Metals Control Activity and Expression of the Heme Biosynthesis Enzyme δ -Aminolevulinic Acid Dehydratase in *Bradyrhizobium japonicum*

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Received 7 April 1997/Accepted 27 June 1997

The heme biosynthesis enzyme δ -aminolevulinic acid dehydratase (ALAD) requires magnesium or zinc for activity, depending on the organism, and the heme moiety contains iron. Thus, metals are important for heme formation in at least two different ways. *Bradyrhizobium japonicum* ALAD* is an engineered derivative of wild-type ALAD that requires Zn^{2+} for activity rather than Mg^{2+} (S. Chauhan and M. R. O'Brian, J. Biol. Chem. 270:19823–19827, 1995). The pH optimum for ALAD* activity was over 3.5 units lower than for that of the wild-type enzyme, and ALAD* activity was inhibited by lead and cadmium, as reported for the zinc-containing dehydratases of animals. In addition, ALAD* was significantly more thermostable than ALAD; the temperature optima are 50 and 37°C, respectively. These observations strongly suggest that the metal contributes to both catalysis and structure, and this conclusion may be extrapolated to ALADs in general. Although iron did not affect the activity of the preformed protein, enzyme assays and immunoblot analysis demonstrated that the iron concentration in which the cells were grown had a strong positive effect on ALAD activity and the protein level. RNase protection analysis showed that the transcript quantity of *hemB*, the gene encoding ALAD, was iron dependent; thus, iron regulates *hemB* at the mRNA level. Induction of *hemB* mRNA in response to iron was rapid, suggesting that the factor(s) needed to mediate iron control was present in iron-limited cells and did not need to be synthesized de novo. ALAD protein levels and enzyme activities were similar in cells of the wild type and a heme-defective strain, indicating that control by iron is not an indirect effect of the cellular heme status. We conclude that the heme biosynthetic pathway is coordinated with cellular iron levels and that this control may prevent the accumulation of toxic porphyrin intermediates.

Rhizobia exist as free-living soil bacteria or in symbiosis with leguminous plants within cells of a specialized organ called a root nodule (reviewed in references 12 and 23). The symbiotically differentiated bacteria (bacteroids) fix atmospheric nitrogen to ammonia with a large expenditure of ATP. As with all aerobic organisms, rhizobia use heme proteins for oxidative phosphorylation and other oxygen-dependent cellular processes. Alterations in heme protein expression by both plant and bacterial symbionts occur during nodule ontogeny to permit efficient respiration in the microaerobic milieu of the plant organ (reviewed in references 1, 17, and 24). Legume hemoglobin facilitates O_2 diffusion to the bacteroids and buffers O_2 at a low tension to prevent inactivation of the nitrogenase complex. The prokaryote synthesizes cytochromes specific to symbiosis and microaerobic metabolism, at least some of which are part of a cytochrome oxidase complex with a very high affinity for O_2 (17). Recent evidence suggests that the induction and repression of specific cytochromes in response to O_2 limitation is under global control in *Bradyrhizobium japonicum* (6) and is coordinated with heme biosynthesis (6, 26). We have focused on *B. japonicum* δ -aminolevulinic acid (ALA) dehydratase (ALAD); the enzyme that catalyzes the second step in heme synthesis, because evidence suggests that it, rather than ALA synthase, is the first essential bacterial enzyme in the pathway for *B. japonicum* heme formation in nodules (4, 27). ALA synthase is not necessary for symbiosis (16) or for heme

synthesis in nodules (22, 27), and we have provided evidence that an ALA synthase mutant is rescued by provision of ALA by the plant host (20, 22, 27). ALADs are metal enzymes, and heme is an iron protoporphyrin; thus, we are interested in the role of metals in the function of *B. japonicum* ALAD and in the regulation of *hemB*, the gene which encodes it.

ALAD is a zinc enzyme in animals and in most bacteria but a magnesium-dependent enzyme in plants. Interestingly, *B. japonicum* ALAD is an Mg^{2+} enzyme (4) and thus is similar to the plant enzymes in that regard. By altering four proximal amino acids, we changed *B. japonicum* ALAD from an Mg^{2+} -dependent enzyme to a Zn^{2+} -requiring one (5), thereby identifying residues involved in metal binding (see Fig. 1). Because amino acids conserved in plants were changed to those conserved in animals, the observations made for *B. japonicum* can be extrapolated to those organisms, thus allowing the tentative identification of residues involved in metal binding in the respective eukaryotes.

Iron availability to microorganisms is extremely limited in soils because the metal is oxidized and therefore almost completely insoluble at pH 7. The source of iron available to rhizobia within nodules has not been established, but iron is unlikely to be free in solution, as it is complexed with proteins in eukaryotes. In either situation, iron may be limiting to rhizobia and is therefore a potential regulator of heme formation. Protoporphyrin, the immediate heme precursor, catalyzes the formation of toxic reactive oxygen species in the presence of light, and thus porphyrin synthesis in an excess of available iron could be deleterious to cells. In mammalian cells, translation of erythroid ALA synthase mRNA is controlled by iron, thus possibly coordinating heme formation with cellular iron levels

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(7, 9). Mammalian ferrochelatase contains an iron-sulfur cluster (8, 11) that may have a regulatory role by conferring sensitivity to nitric oxide (28). No analogous mechanisms for iron control of heme biosynthesis have been described in bacterial systems, and little is known about the effects of iron on the heme pathway in prokaryotes. Herein, we show that iron regulates *hemB* expression in *B. japonicum* and define properties of ALAD that can be ascribed to its Mg^{2+} requirement.

MATERIALS AND METHODS

Bacterial strains, media, and growth. *B. japonicum* I110 is the parent strain used in this study. *B. japonicum* MLG1 is a *hemA* mutant of I110 (16). These strains were routinely maintained in GSY medium as described previously (14). *Escherichia coli* strains were grown in LB medium (2) and supplemented with ampicillin at 200 and 50 μ g/ml on solid and liquid media, respectively, to maintain plasmids derived from pBluescript SK(+). Plasmids used in the present study were maintained and propagated in *E. coli* DH5 α or XL1-Blue. *E. coli* RP523 is a *hemB* mutant of strain C600 (21) and was grown in LB supplemented with hemin at 1 μ g/ml to satisfy the heme auxotrophy. The hemin was omitted when the strain harbored plasmids bearing *B. japonicum hemB* derivatives.

Analysis of *B. japonicum* ALAD and ALAD*. pZEN and pSTAR carry genes encoding wild-type ALAD and the Zn-dependent derivative ALAD*, respectively. Construction of these plasmids was described previously (5). Expression of the plasmid-borne genes in *E. coli hemB* mutant RP523 and preparation of extracts for enzyme analysis were carried out as described previously (5). For the pH profile, the assays were carried out in buffer containing 50 mM morpholineethanesulfonic acid (MES) and 50 mM bis-Tris propane at the specified pH. ALAD activity was measured spectrophotometrically at 37°C as porphobilinogen formation from ALA as described previously (4). For temperature profile and heavy metal analyses, the reactions were carried out at pH 6.0 in MES buffer with 50 μ M ZnCl₂ for ALAD* and at pH 8.0 in Tris buffer with 100 μ M MgCl₂ for ALAD.

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 the culture were resuspended in 600 μ l of lysis mixture containing 10 mM NaCl, 10 mM Tris (pH 8), 5% sodium dodecyl sulfate, and 200 μ g of proteinase K per ml and incubated at 37°C for 5 min. A 300- μ l volume of 5 M NaCl was added to the lysed cells, and the mixture was kept on ice for 10 min and then centrifuged at 13,000 $\times$ g at 4°C. RNA in the supernatant was precipitated by 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 and then ethanol precipitated. This preparation was treated with DNase I in the presence of human placental RNase inhibitor to eliminate any contaminating DNA. 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* mRNAs. RNA levels of specific genes were determined by a quantitative RNase protection assay. pR-PhemB1, containing a portion of the *hemB* gene, was derived by deletion of a 0.7-kb *SphI-KpnI* fragment from pZEN (4). pRPhemH2 contains a portion of the *hemH* gene and was constructed by deletion of a 0.3-kb *SphI-PstI* fragment of pKShemH18 (13). Each plasmid was linearized with *StuI* and used for in vitro transcription from the vector-borne T7 promoter of non-template strand DNA for synthesis of antisense riboprobes of 489 and 188 bases for the *hemB* and *hemH* genes, respectively. The transcription reaction was carried out by using a MAXIScript in vitro transcription kit (Ambion Inc.) in accordance with the manufacturer's instructions. The riboprobes were purified by electrophoresis on a 5% denaturing polyacrylamide gel, followed by elution of excised fragments containing the RNA for 1 to 3 h at 37°C. RNase protection analysis to quantify *hemB* and *hemH* mRNAs was performed by using the Hybspeed RPA kit (Ambion Inc.) in accordance with manufacturer's instructions. A total of 1 to 4 μ g of total RNA was used for each reaction. The protected regions of the *hemB* and *hemH* fragments were 478 and 92 bp, respectively, and were analyzed by polyacrylamide gel electrophoresis as described previously (2). The bands on autoradiograms were quantified by using a Bio-Rad GS-700 imaging densitometer in the transmittance mode and the Molecular Analyst software package, version 1.4.1. Several exposures of a single blot were analyzed to be certain that the data were examined within the linear range of the densitometer.

Effects of iron and heme on *hemB* expression. Cells were grown to the mid-log phase in modified GSY medium containing 0.5, rather than 1 g of yeast extract per liter and supplemented with 0 to 8 μ M FeCl₃. Medium with no added iron contained 0.3 μ M total Fe as determined with a Perkin Elmer 1100B atomic absorption spectrometer. Cells were harvested and analyzed for *hemB* or *hemH* mRNAs by the RNase protection assay described above. ALAD protein was detected in cell extracts by Western blot analysis using anti-ALAD antibodies as described previously (5). To measure the iron-dependent kinetics of *hemB* mRNA induction, cells were grown in the absence of exogenous iron and then 6 μ M FeCl₃ was added at time zero. Cells were harvested at subsequent times and analyzed for mRNA and protein as described above. To assess the effects of

<u>PLANTS</u>																	Metal
Pea	Y	T	D	V	A	L	D	P	Y	S	S	D	G	H	D	G	Mg
Spinach	Y	T	D	V	A	L	D	P	Y	Y	Y	D	G	H	D	G	Mg
 <u>B. JAPONICUM</u>																	
Wild type (ALAD)	L	C	D	V	A	L	D	P	F	T	S	H	G	H	D	G	Mg
Mutant (ALAD*)	L	C	D	V	C	L	C	P	Y	T	S	H	G	H	C	G	Zn
 <u>NON-PLANTS</u>																	
Human	A	C	D	V	C	L	C	P	Y	T	S	H	G	H	C	G	Zn
Bovine	A	C	D	V	C	L	C	P	Y	T	S	H	G	H	C	G	Zn
Yeast	A	C	D	V	C	L	C	E	Y	T	S	H	G	H	C	G	Zn
E. coli	A	S	D	T	C	F	C	E	Y	T	S	H	G	H	C	G	Zn

FIG. 1. Putative metal-binding domains of *B. japonicum* ALAD and ALAD* and comparison of dehydratases from several organisms. Boxed residues are those common to the respective plant or nonplant organisms and found in the *B. japonicum* enzymes.

heme on *hemB* expression, ALAD protein and enzyme activity were measured in cells of strains I110 and MLG1 grown in GSY medium supplemented with 0 or 15 μ M hemin.

***hemB* mRNA turnover assay.** Cultures were grown to the mid-log phase in medium supplemented with 6 μ M FeCl₃, harvested, divided into two cultures, and resuspended in medium containing either 0 or 6 μ M FeCl₃. After 30 min, 200 μ g of rifampin per ml was added to prevent de novo mRNA synthesis. RNA was isolated from cells harvested at various times after rifampin addition, and the *hemB* message was analyzed by the RNase protection assay described above.

RESULTS

Magnesium affects catalytic and structural properties of *B. japonicum* ALAD. ALAD proteins from diverse organisms require either zinc or magnesium for activity, but the role of the latter metal has not been described. By site-directed mutagenesis, we changed four amino acids of the Mg-dependent enzyme of *B. japonicum* and changed it to a Zn²⁺-requiring dehydratase (5; Fig. 1) Because these proteins are otherwise identical, differences between them can be ascribed to their respective metals and the altered residues. Wild-type ALAD had a high pH optimum for activity of at least 9.5 (Fig. 2), whereas the modified enzyme, designated ALAD*, showed a sharp pH optimum of 6. The pH profile of an enzyme's activity is generally dependent upon the protonation status of residues important for catalysis; thus, the pH shift of 3.5 units in ALAD* compared with the wild-type protein is strongly indicative of a change in the amino acids involved in substrate binding or catalysis.

FIG. 2. pH profiles of enzyme activities of *B. japonicum* wild-type ALAD and mutant ALAD*. Activities are presented as nanomoles of porphobilinogen formed from ALA per hour per milligram of protein. The data are averages of triplicate samples. The standard deviation was less than 10% of each average.

FIG. 3. Effects of heavy metals on the activity of ALAD (open bars) or ALAD* (closed bars). The data are presented as percent remaining activity compared with control samples containing no added heavy metal. The data are averages of triplicate samples, and the standard deviations were less than 10%.

ALAD is a primary target of lead toxicity in humans and other mammals, and that toxicity is caused by substitution of lead for the catalytic zinc (18). Replacement of the structural zinc with lead, however, does not inhibit activity (10). We found that wild-type ALAD was not inhibited by lead or other heavy metals (Fig. 3). However, ALAD* activity was inhibited by lead (Fig. 3), suggesting that Zn plays a catalytic role in the engineered enzyme, as it does in the naturally occurring dehydratase from mammals. Other metals were tested, and cadmium was found to inhibit ALAD* as well, as observed for the human enzyme (15). Thus, susceptibility of ALAD is dependent upon the metal in the enzyme, and magnesium confers insensitivity to lead and cadmium.

We measured ALAD activity as a function of assay temperature in ALAD and ALAD* and found that the engineered enzyme had a drastically different temperature optimum than the wild type. ALAD activity was thermolabile, had a temperature optimum of 37°C, and declined sharply at higher temperatures (Fig. 4). By contrast, ALAD* activity was significantly more thermostable, had an optimum of 50°C, and retained a high level even at 55°C (Fig. 4). Thus, the amino acid substitutions conferring Zn²⁺ dependence had a stabilizing effect on ALAD* that is not found in the wild-type enzyme. Collectively, the data show that ALAD and ALAD* differ greatly in properties associated with catalysis and structure.

Iron-dependent expression of ALAD. Iron is part of the heme moiety; thus, it is germane to ask whether iron affects ALAD. Although iron had no effect on the preformed enzyme

FIG. 4. Temperature profiles of the activities of ALAD and ALAD*. The data are expressed as nanomoles of porphobilinogen formed from ALA per hour per milligram of protein and are averages of triplicate samples. The standard deviations were less than 10%, except for those of the ALAD activities at 55 and 65°C, which were less than 20%.

FIG. 5. Effects of iron on ALAD protein (A) and enzyme activity (B) in *B. japonicum* cells. ALAD protein was detected by immunoblot analysis using anti-ALAD antibodies. Thirty micrograms of protein was loaded in each lane. Activity is expressed as nanomoles of porphobilinogen formed from ALA per hour per milligram of protein, and averages of triplicate samples are shown. The standard deviations were less than 10%.

in vitro (Fig. 4), we addressed the question of whether the metal controls its synthesis. *B. japonicum* cells were grown in medium supplemented with FeCl₃ to final concentrations ranging from 0 to 8 μM iron. The iron concentration of the medium without added FeCl₃ was 0.3 μM as determined by atomic absorption spectroscopy. The results show that iron had a strong positive effect on ALAD activity, with an over 80-fold higher level found in cells grown in 6 μM added iron than in those grown in the absence of the exogenous metal (Fig. 5B). To determine whether the effects of iron were due primarily to enzyme synthesis or to action on the extant protein, a Western blot analysis was carried out by using antibodies raised to *B. japonicum* ALAD (Fig. 5A). Cellular protein levels were clearly dependent upon the iron concentration in which the cells were grown, and immunologically reactive material correlated well with enzyme activity. Thus, ALAD synthesis is regulated by iron. In principle, this regulation could occur at the RNA or protein level; thus, message levels of *hemB*, the gene encoding ALAD, were measured by an RNase protection assay (Fig. 6). These data show that *hemB* mRNA was expressed strongly in cells grown in iron-replete medium but very weakly in those grown in low-iron medium. Unlike those of *hemB* mRNA, levels of *hemH* mRNA, which encodes the heme synthesis enzyme ferrochelatase, were expressed independently of iron (Fig. 6), showing that iron-dependent control of *hemB* is not a general phenomenon of heme synthesis genes. Moreover, cells grown in low-iron medium expressed *hemH* (Fig. 6) and grew well (data not shown), indicating that the low *hemB* expression in those cells was not an indirect consequence of nutritional iron deficiency. We conclude that *B. japonicum* *hemB* expression is regulated by iron at the level of mRNA accumulation.

FIG. 6. Expression of *hemB* and *hemH* mRNAs in *B. japonicum* cells grown in various concentrations of Fe. mRNA was analyzed by an RNase protection assay. Two micrograms of total RNA was analyzed at each Fe concentration.

FIG. 7. Kinetics of *hemB* mRNA induction (A) and turnover (B) as a function of iron levels. For induction, cells were grown to the mid-log phase in medium with no added iron, and then 6 μM FeCl_3 or an equivalent volume of buffer was added at time zero. Cells were harvested at the time points shown, and the RNA was extracted and analyzed by an RNase protection assay. For turnover, cells were treated with rifampin as described in the text at time zero and mRNA was analyzed in cells harvested at the times shown. Two micrograms of total RNA was analyzed at each time point.

To monitor the kinetics of *hemB* mRNA induction, *B. japonicum* cells were grown in iron-depleted medium to the mid-log phase and then 6 μM FeCl_3 was added. Cells were harvested at subsequent times, and *hemB* mRNA levels were determined by RNase protection analysis. Strong message induction was observed by 15 min after iron addition and was maintained for at least 8 h (Fig. 7A). No induction was observed in control cells in which buffer without the metal was added. The rapid response indicates that the regulatory component(s) that perceives iron and activates *hemB* is not synthesized de novo upon addition of the metal but is likely present in iron-limited cells and activated in some way. *hemB* mRNA turnover was determined by measuring message levels at various times after the addition of rifampin, an RNA synthesis inhibitor (Fig. 7B). No *hemB* mRNA was detected in cells by 15 min after rifampin addition, regardless of whether the cells were incubated with 6 μM FeCl_3 minutes prior to addition of the inhibitor. The decay was too rapid for half-life measurement; thus, we could not determine whether iron affected the rate of decay. However, if induction of *hemB* mRNA by 15 min after iron addition (Fig. 7A) were due primarily to increased stability, a substantial amount of message should have remained in the iron-replete cells 15 min after rifampin treatment.

Iron control of *hemB* is not an indirect result of the cellular heme level. Because ALAD is part of the heme pathway and heme is an iron porphyrin, it is plausible that the iron-dependent control of *hemB* described herein is actually an indirect consequence of cellular heme status. Thus, we examined ALAD protein and enzyme activity in the heme-defective strain MLG1. Strain MLG1 is mutated in the *hemA* gene (10), and we showed previously that this strain contains an undetectable quantity of heme when grown in yeast extract medium (14). Furthermore, addition of exogenous hemin (heme hydrochloride) to the growth medium restores the cellular heme level of the mutant to approximately 20% of the wild-type level (13). We found that the protein levels and activities in parent strain I110 and mutant strain MLG1 were similar and that addition of exogenous hemin to the growth medium did not substantially affect those levels in either strain (Fig. 8). From this, we conclude that iron control of *hemB* expression is not an indirect effect of cellular heme status.

FIG. 8. ALAD protein and enzyme activity in parent strain I110 and heme-defective strain MLG1 cells grown in iron-replete medium supplemented with either 0 or 15 μM added hemin. ALAD protein was detected by an immunoblot analysis using anti-ALAD antibodies. Thirty micrograms of protein was loaded in each lane. Activity is expressed as nanomoles of porphobilinogen formed from ALA per hour per milligram of protein, and averages of triplicate samples are shown.

DISCUSSION

In the present work, we describe roles of metals for *B. japonicum* ALAD in two contexts: conferral of properties on the enzyme and expression of the gene that encodes it. A four-amino-acid change that converts *B. japonicum* ALAD from an Mg^{2+} -dependent enzyme to a Zn^{2+} -requiring one also changed properties associated with both catalysis and structure. The normal ALAD from *B. japonicum* showed a high pH optimum, insensitivity to heavy metals, and thermolability, whereas the zinc derivative was drastically different in those parameters. There is some indication that the conclusions for the Mg^{2+} - and Zn^{2+} -dependent enzymes studied herein can be extrapolated to plant and animal ALADs, respectively. Firstly, the substitutions made to construct ALAD* were based on comparisons of the plant and animal sequences. Secondly, plant ALADs have been reported to have a higher pH optimum than those of animals (19); thus, the metal and its coordination residues of the protein may contribute directly to those differences, as they do for *B. japonicum* ALAD and ALAD*. Thirdly, prior studies have implicated both structural and catalytic roles for zinc in mammalian ALAD (10) but no direct evidence for the participating amino acids has been provided. It was further postulated that the domains may overlap in a mutually exclusive way, resulting in an octamer in which each subunit contains only one type of zinc (10). Comparisons of *B. japonicum* ALAD and ALAD* suggest that a single region of the enzyme conserved in the mammalian dehydratase is involved in catalysis and structure; thus, that region may have a similar function in the naturally occurring zinc enzyme.

We found that iron controls ALAD at the synthesis level; expression was very low in cells from iron-limited cultures and increased as a function of the iron concentration. Iron must be acquired exogenously and may be limiting; thus, regulation of heme biosynthesis by iron is physiologically relevant. Protoporphyrin is toxic; thus, it would be deleterious to synthesize it unless there were a sufficient quantity of iron to be chelated by the porphyrin to form heme. Therefore, the positive correlation between iron and ALAD expression may prevent porphyrin accumulation in cells under iron deprivation. The induction of *hemB* mRNA occurred within 15 min after iron addition, suggesting that the factor(s) needed to activate *hemB* is present in iron-limited cells. The transcriptional regulator Fur, which is found in many gram-negative bacteria (3), is synthesized constitutively but is activated upon binding to iron, which allows a fast response to the metal. The *B. japonicum hemA* gene promoter contains an element with weak homology to an *E. coli* Fur-binding consensus sequence and is moderately controlled by iron (25). However, *E. coli* Fur does not bind to the *hemA*

promoter (25); therefore, the presence of Fur in rhizobia remains to be demonstrated. The ALAD level was not significantly different in heme-deficient cells grown in the presence of iron compared with the wild type, suggesting that the observed effects of iron are not an indirect consequence of the cellular heme status. This conclusion is consistent with the fact that the response of *hemB* to iron is rapid and is unlikely to rely on the de novo synthesis of a macromolecule. In addition, one would predict that regulation by heme, if any, would be negative towards a heme synthesis gene and that iron would correlate positively with heme. Thus, the activation of *hemB* in response to iron would be difficult to reconcile in terms of heme-mediated control.

ACKNOWLEDGMENT

This work was supported by a grant from the National Science Foundation to M.R.O.

REFERENCES

- Appleby, C. A. 1992. The origin and functions of haemoglobins in plants. *Sci. Prog. Oxf.* **76**:365–398.
- 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.
- Braun, V., and V. Hantke. 1991. Genetics of bacterial iron transport, p. 107–138. *In* G. Winkelmann (ed.), *CRC handbook of microbial iron chelates*. CRC Press, Inc., Boca Raton, Fla.
- 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.
- 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.
- Chauhan, S., and M. R. O'Brian. 1997. Transcriptional regulation of δ -aminolevulinic acid dehydratase by oxygen in *Bradyrhizobium japonicum* and evidence for developmental control of the *hemB* gene. *J. Bacteriol.* **179**:3706–3710.
- Cox, T. C., M. J. Bawden, A. Martin, and B. K. May. 1991. Human erythroid δ -aminolevulinic acid synthase: promoter analysis and identification of an iron-responsive element in the mRNA. *EMBO J.* **10**:1891–1902.
- Dailey, H. A., M. G. Finnegan, and M. K. Johnson. 1994. Human ferrochelatase is an iron-sulfur protein. *Biochemistry* **33**:403–407.
- Dandekar, T., R. Stripecke, N. K. Gray, B. Goossen, A. Constable, H. E. Johansson, and M. W. Hentze. 1991. Identification of a novel iron-responsive element in murine and human erythroid δ -aminolevulinic acid synthase mRNA. *EMBO J.* **10**:1903–1909.
- Dent, A. J., D. Beyersmann, C. Block, and S. S. Hasnain. 1990. Two different zinc sites in bovine 5-aminolevulinic acid dehydratase distinguished by extended X-ray absorption fine structure. *Biochemistry* **29**:7822–7828.
- Ferreira, G. C., R. Franco, S. G. Lloyd, A. S. Pereira, I. Moura, J. J. G. Moura, and B. H. Huynh. 1994. Mammalian ferrochelatase, a new addition to the metalloenzyme family. *J. Biol. Chem.* **269**:7062–7065.
- Fisher, R. F., and S. R. Long. 1992. *Rhizobium*-plant signal exchange. *Nature* **357**:655–660.
- 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.
- Frustaci, J. M., I. Sangwan, and M. R. O'Brian. 1991. Aerobic growth and respiration of a δ -aminolevulinic acid (*hemA*) mutant of *Bradyrhizobium japonicum*. *J. Bacteriol.* **173**:1145–1150.
- Gibbs, P. N. B., and P. M. Jordan. 1981. 5-Aminolevulinic acid dehydratase. 65 Zinc binding and exchange with enzyme from human erythrocytes. *Biochem. Soc. Trans.* **9**:232–233.
- Guerinot, M. L., and B. K. Chelm. 1986. Bacterial δ -aminolevulinic acid synthase activity is not essential for leghemoglobin formation in the soybean/*Bradyrhizobium japonicum* symbiosis. *Proc. Natl. Acad. Sci. USA* **83**:1837–1841.
- 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.
- Jaffe, E. K., S. Bagla, and P. A. Michini. 1991. Reevaluation of a sensitive indicator of early lead exposure. Measurement of porphobilinogen synthase in blood. *Biol. Trace Element Res.* **28**:223–231.
- Jordan, P. M. 1990. The biosynthesis of 5-aminolevulinic acid and its transformation into coproporphyrinogen in animals and bacteria, p. 55–121. *In* H. A. Dailey (ed.), *Biosynthesis of heme and chlorophylls*. McGraw-Hill Publishing Co., New York, N.Y.
- King, N. D., and M. R. O'Brian. 1997. Identification of the *lrp* gene in *Bradyrhizobium japonicum* and its role in regulation of δ -aminolevulinic acid uptake. *J. Bacteriol.* **179**:1828–1831.
- Li, J. M., H. Umanoff, R. Proenca, C. S. Russell, and S. D. Cosloy. 1988. Cloning of the *Escherichia coli* K-12 *hemB* gene. *J. Bacteriol.* **170**:1021–1025.
- 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.
- Mylona, P., K. Pawlowski, and T. Bisseling. 1995. Symbiotic nitrogen fixation. *Plant Cell* **7**:869–885.
- O'Brian, M. R. 1996. Heme synthesis in the rhizobium-legume symbiosis: a palette for bacterial and eukaryotic pigments. *J. Bacteriol.* **178**:2471–2478.
- Page, K. M., E. L. Connolly, and M. L. Guerinot. 1994. Effect of iron availability on expression of the *Bradyrhizobium japonicum hemA* gene. *J. Bacteriol.* **176**:1535–1538.
- Page, K. M., and M. L. Guerinot. 1995. Oxygen control of the *Bradyrhizobium japonicum hemA* gene. *J. Bacteriol.* **177**:3979–3984.
- 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.
- Sellers, V. M., M. K. Johnson, and H. A. Dailey. 1996. Function of the [2Fe-2S] cluster in mammalian ferrochelatase: a possible role as a nitric oxide sensor. *Biochemistry* **35**:2699–2704.