the level of *b*- and *c*-type cytochromes and the diminution or disappearance of cytochrome aa_3 (2) (Fig. 5). In addition, genetic evidence implicates a role for FixL-FixJ in the induction of *fixNOQP*, the genes encoding the high-O_2-affinity oxidase cytochrome cbb_3 (10, 11). Thus, we addressed the question of whether FixJ is involved in O_2-dependent changes in the cytochrome profile by spectroscopic analysis of extracts of wild-type and *fixJ* cells grown in 20 or 2% O_2. As expected, the parent strain, I110, showed a level of *b*- and *c*-type cytochromes in cells grown in 2% O_2 that was about twofold greater than that in cells grown in 20% O_2, as discerned by the spectral features at 561 and 552 nm, respectively (Fig. 5). In addition, the broad peak at 603 nm, due to cytochrome aa_3, in strain I110 cells grown aerobically was absent in cells grown in 2% O_2. The spectral changes associated with O_2 limitation in the wild-type cells were not observed in strain 7360; the levels of *b*- and *c*-type cytochromes

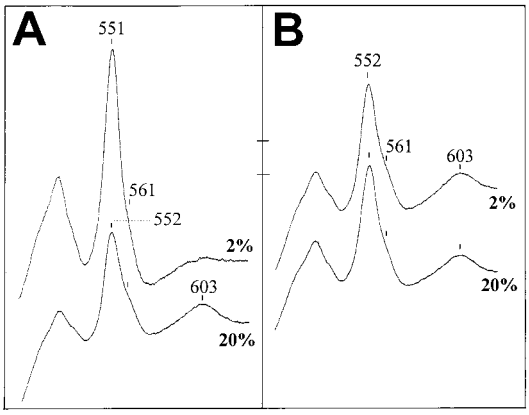

FIG. 5. Reduced-minus-oxidized absorption spectra of extracts of *B. japonicum* parent strain I110 (A) or *fixJ* strain 7360 (B) cells grown under 20 or 2% O_2 tension. Cells were reduced with sodium dithionite and oxidized in air. The hatch marks between the two panels represent a change in absorbance of 0.007. The extracts contained 4.3 mg of protein per ml. Spectral features denoted by unlabeled ticks represent the same absorption wavelengths as those indicated by the labeled tick marks above.

did not increase, and this is likely to be due, at least in part, to the failure to induce the cytochrome cbb_3 complex. Interestingly, cytochrome aa_3 persisted in the mutant cells grown in 2% O_2 (Fig. 5), implying a role for FixJ in the O_2-dependent repression of that oxidase complex. Thus, FixJ appears to affect both the induction and the repression of the appropriate cytochrome, suggesting that overall changes in the cytochrome pattern in response to O_2 deprivation are coordinated.

DISCUSSION

The de novo synthesis of specific cytochromes and the overall increase in heme protein content in rhizobia that occur in cells subjected to the hypoxic environment of root nodules and in O_2-limited cultures predict that heme synthesis is regulated positively under these conditions. Furthermore, data imply that ALA dehydratase, the product of *hemB*, catalyzes the first bacterial step of *B. japonicum* heme formation in nodules (reviewed in reference 26), suggesting that *hemB* may be a control point for heme synthesis. Indeed, the present work shows that *hemB* is a regulated gene in *B. japonicum*; *hemB* mRNA and protein were rapidly induced and maintained in response to O_2 deprivation. Furthermore, the rate of *hemB* transcription was under O_2 control, as determined by runoff experiments. The effects of O_2 on *hemB* are not common to the heme synthesis pathway genes as a whole, because neither the rate of *hemH* mRNA transcription nor its steady-state mRNA level was induced by O_2 limitation. The steady-state level of *hemB* mRNA in induced cells exceeded that of protein, indicating that the full expression of the gene was not proportional to the transcript level. The translation of *hemB* message or protein turnover may become limiting once the mRNA level is elevated, but subsequent steps need not be under O_2 control.

The conclusion that O_2 control of *hemB* is mediated by FixJ bears on two important issues relevant to the coordination of cellular functions. Firstly, O_2 is a key developmental regulator of bacterial differentiation, and the FixL-FixJ system is essential for sensing O_2 status and for initiating a regulatory cascade leading to the expression of genes necessary for nodule ontogeny and microaerobic metabolism (10, 11, 18). Thus, *hemB*, and therefore heme synthesis, is under developmental control in *B. japonicum*. The regulation of heme synthesis by FixJ was also implicated by experiments with *hemA-lacZ* fusion, which showed that an O_2-dependent threefold increase in β-galactosidase activity in wild-type cells was not observed in a *fixJ* mutant (27). Secondly, previous work shows that the induction of the *fixNOQP* operon that encodes the peptides of the cytochrome cbb_3 oxidase is regulated by FixJ (10, 11), and thus, the present work implicates a role for FixJ in the coordination of heme and apoprotein syntheses in O_2-limited cells. There are several examples in which apoprotein synthesis is controlled by heme availability, such as control of globin synthesis by heme in mammalian reticulocytes (20), the heme-dependent transcriptional activator *HAP1* in *Saccharomyces cerevisiae* (28), and the coupling of *cycA* transcription with tetrapyrrole availability by ChrR in *Rhodobacter sphaeroides* (34). The present work demonstrates that apoprotein expression is coordinated specifically with that of a heme biosynthesis gene in *B. japonicum*. The rapid induction of *hemB* mRNA in response to anaerobiosis (Fig. 3) suggests that its activation does not require de novo protein synthesis; therefore, it is improbable that *hemB* control by FixJ is an indirect effect of cytochrome apoprotein synthesis.

Changes in the cytochrome composition during differentiation for microaerobic metabolism involve repression as well as activation. Cytochrome aa_3 is a terminal oxidase necessary for normal aerobic respiration; its expression is diminished or absent in microaerobically cultured cells and in symbiotic bacteroids, and the message level of a gene encoding a subunit of the oxidase is under O_2 control (17). The persistence of cytochrome aa_3 in O_2-restricted cells of mutant strain 7360 (Fig. 5) shows that FixJ is involved in the repression of the oxidase, and therefore the positive and the negative expression of cytochromes share a regulatory feature. FixJ activates genes encoding other transcriptional regulators; thus, there are numerous ways in which this control could be achieved. The situation may be more complex than a scenario of transcriptional control of cytochrome genes by members of a signal transduction pathway. For example, cytochrome aa_3 repression could depend on an altered cellular redox state caused by microaerobiosis, which would be abnormal for the *fixJ* mutant. Nevertheless, work with the *fixJ* strain shows clearly that a mutation in a single gene has a global effect on the O_2-dependent cytochrome profile and on *hemB* expression and that heme protein metabolism is coordinated and is integrated with bacterial differentiation.

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REFERENCES

1. **Anthamatten, D., and H. Hennecke.** 1991. The regulatory status of the *fixL*- and *fixJ*-like genes in *Bradyrhizobium japonicum* may be different from that in *Rhizobium meliloti*. Mol. Gen. Genet. **225:**38–48.
2. **Appleby, C. A.** 1969. Electron transport systems of *Rhizobium japonicum*. I. Haemoprotein P-450, other CO-reactive pigments, cytochromes and oxidases in bacteroids from N_2-fixing root nodules. Biochim. Biophys. Acta **172:**71–87.
3. **Appleby, C. A.** 1969. Electron transport systems of *Rhizobium japonicum*. II. Rhizobium haemoglobin, cytochromes and oxidases in free-living (cultured) cells. Biochim. Biophys. Acta **172:**88–105.
4. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1994. Current protocols in molecular biology. Wiley Interscience, New York, N.Y.
5. **Avissar, Y. J., and K. D. Nadler.** 1978. Stimulation of tetrapyrrole formation in *Rhizobium japonicum* by restricted aeration. J. Bacteriol. **135:**782–789.
6. **Batut, J., and P. Boistard.** 1994. Oxygen control in *Rhizobium*. Antonie Leeuwenhoek **66:**129–150.
7. **Beale, S. I., and J. D. Weinstein.** 1990. Tetrapyrrole metabolism in photo-

synthetic organisms, p. 287–391. *In* H. A. Dailey (ed.), Biosynthesis of heme and chlorophylls. McGraw-Hill Publishing Co., New York, N.Y.
8. **Chauhan, S., and M. R. O'Brian.** 1993. *Bradyrhizobium japonicum* δ-aminolevulinic acid dehydratase is essential for symbiosis with soybean and contains a novel metal-binding domain. J. Bacteriol. **175:**7222–7227.
9. **Chauhan, S., and M. R. O'Brian.** 1995. A mutant *Bradyrhizobium japonicum* δ-aminolevulinic acid dehydratase with an altered metal requirement functions *in situ* for tetrapyrrole synthesis in soybean root nodules. J. Biol. Chem. **270:**19823–19827.
10. **David, M., M. L. Daveran, J. Batut, A. Dedieu, O. Domergue, J. Ghai, C. Hertwig, P. Boistard, and D. Kahn.** 1988. Cascade regulation of *nif* gene expression in *Rhizobium meliloti*. Cell **54:**671–683.
11. **Fischer, H.-M.** 1994. Genetic regulation of nitrogen fixation in rhizobia. Microbiol. Rev. **58:**352–386.
12. **Fisher, R. F., and S. R. Long.** 1992. *Rhizobium*-plant signal exchange. Nature **357:**655–660.
13. **Frustaci, J. M., and M. R. O'Brian.** 1992. Characterization of a *Bradyrhizobium japonicum* ferrochelatase mutant and isolation of the *hemH* gene. J. Bacteriol. **174:**4223–4229.
14. **Frustaci, J. M., and M. R. O'Brian.** 1993. Analysis of the *Bradyrhizobium japonicum hemH* gene and expression in *Escherichia coli*. Appl. Environ. Microbiol. **59:**2347–2351.
15. **Frustaci, J. M., I. Sangwan, and M. R. O'Brian.** 1991. Aerobic growth and respiration of a δ-aminolevulinic acid synthase (*hemA*) mutant of *Bradyrhizobium japonicum*. J. Bacteriol. **173:**1145–1150.
16. **Frustaci, J. M., I. Sangwan, and M. R. O'Brian.** 1995. *gsa1* is a universal tetrapyrrole synthesis gene in soybean and is regulated by a GAGA element. J. Biol. Chem. **270:**7387–7393.
17. **Gabel, C., and R. J. Maier.** 1993. Oxygen-dependent transcriptional regulation of cytochrome aa_3 in *Bradyrhizobium japonicum*. J. Bacteriol. **175:**128–132.
18. **Gilles-Gonzalez, M. A., G. S. Ditta, and D. R. Helinski.** 1991. A haemoprotein with kinase activity encoded by the oxygen sensor of *Rhizobium meliloti*. Nature **350:**170–172.
19. **Hennecke, H.** 1993. The role of respiration in symbiotic nitrogen fixation, p. 55–64. *In* R. Palacios, J. Mora, and W. E. Newton (ed.), New horizons in nitrogen fixation. Kluwer Academic, Boston, Mass.
20. **Hershey, J. W.** 1991. Translational control in mammalian cells. Annu. Rev. Biochem. **60:**717–755.
21. **Kaczor, C., M. W. Smith, I. Sangwan, and M. R. O'Brian.** 1994. Plant δ-aminolevulinic acid dehydratase. Expression in soybean root nodules and evidence for a bacterial lineage of the *Alad* gene. Plant Physiol. **104:**1411–1417.
22. **Keefe, R. G., and R. J. Maier.** 1993. Purification and characterization of an O_2-utilizing cytochrome c oxidase complex from *Bradyrhizobium japonicum* bacteroid membranes. Biochim. Biophys. Acta **1183:**91–104.
23. **Madsen, O., L. Sandal, N. N. Sandal, and K. A. Marcker.** 1993. A soybean coproporphyrinogen oxidase gene is highly expressed in root nodules. Plant Mol. Biol. **23:**35–43.
24. **McGinnis, S. D., and M. R. O'Brian.** 1995. The rhizobial *hemA* gene is required for symbiosis in species with deficient δ-aminolevulinic acid uptake activity. Plant Physiol. **108:**1547–1552.
25. **Mylona, P., K. Pawlowski, and T. Bisseling.** 1995. Symbiotic nitrogen fixation. Plant Cell **7:**869–885.
26. **O'Brian, M. R.** 1996. Heme synthesis in the rhizobium-legume symbiosis: a palette for bacterial and eukaryotic pigments. J. Bacteriol. **178:**2471–2478.
27. **Page, K. M., and M. L. Guerinot.** 1995. Oxygen control of the *Bradyrhizobium japonicum hemA* gene. J. Bacteriol. **177:**3979–3984.
28. **Pfeifer, K., K. S. Kim, S. Kogan, and L. Guarente.** 1989. Functional dissection and sequence of yeast HAP1 activator. Cell **56:**291–301.
29. **Preisig, O., D. Anthamatten, and H. Hennecke.** 1993. Genes for a microaerobically induced oxidase complex in *Bradyrhizobium japonicum* are essential for a nitrogen-fixing endosymbiosis. Proc. Natl. Acad. Sci. USA **90:**3309–3313.
30. **Preisig, O., R. Zufferey, L. Thöny-Meyer, C. A. Appleby, and H. Hennecke.** 1996. A high-affinity cbb_3-type cytochrome oxidase terminates the symbiosis-specific respiratory chain of *Bradyrhizobium japonicum*. J. Bacteriol. **178:**1532–1538.
31. **Sangwan, I., and M. R. O'Brian.** 1991. Evidence for an inter-organismic heme biosynthetic pathway in symbiotic soybean root nodules. Science **251:**1220–1222.
32. **Sangwan, I., and M. R. O'Brian.** 1992. Characterization of δ-aminolevulinic acid formation in soybean root nodules. Plant Physiol. **98:**1074–1079.
33. **Sangwan, I., and M. R. O'Brian.** 1993. Expression of the soybean (*Glycine max*) glutamate 1-semialdehyde aminotransferase gene in symbiotic root nodules. Plant Physiol. **102:**829–834.
34. **Schilke, B. A., and T. J. Donohue.** 1995. ChrR positively regulates transcription of the *Rhodobacter sphaeroides* cytochrome c_2 gene. J. Bacteriol. **177:**1929–1937.
35. **Soupene, E., M. Foussard, P. Boistard, G. Truchet, and J. Batut.** 1995. Oxygen as a key developmental regulator of *Rhizobium meliloti* N_2-fixation gene expression within the alfalfa root nodule. Proc. Natl. Acad. Sci. USA **92:**3759–3763.