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Physical and structural characterization of yersiniophore, a siderophore produced by clinical isolates of *Yersinia enterocolitica*

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Clinical isolates of *Yersinia enterocolitca*, which belong to mouse-lethal serotypes, produce the siderophore yersiniophore. Siderophore production was shown to be iron regulated and to reach maximum production in late log phase. Yersiniophore is a fluorescent siderophore with maximum excitation at 270 nm and a major emission peak at 428 nm. Absorption maxima were seen at 210 and 250 nm with a low broad peak from 280 to 320 nm. Purification of unchelated yersiniophore for structural analysis was made difficult by low yields (1–2 mg mg^{-1}), and susceptibility to acid hydrolysis, oxidation and possibly polymerization. Yersinophore was therefore purified as an Al^{3+} chelate, which was found to be stable in solution for several weeks. To purify Al^{3+}–yersiniophore, unchelated yersiniophore was first extracted from culture supernatants with dichloromethane, concentrated by rotary evaporation and adsorbed to a DEAE–sephacel column. Al^{3+}–yersiniophore was eluted with 0.01 M $AlCl_3$ and further purified by HPLC. The structure was established by a combination of elemental analysis, high resolution mass spectrometry and two-dimensional NMR experiments. Yersiniophore is a phenolate-thiazole siderophore with the formula $C_{21}H_{24}N_3O_4S_3Al$ and a molecular weight of 505.07404 when chelated to Al^{3+}. The structure of yersiniophore was determined to be closely related to the structures of pyochelin, produced by *Pseudomonas aeruginosa*, and anguibactin, produced by *Vibrio anguillarum*.

Keywords: iron, siderophore, *Yersinia enterocolitica*, structure, NMR

Introduction

Iron is an essential nutrient for all bacteria. Microorganisms may encounter conditions where the concentration of free iron is too low to support growth. In response to low iron conditions many bacteria produce high-affinity iron-binding compounds called siderophores (Neilands 1981). Once siderophores have complexed with iron they are recognized by receptors on the bacterial cell surface and the iron is internalized. In human and other mammalian hosts most iron is either sequestered intracellularly or bound extracellularly by transferrin glycoproteins. Consequently the concentration of free iron within the host is very low (Weinberg 1984). Many pathogens utilize siderophores to compete effectively for host iron.

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Yersinia enterocolitica is an invasive bacterial pathogen which can cause symptoms varying from mild diarrhea and abdominal pain to more severe disease where performation of the ileum occurs. Both children and adults are susceptible, but children are the most often infected (Cornelis *et al.* 1987). Septicemia may also occur in rare cases. This complication is usually associated with an underlying disorder such as siderosis, thalassemia or hemochromatosis (Larigakis *et al.* 1990). A variety of *Y. enterocolitica* serotypes are pathogenic to humans; however, only certain of these serotypes are lethal to mice when injected intraperitoneally (Robins-Browne & Prpic 1985). Production of siderophores has been associated with serotypes which are lethal in the mouse model (Hessemann 1987, Chambers & Sokol 1994).

Siderophore produced by *Y. enterocolitica* has been detected using chrome azurol S (CAS) agar (Heesemann 1987, Chambers & Sokol 1994). This sensitive assay detects iron-binding activity of siderophores irrespective of their chemical structure. In solution the CAS–iron complex changes from blue to orange in a semi-quantitative manner as iron is extracted by the siderophore (Schwyn & Neilands

1987). Using this assay, siderophore production has been detected in the mouse lethal serotypes O:4, O:8, O:13, O:20, O:21 and O:40 (Heesemann 1987, Chambers & Sokol 1994).

At least three distinct siderophores are produced by *Y. enterocolitica*. Production of yersiniophore, previously characterized as a phenolate with hydrophobic properties, is associated with clinical isolates of *Y. enterocolitica* (Chambers & Sokol 1994). Yersiniabactin has been purified from the clinical isolate WA-C (O:8) and partially characterized (Haag *et al.* 1993). Although this structure was not determined, yersiniabactin was reported to be a catechol with a molecular weight of 482 Da. Some environmental isolates have been shown to produce a hydrophilic siderophore which is negative for both catechol and hydroxymate assays (Chambers & Sokol 1994).

Yersiniophore and yersiniabactin have both been isolated from clinical strains of serotype O:8. To further examine the clinical significance of yersiniophore production, the frequency of yersiniophore producing strains was determined in a collection of 62 isolates from various sources (Chambers & Sokol 1994). Yersiniophore was found to be produced and utilized by all human isolates belonging to mouse lethal serotypes. The frequency and distribution of yersiniabactin producing strains has not yet been reported. In the present study, yersiniophore produced by YE1165 (O:8) was further characterized and the structure determined.

Materials and methods

Strains

Y. enterocolitica strains UC310 (O:8) and YE1165 (O:8) are human stool isolates from the collection provided by Dr C. H. Pai, Department of Clinical Pathology, Asian Medical Centre, Seoul, Korea. YE1165 was originally obtained from S. Toma at the National Reference Centre for *Yersinia*, Laboratory Services Branch, Toronto, Ontario, Canada. Both UC310 and YE1165 were used for the production of yersiniophore. For structural analysis yersiniophore was isolated from YE1165.

Culture conditions

For structural analysis, a frozen stock of YE1165 was plated onto L agar (Difco, Detroit, MI) and grown overnight at 28°C. A starter culture of 300 ml L Broth (Difco) was inoculated from the plate and grown with shaking for 12–16 h at 28°C. These cells were harvested by centrifugation and used to inoculate 20 l of *Yersinia* minimal media (YMM; Carniel *et al.* 1987) supplemented with 0.4 mM $MgSO_4$ and 75 μM 2,2′-dipyridyl (Sigma, St Louis, MO). Cultures were grown in 1 l volumes in 2 l baffled flasks (Bellco Glass, Vineland, NJ) with shaking for 36 h at 28°C. To prevent iron contamination, all glassware was acid washed and all reagents were prepared with water purified by the Milli-Q System (Millipore, Bedford, MA). Cultures which were used for purification of active yersiniophore, either by thin layer or hydrophobic chromatography, were grown in 2–3 l batches.

Detection of yersiniophore

To detect iron-binding activity of yersiniophore preparations the CAS assay (Schwyn & Neilands 1987) was used. The CAS (Sigma) and hexadecyltrimethylammonium bromide (Fisher Scientific) complex was prepared as originally described. To detect yersiniophore at different steps in the purification process, 0.5 ml CAS solution was mixed with various amounts of siderophore preparations in a final volume of 1 ml.

Yersiniophore purification

Three protocols were developed to purify yersiniophore. Initially, purification was performed using TLC. Iron-poor supernatants from UC310 cultures were acidified with HCl to a pH of 2.5 and extracted (2:5, v/v) with ethyl acetate (B&J Brand, Baxter, Canlab Division, Ontario, Canada). The extract was dried by rotary evaporation, resuspended in methanol (Optima; Fisher Scientific) and chromatographed on thin-layer Silica Gel G plates (Analtech, Newark, DE) using methanol:acetic acid (90:5) as the solvent. A fluorescent yellow/green band ($r_f = 0.98$) visible under UV light was eluted in MeOH and chromatographed on a second TLC plate with chloroform:EtOH:acetic acid (90:5:2.5) as the solvent. Yersiniophore appeared as a large fluorescent green band ($r_f = 0.26$) which was eluted from the silica with MeOH and stored at −20°C (Chambers & Sokol 1994).

An alternative purification protocol was devised using hydrophobic charomatography (Chambers & Sokol 1994). The supernants from iron-poor cultures of YE1165 were extracted at neutral pH (2:5, v/v) with dichloromethane (BDH Assured, ACS, BDH, Ontario, Canada), dried by rotary evaporation and resuspended in 5% MeOH–0.1 M ammonium sulfate. This solution was applied to an octyl sepharose CL-4B (Pharmacia Biotech, Piscataway, NJ) column (22 C × 1.6 cm) previously equilibrated with 5% MeOH–0.1 M ammonium sulfate. The column was eluted with a step gradient of water mixed with increasing quantities of methanol (0, 25 and 80%). Yersiniophore eluted with 25% MeOH–75% H_2O. Both these methods purified biologically active yersiniophore; unfortunately the preparations were unsuitable for structural analysis because of the rapid generation of degradation products.

Chelation of yersiniophore with Al^{3+} followed by HPLC resulted in the purification of a stable Al^{3+}–yersiniophore complex suitable for structural analysis. Yersiniophore was extracted from neutral supernatants with dichloromethane (2:5, v/v) and the dichloromethane was reduced to a few milliliters by rotary evaporation at 35°C. The remaining dichloromethane was removed by twice adding 20 ml MeOH and reducing the solvent volume to a few milliliters. The final 2 3 ml of MeOH was diluted with water to a concentration of 10–20% methanol and most of the insoluble precipitate was filtered with a Nalgene 0.22 μm pore size filter (Baxter Diagnostics, Edmonton, Alberta, Canada). Completely drying the crude yersiniophore during rotary evaporation was found to increase the quantity of

insoluble precipitate and decrease the final yield. The precipitate could be resolubilized in 100% MeOH; however, it never exhibited any iron-binding activity by CAS assay. The precipitate may represent polymerization of yersiniophore similar to that described for anguibactin (Actis *et al.* 1986). Crude yersiniophore in 10–20% MeOH was applied to a DEAE sephacel (Pharmacia) column (1.6 × 25 cm) previously washed with 500 ml 2 M NaCl–2 mM EDTA, 500 ml 2 M NaCl and 500 ml H_2O. Active yersiniophore was eluted with a step gradient of H_2O, 0.01 M NaCl, 0.2 M NaCl and 2.0 M NaCl. Eluent was monitored at A_{250} and by CAS assay.

Al^{3+}–yersiniophore was eluted similarly to unchelated yersiniophore. Once crude yersiniophore was applied to the DEAE–sephacel column, a step gradient of H_2O, 0.01 M $AlCl_3$, 0.2 M NaCl and 2.0 M NaCl was applied. Al^{3+}–yersiniophore, detected at A_{260}, eluted with 0.01 M $AlCl_3$. Fractions from the DEAE column which contained Al^{3+} yersiniophore were pooled, lyophylized and re-solubilized in approximately 5 ml of 40% MeOH–60% H_2O. Al^{3+}–yersiniophore was further purified by HPLC using a Varian 5000 Liquid Chromatograph with a Micropak CN-10 column (30 × 0.8 cm). Al^{3+}–yersiniophore was injected in several batches, 500 µl at a time. Then 40% MeOH–60% H_2O with a flow rate of 3 ml min^{-1} was used to elute Al^{3+}–yersiniophore, which was detected by absorption at A_{260}.

Iron uptake assays

Iron uptake assays were performed as described previously (Chambers & Sokol 1994). Briefly, cultures were grown in iron-poor YMM to a density of 10^8 c.f.u. ml^{-1}. The cells were harvested, washed once with sterile water and resuspended in 10 ml of medium to a final A_{600} of 0.2. Cell suspensions were incubated for 5 min at 28°C with shaking prior to the addition of labeled siderophore. Ferric siderophore was prepared in a 5:1 molar ratio with ^{59}Fe and allowed to equilibrate at room temperature. $^{59}FeCl_3$ was used at 0.5 µCi per assay. The assay was initiated by the addition of labeled yersiniophore. Samples (1 ml) were removed at 0, 2, 5 and 10 min, filtered through 0.2 µm pore size cellulose acetate filters (Sartorius, Goettingen, Germany), and washed with 5 ml of Tris–saline (10 mM Tris, pH 7.5, 0.9% NaCl). The amount of ^{59}Fe accumulated on the filters was measured in an LKB Compugamma counter.

Absorption and fluorescence spectroscopy

Absorption and fluorescence spectra were performed on a 36 µM solution of yersiniophore purified by a combination of anion-exchange and hydrophobic chromatography as well as yersiniophore purified by TLC. Absorption spectra were obtained with a model PU8800 Pye-Unicam spectrophotometer. To determine the absorption spectra of Al^{3+}–yersiniophore, yersiniophore was solubilized in 1 mM $AlCl_3$, whereas for Fe^{3+}–yersiniophore, iron was added to a final concentration of 100 µM. Fluorescence was measured using an Hitachi F2000 fluorescence spectrophotometer to determine both the excitation and emission maxima.

NMR spectroscopy

NMR spectra were recorded at 25°C in CD_3OD solution on a Bruker AMX 500 FT-NMR spectrometer operating at 500.13 MHz for ^1H and 125.77 MHz for ^{13}C NMR. In addition, one-dimensional ^{13}C experiments were recorded on a Bruker AM400 spectrometer at 100.6 MHz. All NMR spectra were referenced to internal TMS (0.0 p.p.m.). The one-dimensional ^{13}C NMR spectra were proton decoupled using a bilevel composite pulse decoupling scheme (2 W during acquisition, otherwise 0.4 W). The two-dimensional NMR experiments, with the exception of the magnitude mode HMBC spectra, were performed in the phase-sensitive mode (TPPI) using standard Bruker software. Typically, 24–112 scans were accumulated per t_1 increment with a recycle time of 2.2 s. For the double quantum filtered COSY experiments, the initial data matrix of 2K × 512 (sweep width of 5000 Hz) was zero-filled to 4K × 2K in the F_2 and F_1 dimensions prior to being apodized with a cosine squared function. The mixing time in the TOCSY experiments varied from 30 to 60 ms, using a spin lock power of 5 W. The ROESY spectrum was recorded with mixing times of 100 and 250 ms and a spin lock power of 5 W. For these two-dimensional experiments, the initial 2K × 400 matrix was zero-filled to 4K × 2K and apodized as before prior to transformation. In the case of the HMQC and HMBC spectra, the data matrix of 1K (^1H, sweep width of 5000 Hz) by 320 (^{13}C, 29 400 Hz) was zero-filled to 4K × 2K and apodized as before. Where necessary, NMR spectra were baseline corrected using a third-order polynmomial function.

Mass spectroscopy (MS)

MS was performed at the University of Alberta, Edmonton, Canada on a Kratos MS50 high resolution (r_p = 10 000) double-focusing mass spectrometer. The spectra were obtained in the electron impact mode with an electron energy of 70 eV. A Mass Spectroscopy Services (MSS) 'MASPEC' data system was used in conjunction with the MS50.

Other analyses

Amino acid analysis was performed by the Protein Sequencing Facility at the University of Calgary. IR spectra were recorded in the Fourier Transform mode (thin film) on a Mattson Instruments 4030 Galaxy Series FT-IR spectrometer. Elemental analyses (EA) were performed at the University of Alberta, Edmonton, Canada.

Results

Production of yersiniophore

Previously it was determined that production of yersiniophore was 5-fold greater in cultures grown at 28°C rather than 37°C (Chambers & Sokol 1994). To determine the phase of growth for optimal yersiniophore synthesis, UC310 was grown in YMM + 50 µM 2,2'-dipyridyl. Cell density was determined by measuring A_{600} and yersiniophore production

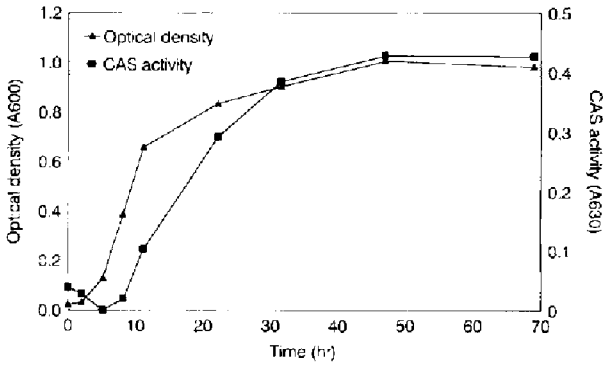

Figure 1. Effect of growth on the production of yersiniophore. Cultures of UC310 (O:8) were grown in YMM + 50 μM 2,2'-dipyridyl at 28°C. Growth was monitored by measuring the optical density (A_{600}) of the culture while yersiniophore production was quantitated by testing culture supernatants with CAS assay solution (A_{630}).

was determined by measuring CAS activity of culture supernatants. CAS activity was first detectable in mid-log phase but peaked along with culture density at around 36–40 h as the culture reached stationary phase (Figure 1). This pattern of siderophore production is similar to other members of the catechol-phenolate class (Neilands 1984).

The effect of iron on yersiniophore production was determined in 50 ml YMM cultures of UC310 grown with various concentrations of 2,2'-dipyridyl (150, 100, 50 and 10 μM) or iron (25, 10, 5 and 0 μM). At 48 h cell density was measured and 25 ml of the supernatant from each culture was extracted with 10 ml ethyl acetate and assayed for iron-binding activity. The extraction was necessary to remove excess iron which would interfere with the CAS assay. Blanks for the CAS assay were prepared from sterile YMM + 50 μM 2,2'-dipyridyl treated in the same manner as the samples. Concentrations of 2,2'-dipyridyl as high as 25 mM had no effect on these CAS assays (data not shown). Culture density remained constant in the iron supplemented cultures, but decreased 4-fold as the concentration of 2,2'-dipyridyl increased to 150 μM. The CAS activity per milliliter of culture increased as 2,2'-dipyridyl concentration increased (Figure 2). In 50 ml cultures, 150 μM 2,2'-dipyridyl resulted in maximum yersiniophore yield; however, when 1 l cultures were grown under these conditions the cell density was too low for yersiniophore purification. It was found that for 1 l cultures, 75 μM 2,2'-dipyridyl provided the optimal combination of growth and yersiniophore production (data not shown). Therefore to purify yersiniophore, cultures were routinely grown in YMM + 75 μM 2,2'-dipyridyl at 28°C for 36–48 h.

Purification of yersiniophore

All purification procedures were initiated by extracting yersiniophore from culture supernatants with either ethyl acetate or dichloromethane followed by removal of the solvent by rotary evaporation. The extract was then solubilized in either methanol or a MeOH–water solution and assayed with CAS assay solution for iron-binding activity. Typically 2–4 μmol of iron-binding activity was extracted per liter of supernatant. It was assumed this activity was due to the presence of yersiniophore present at concentrations of 2–4 μM or 1–2 mg l^{-1}.

Initial purification of yersiniophore was by TLC (see Methods). When extracted supernatants were chromatographed on Silica G plates with chloroform:EtOH:acetic acid (90:5:2.5) as the solvent, a major yellow/green band at $r_f = 0.26$ was resolved along with several other minor fluorescent bands of varying color. In contrast, TLC of extracted supernatants from cultures of non-yersiniophore producing serotypes, such as O:3 and O:9, failed to show any significant fluorescent bands. This suggests the extra bands may be degradation or by-products of yersiniophore biosynthesis. The material eluted from the band at $r_f = 0.26$ was determined by CAS assay to have iron-binding activity and was shown to be biologically active in iron uptake assays with UC310 and YE1165 (data not shown).

^{1}H NMR of the compound which resolved at $r_f = 0.26$ clearly showed four aromatic hydrogens between δ 6.6 and δ 7.4 which integrated in a 1:1:1:1 ratio. The pattern was consistent with an *ortho* di-substituted aromatic compound. The ^{1}H NMR spectrum up field from the aromatic region was not useful for further analysis, suggesting there were still several degradation products in the preparation. This protocol was deemed suitable for the detection of yersiniophore production, but not for the purification of stable yersiniophore.

Yersiniophore was found to adsorb to octyl sepharose (Chambers & Sokol 1994). If yersiniophore was extracted from acidified supernatants with ethyl acetate, iron-binding activity eluted from the octyl sepharose with 80% MeOH–20% H_2O. When neutral supernatants were extracted with dichloromethane the iron-binding activity could be eluted from the octyl sepharose column with 25%

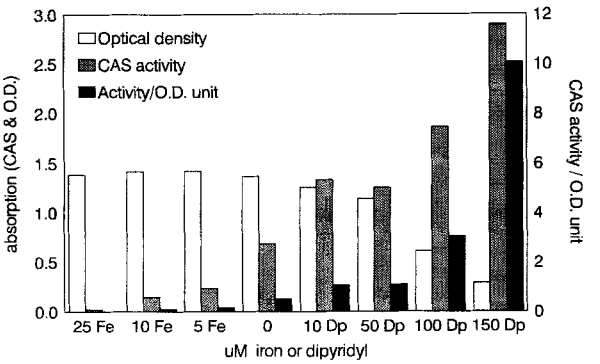

Figure 2. Effect of iron and 2,2'-dipyridyl on yersiniophore production. Cultures were grown in YMM at 28°C for 48 h. Media was supplemented with either $FeCl_3$ or 2,2'-dipyridyl as indicated. Growth was monitored by measuring the A_{600} of the culture. For CAS assays, culture supernants were first extracted with ethyl acetate, dried by rotoevaporation and resuspended in MeOH.

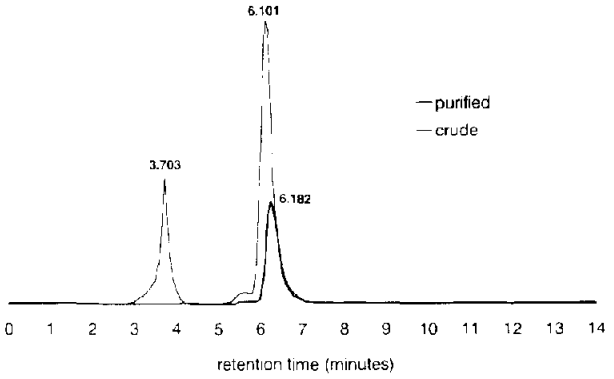

Figure 3. HPLC separation of yersiniophore on a Micropak CN-10 column (30 × 0.8 cm) using an isocratic gradient of 40% MeOH and 60% H_2O at a flow rate of 3 ml min^{-1}.

MeOH–75% H_2O and the yield was greater, approximately 50% of the activity originally loaded onto the column. This suggested that treating supernatants with acid resulted in a degraded product. Interestingly both species were biologically active in the iron uptake assay.

Lyophylizing the product eluted from the octyl sepharose column resulted in a complete loss of iron-binding activity. Low-resolution mass spectroscopy of the lyophylized product gave an observed *m/z* of 142.1 for the base peak. A similar peak (*m/z* of 142.0684) was detected by high-resolution mass spectroscopy of Al^{3+}–yersiniophore. These two peaks likely represent the same degradation product. No peak was seen at the expected *m/z* of 478 for the unchelated siderophore, suggesting that the intact siderophore was not recovered after lyophylizing. Therefore to concentrate the product eluted from the octyl sepharose column, the eluent was extracted with dichloromethane and dried by rotary evaporation. ^1H NMR of this preparation of yersiniophore showed the signature peaks in the aromatic region, but purity was not sufficient for further analysis. When attempts were made to further purify yersiniophore by HPLC no iron-binding activity was ever recovered.

A final purification protocol was devised which resulted in the isolation of stable yersiniophore. Yersiniophore was extracted and prepared for anion-exchange chromatography as described in the methods. Crude sample was loaded onto the DEAE column in a solution of 10–20% MeOH. Water was used to wash through non-adsorbing compounds, which included 2,2′-dipyridyl, followed by a step gradient of 0.01, 0.2 and 2.0 M NaCl. Eluent was monitored at A_{260} for the appearance of yersiniophore. The only peak detected at A_{260}, other than the wash, was a small peak which eluted with 0.2 M NaCl and typically contained 45–55% of the iron-binding activity loaded onto the column. The H_2O wash and the peak which eluted with 0.2 M NaCl exhibited quantitatively similar iron-binding activity by CAS assay; however, only the second peak was biologically active in iron uptake assays (data not shown).

To stabilize yersiniophore it was chelated to Al^{3+}. The DEAE column was loaded as before with crude yersiniophore and washed with water to elute non-adsorbing compounds. Several hundred milliliters of 0.01 M $AlCl_3$ were then applied to the column to elute Al^{3+}–yersiniophore (detected by absorption at A_{260}). Further application of 0.2 and 2.0 M NaCl failed to produce any other peaks, indicating that all the yersiniophore had eluted with the 0.01 M $AlCl_3$ buffer. It was assumed that yersiniophore chelated aluminum causing it to lose its anionic charge and desorb from the DEAE column. Similar concentrations of NaCl were unable to elute the siderophore.

Once the $AlCl_3$ solution was applied to the DEAE column, 25 μl of each fraction eluted from the column was diluted to 1 ml with water and measured for absorption at 260 nm. Absorption measurements showed a slow elution of Al^{3+}–yersiniophore with readings reaching a maximum optical density of approximately 0.3. Fractions containing Al^{3+}–yersiniophore in 10 mM $AlCl_3$ were pooled (final volume approximately 75 ml) and lyophylized overnight. The resulting mixture of aluminum salt and Al^{3+}–yersiniophore was resuspended in approximately 5 ml of 40% MeOH 60% H_2O. Final purification was achieved by HPLC using a CN-10 column (30 × 0.8 cm) with a 500 μl loop for loading sample. An isocratic solvent of 40% MeOH–60% H_2O at a flow rate of 3 ml min^{-1} was used to resolve Al^{3+} yersiniophore from the salt. Al^{3+}–yersiniophore was again detected by absorption at A_{260}. Two major peaks were identified at 3.703 and 6.101 min, the later peak typically containing 80–90% of the total absorption units. Al^{3+}–yersiniophore was assigned to the later peak by comparing absorption profiles for both peaks resolved by HPLC with the profile for active yersiniophore in a 1 mM solution of $AlCl_3$. To confirm purity, 10 μl samples of crude and purified yersiniophore were resolved by HPLC and retention times compared (Figure 3). Finally, the purified Al^{3+}–yersiniophore was lyophylized to a slightly pink powder. A typical yield from 20 l of YMM supernatant was 7–9 mg (14–18 μmol), which indicates that approximately 40% of the original iron-binding activity extracted from the supernatant was recovered as Al^{3+}–yersiniophore.

Absorption and fluorescence spectra

These spectra were determined for active yersiniophore purified by anion-exchange chromatography, followed by hydropyhobic chromatography. Absorption spectra of a 36 μM solution of active yersiniophore showed maxima at A_{210} and A_{250} with a low broad peak occuring from A_{280} to A_{320}. A similar absorption spectra was found for yersiniophore isolated by TLC. Yersiniophore (36 μM) in a 1 mM $AlCl_3$ solution showed maxima at A_{210} and A_{260} with a sloping shoulder from A_{295} to A_{335}. Yersiniophore (36 μM) in a 100 μM $FeCl_3$ solution showed a significantly decreased peak at A_{210}, a second peak at A_{265} and a third broad peak from A_{290} to A_{320}. The addition of iron also caused a charge transfer band at 400 nm not seen with Al^{3+}–yersiniophore (Figure 4A). No strong color change was observed in the iron chelated species.

Fluorescence spectra were obtained by determining the excitation maxima and then scanning for the emission

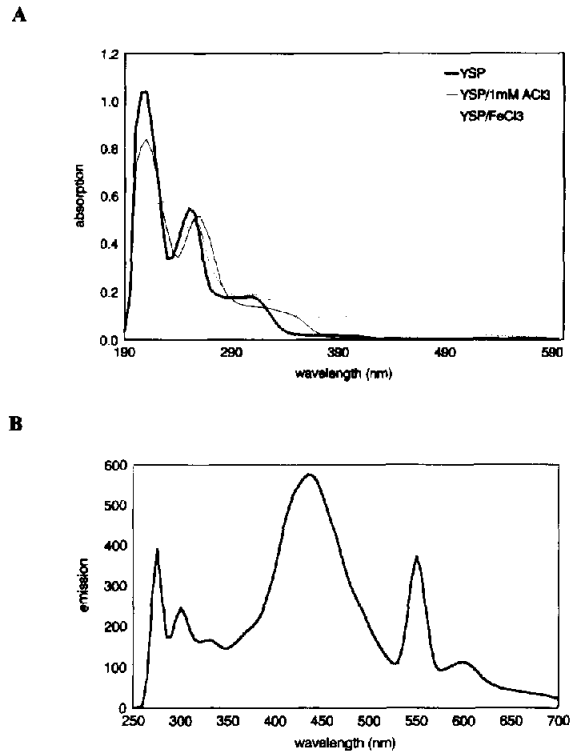

Figure 4. Spectrophotometric characterization of yersiniophore. (**A**) Absorption spectra (190–600 nm) of 36 μM yersiniophore unchelated and solubilized in 1 mM $AlCl_3$ or 100 μM $FeCl_3$. (**B**) Emission spectra (250–700 nm) of 36 μM active yersiniophore solubilized in Milli-Q water and excited at 270 nm.

spectra. Yersiniophore which was purified using hydrophobic chromatography was examined with Milli-Q water as the solvent while yersiniophore isolated by TLC was examined in 100% methanol. Both preparations showed maximum excitation at 270 nm with a major emission peak at 428 nm; however, yersiniophore isolated by TLC was not fluorescent in the presence of H_2O (fluorescence was completely quenched in 90% MeOH–10% H_2O) while yersiniophore which was eluted from octyl sepharose showed a reduced but detectable fluorescence in 25% MeOH–75% H_2O (Figure 4B). Neither Al^{3+}– nor Fe^{3+}–yersiniophore was fluorescent.

NMR Spectroscopy

The signals in the one-dimentional proton NMR spectrum (Figure 5 and Table 1) were broad, therefore only the H-17 geminal coupling constant could be resolved. Signal broadening is most likely due to complexation with traces of ferric iron. The chemical shifts and couplings of the signals in the aromatic region of the spectrum were consistent with an *ortho* di-substituted benzene ring. ^{13}C NMR spectroscopy (Figure 5B and Table 1) indicated that this ring was in fact phenolic. One -NH signal, with a chemical shift dependent on experimental conditions, was also observed in the proton NMR spectrum. The -NH proton was part of a spin system involving four methine and two methylene protons, as revealed by the DQF-COSY and TOCSY spectra. The DQF-COSY spectrum (Figure 6) showed all expected couplings, with the exception of the H-13/H-14 coupling (see Figure 10 for assignments). In particular, the aromatic spin system as well as the spin system involving protons H-8 to H-13, inclusive, were assigned from the DQF-COSY spectrum. These assignments were confirmed by the use of TOCSY spectra recorded at various mixing times (not shown). The three methyl groups did not couple to other protons in the DQF-COSY spectra.

The HMQC (Figure 7) spectrum showed all expected one-bond C–H correlations confirming that H-11 is not bonded to a carbon. In addition, the HMBC (Figure 8) spectrum supported the assignments in the terminal thiazoline ring, which exhibits extensive long range coupling and allows the assignment of the carbon signals at 193.8 and 178.2 p.p.m. to C-16 and C-22, respectively. The positions of C-14 and C-15 are also defined in the HMBC spectrum as H-21 was observed to couple to both C-16 and

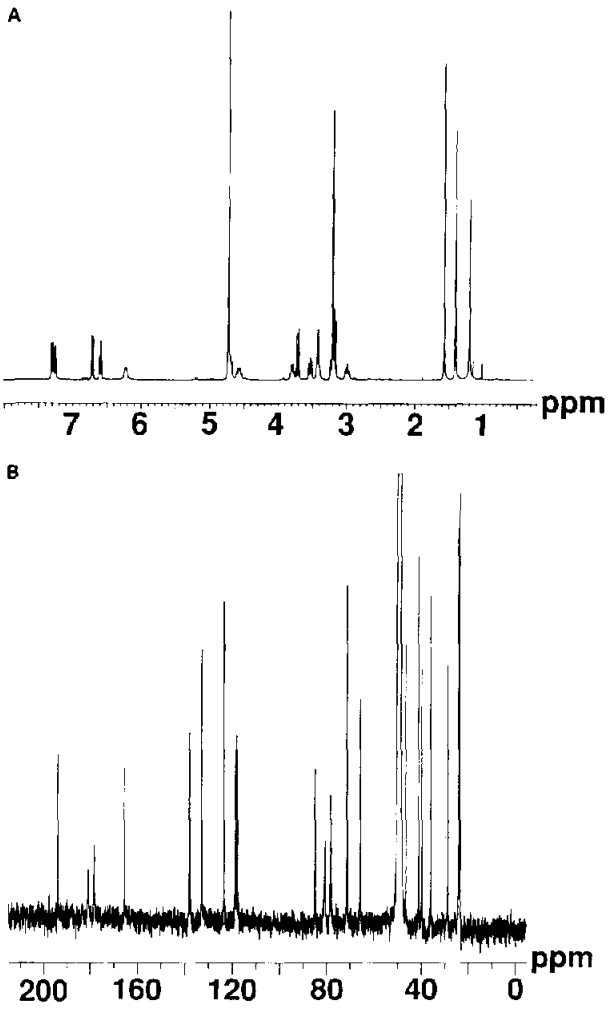

Figure 5. (**A**) ^{1}H and (**B**) ^{13}C one-dimensional NMR spectra of Al^{3+}–yersiniophore.

Table 1. ^{13}C and ^{1}H NMR absorptions for yersiniophore compared with ^{13}C absorptions for pyochelin

Position	Group	Carbon shift	Proton shift	Carbon shifts of pyochelin[a]
1	aromatic C	165.27 (s)		158.8
2	aromatic CH	123.40 (d)	6.71	117.4
3	aromatic CH	132.70 (d)	7.26	133.5
4	aromatic CH	118.49 (d)	6.59	119.2
5	aromatic CH	137.77 (d)	7.30	131.0
6	aromatic C	117.73 (s)		116.5
7	thiazoline C	180.85 (s)		173.2
8	thiazoline CH_2	35.62 (t)	3.24, 3.54	33.6
9	thiazoline CH	78.02 (d)	4.56	74.3
10	thiazolidine CH	71.13 (d)	4.71	77.7
11	thiazolidine NH		6.22	
12	thiazolidine CH_2	39.48 (t)	2.98, 3.41	33.6
13	thiazolidine CH	65.54 (t)	3.78	76.3
14	CHOH	80.42 (t)	3.44	
15	C	46.27 (s)		
16	thiazoline C	193.79 (s)		
17	thiazoline CH_2	40.80 (t)	3.17, 3.718	
18	thiazoline C	84.69 (s)		
19	CH_3	23.26 (q)	1.56	
20	CH_3	28.44 (q)	1.39	
21	CH_3	23.78 (q)	1.18	
22	COOH	178.23 (s)		

[a]Chemical shifts of pyochelin dissolved in $CDCl_3$ (Cox *et al.* 1981).

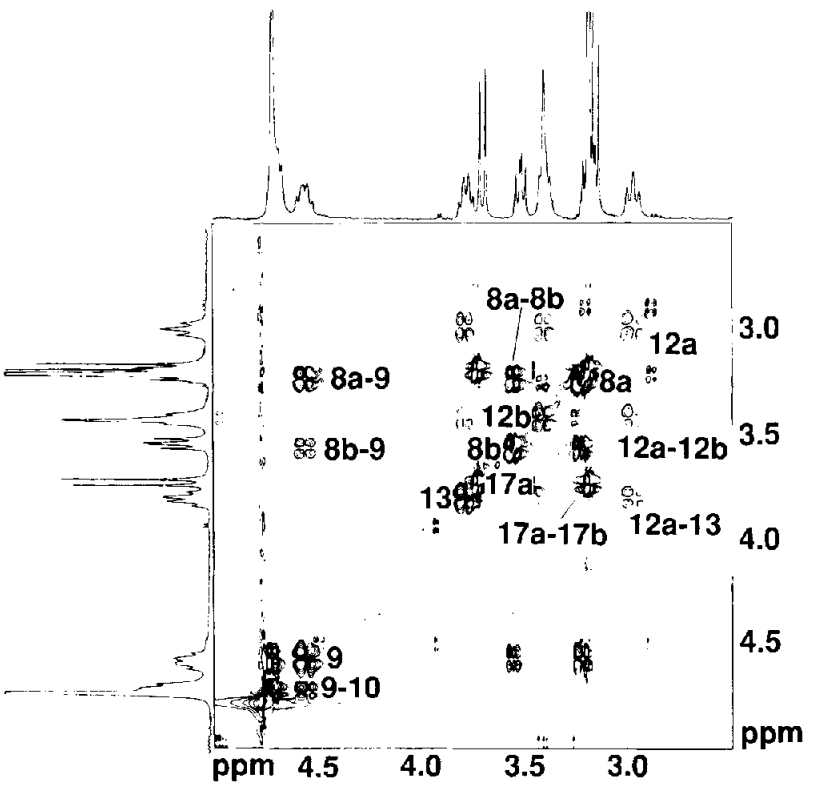

Figure 6. Aliphatic region, methyls excluded, of the phase sensitive DQF-COSY spectrum of Al^{3+}–yersiniophore. Selected diagonal and cross-peaks are labeled with proton number (Figure 10).

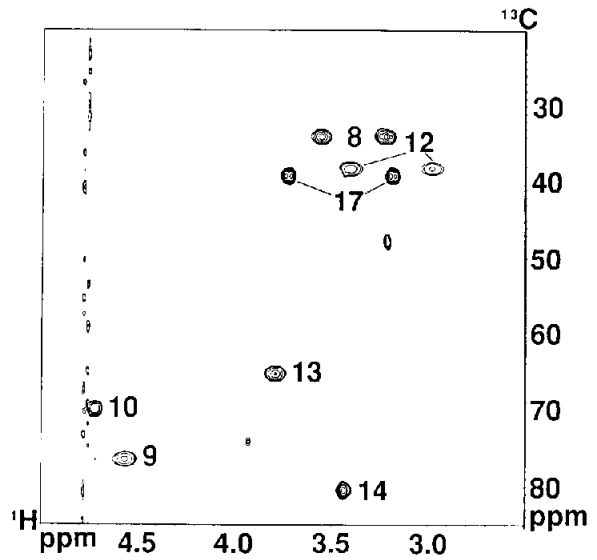

Figure 7. Aliphatic region, methyls excluded, of the phase sensitive HMQC spectrum of Al^{3+}–yersiniophore. Selected diagonal and cross-peaks are labeled with carbon number (Figure 10).

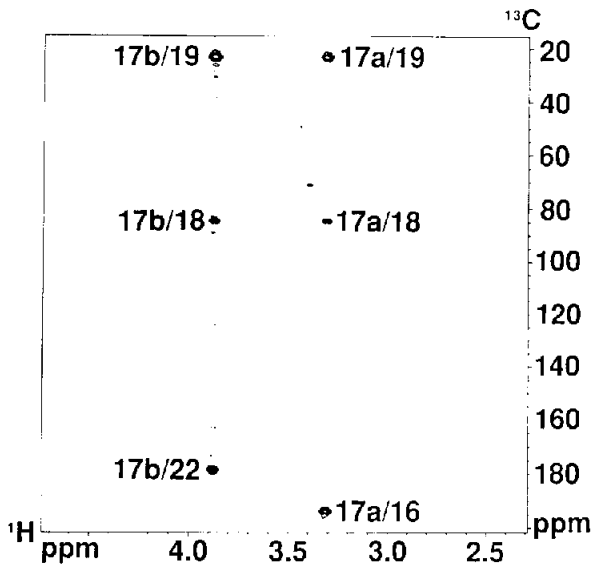

Figure 8. Magnitude mode HMBC spectrum of the terminal thiazoline ring. Delay time 80 ms.

C-14, suggesting that it is attached to a carbon between these two carbons. H-5 was coupled to C-7, thus allowing the assignment of the peak at 180.7 p.p.m. to that carbon. The aromatic system showed the expected long range couplings.

In the ROESY spectrum, correlations were observed from H-14 to H-21 and H-20, as well as between H-13 and H-20. The H-2 proton also exhibited a correlation to H-19, indicating that these protons are in relatively close proximity. A remaining problem was the positioning of C-14 and C-15. In the HMBC spectrum, H-21 is coupled to both C-14 and C-16, which is consistent with the proposed structure. However, the ROESY spectrum shows a correlation between H-20 and both H-13 and H-14. Another unusual observation is the lack of coupling between H-13 and H-14. This may result from the rigid nature of the complex, holding the dihedral angle between these protons near 90° resulting in a very small coupling constant. Also, if O-14 is involved in coordination to the aluminum, this coupling may be reduced so as to make it unobservable.

Other analyses

The amino acid analysis showed one ninhydrin positive peak at very low concentration in the proline position; however, the absorption ratio A_{440}/A_{570} was incorrect for this amino acid. The compound may be a residual contaminant, possibly 2,2′-dipyridyl. It was concluded that the yersiniophore structure did not include any amino acid moieties.

Elemental analysis indicated that the probable formula was $C_{21}H_{24}N_3O_4S_3Al$ which was consistent with NMR data. This was confirmed by high resolution mass spectroscopy (Figure 9), which gave an accurate molecular mass of 505.07404 Da for the molecular ion (calculated molecular weight for $C_{21}H_{24}N_3O_4S_3Al$ is 505.07433 Da). Other major large fragments were observed at m/z values of 461.0844, resulting from a loss of CO_2 due to decarboxylation of the carboxylic acid group (calculated for $C_{20}H_{24}O_2N_3S_3Al$, 461.0846), at m/z 490.0516, resulting from a loss of CH_3 (calculated for $C_{20}H_{21}N_3O_4S_3Al$, 490.0510) and at m/z 446.0609, resulting from a loss of both CO_2 and CH_3 (calculated for $C_{19}H_{21}N_3O_2S_3Al$, 446.0610). A fragment at 319 likely results from fission of the C-14/C-15 bond; however, due to close proximity of the PFK fragment (C_6F_{13}) at m/z 318.9797 it was not possible to get an exact mass (calculated for $C_{12}H_{12}O_2N_2S_2Al$, 319.0189). Major fragments are also observed at m/z 142.684, which likely represents the decarboxylated terminal thiazoline ring with attached isorpropyl group containing carbons 15–21 (calculated for $C_7H_{12}NS$, 142.0690), at m/z 263.9971 (calculated for $C_{10}H_9ON_2S_2Al$, 263.9971) and at m/z 273.0278 (calculated for $C_{12}H_{10}O_2N_2S_2Al$, 273.0278). The last two fragments presumably result from skeletal rearrangements.

The IR spectrum showed no evidence of a ketone carbonyl (not shown). The major peaks present in this spectrum were at 3380 (broad, -OH and -NH), 1667, 1607 (carboxylate), 1583, 1540, 1468, 1452 and 1432 cm^{-1}. The structure shown in Figure 10 was elucidated based on NMR techniques, MS and EA.

Discussion

Contradictory reports of siderophore production by pathogenic *Y. enterocolitica* over the last 20 years (Wake *et al.* 1975, Perry & Brubaker 1979) suggest that these strains may produce a short-lived, unstable siderophore (Heesemann *et al.* 1993), likely found in low concentrations. Our

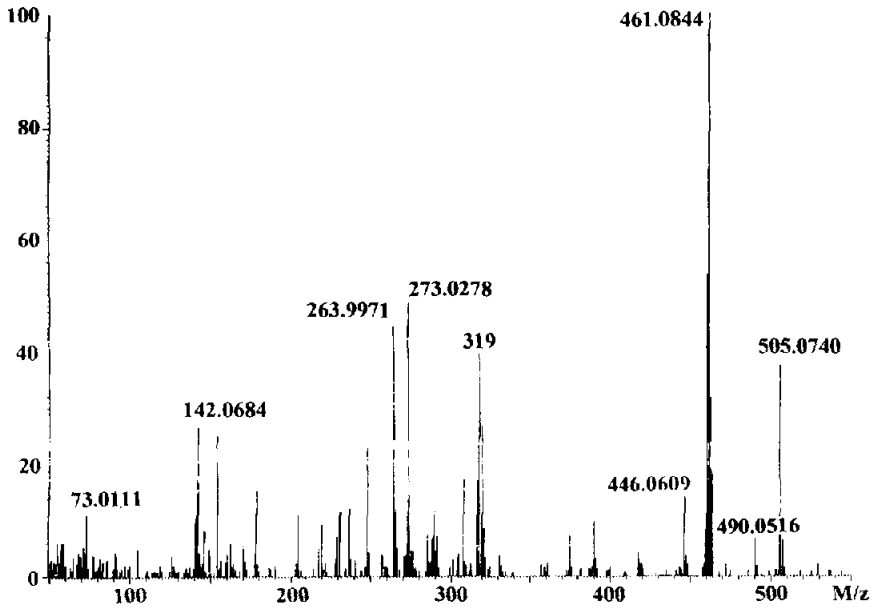

Figure 9. High-resolution electron impact mass spectrum of Al^{3+} yersiniophore.

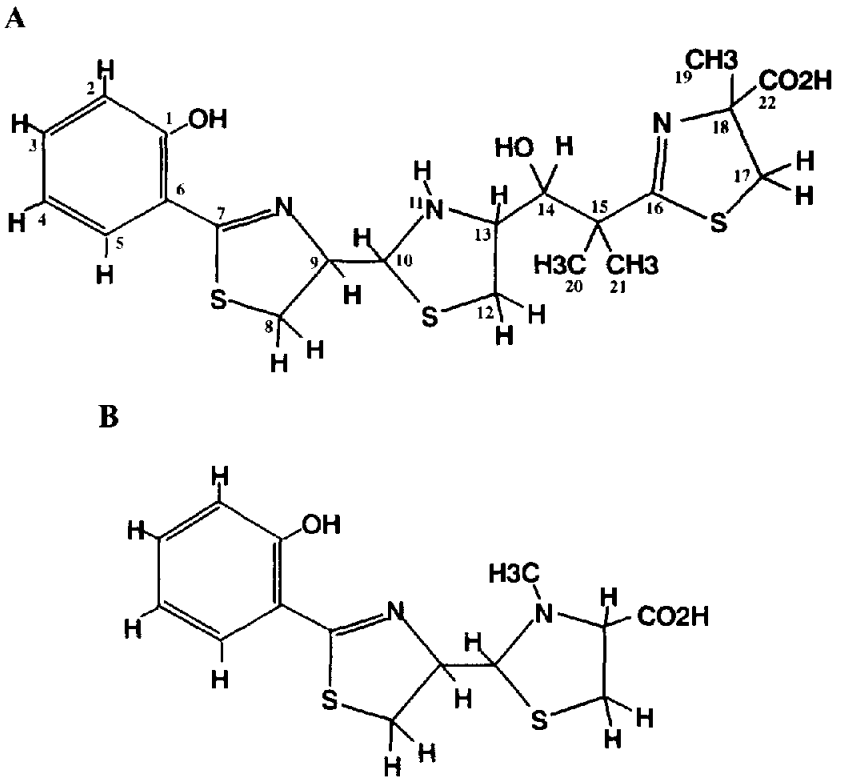

Figure 10. Structures of siderophores. (**A**) Proposed structure of yersiniophore and (**B**) structure of pyochelin (Cox *et al.* 1981). Fission of the C-14/C-15 bond results in two fragments. *m/z* values correlating to fragments of 319 and 142.0684 were detected by high resolution MS (Figure 9).

observations of yersiniophore support these speculations. Indirect evidence of siderophore production by mouse-lethal serotypes of *Y. enterocolitica* has been shown by the secretion of iron-binding compound(s) into CAS agar. Further investigation of these compounds has identified three siderophores produced by the species of *Y. enterocolitica*. One, yersiniabactin, has been isolated from the clinical strain WA-C and is partially characterized as a catechol moiety

of molecular weight 482 Da (Haag *et al.* 1993). Secondly, a hydrophilic siderophore has been partially purified from the supernatant of an environmental isolate. This siderophore was negative for catechol and hydroxamate chemical assays but has not been further characterized (Chambers & Sokol 1994). Finally, yersiniophore, a phenolate siderophore isolated in low yields, is produced by mouse-lethal clinical isolates (Chambers & Sokol 1994). In this study Al^{3+}–yersiniophore was purified to homogeneity and its structure determined.

Although yersiniabactin and yersiniophore have both been purified from clinical strains of serotype O:8 they appear to have distinctly different chemical natures. Yersiniabactin is also produced in far greater quantities, perhaps 50 times more (Haag *et al.* 1993), than yersiniophore, when grown in a defined medium. The relatedness of these two siderophores will be better understood once the structure of yersiniabactin is determined.

Purification of biologically active yersiniophore could be achieved using two different methods. In all protocols yersiniophore was first extracted with organic solvent and concentrated by rotary evaporation. At this step the recovered iron-binding activity was 2–4 μmol l^{-1} of extracted supernatant. This was used as a crude estimate for the concentration of yersiniophore produced in culture since directly assaying such low concentrations of siderophore in the supernatant was not quantitative due to background interference. Further purification by TLC was found to degrade the product but proved to be a simple method for assaying for the production of yersiniophore. Purification utilizing hydrophobic chromatography was used for isolating stocks of yersiniophore to be used in biological assays, chemical assays for catechols and hydroxamates, and absorption and fluorescence spectral analysis. However, NMR spectroscopy of these preparations of yersiniophore indicated that the active siderophore was neither stable nor pure enough for structure determination. Resolution of unchelated yersiniophore by HPLC was unsuitable for determining the purity of these preparations since iron-binding activity could not be recovered, indicating that active yersiniophore degraded during this procedure. Typically about half the iron-binding activity loaded onto either the octyl sepharose or the DEAE columns was recovered. It is not known whether the lost activity was due to degradation during the chromatography step or whether the initial estimate of activity included iron-binding species other than yersiniophore.

For NMR, EA and MS analysis Al^{3+}–yersiniophore was purified by anion-exchange chromatography and HPLC. The chelated product was found to be considerably more stable than the active siderophore: Al^{3+}–yersiniophore could be stored for several weeks at room temperature in a solution of CD_3OD without significant change in the NMR spectra. Interestingly, comparison of ^1H NMR spectroscopy experiments performed on the product several weeks apart showed no observable reduction in the signal corresponding to H-11 (-NH). This suggests that the proton is not exposed to the solvent and therefore not available for exchange when the siderophore is chelated to aluminum.

The procedure resulted in an almost 100% pure preparation of Al^{3+}–yersiniophore. A small amount of contamination was seen in the ^1H NMR causing small peaks to consistently appear at 1.10 and 1.22 p.p.m.; however, they did not interfere with the interpretation of the spectra. It should also be noted that small shifts in the ^1H NMR, particularly H-11 (-NH), were seen when spectra were compared from different sample preparations. These shifts are most likely due to differences in the pH of the sample as it was not standardized during the purification protocol. Broadening of the peaks also varied between samples, likely due to slight contamination of the siderophore with iron. The proposed structure for yersiniophore (Figure 10) is consistent with the spectroscopic data as well as the elemental analysis. Presumably the coordination between yersiniophore and Al^{3+} or Fe^{3+} is hexadentate, the metal ion coordinating in a distorted octahedral fashion (Hider 1984). The detailed three-dimensional structure of this complex is a matter of conjecture; however, from the ROESY data H-2, on the aromatic ring, must be in close proximity to H-19, on the terminal thiazole ring.

Yersiniophore appears to belong to a small sub-group of phenolate siderophores which includes pyochelin and anguibactin. These siderophores all incorporate thiazole rings into their structure. The structural similarity between pyochelin (Cox *et al.* 1981) and yersiniophore is striking (Figure 10) as evidenced by their matched chemical shifts (Table 1). The observed differences in chemical shifts seen may be, in part, due to the changed chemical environment of the chelated species. Although anguibactin does not share as much structural similarity to yersiniophore as pyochelin, many of its physical characteristics appear to be similar. Anguibactin is a catechol-thiazole compound (Jalal *et al.* 1989) with an extended linker region where yersiniophore has a thiazolidine ring. Isolation of anguibactin was made difficult by its susceptibility to hydrolysis in acid and by its oxidation and polymerization in concentrated solution. Yields of anguibactin were also found to be low, about 2 mg l^{-1}. These phenolate siderophores, which are relatively unstable *in vitro*, may eventually demonstrate unique advantages for the microorganism in the host environment.

Acknowledgements

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