Characterization of a 5-Aminolevulinic Acid Synthase Mutant of *Azorhizobium caulinodans* ORS571

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Using a heterologous 5-aminolevulinic acid synthase (ALAS) gene probe from *Rhizobium meliloti*, we cloned an *Azorhizobium caulinodans hemA*-like locus and constructed a *hemA*::Tn*5* insertion mutant via gene replacement. The resulting mutant strain was found to be a strict 5-aminolevulinic acid (ALA) auxotroph, to be nitrogen-fixation proficient in culture (Nif^+) and to induce ineffective, nitrogen fixation-deficient (Fix^-) root nodules, lacking leghemoglobin, on *Sesbania rostrata*. Moreover, the *hemA*::Tn*5* *A. caulinodans* mutant (ALAS122) was found to be unable to induce stem-nodules on *S. rostrata* (Nod^-). Second-site suppressor mutations, capable of growing on full medium (TY) in the absence of additional ALA, were isolated (ALAS122RT strains). *hemA*::Tn*5* strains carrying these secondary mutations were found to be disturbed in free-living nitrogen fixation (Nif^- to $Nif^{+/-}$) and in some cases in nitrogen assimilation ($Ntr^{-/+}$), but displayed the same symbiotic phenotype as the original *hemA*::Tn*5* strain (Fix^- on roots; Nod^- on stems). The $Ntr^-/+$ phenotype of the ALAS122RT strains could be complemented by plasmids carrying the *ntrC* and/or *ntrYX* loci of *A. caulinodans*, suggesting a role for the *ntr* system in controlling the activation of a (normally cryptic) secondary ALA biosynthesis pathway in strains carrying the second-site suppressor mutations. Growth of the ALAS122RT strains on TY medium lacking ALA was found to be inhibited significantly by inhibitors of the C_5 ALA synthesis pathway, such as gabaculine and amino-oxyacetate (AOA), suggesting the possibility that both the Shemin and C_5 ALA synthesis pathways may exist in *A. caulinodans*.

Additional keywords: symbiotic nitrogen fixation, stem- and root nodulation.

Leghemoglobin (Lb) represents an essential component in the *Rhizobium*-legume symbiosis, since it is required for O_2 transport to the actively respiring, nitrogen-fixing bacteroids in the nodule at an intracellular oxygen concentration below that known to irreversibly inactivate the O_2-sensitive nitrogenase enzyme complex (see Appleby 1984). Apo-leghemoglobin is plant encoded (Appleby 1984), whereas the heme prosthetic group is thought to be produced by the bacteroids (Nadler and Avissar 1977).

The essential components of the tetrapyrrole biosynthetic pathway, which leads to the formation of corrins, bilins, chlorophyll, and heme, have been examined in plants, animals, and bacteria and have been found to share many common features. The synthesis of 5-aminolevulinic acid (ALA) constitutes a central step in this pathway, but is carried out via two different routes in members of the α-subgroup of the purple bacteria (e.g., rhizobia) and animal cell mitochondria, versus other bacteria (e.g., *Escherichia coli*) and plant chloroplasts (see Avissar *et al.* 1989). In rhizobia, succinylCoA is condensed with glycine to form 5-aminolevulinic acid (catalyzed by ALA synthase or ALAS; EC 2.3.1.27) via the Shemin (C_4) pathway (Kikuchi *et al.* 1958), whereas in plant chloroplasts, ALA is synthesized in three steps from glutamate via glutamyl-tRNA and glutamate-1-semialdehyde by the enzymes aminoacyl-tRNA-ligase, glutamyl-tRNA-reductase and GSA-aminotransferase (C_5 pathway; Castelfranco and Beale 1983; Kannangara *et al.* 1988; O'Neil and Soll 1990). This pathway appears to be light induced in selected cases (Corriveau and Beale 1986) and is inhibited by pyridoxal phosphate-enzyme inhibitors, such as gabaculine and amino-oxyacetic acid (Weinstein and Beale 1985). Thus far only one organism has been shown to utilize both pathways simultaneously, namely *Euglena gracilis*, which synthesizes ALA via the Shemin (C_4) pathway in mitochondria and the C_5 pathway in chloroplasts (Weinstein and Beale 1983). Genes encoding the enzymes responsible for carrying out ALA synthesis via the Shemin (C_4) and C_5 pathways have been identified and cloned (Leong *et al.* 1982; Elliot 1989; Verkamp and Chelm 1989; Li *et al.* 1989; Drolet *et al.* 1989; Elliot *et al.* 1990; Grimm 1990; Grimm *et al.* 1991). To circumvent a nomenclature problem that has arisen in the literature, here the gene encoding ALAS will be termed *hemA*(C_4) and the gene encoding glutamyl-$tRNA^{glu}$ dehydrogenase will be termed *hemA*(C_5).

hemA(C_4) mutants have been constructed and characterized in different (brady)rhizobial species, namely *Rhizobium meliloti* 102F34 (Leong *et al.* 1982, 1985), *Rhizobium* sp. NGR234 (Stanley *et al.* 1989) and *Bradyrhizobium jap-*

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onicum USDA110 (Guerinot and Chelm 1986; McClung *et al.* 1987). In all three cases, the *hemA*(C_4) mutants were found to be strict ALA auxotrophs. Their symbiotic phenotypes, however, were shown to be different, since *R. meliloti* and *Rhizobium* sp. NGR234 *hemA*(C_4) mutants induced nitrogen fixation-deficient (Fix^-) nodules on their respective host plants, whereas *B. japonicum hemA*(C_4) mutants induced leghemoglobin containing, Fix^+ nodules on soybean. These results suggest the presence of an alternative bacterial pathway of ALA biosynthesis in nodules induced by certain rhizobial strains or symbiotic rescue by plant derived ALA in selected cases. Partial but convincing evidence for the latter hypothesis has been provided by Sangwan and O'Brian (1991), who have suggested an interorganismal pathway for heme biosynthesis in soybean nodules, involving enzymatic steps carried out by both symbiotic partners.

The diazotrophic bacterium *Azorhizobium caulinodans* ORS571 (referred to as ORS571 in this manuscript) induces nitrogen-fixing stem as well as root nodules on its host, the tropical leguminous shrub *Sesbania rostrata* and is capable of N_2-dependent growth in culture at a relatively high O_2 concentration (3%; Dreyfus *et al.* 1988; see de Bruijn 1989). The green stem nodules have been shown to harbor chloroplast containing cells immediately adjacent to bacteroid containing cells and the juxtaposition of potentially oxygen evolving cells and nitrogenase containing cells has been postulated to present an additional challenge to the oxygen transport system (see de Bruijn 1989). Seven major Lb components have been found in both stem- and root nodules, of which one form (LbVI) appears to be more abundant in stem versus root nodules (Bogusz *et al.* 1987). *S. rostrata* Lbs have been found to have the highest oxygen affinity yet recorded for symbiotic leghemoglobins (Wittenberg *et al.* 1985) and allow ORS571 bacteroids to carry out nitrogen fixation at an unusually low oxygen concentration (<10 nM; Bergersen *et al.* 1986). In this context, we have been interested in elucidating the role of ORS571 heme biosynthesis in symbiotic and free-living nitrogen fixation, and in comparing the effect of a *hemA*(C_4) mutation on stem versus root nodule formation or function. Here we report the cloning of the ORS571 *hemA*(C_4) gene and the characterization of a hemA(C_4)::Tn*5* mutation, as well as apparent second-site suppressor mutations. We also discuss the possibility of the existence of an alternative (C_5) ALA synthesis pathway in ORS571. A preliminary report of these results was presented at the 7th International Nitrogen Fixation Congress in Cologne, Germany (Pawlowski *et al.* 1988).

RESULTS

Isolation of the ORS571 *hemA*(C_4) gene.

Hybridization of a 3.1-kb *Sal*I fragment containing the *R. meliloti* 102F34 *hemA*(C_4) gene (Fig. 1A) with chromosomal DNA of ORS571 revealed three hybridizing *Eco*RI fragments, two of ~15 kb and one of ~8 kb (data not shown). A genomic library of ORS571, constructed in the broad host-range cosmid pLAFR1 (Pawlowski *et al.* 1987), was screened for cosmids carrying the *hemA*(C_4) gene, using the *R. meliloti hemA*(C_4) probe. The cosmids hybridizing with this probe could be divided into three classes, two of them containing ~15-kb *Eco*RI hybridizing fragments (pLALAS11, 31), and a third class containing a 7.6-kb *Eco*RI hybridizing fragment (pLALAS21, 22). The three classes of cosmids did not share common *Eco*RI fragments (data not shown). One cosmid clone of each class was chosen for further characterization (pLALAS11, 22 and 31). The regions of homology with the *R. meliloti* probe were subcloned in pACYC184 or pJRD184: A 8.1-kb *Sal*I fragment of pLALAS11 (containing part of the pLAFR1 vector) was cloned into pACYC184 to yield pALAS1, a 7.6-kb *Eco*RI fragment of pLALAS22 into pJRD184 to yield pALAS2 and a 6.2-kb *Eco*RI/*Hin*dIII fragment of pLALAS31 into pJRD184 to yield pALAS3. The regions of homology with the *R. meliloti hemA*(C_4) locus were further delimited by Southern blotting (Fig. 1B). A 1.7-kb *Pvu*I fragment of pALAS1, a 1.1-kb *Pvu*II fragment of pALAS2 and the complete 6.2-kb *Eco*RI-*Hin*dIII insert of pALAS3 (see Fig. 1B) were also used to hybridize back to restriction digests of the 3.1-kb *Sal*I fragment carrying the *R. meliloti hemA*(C_4) locus. The

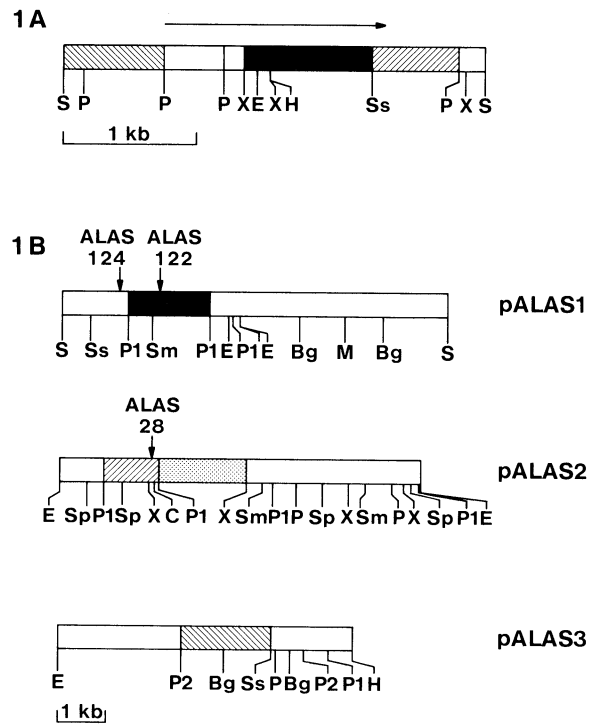

Fig. 1. Structure of the *Rhizobium meliloti hemA*(C_4) probe and the homologous regions from ORS571. **A,** The physical map of the *R. meliloti hemA*(C_4) locus carried by pSL5 (Leong *et al.* 1985) is shown. The region labeled in black delimits the subfragments hybridizing with pLALAS11 and pALAS1, the hatched regions the subfragments hybridizing with pLALAS21,22 and pALAS2 (hatched area on the left) and pLALAS31 and pALAS3 (hatched area on the right), respectively. The arrow indicates the extent of the longer *hemA*(C_4) transcript, as determined by Leong *et al.* (1985). **B,** The inserts of plasmids pALAS1, pALAS2, and pALAS3 are shown. The regions hybridizing against the insert of pSL5 are labeled as described above; the dotted region of pALAS2 denotes weak hybridization. The positions of the Tn*5* insertions are denoted by vertical arrows. Restriction enzyme abbreviations: Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; P1, *Pvu*I; P2, *Pvu*II; S, *Sal*I; Sm, *Sma*I; Sp, *Sph*I; Ss, *Sst*I; X, *Xho*I.

1.7-kb *PvuI* fragment of pALAS1 was found to hybridize with the coding region of the *R. meliloti hemA*(C_4) gene, while the other fragments shared DNA homology with sequences upstream and downstream of the *hemA*(C_4) gene (Fig. 1A; data not shown), suggesting that the ORS571 *hemA*(C_4)-like gene is located on pALAS1. Southern blotting experiments were also carried out to determine if the *hemA*(C_4)-like region carried by pLALAS11 (pALAS1), or the regions carried by pLALAS22 (pALAS2) or pLALAS31 (pALAS3) were linked to previously identified ORS571 loci. No linkage was found with the ORS571 *nifHDKE-nifA* (Elmerich *et al.* 1982; Pawlowski *et al.* 1987), *ntrC* (Pawlowski *et al.* 1987), *nifB, glnA* (de Bruijn *et al.* 1990), or *glt* (Hilgert *et al.* 1987) regions (data not shown).

Complementation experiments.

The cosmid clones pLALAS11, 21, and 31 were examined for their ability to complement the *R. meliloti hemA*(C_4) mutant strain A102, while the wild-type 102F34Nal^R was used as a positive control. A102 transconjugants containing pLALAS11 could grow on full and minimal medium without ALA and induced normal, Fix$^+$ nodules on *M. sativa*, while the other cosmids did not complement either the auxotrophic or symbiotic phenotypes (data not shown).

The cosmid clones and plasmid pALAS1 were also investigated for their ability to complement the ALA auxotrophy of the *E. coli hemA*(C_5) mutant strains SHSP18 and SASX413 (Table 1). The *R. meliloti hemA*(C_4) plasmid pSL5 (Table 1) was included as a positive control. In contrast to pSL5, the ORS571 cosmid or plasmid derivatives were unable to complement the *E. coli* ALA auxotrophy (data not shown). A similar observation has been made with the plasmid borne *hemA*(C_4) locus of *B. japonicum* (Guerinot and Chelm 1986).

Construction and characterization of an ORS571 *hemA*(C_4)::Tn*5* mutant.

Tn*5* mutagenesis experiments were carried out with pALAS1 and pALAS2. Two Tn*5* insertions in pALAS1 and one insertion in pALAS2 were used for gene replacement experiments (see Fig. 1B). The resulting homogenotes (ALAS122, ALAS124, and ALAS28) were examined for

Table 1. Strains and plasmids used in this study

	Relevant characteristics	Source/reference
Strain		
Escherichia coli		
HB101	*recA* strain for cloning experiments	Boyer and Roulland-Dussoix (1969)
SHSP18 (SASX76)	*hemA8, metB1, trpA43, lacY1, malA1, strA134*, λ^R	
SASX413B	*hemA41, relA1, spoT1, metB1*	A. Sasarman *et al.* (1986)
Rhizobium meliloti		
Rm102F34Nal^R	Spontaneous Nal^R derivative of wild-type 102F34; Nod$^+$, Fix$^+$	Ditta *et al.* (1980)
RmA102	*hemA*(C_4)::Tn*5* derivative of 102F34Nal^R, Km^R; Nod$^+$, Fix$^-$	S. Leong and G. Ditta
Azorhizobium caulinodans		
ORS571	Wild-type, Cb^R; Nif$^+$, Nod$^+$, Fix$^+$	Dreyfus *et al.* (1988)
ORS5740	*nifHDK*, Cb^R; Nif$^-$, Nod$^+$, Fix$^+$	Elmerich *et al.* (1982)
A53	*nifA*::miniMu*dII*PR46; Nif$^-$, Nod$^+$, Fix$^-$	Ratet *et al.* (1989)
ALAS122	*hemA*(C_4)::Tn*5*, Cb^R, Km^R; ALA-auxotroph, Nif$^+$, Nod(stem)$^-$, Nod(root) delayed, Fix$^-$, Ntr$^+$	This work
ALAS124	Tn*5* insertion next to *hemA*(C_4), Cb^R, Km^R, Nif$^+$, Nod$^+$, Fix$^+$, Ntr$^+$	This work
ALAS28	Tn*5* insertion, Cb^R, Km^R Nif$^+$, Nod$^+$, Fix$^+$, Ntr$^+$	This work
ALAS122RT1, RT4	ALAS122 second-site revertants, Cb^R, Km^R; ALA-auxotroph on LSO-, but not on TY-medium, Nif$^{-/+}$, Nod(stem)$^-$, Nod(root) delayed, Fix$^-$, Ntr$^{-/+}$	This work
Plasmid		
pLAFR1	Tc^R, cos, Tra$^-$, Mob$^+$, IncP (wide host range vector)	Friedman *et al.* (1982)
pWB5	Tc^R, Km^R (pRK290 derivative with Km^R and polylinker)	W. Buikema and F. M. Ausubel
pACYC184	Tc^R, Cm^R	Chang and Cohen (1978)
pJRD184	Tc^R, Ap^R	Heusterspreute *et al.* (1985)
pSL5	Ap^R; *R. meliloti hemA*(C_4) in pUC9	Leong *et al.* (1985)
p*Src1b1*	Ap^R; *S. rostrata lb* cDNA clone	Metz *et al.* (1988)
pLALAS11, pLALAS21, pLALAS22, pLALAS31	Tc^R; ORS571 genomic DNA in pLAFR1	This work
pALAS1	Cm^R; 8.1-kb *SalI* fragment of pLALAS11 in pACYC184	This work
pWBALAS1	Tc^R; 8.1-kb *SalI* fragment of pALAS1 in pWB5	This work
pALAS2	Tc^R; 7.6-kb *Eco*RI fragment of pLALAS22 in pJRD184	This work
pALAS3	Tc^R, Ap^R; 6.2-kb *Eco*RI/*Hin*dIII fragment of pLALAS31 in pJRD184	
pLRSC1	Tc^R; ORS571 *ntrBC-ntrYX* region in pLAFR1	Pawlowski *et al.* (1987, 1991)
pLRSC1Δ28	Tc^R; *Eco*RI deletion derivative of pLRSC1	Pawlowski *et al.* (1991)
pLRSC1Δ2818	Tc^R, Km^R; Tn*5* insertion in *ntrC* on pLRSC1Δ28	K. Pawlowski and F. J. de Bruijn, unpublished
pLRSC1Δ12	Tc^R; *Eco*RI deletion derivative of pLRSC1	Pawlowski *et al.* (1991)

ALA auxotrophy (growth on medium with and without ALA), nodulation on *S. rostrata* stems and roots (Nod), free living (Nif), and symbiotic nitrogen fixation (Fix) and Ntr phenotype (growth on minimal medium with 0.2% KNO_3, arginine or histidine as sole N source). The Tn*5* insertion mutant ALAS124, located outside the *hemA*(C_4) hybridizing region, and the Tn*5* insertion mutant ALAS28 in the pALAS2 region revealed a wild-type phenotype for all the parameters tested. Strain ALAS122, carrying a Tn*5* insertion in the region of highest homology with the *R. meliloti hemA*(C_4) gene, was found to be a strict ALA auxotroph, both on full and minimal medium. Strain ALAS122 revealed a Ntr^+ phenotype and was able to fix nitrogen at wild-type levels in culture and on plates (Nif^+), in the presence of exogenous ALA. The symbiotic phenotype of strain ALAS122 was examined using *S. rostrata* plants grown in test tubes or in Leonard jars. ALAS122 was shown to be severely affected in its symbiotic properties: Only 'bumps' (swellings of the adventitious roots sites) were found on the stems of mature *S. rostrata* plants (Fig. 2A), while nodule induction at the cotyledonary and primary leaf nodes was not impaired. The latter type of nodules was found to be nitrogen fixation deficient (Fix^-). Root nodule induction by ALAS122 was delayed by several weeks relative to the wild-type ORS571 strain and the nodules formed were strictly Fix^-. The nodules were either very small (smaller than nodules induced by the *nifHDK* mutant ORS5740 or an *nifA*::Tn*5* strain) or bigger than wild-type nodules and in this case often highly irregularly shaped and on the secondary roots rather than the primary root. An example of this class of nodules is shown in Figure 2B. Although all Fix^- mutant strains examined up to now, e.g., *nifHDK* and *nifA* strains (Elmerich *et al.* 1982; Pawlowski *et al.* 1987) reveal a "hypernodulated phenotype" on *S. rostrata* roots, ALAS122 induced root nodules were considerably fewer in number. Occasionally wild-type nodules were observed on roots infected with ALAS122, which were shown to be the result of loss of Tn*5* from the *hemA*(C_4) locus, as demonstrated by examining the antibiotic resistance markers of bacteria reisolated from these nodules. The ALA auxotrophy and defective symbiotic phenotypes of ALAS122 could be complemented by the introduction of a plasmid pWB5 (Table 1) derivative, carrying the 8.1-kb *Sal*I insert of pALAS1 (pWBalas1; data not shown).

To examine whether the *S. rostrata* leghemoglobin (*lb*) genes were expressed in ALAS122 induced root nodules, we isolated RNA from wild-type, ORS5740, and ALAS122 induced nodules of test tube plants 14 wk after infection, separated it by PAGE, and transferred it onto a nitrocellu-

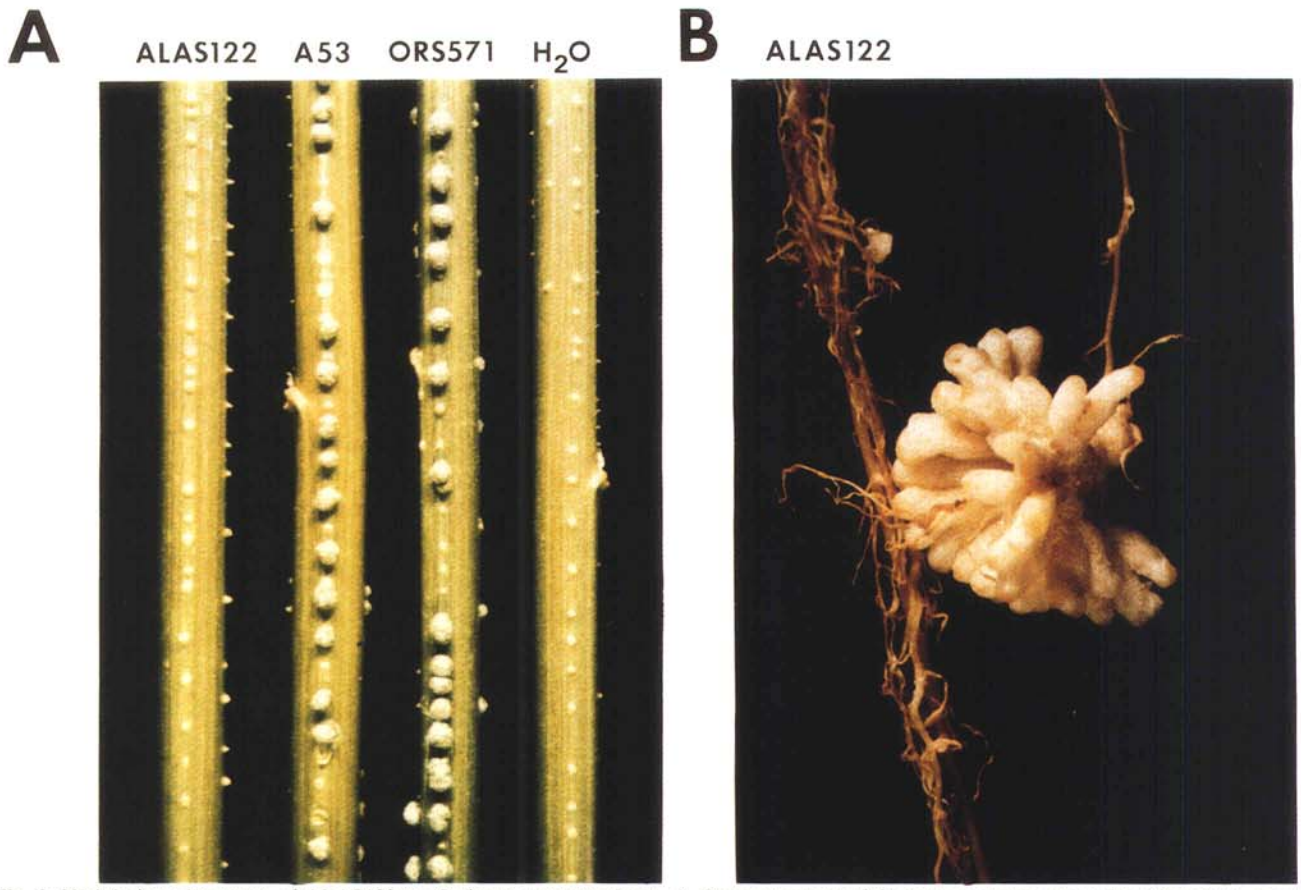

Fig. 2. Nodulation phenotype of ALAS122 on *Sesbania rostrata* plants. **A,** Stem segments of *S. rostrata* plants grown in a Conviron growth chamber are shown 6 wk after infection with *A. caulinodans* strains ALAS122, A53 (*nifA*::MudPR46), ORS571 (wild type) and plants inoculated with water. ALAS122 merely induces swelling of the adventitious root sites. **B,** Ultrastructure of an ALAS122-induced root nodule on *S. rostrata*. This unusual "coral-shape" nodule was observed on the root of a *S. rostrata* plant 14 wk after infection with ORS571ALAS122.

lose membrane. The resulting Northern blot was hybridized with the *lb* cDNA probe p*Srclb*1 (Metz *et al.* 1988). Wild-type and ORS5740 induced nodules contained a high level of *lb* mRNA, but ALAS122 induced nodules were found to lack *lb* mRNA (Fig. 3).

Secondary-site suppressor mutants of ALAS122.

Selection for (pseudo)revertants of strain ALAS122 that were able to grow on full (TY) medium supplemented with Cb and Km, but without ALA, revealed a reversion frequency of $\sim 10^8$. On minimal LSO plates, supplemented with ammonium, glutamine, or nitrate as sole nitrogen sources, no purifiable revertants were found. Revertants found on TY plates (named ALAS122RT) were found to be unable to grow on LSO-ammonium plates in the absence of ALA but grew on LSO-ammonium plates supplemented with 1 g/L of yeast extract (YLS medium) in the absence of ALA. A similar result has been reported for a *hemA* mutant of *B. japonicum*, able to grow in minimal GS medium supplemented with 1 g/L yeast extract (GSY; Frustaci *et al.* 1991).

To demonstrate that the position of the *hemA*(C_4) Tn*5* insertion had remained the same, four randomly picked (pseudo)revertants were selected. Chromosomal DNA was isolated from these strains, digested with *Pvu*I, and hybridized with pALAS1 insert DNA. The same hybridization patterns were observed in the ALAS122RT isolates as in ALAS122 (data not shown). Thus, the ALAS122RT strains appear to constitute second-site (pseudo)revertants or suppressor mutations.

The ALAS122RT strains exhibited slower growth than did the wild-type ORS571 strain on TY medium and formed smaller colonies. Furthermore, the color of the second-site revertant colonies was found to be lighter than the color of the wild-type colonies. The cytochrome content of ALAS122RT4 was found to be substantially lower than that of the wild-type strain (~9%; data not shown). The ALAS122RT strains could be divided in three groups based on nitrogen assimilation and fixation phenotypes. Some of the ALAS122RT strains exhibited altered growth patterns on LSO plates containing nitrate, histidine, arginine, leucine, glutamate, or ammonium as sole nitrogen source ($Ntr^{-/+}$ phenotype; see Table 2). In addition, they were disturbed in free-living nitrogen fixation to varying degrees ($Nif^{-/+}$ to $Nif^{+/-}$; Table 2). All ALAS122RT strains tested revealed the same nodulation/symbiotic nitrogen fixation phenotype as ALAS122. In selected cases, a Fix^+ root nodule was observed, and analysis of the bacteria reisolated from these nodules revealed the loss of the Tn*5* insertion from the *hemA*(C_4) locus (data not shown). These reisolated bacteria were found to be ALA prototrophs and to induce normal Fix^+ nodules on the stems and roots of *S. rostrata*. However, they retained the Nif and Ntr phenotype of the parent ALAS122RT strains. Thus, the symbiotic phenotype of ALAS122 second-site revertants appears to be due to the *hemA*(C_4) mutation alone and does not represent an effect of the second-site suppressor mutations. Moreover, the Nif and Ntr phenotypes of the ALAS122RT strains can be attributed to the second-site suppressor mutations.

The observed Nif and Ntr phenotypes of the ALAS122RT1 (class I) strains are very similar to those observed with *ntrC* or *ntrYX* mutations of ORS571 (Pawlowski *et al.* 1987; 1991). Therefore, the ability of cosmids carrying these loci to complement ALAS122RT mutations was examined. Cosmids pLRSC1 and pLRSC1Δ28, carrying both *ntrBC* and *ntrYX* loci, were introduced into the ALAS122RT1 and RT4 strains and the resulting transconjugants were examined for their ability to grow on nitrate as sole nitrogen source (Ntr phenotype). The ALAS122RT1 and RT4 strains harboring pLRSC1 or pLRSC1Δ28 were found to grow like the wild-type ORS571 strain on nitrate (Ntr^+). The presence of pLRSC1 in the ALAS122RT1 and RT4 strains did not affect growth on TY plates lacking ALA (data not shown). Complementation of the Ntr phenotype by pLRSC1Δ28 was found to be abolished when transposon Tn*5* was inserted in the cosmid-borne *ntrC* locus (pLRSC1Δ2818),

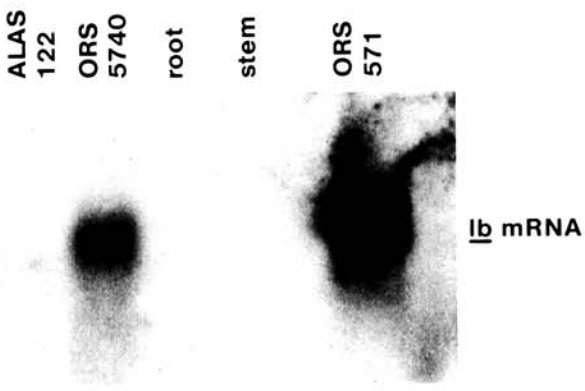

Fig. 3. Analysis of RNA isolated from root nodules induced by ORS571, ORS5740 and ALAS122 by Northern blot hybridization using a *Sesbania rostrata lb* cDNA probe. Total RNA (~20μg) of ORS571-, ORS5740-, and ALAS122-induced root nodules and from roots and stems of uninfected plants was separated on a formaldehyde-agarose gel, transferred onto nitrocellulose, and hybridized with the insert of p*Srclb*1 (Metz *et al.* 1988).

Table 2. Classification of ALAS122RT strains according to their growth on nitrate or ammonium as sole N source[a]

	Class I	Class II	Class III
Growth on			
LSO-ALA-NO_3^-	Slimy[b]	Slimy[b]	Normal[b]
LSO-ALA-NH_4^+	Normal[b]	Slimy[b]	Normal[b]
Nif[c]	0–1%	15%	70%
Fix[d]	...	...	...
Number of revertants per class	7	4	14

[a] Twenty-five independent revertants were analyzed. Nif and Fix phenotypes were examined using one representative of each class only: RT1 (class I), RT4 (class II), and RT3 (class III).

[b] Normal denotes wild-type-like growth on plates, as well as in liquid culture; slimy denotes the formation of slimy, translucent colonies on plates and reduced growth yield and doubling time in liquid cultures.

[c] Free-living nitrogen fixation (Nif) phenotype is expressed in percent of wild-type C_2H_2 reduction activity, as described by Pawlowski *et al.* (1987).

[d] The symbiotic nitrogen fixation (Fix) phenotype of the ALAS122RT strains tested was found to resemble the phenotype of the parent strain ALAS122.

or when a deletion was introduced in the *ntrX* gene (pLRSC1Δ12), suggesting the involvement of the *ntrC* and/or *ntrX* genes in complementing the second-site suppressor mutations that caused the altered growth phenotype on nitrate (RT1 and RT4).

One possible explanation for these results is that a second pathway for ALA biosynthesis, cryptic under normal physiological conditions, may exist in ORS571, which is activated by the generation of a (suppressor) mutation. Because the C_5 pathway is the only other known ALA synthesis pathway described (O'Neill and Soll 1990), the effect of inhibitors of the C_5 pathway on ORS571 wild-type and ALAS122RT strains was tested. Gabaculine and aminooxyacetate (AOA) were found to exert different effects on wild-type and ALAS122RT strains (see Table 3). Growth of ALAS122RT strains was found to be inhibited by gabaculine and AOA to a higher level than the wild-type strain ORS571 and the sensitivity to these inhibitors was relieved when ALA was added to the growth medium.

Experiments to detect enzymatic activity of the C_5 pathway were performed with ALAS122RT4 crude extract, as well as protein fractions purified by serial affinity chromatography. Assays without tRNA, ATP, or NADPH, and assays in the presence of RNase A or gabaculine were carried out as control experiments. However, formation of ^{14}C-ALA from ^{14}C-glutamate could not be demonstrated in wild-type or ALAS122RT bacteria, using the bacterial growth and assay conditions described in Materials and Methods.

DISCUSSION

We have described the cloning and characterization of an ORS571 *hemA*(C_4) gene and the construction and phenotypical analysis of an ORS571 *hemA*(C_4)::Tn*5* mutant (ALAS122). The identity of the *hemA*(C_4) gene was established by DNA hybridization data, functional complementation of a *R. meliloti hemA*(C_4) mutant and the observed ALA auxotrophy of the ALAS122 strain. The ALA auxotrophy of the ORS571 *hemA*(C_4)::Tn*5* strain constitutes a phenotype shared by all *hemA*(C_4) mutant *Rhizobium* and *Bradyrhizobium* strains characterized thus far (Leong *et al.* 1982; Guerinot and Chelm 1986; Stanley *et al.* 1989). The symbiotic phenotype of the ORS571 ALAS122 strain is of particular interest, since the (brady)rhizobial *hemA*(C_4) mutations previously analyzed have been shown to have very different effects on symbiotic nitrogen fixation and Lb production. The *B. japonicum hemA*(C_4) mutant has been found to induce effective, leghemoglobin-containing nodules on its host plant (soybean; Guerinot and Chelm 1986), while the *R. meliloti* and *Rhizobium* sp. NGR234 *hemA*(C_4) mutants induce Fix$^-$ nodules on their respective host plants (Leong *et al.* 1982; Stanley *et al.* 1989). In alfalfa nodules induced by *hemA*(C_4) *R. meliloti* strains, only traces of leghemoglobin mRNA could be found (Leong *et al.* 1982; Dickstein *et al.* 1991) and the soybean *lbc3* gene promoter was shown to be essentially silent in nodules induced by a *hemA*(C_4) strain on transgenic alfalfa plants (de Bruijn *et al.* 1989). The latter observation is interesting in light of the fact that in yeast heme has been found to modulate the translation of chimeric mRNAs consisting of the 5′ upstream region of the same *lbc3* gene fusion to the *cat* reporter gene (Jensen *et al.* 1986), suggesting a role for heme in *lb* gene expression. In contrast, soybean nodules induced by a *hemA*(C_4) *B. japonicum* strain are Fix$^+$ and therefore must contain Lb (Guerinot and Chelm 1986). In addition, because the expression of other late nodulin genes is also blocked in alfalfa nodules induced by a *hemA R. meliloti* mutant strain (Dickstein *et al.* 1991), the observed effect on *lb* gene expression may be general rather than specific.

Sangwan and O'Brian (1991) have presented a possible explanation for the observed Fix$^+$ phenotype of the *B. japonicum hemA*(C_4) mutant, by suggesting that the soybean plant crossfeeds the ALAS deficient bacteroids with ALA, allowing them to complete the biosynthesis of heme and to contribute heme for Lb production. They postulate that this type of "interorganismal heme biosynthesis pathway" may not function properly in other symbiotic systems, resulting in the observed differences in phenotypes of *hemA*(C_4) mutants. The (soybean) host plant does not appear to be able to rescue later blocks in the heme biosynthetic pathway, since *B. japonicum hemH* mutants (defective in the last step of heme biosynthesis) induce ineffective nodules lacking Lb (apoprotein) production (Frustaci and O'Brian 1992).

The results obtained with the ORS571 ALAS122 mutant support the conclusions made studying the other symbiotic systems that bacterial heme production is essential for an effective symbiosis, for Lb production and possibly *lb* gene expression. The inability of *S. rostrata* plants to rescue the ALAS deficiency may be due to a limitation of plant-derived ALA uptake by the ORS571 bacteroids.

However, the ORS571 *hemA*(C_4)::Tn*5* mutation (ALAS122) has some additional interesting characteristics, not observed with the other rhizobial *hemA*(C_4) mutants. The ORS571 ALAS122 strain is severely affected in its nodulation ability, especially on the stem of *S. rostrata* plants. This phenotype is probably not directly related to the auxotrophic nature of the mutant strain, since other auxotrophic and/or nitrogen assimilation deficient strains of ORS571, such as glutamine synthetase (*glnA*) or gluta-

Table 3. Growth of ORS571, ALAS122RT1, RT2, and RT4 on TY plates with or without ALA in the presence of different concentrations of the C_5 pathway inhibitors gabaculine or aminooxyacetate (AOA)

	+/− ALA	ORS571	RT1	RT2	RT4
mM Gabaculine					
...	−	+++	+++	+++	+++
4.5	−	+++	+	+	+
4.5	+	+++	+++	+++	+++
5.0	−	+++	−	+/−	−
5.0	+	+++	+++	+++	+++
6.0	−	++	−	−	−
6.0	+	++	−	−	−
7.0	−	−	−	−	−
mM AOA					
...	−	+++	+++	+++	+++
2.0	−	+++	−	−	−
2.0	+	+++	++	+++	++
4.0	−	−	−	−	−

+++, normal growth; ++, small colonies; + (+/−), (few) small, translucent colonies; −, no growth.

mate synthase (*glt*) mutants (Hilgert *et al.* 1987; de Bruijn *et al.* 1990) do not exhibit this phenotype. However, altered nodulation phenotypes have been observed for auxotrophic mutants of other (brady)rhizobia (Sadowsky *et al.* 1987 and references cited therein). Interestingly (see below), the inability to induce proper stem nodules has been found with ORS571 strains lacking the nitrogen regulatory protein NtrY (Pawlowski *et al.* 1991). These strains, however, do not display the defects in root nodulation observed with strain ALAS122. The aberrant stem- and root-nodulation phenotypes observed with the *hemA*(C_4) and *ntrY* mutations may be a reflection of the different physiological conditions for azorhizobial proliferation/infection at the adventitious root sites on the stem versus in the rhizosphere of *S. rostrata* plants (see de Bruijn 1989; Pawlowski *et al.* 1991).

The second atypical feature of the ORS571ALAS122 mutant strain, which has not been found with other rhizobial *hemA*(C_4) mutants, is the spontaneous appearance of second-site suppressor mutations on full medium lacking ALA. Strains carrying these suppressor mutations (ALAS122RT strains) appear to be able to produce small amounts of ALA, via a pathway that is affected by the C_5 ALA synthesis pathway inhibitors aminooxyacetate (AOA) and gabaculine. While sufficient to allow growth of the ALAS122 mutant strain on full medium in the absence of ALA, and on minimal plates supplemented with yeast extract (YLS), the secondary pathway does not allow the ALAS122 mutant strain to grow on minimal medium in the absence of ALA, nor to induce Fix^+ nodules on *S. rostrata*. Therefore, not enough ALA is produced to restore normal free-living growth, proper nodulation, or symbiotic nitrogen fixation. It is interesting to note that a *B. japonicum hemA* mutant strain is also able to grow in minimal medium in the absence of ALA, when yeast extract is added (Frustaci *et al.* 1991) but produces no detectable levels of heme.

The possible involvement of the nitrogen metabolism regulatory genes *ntrBC* and *ntrYX*, which also influence free-living and symbiotic nitrogen fixation (Pawlowski *et al.* 1987, 1991), in this process suggests that the second pathway may be repressed under normal conditions by the *ntr* system in ORS571. Alternatively, since the *ntrBC-ntrYX* containing cosmid clone pLRSC1 does not disturb the growth of the second-site revertant ALAS122RT4 on full medium without ALA, the second-site mutation could represent a "gain of function," e.g., an extension of promoter-binding specificity in the transcriptional activator proteins NtrC or NtrX. Other explanations, including an indirect role of the *ntr* system in heme- or heme precursor scavenging, cannot be ruled out at this time.

Although the inhibition studies suggest the presence of a C_5-like pathway in ORS571, we were unable to demonstrate ALA synthesis from glutamate in cell-free extracts. Sangwan and O'Brian (1991) also failed to detect C_5 pathway activity in *B. japonicum* bacteroids. These negative results do not allow us to make any conclusive statements about the possible presence of the C_5 ALA synthetic pathway. Additional physiological conditions for growth of the cells or different assay conditions must be tested and ORS571 *hemA*(C_5)- and *hemL*- (glutamate-1-semialdehyde aminotransferase) like genes should be searched for by other means, such as PCR amplification using primers derived from the *hemA* and *hemL* genes of the enteric bacteria (Elliott 1989; Elliott *et al.* 1990; Verkamp and Chelm 1989; Grimm *et al.* 1991), in order to arrive at a firm conclusion. In spite of this reservation, by isolating and characterizing the *A. caulinodans* ORS571 *hemA*(C_4) mutant and its second-site suppressor mutations, we have generated important tools for the further analysis of the unusual *A. caulinodans* ORS571-*S. rostrata* symbiotic system, in terms of rhizobial heme production for symbiotic nitrogen fixation and its involvement in the production of *S. rostrata* Lb proteins.

MATERIALS AND METHODS

Bacterial strains, cosmids and plasmids.

Bacterial strains, cosmids, and plasmids used in this study are listed in Table 1.

Growth media and chemicals.

E. coli strains were grown at 37° C in LB medium (Miller 1972). *(Azo)rhizobium* strains were grown at 37° C (ORS571 and derivatives) or at 28° C (*R. meliloti* strains) in TY (full medium; Beringer 1974), or in LSO (minimal medium; Elmerich *et al.* 1982) supplemented with 0.2% of the desired nitrogen source. Unless indicated otherwise, $(NH_4)_2SO_4$ (LSO-ammonium) or glutamine was used as nitrogen sources for normal growth of ORS571 and derivatives and KNO_3 for examination of the Ntr phenotype. YLS medium (LSO-ammonium supplemented with 1 g/L yeast extract) was also used as growth medium for ORS571 and derivative strains. Antibiotics were used at the concentrations described by Pawlowski *et al.* (1987). ALA was added as a supplement to a final concentration of 50 μg/ml. ALA, aminooxyacetic acid, and succinic thiokinase (succinate:CoA ligase, GDP forming, EC 6.2.1.4.) were purchased from Sigma (St. Louis), gabaculin from Fluka (Buchs, Switzerland). Radioactive chemicals ([2,3-^{14}C]-succinic acid, 5-amino-[4-^{14}C]levulinic acid.HCl, [α-^{32}P]-dCTP) were purchased from Amersham Buchler (Arlington Heights, IL).

DNA manipulations.

Plasmid DNA was prepared by the alkaline lysis method described by Sambrook *et al.* (1989). Chromosomal DNA was prepared as described by Meade *et al.* (1982). Conditions for DNA manipulations, hybridizations, and transformations have been described by Sambrook *et al.* (1989). The enzymes used in these analyses were used according to the specifications of the manufacturers (Boehringer, Mannheim, Indianapolis, IN; Bethesda Research Laboratories, Gaithersburg, MD; New England Biolabs, Boston, MA).

Transposon Tn*5* mutagenesis.

Transposon Tn*5* mutagenesis was carried out as described by de Bruijn and Lupski (1984) and de Bruijn (1987).

Conjugations and gene replacements.

Plasmids were mobilized from *E. coli* to ORS571 using the helper plasmid pRK2013, as described by Ditta *et al.* (1980). Gene replacement experiments with Tn*5* mutagenized regions were carried out as described (de Bruijn 1987).

RNA biochemistry.

Isolation of RNA from nodules was carried out as described by Jones *et al.* (1985). RNA gel electrophoresis and Northern blot experiments were carried out as described by Lehrach *et al.* (1977) and Sambrook *et al.* (1989).

Plant assays.

All nodulation and symbiotic nitrogen fixation tests with *S. rostrata* were performed as described by Pawlowski *et al.* (1987). For growing *Medicago sativa* 'Cardinal', seeds were surface sterilized by soaking them for 3 min in 70% ethanol and for 3 min in 0.1% $HgCl_2$, washed seven times in sterile H_2O and planted into B+D medium solidified with 1% agar (Broughton and Dilworth 1971); inoculation was carried out 4 days after germination with diluted bacterial cultures, as described for *S. rostrata* (Pawlowski *et al.* 1987). To determine the nitrogen-fixation capacity (Fix phenotype), acetylene reduction activity of whole plants was measured by sealing the test tubes with rubber seal stoppers, injecting 10% vol C_2H_2 and by examining the C_2H_4 content of the gas phase after 3–4 hr by gas chromatography. In each experiment, nodules were used for reisolation of bacteria to check their genetic markers. Reisolation of bacteria from the nodules was carried out as described by Pawlowski *et al.* (1987).

Enzyme assays.

Free-living acetylene reduction activity (Nif phenotype) was determined as described by Pawlowski *et al.* (1987). For preparation of crude protein extracts, cells were harvested after growth in full medium at the end of the logarithmic phase, washed, and resuspended in cold suspension buffer (0.1M tricine, 0.3M glycerol, 25mM $MgCl_2$, 1mM DTT; pH 9.0) and fractionated by three passages through a French pressure cell (Aminco, Silver Spring, MD) at 1,000 psi. Cell debris was pelleted by centrifugation at 360,000 × *g* for 15 min. The protein content of the supernatant was determined as described by Bradford (1976). For C_5 pathway assays, protein extract from 10–20 L of culture was separated by serial affinity chromatography, as described by Wang *et al.* (1981), except that chlorophyllin sepharose instead of heme sepharose was used. The activity of the C_5 pathway enzymes was determined using crude protein extract or protein fractions purified by serial affinity chromatography, combined with ORS571 tRNA (chlorophyl-lin sepharose fraction). Assays for ALA formation from ^{14}C-glutamate were performed in suspension buffer containing 1 mM ATP, 1 mM NADPH, 10 mM levulinic acid, and 50μCi of ^{14}C-glutamate. Reaction products were either separated by HPLC as described by Wang *et al.* (1981) or the ALA fraction was isolated for radioactivity determination in the following way: After incubation, 10 mg of nonradioactive ALA was added to the reaction mixture, and the protein was precipitated by adding TCA to a final concentration of 20%. ALA was isolated using DOWEX 50W-X8 columns as described by Wang *et al.* (1981). The pH 5.1 eluate was adjusted to pH 6.9 using H_3PO_4 and boiled with 1/20 volume acetic acid ethyl ester for 20 min. Then the solution was adjusted to pH 7.3 using Na_3PO_4, and the acetic acid ethyl ester left was removed by three to four chloroform extractions. Afterwards, the pH was adjusted to 4.3 using H_3PO_4 and the ALA pyrrol extract with diethyl ether (three times). After adding one drop of 25% NH_3, the combined ether phases were evaporated under N_2 flow. The residue was dissolved in 300 μl of methanol and subjected to thin-layer chromatography on silica gel 60 sheets in methyl acetate/isopropanol/25% NH3 (4/1/1; Irving and Elliott 1969). The ALA-pyrrol containing bands were made visible by application of modified Ehrlich's reagent (Granick *et al.* 1972) at the edge of the chromatogram, scraped off the plate, and eluted with 1 ml of methanol/H_2O/25% NH_3 (5/4/1). 75 μl of the eluate was used to determine the content of cold ALA by reaction with modified Ehrlich's reagent as a measure of recovery. The radioactivity in the remainder was determined by using a scintillation counter.

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LITERATURE CITED

Appleby, C. A. 1984. Leghemoglobin and *Rhizobium* respiration. Annu. Rev. Plant Physiol. 35:433-478.

Avissar, Y. J., Ormerod, J. G., and Beale, S. I. 1989. Distribution of δ-aminolevulinic acid biosynthetic pathways among phototrophic bacterial groups. Arch. Microbiol. 151:513-519.

Bergersen, F. J., Turner, G. L., Bogusz, D., Wu, Y.-Q., and Appleby, C. A. 1986. Effects of O_2 concentrations and various haemoglobins on respiration and nitrogenase activity of bacteroids from stem and root nodules of *Sesbania rostrata* and of the same bacteria from continuous cultures. J. Gen. Microbiol. 132:3325-3336.

Beringer, J. E. 1974. R factor transfer in *Rhizobium leguminosarum*. J. Gen. Microbiol. 84:188-189.

Bogusz, D., Kortt, A. A., and Appleby, C. A. 1987. *Sesbania rostrata* root and stem nodule leghemoglobins: Purification and relationship among the seven major components. Arch. Biochem. Biophys. 254:263-271.

Boyer, J. E., and Roulland-Dussoix, D. 1969. A complementation analysis of the restriction and modification of DNA in *E. coli*. J. Mol. Biol. 41:459-472.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye-binding. Anal. Biochem. 72:248-254.

Broughton, W. J., and Dilworth, M. Y. 1971. Control of leghaemoglobin synthesis in snake beans. Biochem. J. 125:1075-1080.

Castelfranco, P. A., and Beale, S. I. 1983. Chlorophyll biosynthesis: Recent advances and areas of current interest. Annu. Rev. Plant

Physiol. 77:532-535.
Chang, A. C. Y., and Cohen, S. N. 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. 134:1141-1156.
Corriveau, P. A., and Beale, S. I. 1986. Influence of gabaculine on growth, chlorophyll synthesis, and δ-aminolevulinic acid synthase activity in *Euglena gracilis*. Plant Sci. 45:9-17.
de Bruijn, F. J. 1987. Tn*5* mutagenesis to map genes. Methods Enzymol. 154:175-196.
deBruijn, F. J. 1989. The unusual symbiosis between the diazotrophic stem-nodulating bacterium *Azorhizobium caulinodans* ORS571 and its host, the tropical legume *Sesbania rostrata*. Pages 457-493 in: Plant-Microbe Interactions. Vol. 3. E. Nester, and T. Kosuge, eds. McGraw-Hill, New York.
de Bruijn, F. J., Felix, G., Grunenberg, B., Hoffmann, H.-J., Metz, B., Ratet, P., Simons-Schreier, A., Szabados, L., and Schell, J. 1989. Regulation of plant genes specifically induced in nitrogen-fixing nodules: Role of *cis*-acting elements and *trans*-acting factors in leghemoglobin gene expression. Plant Mol. Biol. 13:319-325.
de Bruijn, F. J., Hilgert, U., Stigter, J., Schneider, M., Meyer Z. A., H., Klosse, U., and Pawlowski, K. 1990. Regulation of nitrogen fixation and assimilation genes in the free-living versus symbiotic state. Pages 33-44 in: Nitrogen Fixation: Achievements and Objectives. P. Gresshoff, E. Roth, G. Stacey, and W. Newton, eds. Chapman and Hall, New York.
de Bruijn, F. J., and Lupski, J. R. 1984. The use of transposon Tn*5* mutagenesis in the rapid generation of correlated physical and genetical maps of DNA segments cloned into multicopy plasmids—A review. Gene 27:131-149.
Dickstein, R., Scheirer D. C., Fowle, W. H., and Ausubel, F.M. 1991. Nodules elicited by *Rhizobium meliloti* heme mutants are arrested at an early stage of development. Mol. Gen. Genet. 230:423-432.
Ditta, G., Stanfield, S., Corbin, D., and Helinski, D. R. 1980. Broad host range DNA cloning system for gram-negative bacteria: Construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA 77:7347-7351.
Dreyfus, B., Garcia, J. L., and Gillis, M. 1988. Characterization of *Azorhizobium caulinodans* gen. nov., sp. nov., a stem-nodulating nitrogen-fixing bacterium isolated from *Sesbania rostrata*. Int. J. System. Bacteriol. 38:89-98.
Drolet, M., Peloquin, L., Echelard, Y., Cousineau, L., and Sasarman, A. 1989. Isolation and nucleotide sequence of the *hemA* gene of *Escherichia coli* K12. Mol. Gen. Genet. 216:347-352.
Elliott, T. 1989. Cloning, genetic characterization, and nucleotide sequence of the *hemA-prfA* operon of *Salmonella typhimurium*. J. Bacteriol. 171:3948-3960.
Elliott, T., Avissar, Y. J., Rhie, G.-E., and Beale, S. I. 1990. Cloning and sequence of the *Salmonella typhimurium hemL* gene and identification of the missing enzyme in *hemL* mutants as glutamate-1-semialdehyde aminotransferase. J. Bacteriol. 172:7071-7084.
Elmerich, C., Dreyfus, B., Reysset, G., and Aubert, J.-P. 1982. Genetic analysis of nitrogen fixation in a tropical fast-growing *Rhizobium*. EMBO J. 5:441-447.
Friedman, A. M., Long, S. R., Brown, S. E., Buikema, W. J., and Ausubel, F. M. 1982. Construction of a broad host range cosmid cloning vector and its use in the genetic analysis of *Rhizobium* mutants. Gene 18:289-296.
Frustaci, J. M., Sangwan, I., and O'Brian, M. 1991. Aerobic growth and respiration of a δ-aminolevulinic acid synthase (*hemA*) mutant of *Bradyrhizobium japonicum*. J. Bacteriol. 173:1145-1150.
Frustaci, J. M., and O'Brian, M. R. 1992. Characterization of a *Bradyrhizobium japonicum* ferrochelatase mutant and isolation of the *hemH* gene. J. Bacteriol. 174:4223-4229.
Granick, S., Sassa, S., Granick, J. L., Levene, R. D., and Kappas, A. 1972. Assays for porphyrins, δ-aminolevulinic acid dehydratase and porphobilinogen synthetase in microliter samples of whole blood: Application to metabolic defects involving the heme pathway. Proc. Natl. Acad. Sci. USA 69:2381-2385.
Grimm, B. 1990. Primary structure of a key enzyme in plant tetrapyrrole synthesis: Glutamate 1-semialdehyde aminotransferase. Proc. Natl. Acad. Sci. USA 87:4169-4173.
Grimm, B., Bull, A., and Breu, V. 1991. Structural genes of glutamate 1-semialdehyde aminotransferase for porphyrin synthesis in a cyanobacterium and *Escherichia coli*. Mol. Gen. Genet. 225:1-10.
Guerinot, M. L., and Chelm, B. K. 1986. Bacterial δ-aminolevulinic acid synthase activity is not essential for leghemoglobin formation in soybean/*Bradyrhizobium japonicum* symbiosis. Proc. Natl. Acad. Sci. USA 83:1837-1841.
Heusterspreute, M., Ha-Thi, V., Emery, S., Tournis-Gamble, S., Kennedy, N., and Davison, J. 1985. Vectors with restriction site banks, IV. pJRD184, a 3793-bp plasmid vector having 43 unique cloning sites. Gene 39:299-304.
Hilgert, U., Schell, J., and de Bruijn, F. J. 1987. Isolation and characterization of Tn*5* induced NADH-glutamate synthase (GOGAT-) mutants of *Azorhizobium sesbaniae* ORS571 and cloning of the corresponding *glt* locus. Mol. Gen. Genet. 210:195-202.
Hoober, J. K., Kahn, A., Ash, D. E., Gough, S., and Kannangara, C. G. 1988. Biosynthesis of δ-aminolevulinate in greening barley leaves. IX. Structure of the substrate, mode of gabaculine inhibitions, and the catalytic mechanism of glutamate 1-semialdehyde aminotransferase. Carlsberg Res. Commun. 53:11-25.
Irving, E. A., and Elliot, W. H. 1969. A sensitive radiochemical assay method for δ-aminolevulinic acid synthetase. J. Biol. Chem. 244:60-67.
Jensen, E. O., Marcker, K. A., and Villadsen, I. S. 1986. Heme regulates the expression in *Saccharomyces cerevisiae* of chimaeric genes containing 5′-flanking soybean leghemoglobin sequences. EMBO J. 5:843-847.
Jones, J. D. G., Duinsmuir, P., and Bedbrook, J. 1985. High level expression of introduced chimeric genes in regenerated transformed plants. EMBO J. 4:2411-2418.
Kannangara, C. G., Gough, S. P., Bruyant, P., Hoober, J. K., Kahn, A., von Wettstein, D. 1988. $tRNA^{Glu}$ as a cofactor in δ-aminolevulinate biosynthesis: Steps that regulate chlorophyll synthesis. Trends Biochem. Sci. 13:139-143.
Kikuchi, G., Kumar, A., Talmage, P., and Shemin, D. 1958. The enzymatic synthesis of δ-aminolevulinic acid. J. Biol. Chem. 233:125-135.
Lehrach, H., Diamond, D., Woznay, J. M., and Boedtker, H. 1977. RNA molecular weight determinations by gel electrophoresis under denaturing conditions, a critical reexamination. Biochemistry 16:4743.
Leong, S. A., Ditta, G. S., and Helinski, D. R. 1982. Heme biosynthesis in *Rhizobium*: Identification of a cloned gene coding for δ-aminolevulinic acid synthetase from *Rhizobium meliloti*. J. Biol. Chem. 257:8724-8730.
Leong, S. A., Williams, P. H., and Ditta, G. S. 1985. Analysis of the 5′ regulatory region of the gene for 5-aminolevulinic acid synthetase from *Rhizobium meliloti*. Nucleic Acids Res. 13:5965-5976.
Li, J.-M., Russell, C. S., and Cosloy, S. D. 1989. Cloning and structure of the *hemA* gene of *Escherichia coli* K12. Gene 82:209-217.
McClung, C. R., Somerville, J. E., Guerinot, M. L., and Chelm, B. K. 1987. Structure of the *Bradyrhizobium japonicum* gene *hemA* encoding 5-aminolevulinic acid synthase. Gene 54:133-139.
Meade, H. M., Long, S. R., Ruvkun, G. B., Grown, S. E., and Ausubel, F. M. 1982. Physical and genetic characterization of symbiotic and auxotrophic mutants of *Rhizobium meliloti* induced by transposon Tn*5* mutagenesis. J. Bacteriol. 149:114-122.
Metz, B. A., Welters, P., Hoffmann, H.-J., O. Jensen, E., Schell, J., and de Bruijn, F. J. 1988. Primary structure and promoter analysis of leghemoglobin genes of the stem-nodulated tropical legume *Sesbania rostrata*: Conserved coding sequences, *cis*-elements and *trans*-acting factors. Mol. Gen. Genet. 214:181-191.
Miller, J. H. 1972. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
Nadler, K. D., and Avissar, Y .J. 1977. Heme synthesis in soybean root nodules. Plant Physiol. 60:433-436.
O'Brian, N. R., Kirshbom, P. M., and Maier, R .J. 1987. Bacterial heme synthesis is required for expression of the leghemoglobin holoprotein but not the apoprotein in soybean root nodules. Proc. Natl. Acad. Sci. USA 84:8390-8383.
O'Neill, G. P., and Soll, D. 1990. Transfer RNA and the formation of the heme and chlorophyll precursor, 5-aminolevulinic acid. BioFactors 2:227-235.
Pawlowski, K., Klosse, U., and de Bruijn, F. J. 1991. Characterization

of a novel *Azorhizobium caulinodans* ORS571 two-component regulatory system, NtrY/NtrX, involved in nitrogen fixation and metabolism. Mol. Gen. Genet. 231:124-138.

Pawlowski, K., Ratet, P., Schell, J., and de Bruijn, F.J. 1987. Cloning and characterization of *nifA* and *ntrC* genes of the stem nodulation bacterium ORS571, the nitrogen fixing symbiont of *Sesbania rostrata*: Regulation of nitrogen fixation (*nif*) genes in the free living versus symbiotic state. Mol. Gen. Genet. 206:207-219.

Pawlowski, K., Schell, J., and de Bruijn, F. J. 1988. Construction and characterization of heme biosynthesis *hemA* mutants of *Azorhizobium caulinodans* ORS571: *hemA* is essential for nodulation and symbiotic nitrogen fixation. Page 380 in: Nitrogen Fixation: Hundred Years After. H. Bothe, F. J. de Bruijn, and W. E. Newton, eds. Gustav Fischer, Stuttgart.

Ratet, P., Pawlowski, K., Schell, J., and de Bruijn, F. J. 1989. The *Azorhizobium caulinodans* nitrogen-fixation regulatory gene, *nifA*, is controlled by the cellular nitrogen and oxygen status. Mol. Microbiol. 3:825-838.

Sadowsky, M. J., Rostas, K., Sista, P. R., Bussey, H., and Verma, D. P. S. 1986. Symbiotically defective histidine auxotrophs of *Bradyrhizobium japonicum*. Arch. Microbiol. 144:334-339.

Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Sangwan, I., and O'Brian, M. R. 1991. Evidence for an inter-organismic heme biosynthetic pathway in symbiotic soybean root nodules. Science 251:1220-1222.

Sasarman, A., Surdenau, M., and Horodniceanu, T. 1968. Locus determining the synthesis of δ-aminolevulinic acid in *Escherichia coli* K-12. J. Bacteriol. 96:1882-1884.

Stanley, J., Dowling, D., and Brougton, W. J. 1988. Cloning of *hemA* from *Rhizobium* sp. NGR234 and symbiotic phenotype of a gene-directed mutant in diverse legume genera. Mol. Gen. Genet. 215:32-37.

Verkamp, E., and Chelm, B. K. 1989. Isolation, nucleotide sequence and preliminary characterization of the *Escherichia coli* K-12 *hemA* gene. J. Bacteriol. 171:4728-4735.

Wang, W.-Y., Gough, S. P., and Kannangara, C. G. 1981. Biosynthesis of 5-aminolevulinate in greening barley leaves IV. Isolation of three soluble enzymes required for the conversion of glutamate to 5-aminolevulinate. Carlsberg Res. Commun. 46:243-257.

Weinstein, J. D., and Beale, S. I. 1983. Separate physiological roles and subcellular compartments for two tetrapyrrole biosynthesic pathways in *Euglena gracilis*. J. Biol. Chem. 258:6799-6807.

Weinstein, J. D., and Beale, S. I. 1985. Enzymatic conversion of glutamate to δ-aminolevulinate in soluble extracts of the unicellular green alga, *Chlorella vulgaris*. Arch. Biochem. Biophys. 237:454-464.

Wittenberg, J. B., Bergersen, F. J., Appleby, C. A., and Turner, G. L. 1985. Facilitated oxygen diffusion. The role of leghemoglobin in nitrogen fixation by bacteroids isolated from soybean root nodules. J. Biol. Chem. 249:4057-4066.