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Evidence for polynuclear iron(III) clusters in the root nodule bacterium, *Rhizobium leguminosarum* bv. *viciae* WSM710.

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Cells of the root nodule bacterium *Rhizobium leguminosarum* bv. *viciae* WSM710 were cultured in a medium containing 20 μM ^{57}Fe. Mössbauer spectra of the cells at 5.5 and 3.7 K indicated that the major form of iron present in the cells was in the form of polynuclear iron(III) clusters. At 5.5 K the spectral component associated with these clusters was in the form of a superposition of a broad feature (large magnetic hyperfine field distribution) and a doublet. On lowering the temperature of the cells to 3.7 K, the spectral component was transformed into resolved magnetic hyperfine field splitting which yielded a magnetic hyperfine field of 42.4 T when fitted with broad Lorentzian peaks. These spectral characteristics are typical of the hydrated iron(III) phosphate cores of several bacterioferritins. A small fraction (11%) of the Mössbauer spectral area of the cells was in the form of a doublet which yielded parameters (δ = 1.35 mm/s; ΔE_Q = 3.15 mm/s) indicative of iron(II). The parameters are very similar to those of a spectral component previously observed in several other microbes (R. Böhnke and B.F. Matzanke (1995) *BioMetals* 8, 223–230) and which has been associated with a 2.2 kDa oligomeric iron(II) carbohydrate phosphate.

Keywords: Mössbauer spectroscopy; iron; root nodule bacteria

Introduction

The symbiosis in legume root nodules between root nodule bacteria and legume cells is a major contributor to the biological fixation of atmospheric dinitrogen (Dilworth & Glenn, 1991). Iron is a critical element in this relationship because it is a component of key proteins such as nitrogenase, leghemoglobin, and hydrogenase.

Iron deficiency in legumes occurs mainly on highly leached or alkaline calcareous soils. For the latter, iron availability may be extremely poor despite the presence of iron in the soil. Such iron deficiency is a worldwide problem for crop production, including legumes. Poor nodulation caused by iron deficiency affects such common agricultural crops as chick pea (Rai *et al.*, 1982), French bean (Hemantaranjan & Garg, 1986) and peanut (O'Hara *et al.*, 1988). The primary effect of the iron deficiency may be a failure of nodule initiation, as with lupins (Tang *et al.*, 1991), or of nodule development after nodule initiation, as in peanuts (O'Hara *et al.*, 1988).

The iron nutrition of root nodule bacteria has been little studied but is clearly of agricultural importance. These bacteria have evolved mechanisms and/or strategies to enable them to survive in soils which are iron-deficient for the growth of their legume hosts (O'Hara *et al.*, 1988). One mechanism allowing root nodule bacteria to take up iron(III) from low concentrations in the environment is siderophore production and a range of types of siderophore (carboxylates, catechols, hydroxamates, and others) has been reported for these bacteria (Guerinot *et al.*,

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1990; Rioux *et al.*, 1986; Modi *et al.*, 1985; Nambiar & Sivaramakrishnan, 1987; Patel *et al.*, 1988; Jadhav *et al.*, 1994; Roy *et al.*, 1994; Persmark *et al.*, 1993; Carson *et al.*, 1992a; Leseur *et al.*, 1993). For example, *Rhizobium leguminosarum* biovar *viciae* (hereafter called *R. leguminosarum*) WSM710 produces a trihydroxamate siderophore (vicibactin) (Dilworth *et al.*, 1998) under low iron conditions. Other studies of this organism (Carson *et al.*, 1992b, 1994) have shown cellular iron concentrations in cells grown with limiting and luxury levels of iron increasing from 170 to 250 μg $Fe.g^{-1}$ dry weight. Growth experiments switching cells from low to high iron, or *vice versa*, suggest two pools of intracellular iron (Gill & Neilands, 1989; Carson *et al.*, 1992b). One pool appears to comprise immediately available iron, controlling siderophore production, while the other comprises much less rapidly available iron and possibly acts as an iron store. This putative iron store could possibly involve a bacterioferritin. Bacterioferritins have been reported in a number of bacteria (Andrews, 1998) and their mineral cores are generally composed of a nanoscale particle of noncrystalline hydrated iron(III) phosphate.

This study aimed therefore to investigate what chemical forms of iron occur in cells of *R. leguminosarum* WSM710 grown at high iron concentration through the use of ^{57}Fe Mössbauer spectroscopy of whole cells. The results suggest that most of the intracellular iron is iron(III) in the form of polynuclear clusters with a bacterioferritin-like mineral structure while a smaller fraction of the iron is in the iron(II) form.

Methods

All glassware was soaked in 16% (w/v) aqueous HCl and rinsed in deionised water from a reverse osmosis desalinator (Osmotron, Australia). The same deionised water was used in all growth media.

Cells of *R. leguminosarum* WSM710, grown in the minimal salts medium (MSM) of Brown and Dilworth (1975) containing 0.3 mM phosphate, 50 μM EDTA, 20 μM Fe (90% isotopically enriched in ^{57}Fe, obtained from Oakridge National Laboratory) and buffered with 20 mM HEPES (pH 6.8), were used to inoculate four 500 mL cultures of the same medium in 5 L flasks. The cells were grown with shaking at 28°C to early stationary phase ($A_{600\ nm}$ = approx 1.0) and harvested by centrifugation at 10,000 *g* and 4°C. They were washed twice at 4°C with 1 L of MSM with no added iron and the cells harvested. Approximately 1 ml of cell paste (equivalent to 500 mg dry weight of cells) was placed in a 12.5 mm diameter Perspex sample holder and quickly frozen in liquid nitrogen ready for Mössbauer spectroscopic measurements.

Mössbauer spectra were recorded in transmission geometry using a ^{57}Co in Rh source driven at constant acceleration with a double ramp waveform. The spectra were subsequently folded to eliminate the parabolic background (which results as a consequence of the varying solid angle between source and detector as the source is moved backwards and forwards) yielding a 250 data point spectrum. Cryogenic sample temperatures were obtained using a liquid helium cryostat for 5.5 K, and a pumped liquid helium cryostat for temperatures below 5.5 K. Spectra were fitted with sextets and doublets of Lorentzian absorption peaks using a sum of squares minimization routine.

Results and discussion

Iron-rich cells of *R. leguminosarum* WSM710 at 5.5 and 3.7 K yielded the Mössbauer spectra shown in Figure 1. At 5.5 K the Mössbauer spectrum consists of a broad component (A1) superimposed on at least two doublets (A2 and C). Lowering the tem-

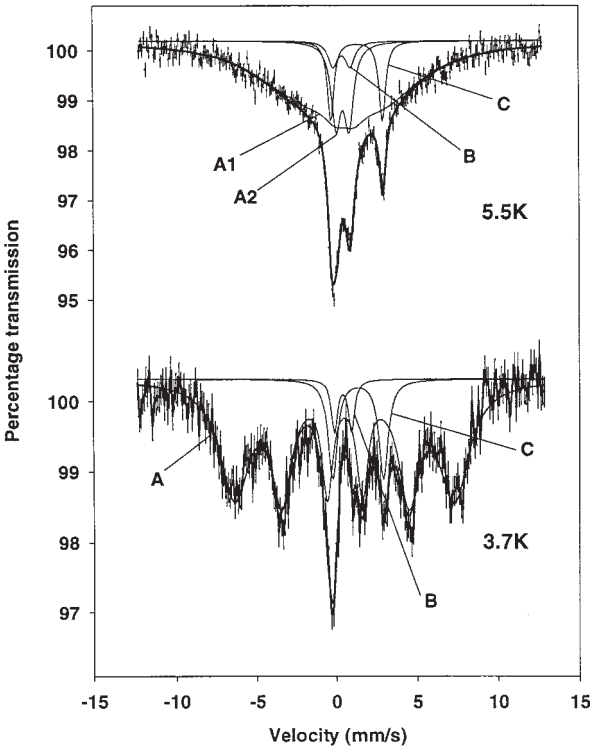

Figure 1. Mössbauer spectra of *Rhizobium leguminosarum* cells at 5.5 and 3.7 K. The vertical dashes indicate error bars of magnitude $(\sqrt{N})/N$ on each of the data points where N is the number of γ-ray counts for the data point. The continuous lines are the spectral subcomponents and their sum. The labels A, A1, A2, B, and C mark the subcomponents described in Table 1 and the text.

Table 1. Mössbauer spectral parameters for *Rhizobium leguminosarum* cells

Temp		Component A					Component B				Component C			
		δ	ΔE_Q	B_{hf}	Γ	%A	δ	ΔE_Q	Γ	%A	δ	ΔE_Q	Γ	%A
5.5K	(A2)	0.49	0.80*	-	0.70*	13	0.42**	1.06**	0.70	4**	1.35	3.19	0.48	9
	(A1)	-	-	≈23	-	74								
3.7K		0.49	–0.08	42.4	2.22	85	0.42**	1.06**	0.45	4	1.35	3.15	0.69	11

Mössbauer spectral parameters derived from fitting Lorentzian absorption peaks to the data with a sum of squares minimization routine. δ is the chemical isomer shift (mm/s), ΔE_Q is the quadrupole splitting (or the quadrupole perturbation on the magnetic hyperfine field splitting), B_{hf} is the magnetic hyperfine field splitting (T), Γ is the linewidth (mm/s) (for the outer lines in the case of a sextet), and %A is the percentage spectral area of each component. Errors are at least ±0.02 mm/s for δ, ±0.03 mm/s for ΔE_Q, ±0.5 T for B_{hf}, ±0.02 mm/s for Γ and ±1 for %A. Errors will tend to be larger for components with smaller values of %A and where significant overlapping of peaks occurs. * denotes parameters that were constrained with an upper or lower limit during the sum of squares minimization procedure while ** denotes parameters that were completely constrained.

perature of the cells to 3.7 K transforms the broad component into resolvable magnetic hyperfine field splitting. There also appears to be a decrease in the relative intensity of the central doublet (A2) while doublet (C) appears to remain unchanged by the lowering of temperature. Fitting the 5.5 K spectrum with either two (A2 and C) or three (A2, C, and B) doublets in addition to the broad component resulted in similar values for both fits for spectral parameters of doublets A2 and C. Values of these parameters derived from the fit shown in Figure 1 are given in Table 1. Fitting of a sextet of peaks to the magnetic hyperfine field splitting of the 3.7 K spectrum yielded spectral parameters that are also given in Table 1. The magnetic hyperfine field splitting of 42.4 T at 3.7 K is indicative of the presence of magnetically ordered polynuclear iron(III) with a magnetic ordering temperature in the region of 6 K. The reduction in intensity of doublet A2 on lowering the temperature indicates that it also is due to the polynuclear iron(III), being transformed to the sextet signal at the lower temperature.

The spectral characteristics of component A are typical of the hydrated iron(III) phosphate cores of bacterioferritins isolated from *Escherichia coli* (Bauminger *et al.*, 1980; Yariv *et al.*, 1981), *Pseudomonas aeruginosa* (St. Pierre *et al.*, 1986), and *Streptomyces olivaceus* (Winkler *et al.*, 1994). This is in contrast to the superparamagnetic behaviour of the hydrated iron(III) oxyhydroxide cores typically found in mammalian ferritins which yield Mössbauer spectra that consist of a coexistence of a sextet and a doublet over a large temperature range (20 to 60 K). In addition, the iron(III) oxyhydroxide cores tend to have a larger magnetic hyperfine field splitting of about 49 to 50 T below 4 K (St. Pierre *et al.*, 1989).

Doublet C has spectral characteristics typical of paramagnetic iron(II). It is interesting to note that the parameters match those measured for iron(II) Mössbauer signals in several microbial systems (Bauminger *et al.*, 1980; Matzanke *et al.*, 1992; Hudson *et al.*, 1993). More recently, this signal has been associated with a 2.2 kDa oligomeric iron(II) carbohydrate phosphate isolated from *E. coli* (Böhnke & Matzanke, 1995). This component has been detected by Mössbauer spectroscopy in a variety of microbes grown in low-iron media supplemented with siderophores (Böhnke and Matzanke, 1995; Matzanke *et al.*, 1992). It is usually accompanied by an iron(III) compound with a Mössbauer spectral centre shift of 0.42 mm/s and quadrupole splitting of 1.06 mm/s at low temperatures (Matzanke *et al.*, 1992). As such, a doublet (B) with these parameters was included in the fitting of the two Mössbauer spectra with only the area and linewidths being allowed to vary (Figure 1 and Table 1).

Since the Mössbauer spectra were measured at very low temperatures it can be assumed that the recoil-free-fraction (or Mössbauer-effect-efficiency) for each form of iron is approximately equal. Therefore the data indicate that about 86% of the total iron is bound in polynuclear clusters (possibly a bacterioferritin-like form), about 10% in a form that is possibly similar to a previously identified 2.2 kDa oligomeric iron(II) carbohydrate phosphate (Böhnke and Matzanke, 1995; Matzanke *et al.*, 1992) and about 4% in a form that is possibly similar to an iron(III) compound previously identified in other microbes (Matzanke *et al.*, 1992).

While the role of the polynuclear iron clusters in *R. leguminosarum* has yet to be elucidated, it is possible that these iron clusters are associated with the less available form of iron inferred from growth studies with *R. leguminosarum* WSM710 to be associated with long term storage (Carson *et al.*, 1992b). In addition, the iron(II) component may be associated with the immediately available pool of iron suggested by the experiments involving switching cells from low to

high iron concentration media and *vice versa* (Carson *et al.*, 1992b). Isolation and characterisation of these types of iron will be needed to further identify their structures and to indicate whether or not the iron(III) component is a bacterioferritin.

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