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Ferric reductases of *Legionella pneumophila*

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Ferric reductase enzymes requiring a reductant for maximal activity were purified from the cytoplasmic and periplasmic fractions of avirulent and virulent *Legionella pneumophila*. The cytoplasmic and periplasmic enzymes are inhibited by zinc sulfate, constitutive and active under aerobic or anaerobic conditions. However, the periplasmic and cytoplasmic reductases are two distinct enzymes as shown by their molecular weights, specific activities, reductant specificities and other characteristics. The molecular weights of the cytoplasmic and periplasmic ferric reductases are approximately 38 and 25 kDa, respectively. The periplasmic reductase (K_m = 7.0 μM) has a greater specific activity and twice the affinity for ferric citrate as the cytoplasmic enzyme (K_m = 15.3 μM). Glutathione serves as the optimum reductant for the periplasmic reductase, but is inactive for the cytoplasmic enzyme. In contrast, NADPH is the optimum reductant for the cytoplasmic enzyme. Ferric reductases of avirulent cells show a 2-fold increase in their activities when NADPH is used as a reductant in comparison with NADH. In contrast, ferric reductases from virulent cells demonstrated an equivalent activity with NADH or NADPH as reductants. With the exception of their response to NADPH, the ferric reductase at each respective location appears to be similar for avirulent and virulent cells.

Keywords: ferric reductase, *Legionella pneumophila*

Introduction

The acquisition of iron by microorganisms is complicated by the limited availability of iron under physiological conditions. At neutral pH, ferrous iron spontaneously oxidizes to its ferric state forming an insoluble complex with hydroxide or oxyhydroxide. In the mammalian host, iron is chelated in body fluids by iron-binding glycoproteins including transferrin and lactoferrin or intracellulary by iron-containing proteins (hemoglobin or ferritin). Most bacteria obtain iron from the hosts iron-binding proteins or by producing siderophores (Griffiths 1987). A few bacterial species are able to use transferrin and/or lactoferrin as a source for iron. Receptors for lactoferrin and transferrin have been observed in the outer membranes of *Neisseria gonorrheae* (McKenna *et al.* 1988) and *Bordetella pertussis* (Redhead *et al.* 1987). *Yersinia pestis* is able to sequester iron from heme or hemin (Perry & Brubaker 1979). However, the majority of bacterial species overcome this iron deficit by using low molecular weight iron chelators (siderophores) which possess high affinities for ferric iron (Neilands 1981). Some bacteria are able to use siderophores produced by other species as well as their own siderophores (Leong & Neilands 1976).

Legionella pneumophila, which requires a large amount of ferric iron for growth, is unable to use transferrin or lactoferrin as a source of iron (Quinn & Weinberg 1988, Bortner *et al.* 1989, Johnson *et al.* 1991). Furthermore, the iron-free form of these natural iron chelators inhibits the growth of this bacterial species. *L. pneumophila* does not produce siderophores or use siderophores produced by other species (Reeves *et al.* 1983). Therefore, it appears that *L. pneumophila* must use a system of iron uptake independent of iron chelators. Most bacterial species that produce siderophores possess a ferric reductase specific for that particular iron chelator; *Argobacterium tumefaciens* (Lodge *et al.* 1982), *Azotobacter vinelandii* (Huyer & Page 1989), *Bacillus subtilis* (Gaines *et al.* 1981) and *Mycobacterium smegmatis* (Brown & Ratledge 1975). Although some of these ferrisiderophore reductases have

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marginal activity with ferric citrate, reductases specific for ferric citrate have been identified in several bacterial species (Lefaou & Morse 1991). Our laboratory has previously reported the presence of ferric citrate reductase activity in *L. pneumophila* (Johnson *et al.* 1991). Although significant activity was demonstrated in the cytoplasmic extract, the highest specific activity was found in the periplasmic extract.

In this study we examined characteristics and properties of a cytoplasmic and periplasmic ferric reductase isolated from avirulent and virulent *L. pneumophila*. The kinetic properties and optimal conditions for activity of the cytoplasmic and periplasmic ferric reductases are described. Our results indicate that the cytoplasmic and periplasmic ferric reductases of *L. pneumophila* are distinct enzymes.

Materials and Methods

Bacteria and growth conditions

L. pneumophila serogroup 1 strain Philadelphia 2 was obtained from the Centers for Disease Control and Prevention (Atlanta, GA). An avirulent culture was produced by passaging the originally virulent culture on supplemented Mueller-Hinton agar as described by Catrenich & Johnson (1989). Both cultures were checked for purity by their inability to grow on blood agar at 37 °C after 48 h. In addition, virulence was confirmed by the growth of avirulent, but not virulent cells, on supplemented Mueller-Hinton agar. Both avirulent and virulent cultures were maintained on buffered charcoal yeast agar (BCYE; Morris *et al.* 1979). Growth under iron deficient conditions was accomplished by passaging the *L. pneumophila* cells at least three times on buffered cyclodextrin yeast agar (BCYDE) containing 13 and 3 μM iron for avirulent and virulent cells, respectively (Johnson *et al.* 1991). Bacterial cultures were harvested after growth at 37 °C for 48 h.

Preparation of cell extracts

Bacterial cells were fractionated into cytoplasmic and periplasmic extracts using a modification of the method described by Cox (1980) with the following exceptions. After the periplasmic extract was isolated, spheroplasts were resuspended in 25 ml of 10 mM Tris–HCl, pH 7.6 containing 1 mM $MgCl_2$. One milligram each of RNase and DNase was added and the suspension was incubated at room temperature for 30–60 min. The spheroplast suspension was then placed in an ice bath and sonicated with a cell disrupter (Model WW 375 Ultrasonics, Inc.). Particulate matter was removed by centrifugation at 5000 × *g* at 4 °C for 15 min. The supernatant was removed and centrifuged at 140 000 × *g* at 17 °C for 2 h to separate the membrane (pellet) and cytoplasmic (soluble) extracts. All extracts were dialyzed at 4 °C for 2 days against several changes of deionized water, lyophilized and stored at 4 °C. This procedure has been shown to produce relatively pure extracts with minimal contamination by other extracts (Cox 1980).

Reductase assay and protein determination

Ferric reductase activity was measured using a modification of the method of Dailey & Lascelles (1977). The reaction is based on the formation of a colored complex, detectable at 562 nm, from a colorless bipyridyl compound (ferrozine) and ferrous iron. The assay mixture (final volume: 2.0 ml) contained 0.4 ml of various fractions derived from cell extracts, 2 μM reductant (NADH, NADPH or reduced glutathione), 5 μM ferrozine and 100 μg of ferric citrate in 10 mM Tris–HCl (pH 7.6). The absorbance at 562 nm was measured on a Varian DMS-90 spectrophotometer at 0, 10 and 30 min against blanks without reductant. Unless otherwise specified, the assay was performed at room temperature (25 °C). Sample absorbance values were corrected for spontaneous reduction of ferric iron by the reductant itself at determined time points. Activity was defined as nmol Fe^{2+} formed per 30 min per milligram of protein, as determined using the molar extinction coefficient for ferrozine of 28 000 $M^{-1} cm^{-1}$. All final determinations represent the average of at least three experiments performed in duplicate. Glass Thunberg cuvettes were used in assays to determine the effect of anaerobic conditions (N_2) on enzymatic activity. The effect of pH on enzymatic activity was examined using 3-(*N*-morpholine)propanesulfonic acid (MOPS; pH 4.0, 5.0 and 6.0) and Tris–HCl (pH 7.0, 8.0 and 9.0). Inhibition by zinc sulfate was tested by performing the assay in the presence of concentrations of zinc sulfate ranging from 0 to 160 μM. Enzymatic activity of column fractions was measured using a microversion of the ferrozine assay (final volume: 0.20 ml) in microtiter plates. Absorbance values at 562 nm were read on a Fisher Biotech BT-100 microplate reader.

Protein concentrations were determined using the Biorad protein assay kit with bovine serum albumin as a standard.

Purification of ferric reductase from cell extracts

Unless otherwise specified, all procedures were performed at 4 °C. Resuspended extract (200 mg) was applied to a gel filtration column (28 × 1.5 cm) of Sephadex G-75 equilibrated with 100 mM Tris–HCl, pH 7.6, and eluted with the same buffer. Three milliliter fractions were collected at a flow rate of 0.5 ml min^{-1}. Each fraction was monitored for protein by measuring absorbance at 280 nm and ferric reductase activity by the ferrozine assay. The active fractions were combined, dialyzed against water and lyophilized. The lyophilized sample was resuspended in a minimal volume of 10 mM Tris–HCl, pH 9.1, and separated on an anion exchange column (46 × 1.0 cm) of DEAE A-25-120 (Sephadex) equilibrated with the same buffer. After washing with 100 ml buffer, the column was eluted with a 0.1–0.25 gradient of NaCl in 10 mM

Tris–HCl, pH 7.0. Three milliliter samples were collected at a flow rate of 0.5 ml min^{-1} at 4 °C. The eluant was monitored at 280 nm and assayed for enzymatic activity. The ferric reductase active fractions were combined, dialyzed against water and lyophilized.

Fractions having ferric reductase activity were further separated on a Biorad Biosil Sec-250 HPLC column (300 cm × 7.8 cm) with a fractionation range of 10 000–300 000 kDa. Fifty microliters of sample, containing 1 mg of protein, was eluted isocratically with 0.1 M Tris–HCl, pH 6.8. A flow rate of 0.5 ml min^{-1} with a pressure of 1200–1500 p.s.i. was maintained with a Waters model 510 pump. The eluant was monitored at 280 nm using a Hewlett Packard 1040A diode array detector system. Fractions of 0.5 ml from several runs of the same sample were collected and a 100 μl portion of each fraction was analyzed for enzyme activity. Enzymatically active fractions were pooled, dialyzed against water, lyophilized and stored at 0 °C.

The active fractions from the Sephadex G-75 column were also separated on a discontinuous polyacrylamide gel without sodium dodecyl sulfate (SDS). Samples containing 5 mg protein were resuspended in 10 μl sample buffer (without 2-mercaptoethanol or SDS) and separated on the non-denaturing gel. SDS was omitted in the upper and lower buffers. The gel was removed and stained for reductase activity as described by Moody & Dailey (1984). Active bands on the stained gel were removed, macreated by boiling for 10 min in denaturing sample buffer and analyzed using SDS–PAGE.

Determination of enzyme kinetics

A Beckman DU-65 spectrophotometer was used to determine the apparent affinity constants (K_m) for the enzymes isolated from the cytoplasmic and periplasmic extracts of avirulent and virulent cells. A Beckman Kinetics Soft-Pak module was used for analysis of enzyme activity at 2 min intervals for 30 min.

Analytical procedures

Molecular weight determinations of the enzymes were performed using size exclusion HPLC and SDS–PAGE on 12% polyacrylamide gel as described by Laemmeli (1970). Isoelectric focusing was performed on Pharmacia Ampholine PAG plates (pH range 3.5–9.5) using an LKB 2117 Multiphor II electrophoretic system. The plates were stained with ferrozine or silver stain. IEF Biorad standards (pH 3.5–9.5) were used to determine the isoelectric points of the reductases.

Chemicals

The following chemicals and materials were purchased from Sigma (St Louis, MO): $CaCl_2$, ferric state citrate, ferrozine, FMN, glutathione, GSH (reduced), $MgCl_2$, $MgSO_4$, $MnCl_2$, NADH, NADPH, $AgNO_3$, sodium azide, TEMED and $ZnSO_4$.

Results

Specificity for reductant of ferric reductases in the cytoplasmic and periplasmic extracts

In our previous study (Johnson *et al.* 1991), ferric reductase activity was observed in the periplasmic, cytoplasmic and membrane extracts of avirulent and virulent *L. pneumophila*. Minimal ferric reductase activity was found in the absence of reductant. Consequently, reduced nicotinamide adenine dinucleotide (NADH) was used as a reductant in these preliminary assays.

In our current investigation, NADH, NADPH and GSH were each tested at 2 μM to determine the optimal reductant for ferric reductase activity in the cytoplasmic and periplasmic extracts from both avirulent and virulent cells. NADH was found to be an effective reductant for the enzyme in both extracts tested for activity (Table 1). Although ferric reductase activity of the avirulent *L. pneumophila* cell-free extracts nearly doubled when NADPH was used as reductant, ferric reductase activity of virulent *L. pneumophila* cell-free extracts had nearly identical activities using either NADH or NADPH

Table 1. Ferric reductase activities[a] of the cytoplasmic and periplasmic extracts of avirulent and virulent *L. pneumophila* with NADH, NADPH and GSH

	Cytoplasmic		Periplasmic	
Reductant	avirulent	virulent	avirulent	virulent
None	2.33 ± 0.38	4.87 ± 0.22	7.93 ± 2.19	1.92 ± 0.23
NADH	12.25 ± 3.7	8.23 ± 1.05	23.64 ± 4.85	20.05 ± 1.4
NADPH	28.24 ± 3.28	12.71 ± 1.59	44.34 ± 3.28	23.31 ± 3.2
GSH	2.7 ± 1.25	3.74 ± 0.18	157.55 ± 10.35	87.45 ± 4.85

[a]Ferric reductase activities are reported as nmol ferric iron converted to ferrous per milligram protein in 30 min.

as a reductant. The cytoplasmic ferric reductase had minimal activity when GSH was used as reductant. In contrast, ferric reductase activity in the periplasmic fraction increased 3- to 4-fold when GSH was used as a reductant when compared to its activity with NADPH. The increase in ferric reductase activity when glutathione was used as a reductant was greater for the avirulent periplasmic extract in comparison with the virulent periplasmic extract. Cysteine–HCl was unable to act as a cofactor for ferric reductases in all extracts tested for activity (data not shown).

Purification of the cytoplasmic and periplasmic ferric reductases

The cytoplasmic and periplasmic extracts from avirulent or virulent cells were fractionated by gel filtration (Sephadex G-75), ionic exchange (DEAE A-25-120) and HPLC (Biosil Sec-250) chromatography. Elution profiles of the ferric reductase in the cytoplasmic extract separations were similiar for the avirulent and virulent cells. Likewise, a similiar elution profile of the ferric reductase in the periplasmic extract separations was observed for the avirulent and virulent cells (data not shown). The purification schemata for the cytoplasmic and periplasmic ferric reductase representing the average of the avirulent and virulent cells are shown in Table 2. Ferric reductase activity was confined to a single fraction in all separations of the cytoplasmic and periplasmic extracts. The HPLC-purified cytoplasmic ferric reductase (360.38 ± 34.52 nmol ferrous iron mg protein^{-1} 30 min^{-1}) demonstrated a lower specific activity than the HPLC-purified periplasmic ferric reductase (9907.54 ± 610 nmol ferrous iron mg protein^{-1} 30 min^{-1}).

Activity-staining of the cytoplasmic and periplasmic ferric reductases on a non-denaturing polyacrylamide gel

The active Sephadex G-75 fractions of the cytoplasmic and periplasmic extracts were separated on a nondenaturing gel and stained for ferric reductase activity (Figure 1). The active cytoplasmic fraction of avirulent (lane 1) and virulent (lane 2) *L. pneumophila* revealed a single band with an R_f value of approximately 0.39. The active periplasmic fraction of the avirulent and virulent cells did not stain as well on the activity gel and was observed only when the background of the gel was too dark to photograph. Although an active band cannot be seen for the avirulent periplasmic fraction (lane 3), a faint band with activity can be seen for the virulent periplasmic fraction (lane 4) on the nondenaturing gel. The active bands from the stained gel were cut out and macreated in denaturing sample buffer for SDS–PAGE analysis.

Determination of molecular weights of the cytoplasmic and periplasmic ferric reductases

The active bands from the non-denaturing gel and peaks from the HPLC column separation of the cytoplasmic and periplasmic extracts were analyzed using SDS–PAGE (Figure 2). The silver-stained SDS–PAGE separation profile revealed the cytoplasmic (lanes 3 and 5) and periplasmic (lanes 7 and 9) ferric reductases had molecular weights of approximately 38 and 25 kDa, respectively. Furthermore, ferric reductase activities of the cytoplasmic extracts of avirulent and virulent *L. pneumophila* were both found in a single peak that eluted at an average retention time of 17.84 min on the Biosil Sec-250 HPLC column. This corresponded to an M_r of

Table 2. Purification of the cytoplasmic and periplasmic ferric reductases of *L. pneumophila*

	Sephadex G-75		DEAE A-25-120		HPLC: Biosil SEC-250	
	cytoplasm	periplasm	cytoplasm	periplasm	cytoplasm	periplasm
Percent recovery	25.55 ± 6.35	42.5 ± 2.5	11.99 ± 1.43	4.34 ± 1.17	6.97 ± 0.55	2.78 ± 0.82
Specific activity[a]	37.76 ± 6.31	1579.75 ± 56.25	261.44 ± 38.0	4021.39 ± 95.23	360.38 ± 34.12	9907.54 ± 610.0
Purification (fold)	3.7	14.2	21.3	25.5	29.32	82.7

[a]Ferric reductase activities are reported as nmol ferric iron converted to ferrous per milligram protein in 30 min. NADPH and glutathione were used as the reductants for testing enzyme activities of the cytoplasmic and periplasmic enzymes, respectively.

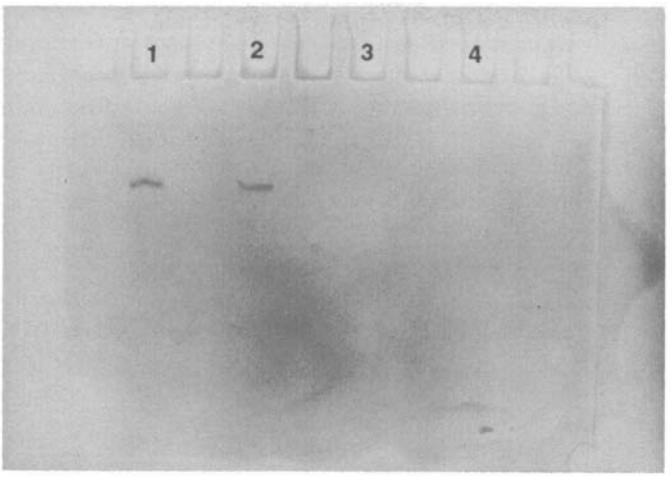

Figure 1. Non-denaturing PAGE of cytoplasmic and periplasmic ferric reductases isolated from avirulent and virulent *L. pneumophia* stained with activity stain (ferrozine). The Sephadex G-75 ferric reductase active fractions from cytoplasmic extracts of avirulent (lane 1) and virulent (lane 2) cells and periplasmic extracts from avirulent (lane 3) and virulent (lane 4) cells were separated on a 12% non-denaturing gel and stained for activity.

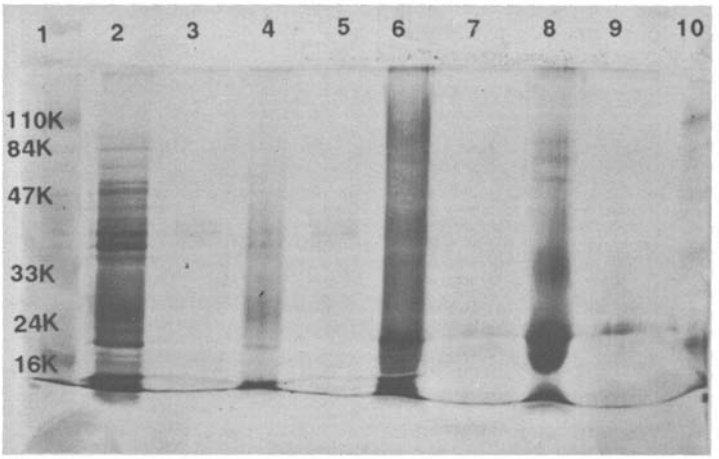

Figure 2. Silver-stained SDS-PAGE of the cytoplasmic and periplasmic ferric reductases isolated from avirulent and virulent *L. pneumophila*. The profiles of the active band macreated from the nondenaturing gel are shown for the cytoplasmic fraction isolated from avirulent (lane 2) and virulent (lane 4) cells and periplasmic fraction isolated from avirulent (lane 6) and virulent (lane 8) cells. In addition, the purified reductases from the HPLC separations are shown for the cytoplasmic reductases from avirulent (lane 3) and virulent (lane 5) cells and periplasmic reductases from avirulent (lane 7) and virulent (lane 9) cells. The molecular weights of standards (lanes 1 and 10) are indicated on the left.

37.5 kDa in comparison with standards run on the same column. In contrast, the periplasmic ferric reductases from avirulent and virulent cells had an apparent M_r of 22 kDa when compared with standards on the HPLC column.

Optimal conditions for enzyme activities

No significant differences were observed in optimal conditions for enzyme activity of the cytoplasmic and periplasmic ferric reductases from avirulent cells and the corresponding reductase in virulent cells. Ferric reductase activities of both cytoplasmic and periplasmic extracts were the same when tested under aerobic or anaerobic (N_2) conditions. Flavine monophosphate nucleotide (15 μM) and Mg^{2+} (1 mM) had no effect on reductase activities of either extract. The optimal temperature and ionic strength for ferric reductase activity was nearly identical for both the cytoplasmic and periplasmic extracts. At 37 °C, enzyme activity was only slightly higher than at room temperature (25 °C). Minimal activity was

observed at 4 °C and there was no activity at 60 °C. Ferric reductase activity remained unchanged at an ionic strength of $I = 0.01–0.1$ M Tris–HCl. Ferric reductase activities of extracts from avirulent and virulent cells were tested at a pH range of 3.0 to 11.0. The pH optima were similar for the cytoplasmic (pH 7.8) and periplasmic enzymes (pH 8.0).

Affinities of the cytoplasmic and periplasmic ferric reductases for ferric citrate and reductants

The cytoplasmic ferric reductases of the avirulent and virulent cells had similiar affinities for ferric citrate and reductants. Likewise, the periplasmic ferric reductase of the avirulent cells had similiar affinities for ferric citrate and reductants as the periplasmic reductase of virulent cells. The average affinities (apparent K_m) of the avirulent and virulent cytoplasmic and periplasmic ferric reductases for ferric iron and reductants are shown in Table 3. Although the cytoplasmic ferric reductase had only one-half the affinity for ferric citrate as the periplasmic ferric reductase, both reductases had similar affinities for NADH. The cytoplasmic reductase had approximately 12-fold the affinity for NADPH when compared with its affinity for NADH. In contrast, the affinity of the periplasmic ferric reductase for GSH was actually less than its affinity for NADH.

Characteristics of the cytoplasmic and periplasmic ferric reductases

Although the cytoplasmic and periplasmic reductases appear to be different enzymes based on SDS–PAGE analysis, some common characteristics were found for both enzymes. The specific activities of the cytoplasmic and periplasmic enzymes were not affected by the concentration of iron in the growth medium (data not shown). Thus, both ferric reductases are most likely constitutive in their expression. Sodium azide (0.05%) did not inhibit the activities of either enzyme. In addition, a hydrophobic (e.g. membrane) environment appears not to be necessary as Triton X-100 (0.066%) did not enhance the activities of either reductase. Zinc was able to inhibit both the cytoplasmic and periplasmic reductase by approximately 50% when zinc sulfate was added at an equimolar quantity as iron. The K_i values for zinc were nearly the same for the periplasmic and cytoplasmic reductases averaging 415.00 ± 35.00 and 370.50 ± 3.50 μM, respectively. In the presence of zinc, both the K_m and V_{max} values of the periplasmic and cytoplasmic enzymes are affected which implicates a mixed inhibition by zinc common to both reductases. Other divalent cations including $CaCl_2$, $MgSO_4$ and $MnCl_2$ had no affect on enzymatic activity. The cytoplasmic and periplasmic ferric reductases were determined to have isoelectric points of 4.30 and 7.10, respectively (data not shown).

Discussion

We have previously reported ferric reductase activity in crude periplasmic, cytoplasmic and membrane extracts from avirulent and virulent *L. pneumophila* cells (Johnson *et al.* 1991). Results of our current study indicate that the cytoplasmic and periplasmic reductases are two distinct enzymes as summarized in Table 4. Differences were observed between both enzymes in their reductant specificities, molecular retention, isoelectric points and activity staining on non-denaturing gels. The periplasmic reductase has a greater specific activity and twice the affinity for ferric citrate when compared to the cytoplasmic enzyme. The highest specific activity was observed when GSH was used as a reductant for the periplasmic reductase. The periplasmic reductases isolated from both avirulent and virulent cells exhibited a 3- to 4-fold increase when GSH was used as a reductant in comparison with NADPH. Although an uncommon reductant for bacterial reductases, GSH was found to be a suitable reductant for the periplasmic reductase of *Pseudomonas aeruginosa*

Table 3. Apparent affinity constants (K_m) of the cytoplasmic and periplasmic ferric reductases of *L. pneumophila* for ferric citrate and reductants

	Affinity for ferric citrate (μM)[a]	Affinity for reductant (μM)		
		NADH	NADPH	GSH
Cytoplasmic reductase	15.45 ± 0.85	11.35 ± 4.65	8.85 ± 0.25	ND[b]
Periplasmic reductase	7.00 ± 0.2	114.8 ± 2.7	ND	159.2 ± 12.5

[a]The reductant used for the determination of affinities for ferric citrate was NADH.
[b]Not determined.

Table 4. Characteristics that differ between the cytoplasmic and periplasmic reductases of *L. pneumophila*

Characteristic	Cytoplasmic reductase	Periplasmic reductase
Molecular weight (kDa)	38	25
Optimum reductant	NADH[a]	Glutathione
Ferric citrate affinity (K_m)	15.30 ± 0.85 μM	7.00 ± 0.20 μM
IEF point	4.30	7.10
Average specific activity[b]	360.38 ± 39.12	9907.54 ± 610

[a]Although the virulent cytoplasmic reductase has equivalent activity using NADH or NADPH as the reductant, the avirulent cytoplasmic reductase has a higher specific activity with NADPH.
[b]Ferric reductase activities are reported as nmol ferric iron converted to ferrous per milligram protein in 30 min. The reductant with the highest specific activity was used for testing enzyme activities.

(Cox 1980). In contrast with the periplasmic reductase, the cytoplasmic reductase had only minimal activity using GSH. A difference in the optimum reductant was observed for cytoplasmic ferric reductases of avirulent and virulent *L. pneumophila*. The reductase isolated from virulent, but not avirulent cells had greater activity with NADPH as reductant than NADH. This difference might have some significance on the survival of virulent cells in macrophages. Although the NADH concentration in macrophages remains constant during phagocytosis, the NADPH level decreases due to an increase in NADPH oxidase activity (Rossi *et al.* 1972). Consequently, only the cytoplasmic ferric reductase from avirulent cells would be affected by this decrease in NADPH concentration.

Both ferric reductases of *L. pneumophila* have characteristics that are common to other bacterial reductases. The optimal conditions in temperature and pH and constitutive nature of *L. pneumophila* ferric reductases are representative of nearly all bacterial reductases. The existence of multiple ferric reductases has been demonstrated in several bacterial species; *B. subtilis* (Gaines *et al.* 1981), *P. aeruginosa* (Cox 1980) and *Rhodopseudomonas sphaeroides* (Moody & Dailey 1985). Although the molecular weight values of the enzymes isolated from *L. pneumophila* are slightly lower than that reported for other bacterial reductases, their specific activities and affinity values for ferric citrate are within the range of most reductases (Lefaou & Morse 1991). Both ferric reductases have characteristics that are representative of some bacterial reductases but not others. They are equally active under aerobic or anarobic conditions and have reductant specificities similar to other bacterial reductases. Flavine monophosphate nucleotide and magnesium do not enhance the activity of either reductase. The ferric reductases of *L. pneumophila* are similar to the ferric reductase of *Azotobacter vinelandii* (Huyer & Page 1989) in that zinc sulfate inhibits the enzymes in a mixed inhibitor fashion. Periplasmic ferric reductases are uncommon, having been found in *P. aeruginosa* (Cox 1980) and only suggested to exist in *A. vinelandii* (Huyer & Page 1989). Furthermore, ferric reductases with specificities for ferric citrate have been found almost exclusively in the cytoplasmic fraction of bacteria (Cox 1980, Gaines *et al.* 1981, Moody & Dailey 1985, Huyer & Page 1989). The periplasmic ferric reductase of *L. pneumophila* is unique in that it represents a periplasmic reductase with a specificity for ferric citrate. As *L. pneumophila* was found not to utilize siderophores (Reeves *et al.* 1983) or transferrin and lactoferrin (Quinn & Weinberg 1988, Johnson *et al.* 1991), the only substrate used when testing for reductase activity was ferric citrate. In addition, the only known bacterial reductase specific for transferrin or lactoferrin was found in *Listeria monocytogenes* (Cowart & Foster 1985). However, there is the possibility that ferric reductases of *L. pneumophila* have even greater specific activities for a yet undiscovered siderophore or iron-binding protein.

With the exception of their response to NADPH, the ferric reductase at each respective location appears to be similar for avirulent and virulent cells. Thus, the difference in requirements of iron for avirulent and virulent cells does not appear to involve differences in their ferric reductases.

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