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Structure of acinetoferrin, a new citrate-based dihydroxamate siderophore from *Acinetobacter haemolyticus*

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From low-iron cultures of *Acinetobacter haemolyticus* ATCC 17906, a new hydroxamate siderophore was purified by XAD-7 adsorption followed by preparative thin layer chromatography. The siderophore, named acinetoferrin, released citric acid, 1,3-diaminopropane and (*E*)-2-octenoic acid upon hydrolysis with HCl, reductive hydrolysis with HI and oxidation with periodate, respectively. Structure elucidation by a combination of NMR spectroscopy and positive fast atom bombardment mass spectrometry revealed that acinetoferrin is a derivative of citric acid, both of its terminal carboxyl groups being symmetrically amide-linked with the 1-amino-3-(*N*-hydroxy-*N*-2-octenylamino)propane residues. The (*E*)-2-octenoic acid is novel as a component of the siderophores.

Keywords: *Acinetobacter haemolyticus*, acinetoferrin, iron transport, siderophore

Introduction

In response to iron limitation, many microorganisms produce highly specific chelators for Fe(III), called siderophores, and the iron complexes formed by these molecules are taken up via specific transport systems to promote the growth (Neilands 1993, and the references cited therein). In animals, free iron is kept at very low levels by iron-sequestering proteins such as transferrin and lactoferrin (Weinberg 1978, Crichton & Charloteaux-Wauters 1987). It has frequently been reported that the siderophores can compete with these glycoproteins for iron. Therefore, it is postulated that this iron acquisition system is one of the factors associated with bacterial pathogenicity (Griffith *et al.* 1988, Payne 1988, Martínez *et al.* 1990).

The members of the genus *Acinetobacter* are Gram-negative coccobacilli highly prevalent in nature (Juni 1978), and generally considered to have low pathogenic potential for humans (Henriksen 1973). However, an increasing number of reports deal with *Acinetobacter* strains responsible for hospital infections, including respiratory tract infections, bacteremia and meningitis, and sometimes highly resistant to a wide range of antibiotics (Glew *et al.* 1977, Retailliau *et al.* 1979, Bouet & Grimont 1987, Gerner-Smidt 1987, Beck-Sagué *et al.* 1990).

Few reports have appeared in the literature concerning the siderophore production in this genus. Smith *et al.* (1990) reported that *A. calcoaceticus* secreted 2,3-dihydroxybenzoic acid in response to low iron availability. More recently, Echenique *et al.* (1992) isolated from *A. baumannii* a new catecholate siderophore capable of promoting growth in the presence of transferrin, but the structure was not determined.

In this paper, we describe the structure elucidation of a siderophore isolated from *A. haemolyticus*. The chemical and instrumental analyses revealed that the siderophore, named acinetoferrin, was a novel citrate-based dihydroxamate having the structure as shown in Figure 1.

Materials and methods

Materials

Ethylenediamine-di-(*o*-hydroxyphenyl)acetic acid (EDDA) was obtained from Sigma (St Louis, MO); (*E*)-2-octenoic acid was from Aldrich (Milwaukee, WI); Amberlite XAD-7 resin was from Nacalai Tesque (Kyoto,

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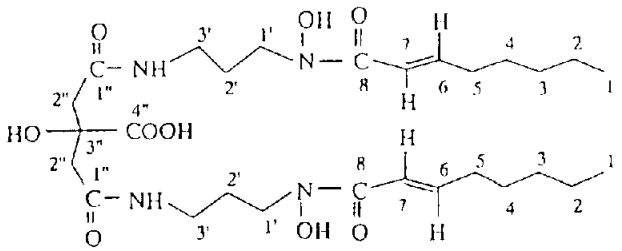

Figure 1. Structure of acinetoferrin.

Japan) and purified as described by Actis *et al.* (1986); pre-coated silica gel 60 plates (thickness 0.25 mm) were from Merck (Darmstadt, Germany) and before use they were developed in methanol:acetone:10 M HCl (45:45:10) and air-dried.

Bacterial strain and culture conditions

The type strain *A. haemolyticus* ATCC 17906 was used. It was routinely maintained on nutrient agar slopes at 30 °C and pre-cultured for 20 h in a chemically defined (CD) medium (pH 7.4) consisting of Tris, 12.1; KCl, 3.7; NH_4Cl, 1.1; KH_2PO_4, 0.272; $CaCl_2 \cdot 2H_2O$, 0.15; Na_2SO_4, 0.142; $MgCl_2 \cdot 6H_2O$, 0.1; NaCl, 5 and sodium succinate, 5 (in $g\,l^{-1}$). For production of acientoferrin, the pre-culture was added to the autoclaved CD medium (1:50, v/v), which was then fortified with sterile $FeCl_3$ solution at a final concentration of 0.1 μM to enhance siderophore yields. The bacterium was grown at 30 °C for 28 h. During purification of the siderophore, its bioactivity was assayed according to the method of Miles & Khimji (1975). Briefly, an aliquot (10–20 μl) of the solution to be tested was spotted on an 8 mm sterile EDTA-treated paper disk and allowed to dry before the disk was applied to agar plates, containing MMOF medium (Yamamoto *et al.* 1979) and 150 μg EDDA ml^{-1}, that had been seeded with the producer strain (about 10^4 c.f.u. ml^{-1}). The growth halo surrounding the disk was examined after incubation at 30 °C for 10–15 h.

Isolation and purification of siderophore

The supernatant was recovered from 4 l batches of culture by centrifugation at 10000 × ***g*** for 10 min, and adjusted to pH 6 with 60% citric acid. An XAD-7 resin (80 g) was added and the suspension was shaken slowly on a rotary shaker for 1 h to adsorb the siderophore. The suspension was filtered with a glass filter funnel and the resin was washed three times with 1 l of distilled water. The resin was soaked in 200 ml of methanol for 30 min to deadsorb the siderophore, filtered and then washed with 100 ml of methanol. This procedure was repeated twice more. The filtrate was evaporated to dryness at 30 °C under reduced pressure and the residue was dissolved in 100 ml of water. The aqueous solution adjusted to pH 2.0 with solid citric acid was immediately extracted three times with 200 ml of ethyl actate. After washing three times with 0.1 M sodium citrate, pH 5.5, the organic layer was evaporated to dryness at 30 °C under reduced pressure to give a crude siderophore fraction. This was positive both in the plate bioassay and the Csáky test (Csáky 1948) for the presence of hydroxamic acid residue(s), and stored at −20 °C until further purification. The combined crude siderophore fraction from 24 l of supernatant was dissolved in 5 ml of methanol and the insoluble materials were removed by centrifugation. The resulting solution was subjected to preparative thin layer chromatography (TLC) on silica gel 60 plates in chloroform:methanol (4:1). A strip was taken from each plate and sprayed with 0.1% $FeCl_3$ in 0.1 M HCl to locate the iron-binding compounds. Corresponding regions of the remaining plates were excised and extracted with chloroform:acetone (1:1). Only an intensively colored band of R_f 0.58 exhibited siderophore activity in the plate bioassay. The concentrated extract was rechromatographed on the same matrix in benzene:acetic acid:water (125:72:3), and a band of R_f 0.31 was extracted with chloroform:acetone (1:1). After evaporation of the solvent, the residue was dissolved in acetone, from which a colorless precipitate, acinetoferrin, was obtained by addition of *n*-hexane. The yield was 62 mg (~ 4.5 μM in supernatant). The melting point of acinetoferrin could not be measured owing to its hygroscopicity.

Compositional analysis of acinetoferrin

Purified acinetoferrin was hydrolyzed *in vacuo* with 6 M HCl or with 50% HI for 15 h at 110 °C (Gibson & Magrath 1969). Excess mineral acid was removed by repeated evaporations under reduced pressure. The residue from HCl hydrolysis was esterified with diazomethane in diethyl ether (Konetschny-Rapp *et al.* 1990), while that from HI hydrolysis was treated with ethyl chloroformate to prepare the *N*-ethoxycarbonyl derivatives of amines (Yamamoto *et al.* 1984). Samples thus obtained were analyzed by gas chromatography–mass spectrometry (GC-MS) with citric acid and 1,3-diaminopropane as references. For identification of (*E*)-2-octenoic acid, purified acinetoferrin was oxidized with 0.05% periodic acid (Emery & Neilands 1960) and the carboxylic acid released was extracted with ether after acidification with HCl. After evaporation of ether, the residue was esterified with diazomethane and analyzed by GC-MS.

Absorption spectroscopy

UV and visible spectra of acinetoferrin and its ferric complex were taken on a Shimadzu UV-160A spectrophometer.

Mass spectrometry

GC-MS and positive fast atom bombardment mass spectrometry (FAB-MS) were performed with a VG Analytical 70SE mass spectrometer. For GC-MS, a Quadrex bonded-fused silica capillary column coated with OV-1 (25 m × 0.25 mm ID) was used and the instrument was operated in the electron impact ionization mode (70 eV). FAB-MS spectra were measured from a glycerol matrix.

NMR spectroscopy

NMR spectra were recorded on a Varian VXR 500 spectrometer (500 MHz for ^1H and 125.7 MHz for ^{13}C) each with an exclusive probe. Samples were dissolved in CD_3OD. The chemical shifts are given in δ values (p.p.m.) relative to tetramethylsilane, and the coupling constants are expressed as J values in Hz. Varrian's standard programs were used for ^1H–^1H shift correlation spectroscopy (COSY), ^1H–^{13}C COSY, and ^1H–^{13}C long-range COSY. ^1H–^{13}C COSY and long-range COSY were obtained with average J_{CH} values of 140 and 7 Hz for overnight runs, respectively.

Reversal of EDDA-inhibited growth of A. haemolyticus *by acinetoferrin*

Log-phase cells grown in CD medium were added at a starting inoculum of 10^5 c.f.u. ml^{-1} to flasks containing either CD medium containing 0.5 μM $FeCl_3$ (A), (A) supplemented with 100 μM EDDA or (A) supplemented with 100 μM EDDA plus 5 μM acinetoferrin. For preparation of the latter two media, iron and EDDA were added at least 24 h before use to permit the chelation of all adventitious iron. Growth curves were measured at intervals at A_{660}.

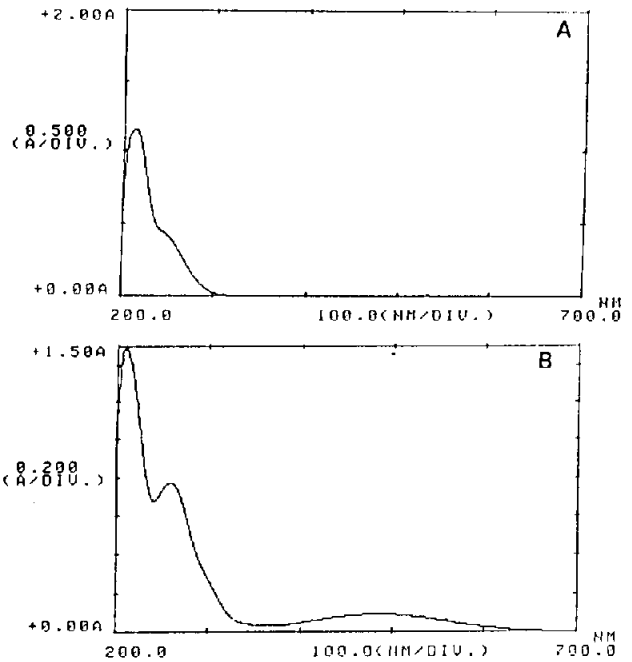

Figure 2. Absorption spectra of acinetoferrin (**A**) and its ferric complex (**B**) in methanol (each 4×10^{-2} M).

Results

Production of acinetoferrin by *A. haemolyticus*, when grown in CD medium supplemented with iron at the range of 0–10 μM, was examined by the Csáky test for the respective supernatants. The results showed that its production was tightly regulated by the availability of iron in the growth medium, being effectively synthesized at around 0.1 μM added iron. No acinetoferrin was detected when the organism was grown at 10 μM added iron. Catecholate siderophore was not detected in any of the supernatants by the chemical assay of Arnow (1937).

Purified acinetoferrin showed an absorption maximum at 212 nm (2.9×10^4 M^{-1} cm^{-1}) with a shoulder at 250 nm, and upon addition of an aqueous solution of $FeCl_3$ to the methanolic acinetoferrin solution, a red-brown color developed immediately; the maximum of the broad absorption band of low intensity was observed at 486 nm (9.8×10^2 M^{-1} cm^{-1}) (Figure 2), showing a characteristic charge-transfer band.

Citric acid was detected in the 6 M HCl hydrolyzate of acinetoferrin. This together with a positive Csáky reaction suggested that acinetoferrin might be a citrate-based hydroxamate siderophore. Then, to detect the other components, acinetoferrin was reductively hydrolyzed with HI or oxidized with periodate according to Gibson & Magrath (1969). As a result, reductive hydrolysis gave a ninhydrin positive compound, which was identified as 1,3-diaminopropane by GC-MS, while oxidation with periodate eliminated (*E*)-2-ocenoic acid, identified by GC-MS (Figure 3). These findings indicated that the one nitrogen atom of 1,3-diaminopropane was hydroxylated, which was amide-linked to (*E*)-2-octenoic acid, since it is known that the acyl residue of a hydroxamic acid grouping is released by the periodate oxidation as the free carboxylic acid (Emery & Neilands 1960).

NMR spectra of acinetoferrin were measured to evaluate how the components identified are connected. The ^1H- and ^{13}C-NMR spectral data are shown in Table 1, and the ^1H–^{13}C COSY spectrum in Figure 4. The two doublets at δ2.69 and 2.79 in the ^1H-NMR spectrum, each integrated to two protons, could be assigned unequivocally to the magnetically equivalent methylene protons in the citric acid moiety. Their chemical shift values and geminal coupling constants (J = 14.5) corresponded well to the reported values for aerobactin, a symmetrically substituted citrate-based siderophore (Gibson & Magrath 1969). Therefore, we assumed that the terminal carboxyl groups in acinetoferrin are symmetrically substituted in amide linkage to the amino groups of two residues of 1-amino-3-(*N*-hydroxy-*N*-(*E*)-2-octenyl)aminopropane. This assumption was supported by the fact that all other signals in the methylene proton region were integrated to four

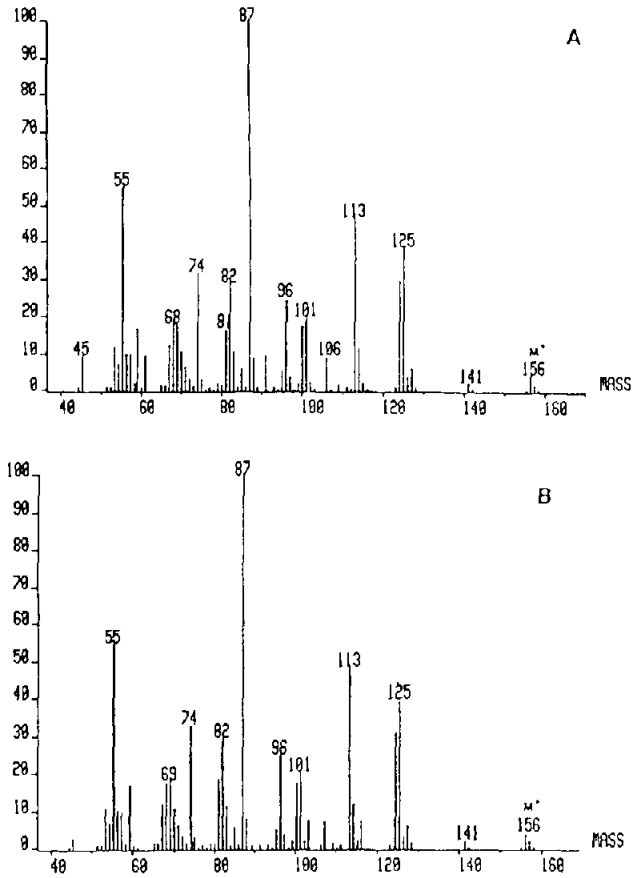

Figure 3. GC-MS identification of (*E*)-2-octenoic acid released from acinetoferrin upon periodate oxidation. Authentic (*E*)-2-octenoic acid (**A**) and oxidation product from acinetoferrin (**B**) were esterified with diazomethane in ether, and the peaks eluted at 108 °C were analyzed. GC conditions: Quadrex bonded-fused silica capillary column coated with OV-1 (25 m × 0.25 mm ID); temperature-programmed from 80 to 150 °C at 7 °C min^{-1}.

protons except for the signals at δ0.96 (H-1) and 1.38 (H-2 and H-3), which were integrated to six and eight protons, respectively. The proton signals due to the (*E*)-2-octenoic acid residue could be assigned by their sequential correlation in the ^1H–^1H COSY spectrum (data not shown). The characteristic appearance as a doublet and a doublet of triplets, and their coupling constants (J = 15.5) and chemical shift values indicated that the signals at δ6.66 and 6.89 corresponded to two vinyl protons (H-7 and H-6), which were positioned *trans* next to a carbonyl group. The multiple signal at δ1.87 was correlated with the signals at δ3.24 and 3.75 by ^1H–^1H COSY (data not shown), indicating that these signals are attributed to the presence of two propylene groups. The assignment was further confirmed by comparing the chemical shift values with the reported ones for

Table 1. ^1H and ^{13}C NMR spectral data of acinetoferrin

Carbon number[a]	δH[b]	δC[b]
1	0.96 (t, 6H, J = 6.5)	13.3
2	1.38 (m, 4H)	22.5
3	1.38 (m, 4H)	31.5
4	1.52 (m, 4H)	28.1
5	2.29 (dt, 4H, J = 7.5, 7.0)	32.4
6	6.89 (dt, 2H, J = 15.5, 7.0)	147.3
7	6.66 (d, 2H, J = 15.5)	119.4
8		167.6
1′	3.75 (t, 4H, J = 6.5)	46.0
2′	1.87 (m, 4H)	26.6
3′	3.24 (t, 4H, J = 6.5)	36.7
1″		171.2
2″	2.69 (d, 2H, J = 14.5)	44.1
	2.79 (d, 2H, J = 14.5)	44.1
3″		74.1
4″		176.0

Spectra were measured at 500 MHz for ^1H and at 126 MHz for ^{13}C using CD_3OD as a solvent and TMS as an internal standard. Multiplicity, integration and coupling constants in Hz are in parentheses: m, multiple; t, triplet; d, doublet.
[a]The numbering of carbon atoms, see Figure 1.
[b]Assignments are based on the ^1H–^1H and ^1H–^{13}C COSY spectrum.

schizokinen, a siderophore containing the *N*-hydroxy-1,3-diaminopropane moieties (Mullis *et al.* 1971).

The signal at δ44.1 in the ^{13}C-NMR spectrum was assigned to C-2″ by ^1H–^{13}C COSY (Figure 4). The signal at δ74.1 was assigned by ^1H–^{13}C COSY to C-3″ , which showed no correlation with any of the protons. The assignment of the carbonyl carbons, C-1″ and C-4″ could be established by the ^1H–^{13}C long-range COSY spectrum, where two- or three-bond coupling with the methylene protons (C-2″) in the citric acid residue was detected (data not shown). Therefore, the remaining carbonyl carbon signal at δ167.6 could be assigned to C-8 next to the vinyl group. The other alkyl carbons were assigned by the ^1H–^{13}C COSY spectrum shown in Figure 4.

FAB-MS of acinetoferrin revealed peaks at m/z = 585 (100), 569 (40) and 567 (35) for $[MH]^+$, $[MH-O]^-$ and $[MH-H_2O]^+$, respectively (Figure 5A). The peak at m/z = 569 agreed with the finding by Dell *et al.* (1982) that the hydroxamino oxygen is characteristically eliminated by mass spectrometry of hydroxamate siderophores. The accurate mass for the $[MH]^+$ ion was determined by high-resolution FAB-MS to be 585.3516, which was consistent with a molecular formula, $C_{28}H_{49}O_9N_4$ (585.3500), deduced from the NMR data. FAB-MS of the ferric

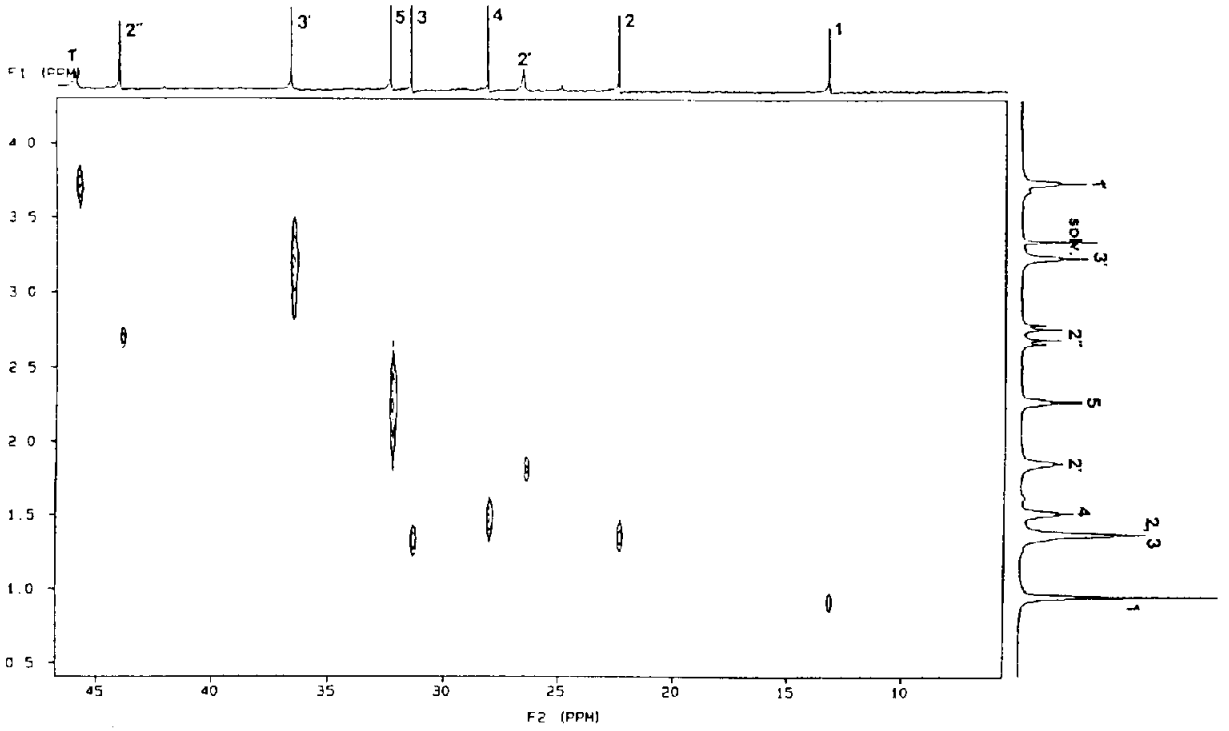

Figure 4. Alkyl region of the $^{1}H-^{13}C$ shift correlation spectrum of acinetoferrin in CD_3OD (22 mg ml^{-1}).

complex of acinetoferrin yielded a molecular ion at $m/z + 638$, corresponding to $[MH + Fe-3H]^+$ (Figure 5B). The difference of 53 mass units between the free ligand and its iron complex indicated a 1:1 stoichiometry.

Figure 6 depicts the growth kinetics in liquid media of the producer strain under conditions in which all added iron was sequestered by EDDA. The addition of acinetoferrin at 5 μM significantly shortened the prolonged growth lag phase observed in the iron-deficient medium supplemented with a 200-fold molar excess of EDDA over added iron, indicating that acinetoferrin can assimilate iron from the EDDA–iron complex.

Discussion

In the preliminary experiments, many strains of *Acinetobacter*, including all seven named species and 30 clinical isolates, were screened for siderophore production by the Chrome Azurol S test (Schwyn & Neilands 1987). Only the strains of *A. baumannii* and *A. haemolyticus* gave strongly positive results. Consistent with the recent report by Echenique *et al.* (1992), *A. baumannii* produced an unknown catecholate siderophore. While *A. baumannii* is the main species associated with nosocomical infections, *A. haemolyticus* is also isolated from clinical specimens (about 3% of total *Acinetobacter*

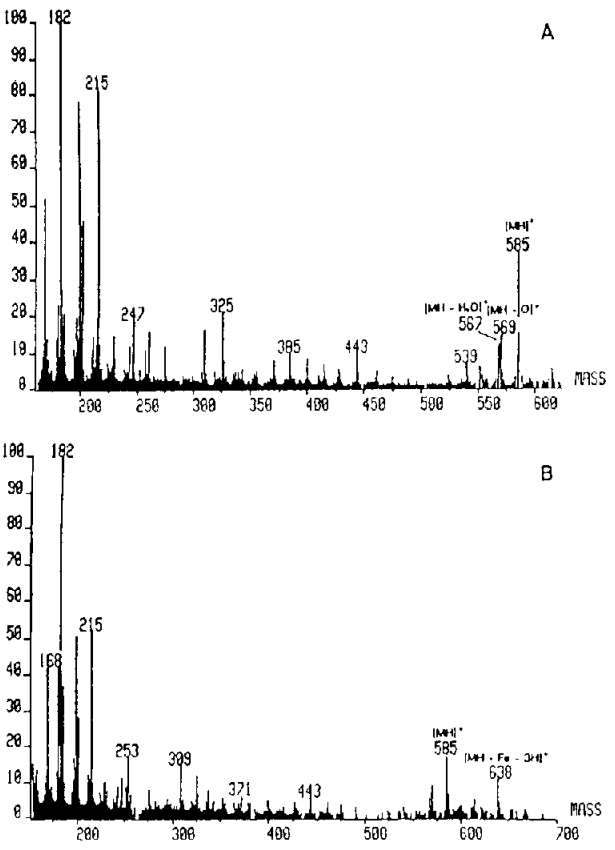

Figure 5. FAB mass spectra of acinetoferrin (**A**) and its ferric complex (**B**) in glycerol matrix, positive mode.

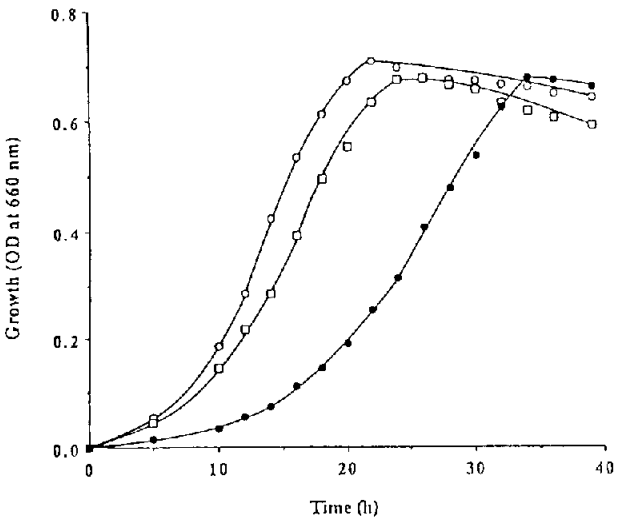

Figure 6. Reversal of EDDA-inhibited growth of *A. haemolyticus* by acinetoferrin. Strain ATCC 17906 was grown in CD medium containing 0.5 μM $FeCl_3$ (A) (○), (A) supplemented with 100 μM EDDA (●) and (A) supplemented with 100 μM EDDA plus 5 μM acinetoferrin (□). Growth was monitored at OD_{660}.

isolates) (Bouvet & Grimont 1987). In this work, the siderophore, named acinetoferrin, synthesized by strain ATCC 17906 under the influence of the iron available for growth was purified and characterized by compositional and instrumental analyses, and this set of analytical data demonstrates that it is a citrate-based dihydroxamate containing an α,β-unsaturated fatty acid, (*E*)-2-octenoic acid.

The well-known siderophores, aerobactin (Gibson & Magrath 1969), schizokinen (Mullis *et al.* 1971) and anthrobactin (Linke *et al.* 1972), share a common feature in that the terminal carboxyl groups of citric acid are symmetricaly substituted with *N*-acetylated *N*-hydroxydiamines or N^6-hydroxylysine. Recently, nannochelins which carry cinnamoyl instead of alcy groups were isolated from *Nannocystis exedens* (Kunze *et al.* 1992). More recently, when the structural elucidation of acinetoferrin was almost accomplished, Permark *et al.* (1993) isolated from *Rhizobium meliloti* 1021 an asymmetrical (chiral) citrate-based dihydroxamate, rhizobactin 1021, carrying two different acly groups, acetyl and (*E*)-2-decenoyl. Both of (*E*)-2-decenoic acid and (*E*)-2-octenoic acid are unprecedented as the siderophore constituents, although various carboxylic acids have been found in non-citrated-based siderophores such as mycobactins (Snow 1970) from mycobacteria, palmitoylcoprogen from *Trichoderma* (Anke *et al.* 1991) and ornibactin-C8 from *Pseudomonas cepacia* (Stephan *et al.* 1993). It is of interest that ornibactin-C8 contains a C_8-fatty acid, 3-hydroxyoctanoic acid, analogous to 2-octenoic acid. Interestingly, any of nannochelin, rhizobactin 1021 and acinetoferrin was sucessfully concentrated from the culture supernatants by use of the Amberlite XAD resin. The hydrophobic acyl groups in these siderophores seem to play an important role in adsorbing them onto the resin. Like schizokinen and rhizobactin 1021, acinetoferrin contained *N*-hydroxy-1,3-diaminopropane as a hydroxylamine residue.

Considering the structural similarity to aerobactin, acinetoferrin may chelate iron in a hexadentate fashion. This was supported by the FAB-MS spectrum showing the formation of a 1:1 siderophore–iron complex. Also, the observation that acinetoferrin relieved the growth inhibition caused by EDDA, a type of activity generally regarded as indicative of a true siderophore (Neilands 1984), strongly suggests that acinetoferrin is able to deliver iron to *A. haemolyticus* cells and in fact it has a higher affinity for iron than EDDA. Thus, the high-affinity iron uptake system mediated by acinetoferrin may be of use for acquisition of iron from host's iron-binding proteins such as transferrin and lactoferrin, as reported for aerobactin in *Escherichia coli* (Neilands *et al.* 1987), and may enhance the survival of this bacterium in the host. Therefore, the detailed function, genetics and biosynthesis of this siderophore deserve more study.

Acknowledgments

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