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Mössbauer spectroscopy and electron paramagnetic resonance studies of iron metabolites in *Pseudomonas aeruginosa*: Fe^{2+} and Fe^{3+} ferritin in ^{57}ferripyoverdine incubated cells and ^{57}ferric citrate fed cells

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***Pseudomonas aeruginosa* samples were studied using Mössbauer spectroscopy and electron paramagnetic resonance (EPR). Samples included whole cells, membranes, and soluble fractions from cells which had been grown with ^{57}ferric chloride, ^{57}ferric citrate or incubated with ^{57}ferripyoverdine. These experiments show for the first time that *P. aeruginosa* can accumulate iron in a bacterioferritin when grown under conditions of iron limitation and incubated with its cognate ferrisiderophore, ferripyoverdine. Soluble fraction from *P. aeruginosa* cells which were grown iron starved and incubated with ^{57}ferripyoverdine for 120 min showed the presence of both a ferric and ferrous complex whose Mössbauer spectra matched that of bacterioferritin extracted from *Azotobacter vinelandii* and whose EPR spectra showed a characteristic ferritin-like resonance. A second soluble fraction sample from cells which had been grown with ^{57}ferric citrate also showed the presence of a species with the same EPR and Mössbauer parameters. In addition Western blotting confirmed the presence of bacterioferritin in the soluble fraction of the cells which had been incubated with ferripyoverdine.**

Keywords: Mössbauer, EPR, ferritin, ferripyoverdine, *Pseudomonas aeruginosa*

Introduction

Pseudomonas aeruginosa is a Gram-negative aerobic bacterium present in soil. The siderophore associated with its high affinity iron transport system is pyoverdine (Myer & Abdallah 1978). In addition it can take iron from citrate constitutively, i.e. citrate can serve as a functional siderophore (Harding & Royt 1990). Previous studies on *P. aeruginosa* showed that the bacterioferritin extracted from the microorganism (Moore *et al.* 1986) is similar to *Escherichia coli* bacterioferritin both in its immunological character and in the size of its constituent subunits (Andrews *et al.* 1991). Bacterioferritin is typically purified from bacteria grown in relatively iron rich media (*E. coli*, 2 mgl^{-1}, Bauminger *et al.* 1980; *P. aeruginosa*, anerobic growth 10 mgl^{-1}, Moore 1991). These growth conditions may stimulate the cells to accumulate iron within bacterioferritin.

In the studies reported here cells were grown aerobically (Mielczarek *et al.* 1990a), with either ferric citrate, 2 mgl^{-1} (Mielczarek *et al.* 1989), or were incubated with ferripyoverdine, 1 mgl^{-1} (Mielczarek *et al.* 1990a,b). These experiments suggest, for the first time, that *P. aeruginosa* accumulates iron in a bacterioferritin when grown under conditions of iron limitation and incubated with its cognate ferrisiderophore. Under these conditions, the modality of iron transport mimics that of an iron-stressed environment. Previously, the accumulation of iron in a bacterioferritin within *P. aeruginosa* had only been demonstrated in growths at relatively high levels of non-siderophore-associated-iron in the media. Furthermore, the presence of both ferric and ferrous bacterioferritin iron is indicated in two of the samples studied. Sample I is the soluble fraction, i.e. cytoplasmic and periplasmic

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fraction, of cells incubated with ferripyoverdine for 120 min; sample II is the soluble fraction of cells grown in ferric citrate and harvested at stationary phase.

Previous Mössbauer studies of *P. aeruginosa*, at 91 K, incubated with ^{57}ferric citrate, ^{57}ferric chloride and ^{57}ferripyoverdine (Mielczarek *et al.* 1989, 1990a) revealed the presence in some samples of an iron metabolite whose Mössbauer parameters match those of bacterioferritin extracted from *Azotobacter vinelandii* (Watt *et al.* 1986), see Table 1. This paper presents the results of electron paramagnetic resonance (EPR), 4.2 K Mössbauer spectroscopy and Western blot studies on several of these *P. aeruginosa* samples.

Materials and methods

Western blotting analysis

Proteins were fractionated by polyacrylamide gel electrophoresis (PAGE) under denaturing conditions [0.1% sodium docecyl sulphate (SDS) and 15% acrylamide] (Laemmli 1970). Bacterial cell pellets corresponding to 0.25–0.5 mg of dry cell weight were suspended in 20 μl of loading buffer [0.1 M Tris–HCl, pH 6.8, 10% (v/v) glycerol, 2.3% (w/v) SDS, 5% (v/v) β-mercaptoethanol] and then heated at 100 °C for 10 min prior to SDS–PAGE. Following electrophoresis, fractionated proteins were transferred to nitrocellulose membranes using a Bio Rad Transblot Electrophetic Transfer Cell according to the manufacturers instructions. Subsequent immunodetection of bacterioferritin was as described by Blake *et al.* (1984) using as first antibody a 1 in 400 dilution of rabbit anti-*E. coli*-bacterioferritin serum and then as second antibody a 1 in 1000 dilution of goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma).

Bacterial cultures

E. coli K-12 strain W3110 was grown for 16 h in L-broth.

EPR

Figures 1 & 2 show the EPR spectra of sample I, a soluble fraction from *P. aeruginosa* grown to late log phase which had been incubated with ferripyoverdine for 120 min; and sample II, a soluble fraction from ferric citrate fed cells harvested at stationary phase. The EPR spectrum (Figure 3) of these samples shows the characteristic ferritin-like resonance (Weir *et al.* 1984, 1985). The iron-dense core of ferritin is composed of antiferromagnetically

Table 1. Mössbauer parameters for extracted bacterioferritin samples I and II

	T (K)	Isomer shift (mm s^{-1})	Quadrupole splitting (mm s^{-1})	Hyperfine field (T)
A. vinelandii[a]				
bacterioferritin	4.2	0.45		49.0
	80	0.48	0.78	
reduced bacterioferritin	80	0.47	0.66	
	80	1.28	2.88	
Sample I—soluble fraction from *P. Aeruginosa* incubated with ferric pyoverdine for 2 h[d]				
ferric component 1	91	0.35 ± 0.01	0.77 ± 0.03	
ferric component 2[c]	91	0.57 ± 0.04	0.77 ± 0.01	
ferrous component[c]	91	1.23 ± 0.06	2.81 ± 0.10	
ferric component 1	4.2	0.46 ± 0.04	1.02 ± 0.05	
ferric component 2[c]	4.2	0.53 ± 0.05		48.2
Sample II—soluble fraction from *P. Aeruginosa* grown in ferric citrate to stationary phase[b]				
ferric component 1[c]	91	0.46 ± 0.01	0.77 ± 0.08	
ferrous component[c]	91	1.23 ± 0.03	2.88 ± 0.02	
ferric component 1	4.2	0.47 ± 0.03	0.89 ± 0.04	
ferric component 2[c]	4.2	0.48 ± 0.07		46.7
ferrous component[c]	4.2	1.25 ± 0.07	2.88 ± 0.09	

[a]Watt *et al.* (1986) (errors associated with these parameters are nominally 0.05 mm s^{-1}; R. Frankel, private communication).
[b]Mielczarek *et al.* (1989).
[c]Parameters match those of bacterioferritin extracted from *A. vinelandii* to within experimental error.
[d]Mielczarek *et al.* (1990a).

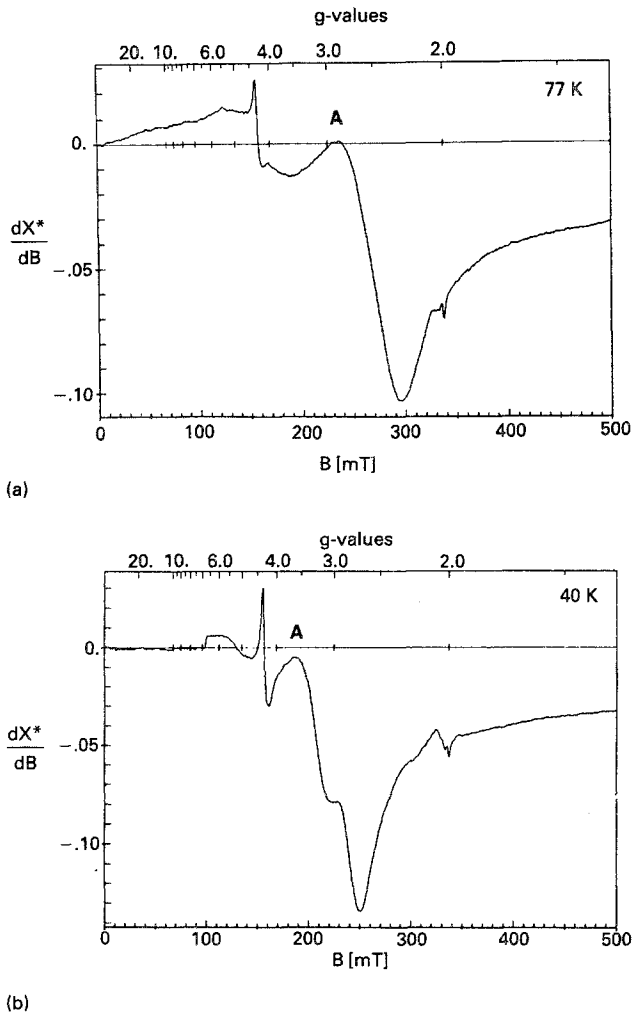

Figure 1. EPR spectra for the soluble fraction from *P. aeruginosa* which had been incubated with ^{57}ferripyoverdine for 120 min. Sample I: (**a**) 77 K; (**b**) 40 K.

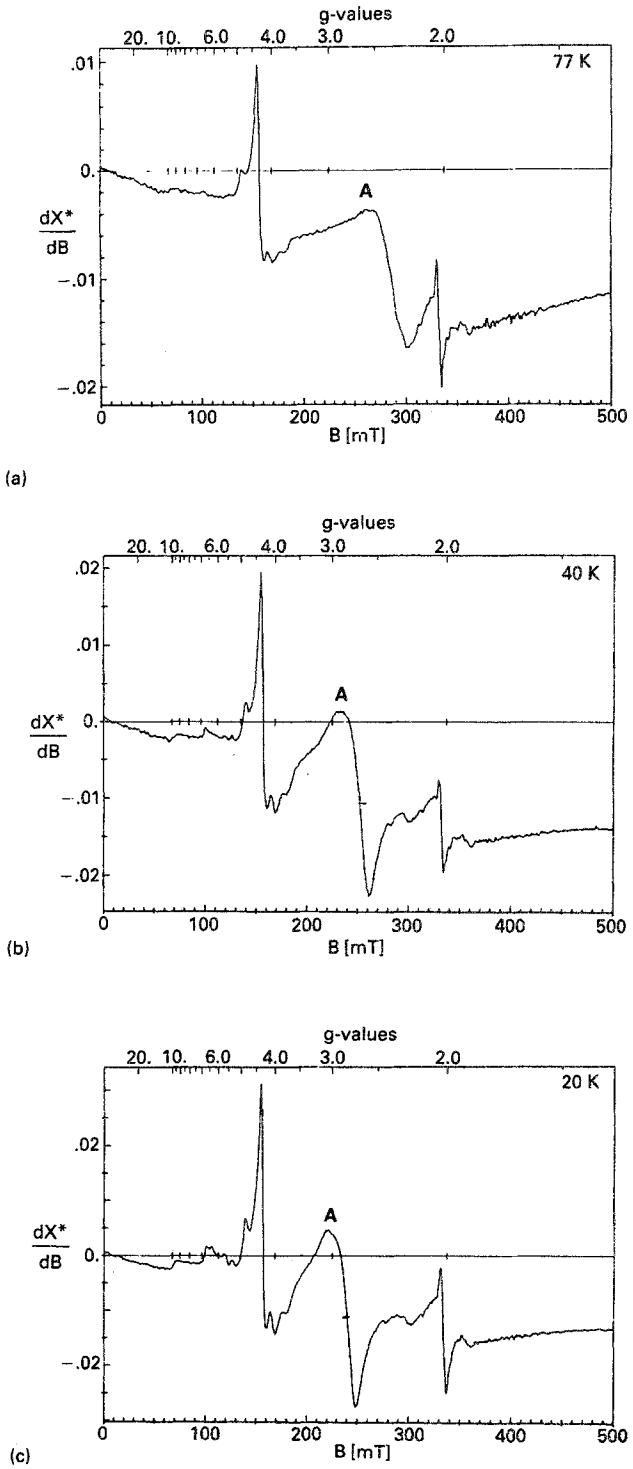

Figure 2. Electron paramagnetic resonance spectra for the soluble fraction from *P. aeruginosa* which had been grown in ^{57}ferric citrate and harvested at stationary phase. Sample II: (**a**) 77 K; (**b**) 40 K; (**c**) 20 K.

ordered magnetic particles, magnetic microcrystals, which exhibit superparamagnetism (Boas & Troup 1971, Weir *et al.* 1984, 1985, Watt *et al.* 1986, St Pierre *et al.* 1989). At low temperatures the magnetization vector of these magnetic microcrystals is frozen into a particular direction and the Mössbauer spectrum is a six line hyperfine spectrum. Analysis and experiments (Weir *et al.* 1984, 1985) showed that the EPR spectrum is the result of two overlapping broad lines which arise from contributions from superparamagnetic cores of varying densities. Such a spectrum is characteristic of a sample of superparamagnetic particles with a distribution of core sizes and/or iron protein ratio. In Figure 3 the peak labeled A is a contribution from ferritin cores which are below their blocking temperature; the dip seen at about 300 mT which is very broadened in the 295 K spectra arises from the overlapping line contributed from cores above their blocking temperature. As T decreases, more cores are below the blocking temperature and peak A becomes more prominent. The sum of these two contributions

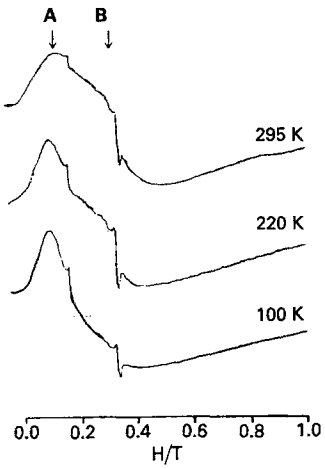

Figure 3. Temperature dependence of the ferritin EPR spectrum. Temperature given on figure. Baseline crossovers [interrupted lines occur at $H = 330$ mT (295 K), 319 mT (220 K) and 200 mT (200 K)]. Broad features (A and B) are arrowed; A increases relative to B as the temperature is lowered, accompanied by a downfield shift in the baseline crossover and greater intensity at high field (reprinted with permission from Weir *et al.* 1985).

yields a spectrum in which as the temperature is lowered the baseline crossover moves to lower fields. These features are present in the EPR spectra of samples I and II (Figures 1 & 2). In sample I, as T decreases from 77 to 40 K, the baseline crossover point moves from 270 to 210 mT. In sample II, as T decreases from 77 to 20 K the baseline crossover point moves from 280 to 240 mT. Also, the feature labeled A becomes narrower and more prominent, i.e. more easily recognizable as a peak. In contrast, EPR spectra from a third and fourth sample are shown in Figure 4. The characteristics of EPR ferritin resonance are absent from these samples.

Mössbauer spectroscopy

Additional evidence for the presence of a superparamagnetic iron storage molecule in samples I and II is provided by the 4.2 K Mössbauer spectra of these samples (Figure 5). Both of these samples show the major spectral component as being a six line hyperfine spectra. Such a six line spectra arises from iron nuclei in a magnetic field. For samples I and II the fields are measured to be 48.2 and 46.7 T. The isomer shift, center of the Mössbauer spectrum, is 0.53 ± 0.05 mm s^{-1} for sample I and 0.48 ± 0.07 mm s^{-1} for sample II. Bauminger *et al.* (1988) have measured the Mössbauer spectra of horse spleen ferritin molecules which had been loaded with varying amounts of iron. The hyperfine

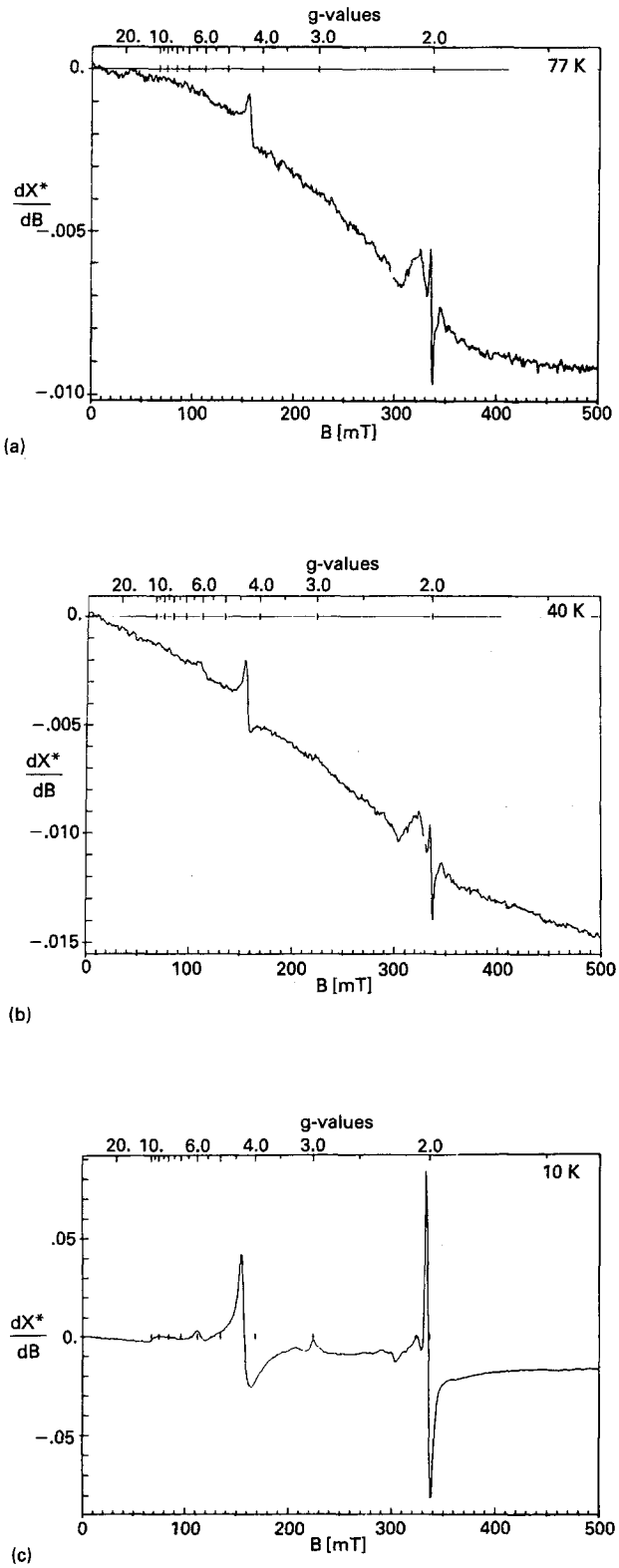

Figure 4. EPR spectra for sample III, inner membranes from *P. aeruginosa* which had been incubated with ^{57}ferripyoverdine for 360 min: **(a)** 77 K and **(b)** 40 K, and sample IV, inner membranes harvested from stationary phase *P. aeruginosa* which had been fed ^{57}ferric chloride: **(c)** 10 K.

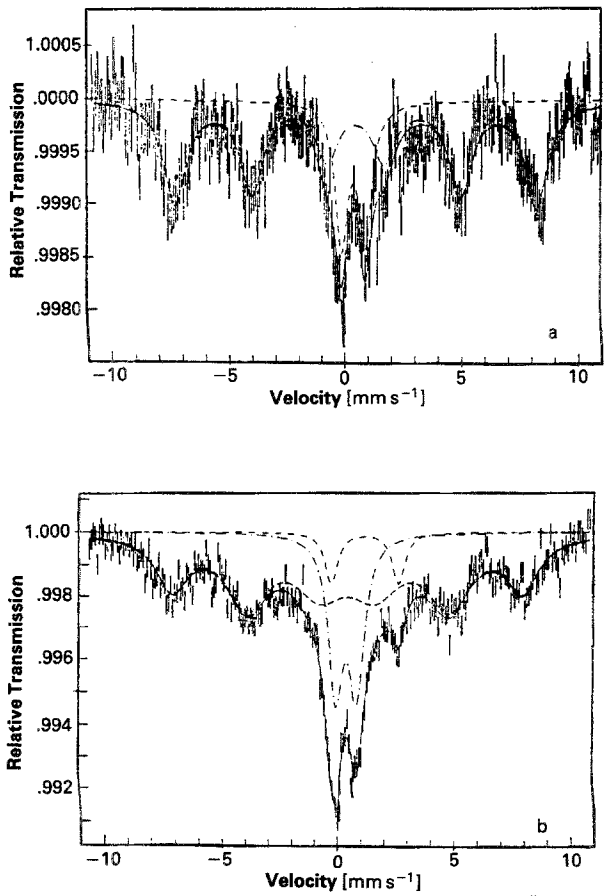

Figure 5. Mössbauer spectra at 4.2 K. (**a**) Sample I; (**b**) sample II.

spectra for those samples which contain iron clusters of 150–480 iron nuclei show an internal magnetic field of 46 T and isomer shifts of 0.50 mm s^{-1}. In addition to the iron storage species producing the hyperfine spectra, sample I shows a second ferric complex. As T increases the six line spectra should coalesce into a quadrupole doublet. At 91 K the species contributing to the Mössbauer spectra of the *P. aeruginosa* samples exhibit only a quadrupole doublet. The temperature at which the intensity of the magnetic sextet equals the intensity of the quadrupole doublet is known as the blocking temperature. It depends on the magnetic anisotropy energy, and the volume of the magnetic particle. Thus the blocking temperature of these samples occurs between 4.2 and 91 K. This transition has been found in mammalian ferritins and in some bacterioferritins to be between 20 and 38 K (St Pierre *et al.* 1989). However a ferric complex, identified as bacterioferritin (Read *et al.* 1990, Kadir *et al.* 1991) in *P. aeruginosa* cells which had been grown in high-iron, high-nitrate medium had a blocking temperature between 1.4 and 4.2 K, and cells grown in high-iron, low-nitrate medium had a blocking temperature above 4.2 K.

Western blotting

Because none of these samples were large enough to allow purification of bacterioferritin, Western blotting analysis was carried out in an attempt to detect the presence of bacterioferritin immunologically (see Materials and methods).

Western blotting confirmed the presence of bacterioferritin in sample I (unfortunately sample II was destroyed in transit). Whole cell extracts of *P. aeruginosa* and *E. coli* were compared and both were found to contain an immuno-reactive component (M_r 18 000–18 500) corresponding to the bacterioferritin (Figure 6). However, this band was not observed in control Western blots developed with an immunostain that included a pre-immune serum in place of the anti-*E. coli*-bacterioferritin serum. In addition to confirming the presence of bacterioferritin these results also confirm previous observations that *P. aeruginosa* contains a bacterioferritin (Moore *et al.* 1986) which is similar to *E. coli* bacterioferritin both in its immunological character and in the size of its constituent subunits (Andrews *et al.* 1991).

Summarizing the experimental evidence: (i) The Mössbauer spectra of these samples shows the presence of an iron complex with bacterioferritin-like parameters. Table 1 reports the parameters for both 4.2 and 91 K Mössbauer spectra. For sample I at 91 K the parameters of the ferric species labeled component 2 and the parameters of the ferrous species match those of *A. vinelandii* bacterioferritin to within experimental error. At 4.2 K one of these ferric species became magnetically ordered. The most likely possibility is that this is component 2, because its 91 K spectral parameters are a better match to *A. vinlandii* bacterioferritin parameters. Only 10% of the iron in this sample is in the ferrous form and its signal at 4.2 K cannot be resolved from the hyperfine spectra. In sample II only one ferric species can be resolved at 91 K. This species and the ferrous species both have parameters which agree with those of *A. vinelandii* bacterioferritin. [The ferrous component in these samples might also be assigned to a non-ferritin species found by Matazanke (1989). However this non-ferritin metabolite is prominent in cells grown under low ferric citrate conditions, 100 μM, in samples with no other corroborative evidence for bacterioferritin.] At 4.2 K some of this ferric iron, component 1, is magnetically ordered. (ii) the EPR spectra show that the

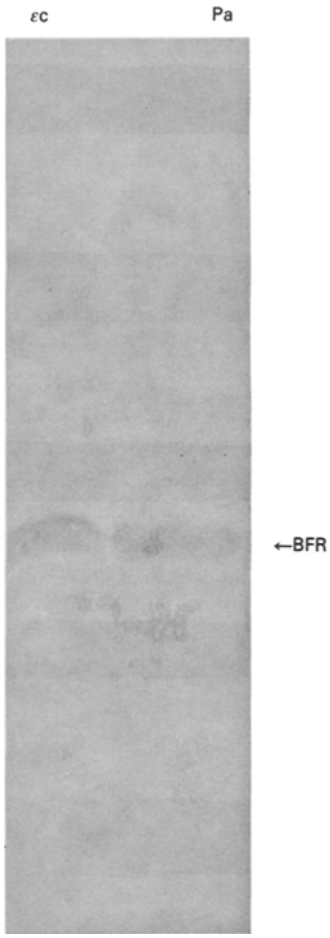

Figure 6. Western blotting analysis of total cell proteins of *P. aeruginosa* (Pa) and *E. coli* (εc). The position of the immunopositive band corresponding to the BFR subunit is indicated.

complex is antiferromagnetically ordered with a spectrum characteristic of that of ferritin. (iii) Western blotting identified the presence of bacterioferritin in sample I. One can conclude from these experiments that not only is bacterioferritin present in samples I and II but that both ferrous and ferric valence states are present in both these soluble fractions.

The other samples studied with EPR and Mössbauer spectroscopy included whole cells, membranes and soluble fractions from cells which had also been incubated with ferric chloride, ferric citrate or ferripyoverdine. Only the two soluble fraction samples discussed in this paper showed both strong EPR ferritin-like lines and Mössbauer spectra which matched the parameters of both Fe^{2+} and Fe^{3+} bacterioferritin which had been extracted from bacteria (Watt *et al.* 1986). In the ferripyoverdine studies, four other samples (inner membranes from cells which had been incubated with ferripyoverdine for 120 min, inner membranes from cells which had been incubated with ferripyoverdine for 40 min, soluble fraction from cells which had been incubated with ferripyoverdine for 60 min, and soluble fraction from cells which had been incubated with ferripyoverdine for 360 min) showed only weak antiferromagnetic resonance EPR lines. In addition none of these samples showed Mössbauer parameters indicative of both a ferrous bacterioferritin-like component and a ferric bacterioferritin-like component.

Because well defined bacterioferritin species were found in only two of these samples these studies strengthen the conclusion reached by previous measurements (Mielczarek *et al.* 1990a, Matazanke *et al.* 1989): cellular processing of iron directly following iron uptake depends not only on the source of extracellular iron but also on the availability of iron.

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