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Heme Compounds as Iron Sources for Nonpathogenic *Rhizobium* Bacteria

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Many animal-pathogenic bacteria can use heme compounds as iron sources. Like these microorganisms, rhizobium strains interact with host organisms where heme compounds are available. Results presented in this paper indicate that the use of hemoglobin as an iron source is not restricted to animal-pathogenic microorganisms. We also demonstrate that heme, hemoglobin, and leghemoglobin can act as iron sources under iron-depleted conditions for *Rhizobium meliloti* 242. Analysis of iron acquisition mutant strains indicates that siderophore-, heme-, hemoglobin-, and leghemoglobin-mediated iron transport systems expressed by *R. meliloti* 242 share at least one component.

The ability to use heme and heme-containing compounds as iron sources under iron-deficient conditions has been documented for several animal-pathogenic bacteria (18). The system shares homology with the uptake and transport of siderophores. Outer membrane receptors, TonB requirement, and Fur regulation seem to be common characteristics (7, 13, 16, 19).

Leghemoglobin can represent up to 30% of total soluble proteins in N_2-fixing nodules (2). Acting as an oxygen transport protein, it is responsible for the diffusion of O_2 to bacteroids (22). Besides this protein, other hemoglobin-like genes have been reported in different soybean tissues (cotyledons, young leaves, etc.) (1) and in some tissues of *Parasponia andersonii* (3), *Trema tormentosa* (5), and *Casuarina glauca* (15). Hardison (12) suggests that hemoglobins are widespread in plants and may have more generalized roles besides those in nodulation.

It is conceivable that porphyrinic compounds may be available in the normal habitat of rhizobia, so we find it interesting to evaluate the use of some heme compounds as iron sources by these nonpathogenic microorganisms. In the present work, we examine the ability to use hemoglobin as an iron source by different nonpathogenic strains of bacteria. We also study the use of hemin, hemoglobin, and leghemoglobin by a *Rhizobium meliloti* strain.

Strains, media, and bacterial growth. The strains employed in this study are listed in Table 1. Rhizobium strains were grown in TY (4) or YEM (25) complex medium. A modified medium B of King (17) (10 instead of 18 g of Difco proteose peptone no. 3) was used as indicated in Table 1. Iron-limited media were obtained by the addition of di-*o*-hydroxyphenylacetic acid (EDDHA). The water used was obtained from a Milli-Q system. Media were supplemented with 100 μg of streptomycin per ml for *R. meliloti* 242 (wild type) and with 50 μg of neomycin per ml for *R. meliloti* 242 iron acquisition mutants (8). EDDHA, hemin, bovine hemoglobin, ferritin, and protoporphyrin IX were purchased from Sigma Chemical Company. Purified and lyophilized ferrichrome was kindly provided by P. Gill and J. B. Neilands (University of California, Berkeley). Soybean leghemoglobin was generously provided by R. Arredondo-Peter (University of Nebraska, Lincoln).

As shown in Table 1, the 14 rhizobium strains and the 5 nonpathogenic pseudomonad strains tested presented very limited growth or no growth at all in highly iron-starved media, but normal growth could be obtained when iron-depleted media were supplemented with hemoglobin. The same results were obtained for *Azotobacter paspali* AX-12, *Agrobacterium tumefaciens* GM I 9023 and *Micrococcus luteus* ATCC 94341, while hemoglobin was unable to support the growth of *Escherichia coli* K-12, *Erwinia carotovora* SCC3193, *Bacillus subtilis* ATCC 6633, *Acetobacter diazotrophus* Pal5, and *M. luteus* ATCC 10240 under iron-limited conditions. The same behavior was observed regardless of whether the assay was performed in liquid or solid media. These results indicate that the use of hemoglobin as an iron source is not restricted to animal-pathogenic bacteria.

With the aim of characterizing the iron sources used by rhizobia, we examined the bacterial growth of an *R. meliloti* strain in iron-limited media supplemented with hemin, hemoglobin, or leghemoglobin. The assay was performed with *R. meliloti* 242 (wild type) and with a siderophore-negative mutant of this strain (mutant 1.3) (8). Iron-depleted medium (IDM) was created by the addition of 200 μM EDDHA to TY-rich medium (for wild-type growth) or with 50 μM EDDHA (for mutant growth). Cells were grown on IDM containing 10 μM hemin, 2.5 μM hemoglobin, 6 μM leghemoglobin, or 10 μM protoporphyrin IX. Both strains exhibited growth rates ranging from 0.16 to 0.12 generations/h when they were grown on iron-sufficient medium or on IDM containing leghemoglobin, hemoglobin, or hemin. Values were less than 0.01 generations/h when bacteria were grown on IDM or IDM supplemented with protoporphyrin IX. These results indicate that hemin, hemoglobin, and leghemoglobin promote the growth of *R. meliloti* 242 under iron-limiting conditions.

Bioassays. *Rhizobium meliloti* 242 and some iron acquisition mutants of this strain (2.1, 1.3, 5.3, and 5.6) (8) (Table 2) were incorporated into solid TY media (15 g/liter) supplemented with 1 mM EDDHA (for wild-type growth) or 500 μM EDDHA (for mutant growth) to a final concentration of 10^5 CFU/ml. After the agar was solidified, 20 μl of the solutions to be tested was added in holes on the agar. Plates were then incubated at 30°C for 78 h.

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TABLE 1. Effect on bacterial growth of the addition of hemoglobin to IDM

Strain	IDM	Growth on IDM[a]		Strain source or reference[b]
		−Hb	+Hb	
Rhizobium meliloti 1021 (ATCC 51124)	TY + EDDHA (1 mM)	−	+	ATCC
Rhizobium meliloti L530	TY + EDDHA (1 mM)	−	+	J. Denarie, France
Rhizobium meliloti 242	TY + EDDHA (1 mM)	−	+	8
Rhizobium etli CE3	TY + EDDHA (1 mM)	−	+	21
Rhizobium tropici CIAT 899	TY + EDDHA (2 mM)	−	+	CIAT
Rhizobium leguminosarum bv. *phaseoli* BR 365	YEM + EDDHA (2 mM)	−	+	CNPBS
Rhizobium leguminosarum bv. *trifolii* WU290	YEM + EDDHA (2 mM)	−	+	WAU
Rhizobium leguminosarum bv. *trifolii* U28	YEM + EDDHA (2 mM)	−	+	ENZUR
Rhizobium sp. strain Adesmia 202	YEM + EDDHA (1 mM)	−	+	9
Rhizobium sp. Oregon 191	YEM + EDDHA (1 mM)	−	+	6
Bradyrhizobium japonicum 29W	YEM + EDDHA (1 mM)	−	+	ENZUR
Bradyrhizobium japonicum 587	YEM + EDDHA (1 mM)	−	+	ENZUR
Bradyrhizobium sp. strain (Vigna) I 700	YEM + EDDHA (1 mM)	−	+	24
Bradyrhizobium sp. strain (Lotus) CC814s	YEM + EDDHA (1 mM)	−	+	DSIR
Azotobacter paspali AX-12	KB + EDDHA (5 mM)	−	+	J. Dobereiner, Brazil
Pseudomonas putida B10 (ATCC 39169)	KB + EDDHA (3 mM)	−	+	ATCC
Pseudomonas fluorescens Pf5	KB + EDDHA (5 mM)	−	+	14
Pseudomonas fluorescens UP 148	KB + EDDHA (5 mM)	−	+	A. Arias, Uruguay
Pseudomonas sp. strain F113	KB + EDDHA (4 mM)	−	+	F. O'Gara, Ireland
Pseudomonas aeruginosa ATCC 29336	KB + EDDHA (1 mM)	−	+	ATCC
Agrobacterium tumefaciens GM I 9023/pSym of CFN299	KB + EDDHA (5 mM)	−	+	CFN
Erwinia carotovora subsp. *carotovora* SCC3193	KB + EDDHA (5 mM)	−	−	23
Escherichia coli K-12	KB + EDDHA (5 mM)	−	−	Laboratory strain
Bacillus subtilis ATCC 6633	KB + EDDHA (5 mM)	−	−	ATCC
Micrococcus luteus ATCC 10240	KB + EDDHA (2 mM)	−	−	ATCC
Micrococcus luteus ATCC 9341	KB + EDDHA (2 mM)	−	+	ATCC
Acetobacter diazotrophicus sp. Pal5	KB + EDDHA (3 mM) + glucose (25 mM)	−	−	J. Dobereiner, Brazil

[a] Growth was determined on solid and liquid media. Hemoglobin (Hb) was added at a final concentration of 3 μM. Growth was recorded as follows: good growth (+) or poor growth or no growth at all (−). All strains tested presented very good growth on media without EDDHA.

[b] ATCC, American Type Culture Collection, Rockville, Md.; CIAT, Centro Internacional de Agricultura Tropical, Cali, Colombia; CNPBS, Centro de Pesquisas em Biología do Solo, Seropédica, Río, Brazil; WAU, Western Australia University, Nedlands, Australia; ENZUR, Inoculant Production Company, Montevideo, Uruguay; DSRI, Department of Scientific and Industrial Research, Palmerston North, New Zealand; CFN, Centro de Investigación sobre Fijación de Nitrógeno, Universidad Nacional Autónoma de México, Cuernavaca, Morelos, México.

The radius of the halo corresponding to the bacterial growth was measured, and the results obtained are shown in Table 2. The wild-type strain presented a soft growth throughout all of the plate, and in this case, the presence of a dense halo of bacterial growth was recorded. As is shown, the wild-type strain and mutants 1.3 and 2.1 but not mutants 5.3 and 5.6 were able to use leghemoglobin, hemoglobin, hemin, and ferrichrome as iron sources under iron-depleted conditions.

As negative controls, protoporphyrin IX and 0.2 μmol of $FeCl_3$ (which is equivalent to a putative 5% of iron chloride contamination in the reagents) were tested. No bacterial growth on either negative control could be detected. It is important to mention that EDDHA produces a reddish color in the presence of iron traces, and this color was not detected around the holes containing hemin, hemoglobin, or leghemoglobin.

The results obtained also show that the rhizobial siderophore-rich supernatant obtained from the 2.1 mutant was able to restore growth to the 1.3 mutant, but it was not utilized by the 5.3 and 5.6 mutants as an iron source, as expected.

In conclusion, our findings show that the ability to use hemoglobin as an iron source under iron-depleted conditions is not restricted to animal-pathogenic bacteria. Moreover, this ability seems to be a common feature among the genera *Rhizobium*, *Bradyrhizobium*, and *Pseudomonas*. As is well known, rhizobia are able to interact with leguminous hosts (20) in which important amounts of leghemoglobin could be ex-

TABLE 2. Ability of some iron acquisition mutants of *Rhizobium meliloti* 242 to grow on solid medium by using different compounds

Compound	Avg radius of halo growth (cm) of strain[a]:				
	242	1.3	2.1	5.6	5.3
Leghemoglobin (0.02 μmol)	2.5	2.2	2.9	0.0	0.0
Hemoglobin (0.01 μmol)	2.0	1.6	2.9	0.0	0.0
Hemin (1 μmol)	1.5	1.3	2.2	0.0	0.0
Ferrichrome (0.05 μmol)	ND[b]	2.4	4.0	0.0	0.0
2.1 supernatant[c]	1.0	1.0	0.0	0.0	0.0
Ferritin (20 μg or 2 mg)	ND	0.0	0.0	0.0	0.0
Protoporphyrin IX (1 μmol)	0.0	0.0	0.0	0.0	0.0
$FeCl_3$ (0.2 μmol)	0.0	0.0	0.0	0.0	0.0
$FeCl_3$ (1.0 μmol)	2.2	1.0	2.5	0.8	0.8
$FeCl_3$ (2.0 μmol)	2.4	1.5	2.8	1.0	1.1

[a] Strains 1.3, 2.1, 5.3, and 5.6 are Tn*5*-induced mutants of *R. meliloti* 242 (8). 1.3 does not produce siderophores. 2.1 has a defect in the siderophore-uptake system. 5.3 and 5.6 are also defective in siderophore uptake, but they are genomically and phenotypically different from 2.1. All mutants produce effective nodules. Results are an average of five independent experiments. The standard deviation is less than 0.2.

[b] ND, not done.

[c] Mutant 2.1 was grown on TY liquid medium supplemented with 25 μM EDDHA. The filtered supernatant was concentrated 10-fold and sterilized by filtration; 20 μl was used.

pressed (2, 22), and we can assume that leghemoglobin and leghemoglobin-derived compounds may be present in the soil as nodule degradation products. Therefore, leghemoglobin could be a physiological candidate to act as an iron source for free-living rhizobia in natural environments.

Frustaci et al. (11) had reported that hemin-supplemented media could enhance the growth of *Bradyrhizobium japonicum* I110. This research group obtained hemin-biosynthetic mutants on the basis of the ability of this bacterium to utilize exogenous hemin (10). In those studies, hemin was used as a porphyrin source and not as an iron source. We can assume that cells need more iron than porphyrins, and it is not surprising to find differences in the hemin uptake according to iron or porphyrin demands (18).

The data presented here also show that mutants 5.3 and 5.6 were unable to utilize iron from the siderophore produced by the mutant 2.1, from hemin, hemoglobin, leghemoglobin, and ferrichrome, but all of these compounds could be used as iron sources by the 1.3 mutant. Moreover, the 2.1 mutant can use hemin, hemoglobin, and leghemoglobin but is unable to internalize its own siderophore. Since these mutants had a single Tn*5* insertion (8), the results obtained suggest that the transport mechanisms of these different iron sources share at least one component. For animal-pathogenic bacteria, there is strong evidence suggesting that transport of siderophores, hemin, and hemoglobin is mediated by the Ton complex (13, 16). Although proteins of this complex have not yet been detected in members of the family *Rhizobiaceae*, our data could be explained according a similar model of transport.

In a previous paper, we showed that mutants 5.3 and 5.6 were able to produce effective nodules on alfalfa plants (8). According to the data obtained in the present paper, we can not determine if the hemin-containing compounds could be used as iron sources in the symbiotic state. We cannot discard the possibility that the transport mechanism could be different in the free-living state and the symbiotic state or that bacteroids could have several mechanisms for obtaining iron. Shutting one or two of them down would not be sufficient to halt nodulation and nitrogen fixation.

As we previously mentioned, this is the first paper showing the utilization of heme compounds as iron sources by nonpathogenic bacteria. However, because globin moieties are more common among animals, plants, protists, and bacteria than previously thought, it would not be surprising that many microorganisms had acquired the ability to obtain iron from these proteins in the same way they acquired the ability to use siderophores they do not produce. We consider that the characterization of leghemoglobin-mediated iron transport in rhizobia could give additional insight into the phylogenesis of these bacteria.

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