Ferric-reductase activities in whole cells and cell fractions of *Vibrio (Listonella) anguillarum*

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The ability of *Vibrio (Listonella) anguillarum* strains from serotype groups O1 and O2 to reduce Fe^{3+} in the form of different chelates was investigated. All strains, grown in M9 minimal medium supplemented with 0.2% Casamino acids, reduced Fe^{3+} complexed by citrate, nitrilotriacetic acid and EDTA. In whole cells, the degree of reduction was dependent on the Fe^{3+} ligand and on the strain, with the greatest values corresponding to ferric dicitrate and serotype group O1 strains, respectively. The ferric-reductase activity increased, over the basal levels, when the cells were grown with iron added as ferric dicitrate, haemin or haemoglobin. All strains also reduced ferricyanide, a compound that is not transported into the bacterial cells. Ferricyanide reduction was also increased when the cells were grown in the presence of an iron source. All of the cell fractions (periplasm, membranes and cytoplasm) showed Fe^{3+} -reducing activity, with the highest values observed in the presence of Mg^{2+} , NADH and FAD in the assay buffer. Cytoplasmic ferric-reductase could be visualized using native polyacrylamide or starch gel electrophoresis, whereas the periplasmic and membrane reductase(s) could only be detected on starch gels. The results indicate the presence of different ferric-reductase activities in *V. anguillarum*, which could be involved in the different iron-acquisition systems present in this micro-organism, i.e. siderophore-mediated systems and siderophore-independent mechanisms.

Keywords: *Vibrio anguillarum*, ferric-reductases, ferric dicitrate, iron uptake

INTRODUCTION

Iron is a basic mineral nutrient for all micro-organisms. However, the ferric form (Fe^{3+}), which is the main source in aerobic environments, is highly insoluble at physiological pH values (Neilands *et al.*, 1987). Thus, micro-organisms have developed different mechanisms to acquire sufficient iron for growth. One mechanism is the secretion of high-affinity, low-molecular-mass compounds known as siderophores, which are internalized through cell surface protein receptors (Crosa, 1989). Once inside the cell, iron must be released from the ferrisiderophore, and this is usually accomplished by means of a ferric-reductase. Ferrisiderophore reductase activities have been reported in several micro-organisms, and in some cases the enzyme has been isolated (Cox, 1980; Moody & Dailey, 1983; Hallé & Meyer, 1989).

Other micro-organisms do not appear to secrete siderophores, e.g. *Saccharomyces cerevisiae*. In this organism, a

membrane ferric-reductase catalyses the transfer of electrons from cytosolic NAD(P)H to extracellular ferric chelates. The soluble Fe^{2+} produced is then transported into the cell (Lesuisse *et al.*, 1987, 1991). Reductase activities in membranes have also been described in other micro-organisms, such as *Spirillum itersonii* (Dailey & Lascelles, 1977), *Staphylococcus aureus* (Lascelles & Burke, 1978) and *Escherichia coli* (Fischer *et al.*, 1990). Thus, in several micro-organisms ferric-reductases could be important to reduce siderophore-bound iron or to acquire it from soluble ferric complexes, including ferric citrate (Johnson *et al.*, 1991).

In *Vibrio (Listonella) anguillarum*, one of the most important marine fish pathogens, two different mechanisms of iron acquisition mediated by siderophores have been described in pathogenic strains belonging to serotype groups O1 and O2 (Lemos *et al.*, 1988; Crosa, 1989). The utilization of haem-containing compounds as iron sources has also been reported in this micro-organism (Mazoy & Lemos, 1991, 1996). The involvement of reductases in the iron-uptake process by these different mechanisms is thus

Abbreviation: NTA, nitrilotriacetic acid.

far unknown. Our goal in the present work was to analyse the presence of ferric-reductase activities in whole cells and different cellular fractions from *V. anguillarum* strains belonging to the main pathogenic serotype groups O1 and O2.

METHODS

Bacterial strains and culture conditions. *V. anguillarum* strains 775(pJM1) and H775-3 (plasmidless derivative of 775) of serotype group O1, and strains RV22 (serotype group O2) and NG-6 (siderophore-deficient derivative of RV22) have been described previously (Lemos *et al.*, 1988; Conchas, 1994). Cells were grown aerobically at 25 °C with shaking at 170 r.p.m. in flasks containing M9 minimal medium (Miller, 1972) supplemented with 0.2% Casamino acids (Difco; CM9) or in CM9 with 5 µM iron added as ferric dicitrate, haemin or haemoglobin. Cells were harvested by centrifugation at the stationary phase of growth (about 10⁹ c.f.u.), washed, resuspended in the same medium, and assayed for ferric-reductase activities.

Preparation of cell fractions. The method described by Angerer *et al.* (1992) was slightly modified and used to release the periplasm fraction and produce spheroplasts. Bacterial cells grown in CM9 (with or without iron added as ferric dicitrate, haemin or haemoglobin at final concentrations of 5 µM) were resuspended in 0.19 M Tris, 0.21 M sucrose and 0.5 mM EDTA (pH 8.0) with lysozyme at 0.5 mg ml⁻¹. The cell suspension was incubated on ice for 5 min and MgSO₄ was added to a final concentration of 0.02 M. The cell suspension was then centrifuged at 10000 *g* for 30 min at 4 °C. The supernatant contained the periplasm and the pellet contained the spheroplasts.

The cytoplasmic and membrane fractions were isolated by disruption of these spheroplasts in 0.19 M Tris, 0.21 M sucrose, 0.5 mM EDTA and 0.02 M MgSO₄ (pH 8.0) (buffer A) by sonication on ice. The sonicated suspension was centrifuged at 10000 *g* at 4 °C for 5 min to remove whole cells and cellular debris. The supernatant was then centrifuged at 80000 *g* for 60 min to obtain the membrane (pellet) and cytoplasmic (supernatant) fractions. Membrane fractions were washed twice in the same buffer and centrifuged again. All fractions were used immediately after preparation.

Assay of ferric-reductase activities in cell fractions. The reduction of Fe³⁺ was assayed with ferrozine [3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine] (Sigma) as Fe²⁺-trapping reagent (Dailey & Lascelles, 1977). Ferrozine is water soluble and does not react with Fe³⁺. The ferrous complex of ferrozine has an absorbance maximum at 562 nm and a molar extinction coefficient of 27900 (Stookey, 1970).

The reaction mixture contained (in a final volume of 2 ml): 1 ml cell extract, 12.5 µM iron as ferric dicitrate, ferric-nitritotriacetic acid (ferric-NTA), ferric-EDTA or ferric chloride; 250 µM ferrozine; 250 µM NAD, reduced form (NADH; Sigma). In some experiments, FAD (Sigma) was added to a final concentration of 50 µM. The reference cuvettes contained all of the reagents except iron. The assay was performed at 24 °C in polystyrene cuvettes in order to minimize non-enzymic reduction (Cox, 1980) and was monitored at 562 nm in a Hitachi U-2000 double-beam spectrophotometer.

Measurements of enzymic stability were made by heating each cell extract at 95 °C. After incubation for 15 min, the extracts were cooled in an ice-bath, equilibrated to 24 °C, and then tested for ferric-reductase activities. Specific activities in each cell fraction were calculated as the amount of product formed (mg protein)⁻¹ min⁻¹. The results represent the means of four experiments performed in duplicate.

In some experiments, the following inhibitors were added as 100 × solutions: 20 mM NaN₃ (Sigma); 10 mM 2,4 dinitrophenol (DNP; Sigma). These inhibitors were also used to treat growing cultures of bacteria.

Ferric-reductase activities of whole cells. Ferric-reductase activities of whole cells, grown, washed and resuspended in CM9, were assayed spectrophotometrically with ferrozine (final concentration of 250 µM) and iron (12.5 µM) as ferric dicitrate, ferric-NTA, ferric-EDTA or ferric chloride. Since ferrozine is sulfonated, it would not be expected to readily traverse the cell membrane and only reduction of external Fe³⁺ should be visualized (Evans *et al.*, 1986). Ferric-reductase activities were calculated as the amount of product formed (mg wet cell weight)⁻¹ min⁻¹. The results represent the means of four experiments performed in duplicate.

Ferricyanide reduction, transport and excretion of soluble reducing reagents were measured by following the decrease in *A*₄₂₀ at room temperature (24 °C) using the methods described by Ecker & Emery (1983), without adjusting pH in transport studies.

Protein determination. Protein concentrations were determined with the Bio-Rad protein assay kit, based on the method of Bradford (1976), with bovine serum albumin as standard.

PAGE. Non-denaturing discontinuous PAGE was performed by following the protocol of Laemmli (1970) without SDS in the gels and buffers and using 12.5% (w/v) acrylamide in the separating gel and 3.5% (w/v) in the stacking gel. The cytoplasmic fraction (in buffer without MgSO₄, plus 10 µg PMSF ml⁻¹ and 0.2 mg DNase II ml⁻¹) (20 µl) was diluted with 20 µl sample buffer containing 10 mM Tris/HCl (pH 7.5), 30% (v/v) glycerol and 0.01% bromophenol blue as the tracking dye. The sample (30 µl) containing 90–100 µg protein was applied to the gel. The gel was run at 32 mA (stacking gel) and 37 mA (separating gel) for 4 h at 4 °C. After electrophoresis, the gel was removed from the apparatus and ferric-reductase activities were detected, at 37 °C with shaking, by adding 100 ml buffer A containing 0.45 mM NADH, 2 mM ferrozine and 250 µM iron as ferric dicitrate.

Starch gel electrophoresis. Starch gels were prepared using 11% (w/v) starch in 50 mM Tris, 385 mM glycine. Each cell fraction (20 µl) (in buffer A, without MgSO₄, plus 10 µg PMSF ml⁻¹) was diluted with 20 µl sample buffer containing 10 mM Tris/HCl (pH 7.5) and 30% (v/v) glycerol, and applied to the gel using a piece of Whatman no. 3 paper. The gels were run at 20 mA for 30 min. The paper was then removed and the electrophoresis was continued for 6 h. Ferric-reductase activities were detected as described above for native polyacrylamide gels.

Materials. Ferric chelates (ferric-NTA and ferric-EDTA) were prepared by mixing NTA or EDTA with equimolar concentrations of FeCl₃ in deionized water. Ferric dicitrate was prepared by mixing sodium citrate with ferric chloride (200:1) in deionized water.

RESULTS

Fe³⁺ reduction by whole cells

Several *V. anguillarum* strains belonging to serotype groups O1 and O2 were assayed for their ferric-reductase activity when grown in iron-deficient and iron-rich conditions using ferrozine as Fe²⁺-trapping reagent (Table 1).

All strains reduced Fe³⁺ in different chelates, with a greater activity on ferric dicitrate in strains belonging to

Table 1. Specific ferric-reductase activities in whole-cell preparations (cell density about 10^9 c.f.u.) of *V. anguillarum* strains grown in CM9 without or with iron added as 5 μ M ferric dicitrate

Specific activities are expressed as nmol Fe^{2+} -ferrozine formed min^{-1} (mg wet wt) $^{-1}$. The results are the means of four experiments in which each sample was determined in duplicate. All standard deviations were within 10% of the mean.

Strains	Reduction of iron compounds							
	Cells grown without iron				Cells grown with iron			
	Fe^{3+} dicitrate	FeCl_3	Fe^{3+} -NTA	Fe^{3+} -EDTA	Fe^{3+} dicitrate	FeCl_3	Fe^{3+} -NTA	Fe^{3+} -EDTA
775	0.17	0.03	0.06	0.05	0.55	0.08	0.21	0.14
H775-3	0.16	0.05	0.07	0.05	0.51	0.17	0.18	0.15
RV22	0.06	0.03	0.04	0.12	0.08	0.03	0.06	0.16
NG-6	0.02	0.01	0.01	0.06	0.04	0.02	0.03	0.09

Fig. 1. Ferric dicitrate reduction by *V. anguillarum* strain H775-3 grown in CM9 medium with 5 μ M (as iron) ferric dicitrate. ■, Reduction at 24 °C; ●, reduction at 4 °C; ○, reduction in the presence of sodium azide (20 mM); □, reduction in the presence of DNP (10 mM). Points represent the means of four experiments carried out in duplicate. Vertical bars indicate standard deviations for each point.

Fig. 2. Reduction of ferricyanide (1.3 mM) by cells of *V. anguillarum* strain H775-3 grown in CM9 without (●) and with (○) 5 μ M (as iron) ferric dicitrate. Points represent the means of four experiments carried out in duplicate. Vertical bars indicate standard deviations for each point.

serotype group O1 and on ferric-EDTA in O2 strains. No spontaneous reduction of Fe^{3+} was observed in cell-free controls or when the bacterial cells were incubated with ferrozine alone without any added iron chelate. Strains from serotype group O1 showed a greater ferric-reductase activity than O2 strains, except on ferric-EDTA.

The ferric-reductase activity after growth in CM9 containing iron (5 μ M) as ferric dicitrate, ferric chloride, haemin or haemoglobin in cells from both serotypes was greater than in cells grown in CM9 alone. Thus, the presence of a source of iron in the growth medium increases the basal ferric-reductase activity of the cells.

The rate of reduction was strongly inhibited at 4 °C and in the presence of the respiratory inhibitor sodium azide (20 mM) or the uncoupler DNP (10 mM), as shown in

Fig. 1 for the strain H775-3. Identical results were obtained with strains 775, RV22 and NG-6.

The possibility that cells could reduce substrates that are not transported was tested by the method of Ecker & Emery (1983), using ferricyanide (final concentration of 1.3 mM) added to a culture of cells previously washed and resuspended in CM9. Strains from both serotypes reduced ferricyanide, with fivefold greater rates in group O1 strains than in serotype group O2 strains. Since ferricyanide is not able to penetrate cellular membranes, it is likely that this reductase capacity was also responsible for reduction of Fe^{3+} chelates (Ecker & Emery, 1983). Ferricyanide reduction was also increased, over the basal reductase activity, when the cells were cultured in CM9 medium with iron added as ferric dicitrate, haemin or haemoglobin, as is shown in Fig. 2 for strain H775-3.

Fig. 3. Reduction of ferricyanide (1.3 mM) (○) and Fe^{3+} (12.5 μM) as ferric dicitrate (●) by cells of *V. anguillarum* strain H775-3 grown in CM9 with 5 μM (as iron) ferric dicitrate. Points represent the means of four experiments carried out in duplicate. Vertical bars indicate standard deviations for each point.

All strains tested reduced ferricyanide, although it appears to remain extracellular, since 97–99% of the ferricyanide colour was recovered in the medium supernatant using the method of Ecker & Emery (1983). The possible excretion to the growth medium of a soluble compound with ferricyanide reductase activity was tested using sterile supernatants obtained from cultures of strains from both serotypes. No decrease in the A_{420} (which indicates ferricyanide reduction) was observed over a period of 60 min in any strain. Thus, the reduction of this compound is associated with the cell surface.

When ferricyanide and ferric dicitrate were added sim-

ultaneously to the bacterial cells resuspended in CM9, ferricyanide was reduced first followed by the reduction of ferric dicitrate, as shown in Fig. 3 for strain H775-3.

Fe^{3+} reduction in cell fractions

Cell-free extracts of *V. anguillarum* H775-3 grown aerobically in CM9 showed high rates of Fe^{3+} reduction, with higher values in the periplasmic and membrane fractions (Table 2). No spontaneous reduction of Fe^{3+} was observed in the absence of cell extracts.

The activities were assayed aerobically in crude extracts without addition of reductant and with NADH as potential electron donor for the enzyme. This compound was an effective cofactor for Fe^{3+} reduction, stimulating a 10–15-fold increase in reduction rates in the different fractions. The reductase activity was greatly inhibited (about 85%) when Mg^{2+} was omitted from the assay buffer. NADPH was as effective as NADH in donating electrons to the reductase.

Ferric-reductase activities were completely destroyed in all fractions when the preparation was heated at 95 °C for 15 min prior to the assay. The presence of iron in the growth medium did not have a clear effect on the ferric-reductase activity in periplasmic and membrane fractions. However, there was an increase in reduction rate in the cytoplasm of cells grown in CM9 without added iron (Table 2). Addition of FAD to the assay buffer increased the ferric-reductase activities fivefold in the periplasm and cytoplasm and 15-fold in the membrane fractions. However, addition of sodium azide or DNP had no effect on the reductase activity of the membrane fractions.

Similar ferric-reductase activities were obtained with strain 775, whereas in strain RV22 (serotype group O2) the reduction rates assayed using ferric dicitrate as iron source were lower: 1/3 lower in the cytoplasm and 1/6 lower in the membranes and periplasm.

Table 2. Specific activities of ferric-reductases in cell fractions from H775-3 grown in CM9 without (– Fe) or with (+ Fe) added iron, in a Mg^{2+} -containing buffer and with NADH as reductant

Specific activity of ferric-reductase is expressed as nmol Fe^{2+} –ferrozine formed min^{-1} (mg protein^{-1}) $^{-1}$. The results are the means of four experiments in which each sample was determined in duplicate. All standard deviations were within 10% of the mean. NT, Not tested.

Cell fraction	Reduction of:			
	Fe^{3+} dicitrate	FeCl_3	Fe^{3+} –NTA	Fe^{3+} –EDTA
Cytoplasm				
– Fe	0.83	0.51	0.39	0.34
+ Fe	0.33	0.20	0.18	0.13
Membranes				
– Fe	1.22	1.08	0.98	NT
+ Fe	1.20	1.15	1.07	NT
Periplasm				
– Fe	1.82	1.71	1.66	NT
+ Fe	1.76	1.63	1.62	NT

Fig. 4. Native discontinuous PAGE of ferric-reductase in the cytoplasmic fraction from (a) *V. anguillarum* strains H775-3 (lanes 1 and 4) and RV22 (lanes 2 and 3) and (b) *E. coli* HB101 (lane 1, bands A, B and C) and *V. anguillarum* H775-3 (lane 2, band D) with ferric dicitrate as iron source.

Detection of ferric-reductases by native PAGE

Assays of ferric-reductase activity in native polyacrylamide gels were performed with concentrated cytoplasmic, membrane and periplasmic fractions of *V. anguillarum* strains H775-3, 775, RV22 and NG-6 and *E. coli* HB101. Reductase activity could not be visualized in polyacrylamide gels of the periplasmic and membrane fractions. However, in the cytoplasmic fraction, a purple band in a pink background began to appear in all of the strain preparations after 20 min and was completely visible after 3 h, as shown in Fig. 4(a) for H775-3 and RV22. In contrast in *E. coli* HB101, used for comparative purposes,

Fig. 5. Starch gel electrophoresis of ferric-reductases from the cytoplasm (A) and membrane/periplasm (B) fractions of *V. anguillarum* strains H775-3 (lane 1) and RV22 (lane 2) grown in CM9 with ferric dicitrate as iron source.

three bands appeared after that time (Fig. 4b). Although these bands were easily visualized by the naked eye, photographs of the gel were of poor contrast. Since H775-3 and RV22 are plasmidless strains of *V. anguillarum*, this cytoplasmic reductase activity may be chromosomally encoded.

Detection of ferric-reductases by starch gel electrophoresis

Only a single band of reductase activity was visible in starch gels of membrane fractions from strains H775-3 and RV22 grown in CM9 with and without 5 μ M (as iron) ferric dicitrate. A band with identical mobility but of poor contrast was observed in the periplasmic fraction. When the whole-cell extracts from H775-3 and RV22 were applied to a starch gel, two bands were clearly visible. The band with the greatest mobility corresponded to the cytoplasmic reductase and the band with the lowest mobility to the membrane/periplasmic reductase(s) (Fig. 5). Identical results were obtained with strains 775 and NG-6 (data not shown).

DISCUSSION

This work demonstrates that *V. anguillarum* strains from serotype groups O1 and O2 can reduce Fe^{3+} in the form of different chelates, including ferric dicitrate. This ferric-reductase activity was observed in whole cells as well as in cell fractions. In whole cells, the degree of reduction was dependent on the Fe^{3+} ligand and on the strain, with the greatest values corresponding to ferric dicitrate and serotype group O1 strains, respectively. The existence of differences in ferric-reductase activity between strains from serotype groups O1 and O2 is not surprising, since it is known that the siderophore-mediated iron-acquisition systems are different between strains of both serotypes (Lemos *et al.*, 1988). Thus, the results reported here do confirm the existence of biochemical differences in the iron metabolism of both serotypes. Ferric-reductase activity in whole cells was induced when cells were grown in the presence of iron in the form of ferric dicitrate (Table 1), which suggests a reductive mechanism for utilization of iron from ferric dicitrate. However, this induction by

iron is not observed in cellular fractions (Table 2). This could be explained by a possible alteration of the ferric-reductase(s) associated with membranes during the cell-fractionation process, in which the membrane structure is damaged. Also, an iron-inducible ferric-reductase could be detected in whole cells but not in cell fractions.

All of the strains tested could also reduce ferricyanide, a compound that is not transported into the cell but can cross the outer membrane of Gram-negative bacteria and thus can be reduced in the periplasm (Ingledew & Poole, 1984). Ferricyanide reduction has also been described for *Ustilago sphaerogena* (Ecker & Emery, 1983) and *S. cerevisiae* (Crane *et al.*, 1982). Ferricyanide reduction was also increased when cells were grown in the presence of added iron, with the fastest reaction rates found in strains belonging to serotype group O1, confirming the existence of differences in ferric-reductase activities between the two serotypes.

Ferric-reductase activity was detected in all of the cell fractions analysed, irrespective of the iron concentration of the growth medium, suggesting that ferric-reductase activity could be constitutive. However, in the cytoplasmic fraction some stimulation of activity (2.2–2.6-fold) occurred when the cells were grown in low-iron medium (Table 2). This could be explained if the cytoplasmic reductase was involved in the release of iron from ferrisiderophores. Since many of the components of the iron-uptake mechanism mediated by siderophores in *V. anguillarum* are regulated by iron (Crosa, 1989; Tolmasky & Crosa, 1991), the cytoplasmic ferrisiderophore reductase could also be inhibited by the presence of Fe^{3+} . Membrane and periplasmic reductases would not be involved in siderophore-based iron-uptake mechanisms, as they are not regulated by iron. In most micro-organisms, the ferric-reductase activity is not regulated by iron (Fontecave *et al.*, 1994), although in *Neurospora crassa* it is inducible under iron deficiency (Ernst & Winkelman, 1977).

The presence of ferric-reductases in different cell fractions is not unusual, although in most micro-organisms the ferric-reductase activity is associated only with the cytoplasm (Fontecave *et al.*, 1994). In *Pseudomonas aeruginosa*, a ferripyochelin reductase was detected mainly in the cytoplasm, although it was also detected in the periplasmic and membrane fractions (Cox, 1980); in *Legionella pneumophila* (Johnson *et al.*, 1991), Fe^{3+} reduction was also detected in all of the cell fractions. The membrane-associated ferric-reductase activity must be located mainly in the inner membrane, since our data (unpublished observations) indicate that ferric-reductase activity is not detectable in the outer membrane of *V. anguillarum* grown under aerobic conditions. Similar observations have been reported previously by Myers & Myers (1992, 1993) for *Shewanella putrefaciens*, where ferric-reductases, which are part of the anaerobic respiratory chain, are detected in the outer membrane of cells grown under anaerobic, but never under aerobic, conditions.

All assays with cell fractions were performed under aerobic conditions, although it is known that in many

micro-organisms ferric-reductase activities can only be detected under strict anaerobiosis (Myers & Myers, 1993; Fontecave *et al.*, 1994). However, in *P. aeruginosa* the cytoplasmic ferric citrate reductase is active in the presence of O_2 (Cox, 1980). This also seems to be true for reductases located in cell fractions of *L. pneumophila* (Poch & Johnson, 1993) and a *Rhizobium* sp. (Roy *et al.*, 1994). Moreover, in this study we found that the reductase activities (with ferric dicitrate as iron source) were maximal when the assay buffer contained NADH, Mg^{2+} and FAD^+ . This is not surprising, since in many other micro-organisms the presence of a free flavin (FMN or FAD) has been shown to actively stimulate the enzymic reduction by NADH (or NADPH) of many ferric ligands (Fontecave *et al.*, 1994).

Neither DNP nor sodium azide inhibited the enzymic activity in the cell fractions assayed. However, Fe^{3+} reduction by whole cells was strongly decreased in the presence of these compounds (Fig. 1). These results could be explained if both compounds were energetic inhibitors of Fe^{3+} transport across the outer membrane to the periplasm/membrane reductases. In fact, the role of DNP as a protonophore is well known, collapsing the cytoplasmic membrane electrochemical potential and thus preventing active transport across the outer membrane (Jaskula *et al.*, 1994).

As discussed above, some of the ferric-reductase activities observed in *V. anguillarum* could be involved in the mechanism of iron release from ferrisiderophores in the cytoplasm, as occurs in other micro-organisms (Emery, 1987). However, this reductive mechanism could also be used by *V. anguillarum* to obtain iron from other ligands (including ferric dicitrate) by a siderophore-independent mechanism. In that case, the function of the reductase would be to reduce external Fe^{3+} , followed by Fe^{2+} transport to the cytoplasm by means of a transmembrane Fe^{2+} -transport system, as has been proposed for *Streptococcus mutans* (Evans *et al.*, 1986), *S. cerevisiae* (Lesuisse *et al.*, 1987, 1991) and *Listeria monocytogenes* (Adams *et al.*, 1990). In fact, our data (unpublished observations) indicate that although *V. anguillarum* cells can grow with ferric dicitrate as the only iron source, and that iron from ferric dicitrate is actually transported into the cell, both activities are strongly inhibited in the presence of 5 mM ferrozine (a strong Fe^{2+} -trapping reagent) (70% inhibition) or 10 mM ferricyanide (a competitive inhibitor) (90% inhibition). However, this growth reduction is not appreciable when a different iron source, such as haemoglobin, is used. These observations would suggest that an external reduction step would be necessary for utilization of Fe^{3+} from ferric dicitrate complexes.

Since *V. anguillarum* can survive for long periods in sea water (Muroga *et al.*, 1986; Hoff, 1989), Fe^{3+} reduction from different iron chelates could constitute an additional mechanism to obtain iron from the marine environment, where the siderophore-mediated systems are probably not very effective.

In conclusion, *V. anguillarum* possesses intrinsic ferric-reductase activities that can be detected in whole cells as

well as in different cell fractions. These could be involved in the different systems of iron uptake by the cell, although other roles in cellular biochemistry cannot be ruled out. Further work is under way to elucidate the precise role of the reductase(s) in iron acquisition by this micro-organism.

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