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Purification, spectroscopic analysis and biological activity of the macrocyclic dihydroxamate siderophore alcaligin produced by *Bordetella pertussis* and *Bordetella bronchiseptica*

Timothy J. Brickman, Jan-Gerd Hansel*, Marvin J. Miller* & Sandra K. Armstrong

*Department of Microbiology and Immunology, East Carolina University School of Medicine, Greenville, NC and *Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, IN, USA*

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Hydroxamate siderophores of virulent *Bordetella pertussis* and *Bordetella bronchiseptica* strains were purified using a simple large-scale isolation procedure, and identified by various spectroscopic techniques as the macrocyclic dihydroxamate siderophore trivially known as alcaligin, 1,8(*S*),11,18(*S*)-tetrahydroxy-1,6,11,16-tetraazacycloeicosane-2,5,12,15-tetrone, which was previously isolated from the taxonomically-related bacterial species *Alcaligenes denitrificans* subsp. *xylosoxydans*. Alcaligin purified from iron-depleted cultures of *B. pertussis* and *B. bronchiseptica* exhibited specific growth-promoting activity under iron-restricted conditions for *Bordetella* indicator strains, and were active in [^{55}Fe]ferric alcaligin transport assays. Evidence suggests that several C_2-symmetric conformations of alcaligin exist simultaneously in both methanolic and aqueous solution.

Keywords: iron, siderophore, alcaligin, *Bordetella pertussis*, *Bordetella bronchiseptica*, siderophore purification, iron transport

Introduction

Sequestration of iron is a host defense against disease-causing infectious agents. The ability of invading pathogenic microorganisms to multiply in host tissues is fundamental to the establishment of infection and successful multiplication of a pathogen depends on its ability to acquire essential nutrients, among which the biometal iron is almost universally limiting in availability.

Microbial strategies aimed at defeating host iron restriction may involve the action of a siderophore. Siderophores are low molecular mass, high-affinity, ferric iron-specific chelators of microbial origin that are synthesized along with their cognate receptors and transport machinery in response to iron starvation (Lankford 1973). Chemically, most siderophores may be arbitrarily classed as hydroxamates or phenolates on the basis of their iron-coordinating groups. The role of siderophores in the virulence of a number of pathogenic microorganisms is well established (Weinberg 1978, 1984).

Bordetella pertussis, the etiologic agent of human whooping cough, and *Bordetella bronchiseptica*, the agent of swine atrophic rhinitis and kennel cough in dogs, are Gram-negative pathogens of the upper respiratory tracts of their mammalian hosts. As mucosal pathogens, these bacteria adhere and multiply in a niche bathed in the iron-sequestering host glycoprotein lactoferrin. In early investigations of nutritional iron sources for *B. pertussis*, siderophore-like compounds were not detected in iron-restricted culture supernatants using chemical assays for phenolates and hydroxamates (Redhead *et al.* 1987). Using the highly versatile and sensitive chrome azurol S (CAS) universal siderophore detection method of Schwyn & Neilands (1987) along with the Csaky assay for hydroxamates (Csaky 1948), *B. pertussis*, *B. bronchiseptica* and the related pathogenic species *Bordetella parapertussis* were later shown to elaborate a putative hydroxamate-class siderophore in response to iron starvation (Gorringe *et al.* 1990). Subsequently, other investigators (Agiato & Dyer 1992) reproduced some of these findings, reporting the detection of CAS-reactive, Csaky-positive material in iron-starved culture supernatants of *B. pertussis*. Absorption spectroscopy of spent *B. pertussis* culture supernatants revealed the presence of a ferric iron-reactive substance, presumably a siderophore, which the authors named bordetellin.

In the first molecular genetic studies of *Bordetella* iron acquisition systems, Armstrong & Clements (1993) generated *B. bronchiseptica* mutants deficient in siderophore activity

Address for correspondence: T. J. Brickman, Department of Microbiology and Immunology, East Carolina University School of Medicine, Greenville, NC 27858, USA. Tel: (+1) 919 816-3798; Fax: (+1) 919 816-3535.

by transposon mutagenesis. High stringency DNA hybridization analysis using DNA probes derived from genomic sequences flanking the transposon insertions revealed that homologs of *B. bronchiseptica* genes required for siderophore production were present in *B. pertussis*. Crude siderophore preparations of *B. pertussis* and *B. bronchiseptica* were biologically active in supplying iron to the opposing species in reciprocal cross-feeding experiments, lending further support to the notion that hydroxamate siderophores produced by these related species were structurally similar if not identical.

A report appearing in the literature while this manuscript was in review described the purification by HPLC of siderophores produced by iron-starved *B. pertussis* and *B. bronchiseptica* (Moore *et al.* 1995). Analysis revealed identity of the purified *Bordetella* siderophores with the known siderophore alcaligin produced by *Alcaligenes denitrificans* subsp. *xylosoxydans* (Nishio *et al.* 1988, Nishio & Ishida 1990). Concurrent studies described in this report corroborate and extend these observations.

We present the purification and structural characterization of biologically active hydroxamate siderophores produced by *B. pertussis* and *B. bronchiseptica* under conditions of iron starvation. Spectroscopic analysis independently determined that siderophores of both *Bordetella* species are identical to the previously described macrocylic dihydroxamate siderophore alcaligin produced by the taxonomically-related bacterial species *A. denitrificans* subsp. *xylosoxydans* (Nishio *et al.* 1988, Nishio & Ishida 1990). We also report the development of a significantly improved purification procedure facilitating the isolation of large quantities of crystalline deferrisiderophores from *Bordetella* culture supernatants. Biological activity of the purified siderophores was evidenced by their ability to reverse the iron restriction-imposed growth inhibition of a diverse collection of *Bordetella* siderophore-deficient mutants, as well as by quantitative ^{55}Fe uptake by iron-starved *Bordetella* cells in [^{55}Fe]ferric alcaligin transport assays. New evidence obtained by high resolution proton and carbon NMR spectroscopy analysis is consistent with the symmetry of alcaligin, although the data suggest that several C_2-symmetric conformations of alcaligin are present at the same time in both methanolic and aqueous solution.

Materials and methods

Bacterial strains, growth media and culture conditions

Virulent-phase. *B. pertussis* UT25 was described previously (Field & Parker 1979). *B. bronchiseptica* strain B013N, a nalidixic acid-resistant derivative of virulent strain B013, and the generation of siderophore deficient Group I and III mutants BRM1, BRM6, BRM9, BRM3 and BRM5 of *B. bronchiseptica* B013N by insertional inactivation were previously described (Armstrong & Clements 1993). *B. bronchiseptica* B013N Mn^{r}4, a *fur* mutant derivative of strain B013N that constitutively expresses siderophore activity, was described by Brickman & Armstrong (1995). *B. pertussis* UT25D, a Phase IV derivative of UT25 adapted to growth on bloodless media (Armstrong & Parker 1986), was maintained on standard Luria Bertani (LB) agar plates. *B. pertussis* UT25 was maintained on Bordet–Gengou agar plates (Bordet & Gengou 1906), and *B. bronchiseptica* on blood agar or LB agar plates with nalidixic acid at 35 μg ml^{-1} and kanamycin at 50 μg ml^{-1} when appropriate. Iron-replete and iron-depleted Stainer–Scholte (Stainer & Scholte 1970, Schneider & Parker 1982) broth (SS) culture conditions were achieved by the methods of Armstrong & Clements (1993). Optical densities of SS cultures were monitored with a Klett-Summerson colorimeter fitted with a no. 54 filter (Klett, Long Island City, NY).

Materials

All glassware was acid-cleaned and rinsed repeatedly in distilled deionized water prior to use. When appropriate, solutions and growth media were rendered iron-free by treatment with Chelex-100 resin (BioRad, Richmond, CA). Ethylenediaminedi[(*o*-hydroxyphenyl)acetic acid] (EDDA), benzyl alcohol, diethyl ether, antibiotics and precoated silica gel TLC plates were from Sigma (St Louis, MO). EDDA was deferrated by the method of Rogers (1973).

Siderophore assays

The CAS universal siderophore detection assay (Schwyn & Neilands 1987) was used to monitor siderophore production by *Bordetella* strains grown in liquid culture as reported previously (Armstrong & Clements 1993) and to measure iron-chelating activities of purified siderophores. Determination of hydroxamates was by the method of Csaky (1948) using hydroxylamine hydrochloride as the standard. Siderophore assays were performed in triplicate in multiple experimental trials.

Purification of siderophores produced by Bordetella *species*

Siderophores were purified from *Bordetella* culture supernatants by a modification of the benzyl alcohol/ether extraction method reported by Neilands (1952) for purification of the prototypic hydroxamate siderophore ferrichrome from cultures of the rust fungus *Ustilago sphaerogena*. *Bordetella* cells were subcultured 1:200 from late logarithmic phase iron-replete SS cultures into 1 l volumes of iron-depleted SS in 4 l Fernbach flasks and grown with vigorous shaking at 37°C. Cultures were sampled at approximately 12 h intervals and assayed for the production of siderophore activity by the CAS method. Maximal levels of siderophore activity were generally attained in 3–4 days for *B. bronchiseptica* B013N and B013N Mn^{r}4, and 4–5 days for *B. pertussis* UT25. Bacterial cells were removed from mature cultures by two successive centrifugation steps and the culture supernatant sterilized by vacuum filtration through cellulose acetate filters (0.2 μm pore size) with glass fiber prefilters. The cell-free sterilized medium was brought to 100% saturation with solid ammonium sulfate at 0°C and then held at 0°C with stirring for 1 h. The resulting precipitate was removed by centrifugation, and the cleared

supernatant adjusted to pH 7.0 at room temperature using 1 N HCl.

A 500 ml aliquot of the cleared supernatant was shaken with 25 ml of benzyl alcohol in a 1 l separatory funnel. This extraction was repeated and the benzyl alcohol layers combined and washed with 5 ml of distilled deionized water. The washed organic phase was returned to a clean separatory funnel, then 150 ml of diethyl ether and enough deionized water to give a volume of approximately 10 ml were added. After vigorous shaking, the orange-colored aqueous bottom layer was drawn off and replaced with an additional 5 ml of H_2O. After this second extraction, the aqueous layers were pooled and shaken with 5 ml of diethyl ether and the ether wash was discarded. The aqueous solution was concentrated to dryness under reduced pressure at room temperature to yield the semipure siderophore.

For small-scale purification of siderophores by preparative TLC, portions of the dried semipure siderophore residue resulting from benzyl alcohol/ether extraction were dissolved in a minimal volume of deionized water and the resulting solution applied to TLC plates precoated with silica gel. The chromatographs were developed with *n*-butanol:acetic acid:water (4:1:5, upper phase) and the positions of Fe(III)-reactive species visualized by spotting a solution of 1% $FeCl_3$ in 1 mM HCl at the edge of the TLC plate, which produced a deep red-colored siderophore complex. The silica gel mixture at the remaining area of the chromatograph containing the colorless deferrisiderophore was scraped from the plate and the siderophore eluted with distilled deionized water. The aqueous siderophore was dried to constant weight under vacuum at room temperature, yielding a white powder that was stored at 4°C.

For simplified large-scale preparation of crystalline deferrisiderophores, the dried semipure residue recovered by benzyl alcohol/ether extraction was dissolved in a minimal volume of hot 95% ethanol. Colorless deferrisiderophore crystals formed readily upon cooling of this solution to room temperature and were recovered by brief centrifugation, whereas orange-colored ferric siderophore complexes formed with residual amounts of contaminating iron remained preferentially soluble. Multiple recrystallization steps from 95% ethanol yielded pure colorless crystalline *Bordetella* deferrisiderophores. Routine recoveries of purified siderophore approximated 25–50 mg l^{-1} of culture. Siderophores were stored in the dark at 4°C as dry crystals or as concentrated aqueous solutions, which retained full biological activity indefinitely.

Absorption spectroscopy

Visible and UV spectroscopy used a Beckman Du®-65 spectrophotometer (Beckman Instruments, Fullerton, CA).

Mass spectrometry

Fast atom bombardment (FAB) mass spectra were recorded on a Finnigan MAT Model 8430 spectrometer using glycerol for sample preparation.

NMR spectroscopy

NMR spectra were obtained at 20°C on a Varian VXR 500-S instrument operating at 500 MHz. Chemical shifts are reported in parts per million (p.p.m.) using tetramethylsilane (in CD_3OD solutions) or a sodium 3-trimethylsilyl-propionate (in D_2O solutions) as reference (0 p.p.m.). Homonuclear shift correlation spectroscopy (COSY) and nuclear Overhauser spectroscopy (NOESY) were performed using standard pulse sequences and parameters (Derome 1987).

TLC

Analytical and preparative thin layer chromatography used 20 × 20 cm glass plates precoated with a 250 μm layer of silica gel (Sigma). Chromatographs were developed with *n*-butanol:acetic acid:water (4:1:5, upper phase) or chloroform:methanol:water (35:12:2) and ferric iron-reactive species localized by treatment with freshly prepared 1% $FeCl_3$ in 1 mM HCl.

Stoichiometry of ferric alcaligin

Job's method of continuous variation was used to examine the stoichiometry of *Bordetella* alcaligin to iron in the ferric alcaligin complex. The molar fraction of iron in a 0.5 mM [Fe + alcaligin] mixture in 20 mM KCl buffer, pH 2.0, or in 20 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer, pH 6.0, was varied from 0.00 to 1.00. After 18 h at room temperature, absorbance values of the resulting solutions at pH 2.0 were recorded at 420, 440 and 460 nm wavelength, or at 440, 460 and 480 nm wavelength for solutions at pH 6.0, and plotted as a function of the molar fraction iron.

Ferric alcaligin crystallization

An aqueous solution containing 83.5 mM alcaligin and 55.6 mM $FeCl_3$ in 20 mM MES buffer, pH 6.0 (a 3:2 molar ratio of alcaligin to iron based on stoichiometry determinations) was held at room temperature and examined microscopically for the presence of crystals after 1 week.

Siderophore bioassays

Bioassays of purified *Bordetella* siderophore used *B. bronchiseptica* siderophore-deficient mutants BRM1, BRM6, BRM9, BRM3 and BRM5, and their wild-type parent strain *B. bronchiseptica* B013N as indicator bacteria in an iron-restricted agar plate growth stimulation assay. Siderophore-deficient *B. bronchiseptica* mutants used as indicators represent three distinct genetic complementation groups, lessening the possibility of cross-feeding of specific indicator mutants by siderophore precursors. *B. pertussis* UT25D was used as a *B. pertussis* indicator strain since virulent Phase 1 *B. pertussis* such as the parent strain UT25 cannot grow on the bloodless medium used in this bioassay system. A sterile solution of the nonutilizable iron chelator EDDA was added to molten LB agar to 100 μg ml^{-1} final concentration. The molten agar was cooled to 50°C, seeded

with approximately 10^5 c.f.u./ml of indicator bacteria grown overnight on LB agar and poured into Petri plates. Six millimeter diameter wells were punched in the seeded solidified agar and 100 μl volumes of the following solutions were added: *Bordetella* siderophores in H_2O, 1.00, 0.50 and 0.25 mg ml^{-1}; 1 mM $FeCl_3$ in 10 mM HCl; and H_2O. Plates were incubated at 37°C and diameters of indicator strain growth zones measured after 24 h.

To examine the effects of added siderophore on growth kinetics in chemically defined liquid culture media, *B. bronchiseptica* siderophore-deficient mutant BRM3 was subcultured from overnight growth on selective LB agar plates into iron-depleted SS at an initial optical density of approximately 25 Klett Units and grown at 37°C with rotary shaking for approximately 18 h. Iron-starved BRM3 cells were harvested from this culture by centrifugation at room temperature, washed twice with iron-depleted SS and used to seed the following 10 ml broth cultures in 250 ml side-arm flasks to an initial optical density of 15 20 Klett Units for growth kinetics analysis: (i) iron-replete SS cultures contained 36 μM iron as $FeSO_4$, (ii) iron-restricted SS cultures used the same medium plus 100 μg ml^{-1} EDDA iron chelator and (iii) iron-restricted SS culture medium containing 100 μg ml^{-1} EDDA, with purified *B. bronchiseptica* siderophore added to 50 μg ml^{-1} final concentration. Cultures were maintained at 37°C with rotary shaking and optical densities were monitored over a 40 h time period.

[^{55}Fe]Ferric alcaligin transport assays

[^{55}Fe]Ferric chloride (7.9 mCi/ml, 200 μg Fe ml^{-1}) was purchased from Amersham Life Sciences (Arlington Heights, IL). *B. bronchiseptica* B013N or BRM3 were cultured in iron-depleted SS until mid- to late-logarithmic phase, then were recovered by centrifugation, washed twice using iron-depleted SS and resuspended in iron-depleted SS (uptake medium) to a cell density giving an absorbance value of 1.0 at 600 nm wavelength. [^{55}Fe]Ferric alcaligin complexes were prepared at various concentrations in portions of the same iron-depleted SS uptake medium by addition of [^{55}Fe]ferric chloride and alcaligin to provide a constant 2:3 molar ratio of ^{55}Fe(III) to alcaligin. The resulting solutions were held at room temperature overnight and filtered through cellulose acetate filters before use. To examine the rate of uptake of the ^{55}Fe label of [^{55}Fe]ferric alcaligin by iron-starved *Bordetella* cells, a final alcaligin concentration of 5 μM, together with 3.3 μM ^{55}Fe(III), was used. Equal volumes of prewarmed cell suspensions and [^{55}Fe]ferric alcaligin solutions in uptake medium were combined at zero time, shaken at 37°C, and 0.5 ml samples of suspension were withdrawn at intervals of 1.0, 2.0, 5.0, 7.5 and 10.0 min, and filtered through cellulose acetate membrane filters (0.45 μm pore size, presoaked in 0.9% NaCl containing 40 μM Fe-EDTA) using a vacuum manifold apparatus. Saturability of uptake of the ^{55}Fe label of [^{55}Fe]ferric alcaligin was examined using a range of final [^{55}Fe]ferric alcaligin concentrations equivalent to 0.04–40 μM alcaligin, and measured at 10 min time. The filters were rinsed twice using 5 ml volumes of 0.9% NaCl containing 1 μM $FeCl_3$, dried briefly, placed in scintillation vials, and counted using EcoLume™ scintillant (ICN Biomedicals, Irvine, CA) in a Beckman LS5000TD liquid scintillation counter (Beckman Instruments). Mock uptake values were determined using no bacterial cells, and were subtracted from disintegrations per minute (d.p.m.) determined for test samples.

Results

Growth of *B. pertussis* UT25 and *B. bronchiseptica* B013N in iron-depleted culture medium was accompanied by the production of Csaky-positive material reacting strongly in the CAS universal siderophore assay (Armstrong & Clements 1993 and this study). Production of this material was repressible by the addition of iron to the culture medium. Given the previously observed growth-promoting activity of *B. pertussis* and *B. bronchiseptica* culture supernatants in reciprocal cross-feeding experiments, and the high degree of DNA homology between genetic regions disrupted by transposon insertions in siderophore-deficient *B. bronchiseptica* mutants and genomic DNA of *B. pertussis* (Armstrong & Clements 1993), comparative structural analysis of purified compounds was essential to determine whether siderophores produced by these related pathogenic species were chemically similar or identical to one another and to ascertain whether the siderophores were novel compounds.

Unidentified hydroxamate siderophores were purified from iron-depleted culture supernatants of *B. pertussis* UT25 and *B. bronchiseptica* B013N, as well as from a recently described *B. bronchiseptica fur* mutant, B013N Mn^{r}4, that constitutively produces siderophore activity, by a modification of the benzyl alcohol/ether extraction method (Neilands 1952). Large-scale purification of deferrisiderophores was accomplished simply and rapidly by multiple recrystallizations from ethanolic solutions of semipure benzyl alcohol/ether-extracted siderophore residues, which resulted in pure crystalline deferrisiderophores (Figure 1). The observation during early solubility trials that deferrisiderophores were quantitatively precipitated from ethanolic solutions of semipure siderophores at room temperature, while contaminating orange-colored ferric complexes remained preferentially soluble, facilitated the isolation of crystalline deferrisiderophores with minimal loss of product and obviated the need for subsequent deferration by 8-hydroxyquinoline treatment or more sophisticated separation techniques. By this procedure, starting with mature, iron-depleted broth cultures of *Bordetella* cells, it was possible to isolate high-purity crystalline siderophore product in less than 12 h time. Overall efficiency of recovery of siderophore from crude supernatants was consistently greater than 85% based on CAS assay data (not shown). Alternatively, purification of small quantities of deferrisiderophores could readily be achieved by preparative TLC of semipure benzyl alcohol/ether extracted residues. In recent preliminary crystallization trials, red–orange colored crystals

(Figure 1), putative ferric siderophore complexes, formed in concentrated, buffered aqueous solutions of purified siderophore and $FeCl_3$ after 1 week at room temperature.

Absorption spectroscopy of aqueous solutions of highly purified siderophores revealed a single maximum at 210 nm at pH 6.0 (Figure 2). In the presence of excess added Fe(III), an additional maximum at 430 nm was observed. *B. pertussis* and *B. bronchiseptica* siderophores displayed identical spectral properties, giving superimposable spectra for equivalent siderophore concentrations.

Analytical silica gel thin layer chromatography of purified siderophores identified a single colorless ferric iron-reactive species with an R_f value of 0.53 using a solvent system composed of *n*-butanol:acetic acid:water (4:1:5, upper phase) and an R_f value of 0.71 in chloroform:methanol:water (35:12:2). Detection of siderophores by spraying TLC plates with ferric iron solution produced a deep red color. Addition of excess ferric iron to purified siderophore solutions prior to TLC converted the colorless ferric iron-reactive species to red–orange colored complexes exhibiting very low mobility in both solvent systems. Purified siderophores of *B. pertussis* and *B. bronchiseptica* were indistinguishable by TLC when examined in parallel using the solvent systems described (data not shown).

Biological activity of purified siderophores was evidenced by their ability to reverse the iron restriction-imposed growth inhibition of all *Bordetella* indicator strains examined. In iron-restricted agar plate bioassays (Table 1), purified *B. bronchiseptica* siderophore isolated from strain

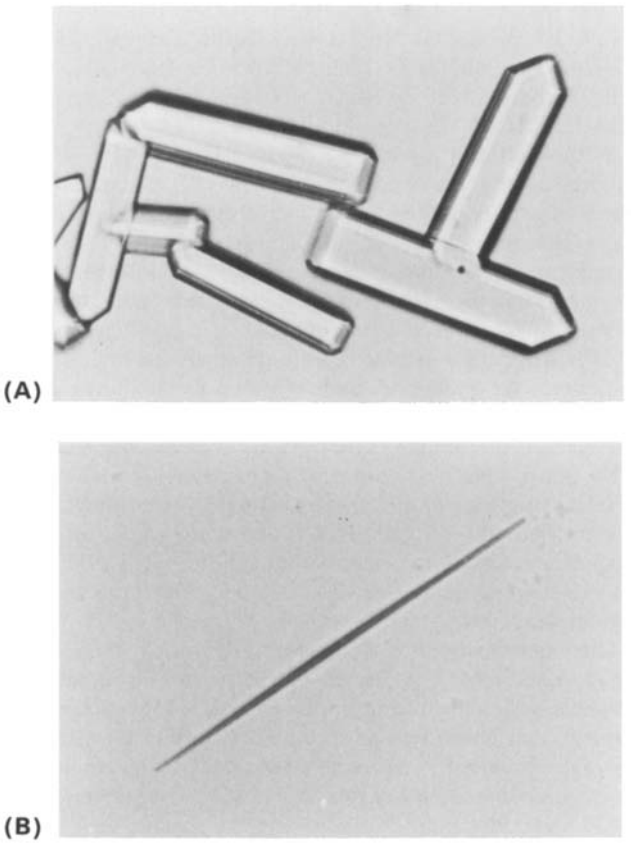

Figure 1. (**A**) Phase-contrast micrograph of crystalline *B. pertussis* UT25 hydroxamate deferrisiderophore. (**B**) Phase-contrast micrograph of a representative red–orange colored crystal formed in concentrated aqueous *B. pertussis* UT25 siderophore solutions in the presence of ferric iron.

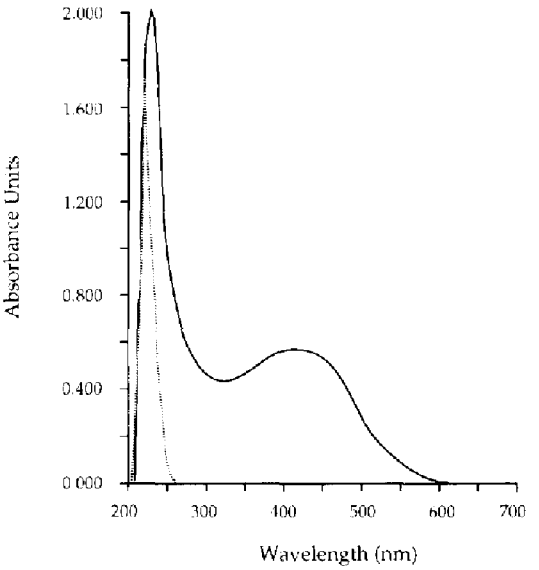

Figure 2. Absorption of purified *B. pertussis* hydroxamate siderophore at approximately 200 $\mu g\ ml^{-1}$ concentration in 20 mM MES buffer, pH 6.0. Dashed line, spectrum of deferrisideropnore solution; solid line, spectrum recorded 30 min after addition of excess $FeCl_3$.

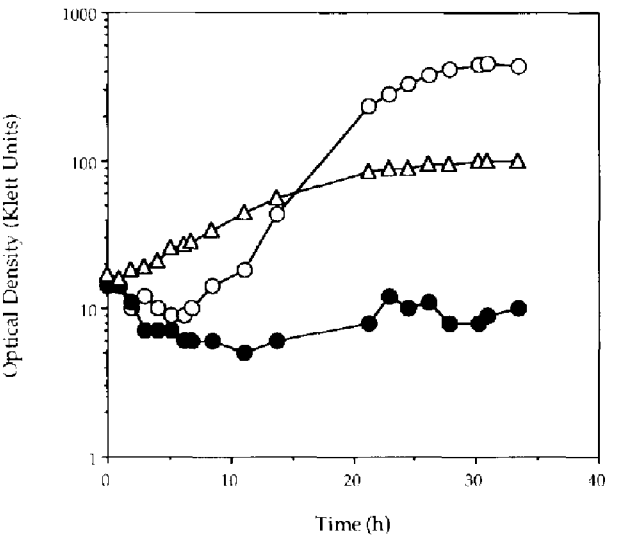

Figure 3. Reversal of iron restriction-imposed growth inhibition of siderophore-deficient *B. bronchiseptica* mutant BRM3 by purified *Bordetella* siderophore. Optical densities of cultures were monitored as a function of time. (○) Iron-replete culture medium, (●) iron-restricted culture medium and (△) iron-restricted culture medium with purified *B. bronchiseptica* B013N siderophore added to 50 $\mu g\ ml^{-1}$ final concentration.

Table 1. Growth stimulation of *Bordetella* species

Iron source	Indicator strain growth zone[a]						
	B. bronchiseptica						*B. pertussis*
	BRM3	BRM5	BRM1	BRM6	BRM9	B013N	UT25D
Siderophore (mg/ml)							
1.00	+ + +	+ − +	+ + +	+ + +	+ + +	+ + +	+ + +
0.50	+ −	+ +	+ +	+ +	+ +	+ +	+ + +
0.25	+	+	+	−	+	+	+ +
$FeCl_3$ (1 mM)	+ + +	+ + +	+ + +	+ + +	+ + −	+ + +	+ + +
H_2O	—		—	—	—	—	

[a] + + +, 20–30 mm diameter; + +, 15–20 mm diameter; +, 10–15 mm diameter; —, no stimulation.

B013N stimulated the growth of all indicator bacteria, including the heterologous *Bordetella* species *B. pertussis* UT25D, in a dose-dependent fashion at physiologically relevant concentrations. Ferric iron likewise stimulated growth when used at EDDA-saturating levels. Identical growth-promoting activities were observed using equivalent amounts of *B. pertussis* or *B. bronchiseptica* siderophores. Monitoring growth kinetics of *B. bronchiseptica* siderophore mutant BRM3 cultured in chemically defined SS broth medium (Figure 3), it was again observed that purified *B. bronchiseptica* deferrisiderophore was able to reverse the growth defect associated with iron starvation imposed by the incorporation of the nonutilizable iron chelator EDDA in the growth medium. Addition of deferrisiderophore to 50 $\mu g\,ml^{-1}$, corresponding to a siderophore concentration attainable after 24 h growth of the wild-type parent strain B013N under iron-restricted conditions, resulted in complete relief of lag phase for the siderophore mutant strain BRM3 compared with the 5–10 h lag phase associated with fully iron-replete cultures without added siderophore. Furthermore, siderophore addition resulted in greatly elevated biomass accumulation for the mutant at stationary phase compared with iron-restricted cultures of BRM3 without added siderophore, which showed no measurable increase over the initial cell density of the culture.

Siderophore purification, absorption spectroscopy, thin-layer chromatography and growth promotion assays were all accomplished without prior knowledge of the identities of the *Bordetella* siderophores. Mass spectrometry and proton NMR spectroscopy were used to identify their chemical structures. Samples isolated from *B. pertussis* UT25, *B. bronchiseptica* B013N and *B. bronchiseptica* B013N Mn^r4 cultures were found to be a single compound, the known macrocyclic dihydroxamate alcaligin (Nishio *et al.* 1988) (1,8(*S*),11,18(*S*)-tetrahydroxy-1,6,11,16-tetraaza-cycloeicosane-2,5,12,15-tetrone, $C_{16}H_{28}N_4O_8$, MW 404). FAB mass spectra of all three preparations showed peaks at $m/z = 427$, 405 and 389 corresponding to $[M+Na]^+$, $[M+H]^+$ and $[M-O+H]^+$ ions, respectively. High resolution ^{1}H-NMR spectra obtained in CD_3OD (Figure 4): ^{1}H-NMR (500 MHz, CD_3OD, 20°C) δ 1.239 (dddd, $J = 14.7$, 11.0, 3.6, 1.5 Hz, 1H, H-19), 1.495 (ddt, $J = 14.3$, 9.5, 4.8 Hz, 1H, H-9), 1.828 (dddd, $J = 14.5$, 9.1, 5.4, 3.2 Hz, 1H, H-9), 2.148 (dt, $J = 13.9$, 3.9 Hz, 1H, H-13 or H-14), 2.224 (dddd, $J = 14.8$, 12.4, 2.5, 1.1 Hz, 1H, H-19), 2.373 (ddd, $J = 15.1$, 7.2, 3.8 Hz, 1H, H-3 or H-4), 2.466 (dt, $J = 17.3$, 3.6 Hz, 1H, H-13 or H-14), 2.547 (m, 3H, H-3 or H-4, H-13 or H-14, H-17), 2.673 (ddd, $J = 17.0$, 7.1, 3.6 Hz, 1H, H-3 or H-4), 2.907 (ddd, $J = 16.9$, 10.5, 3.8 Hz, 1H, H-13 or H-14), 2.925 (dd, $J = 13.9$, 7.6 Hz, 1H, H-7), 2.960 (ddd, $J = 17.2$, 12.7, 3.5 Hz, 1H, H-3 or H-4), 3.350 (m, 3H, H-7, H-10, H-20), 3.493 (dddd, $J = 11.2$, 10.2, 3.5, 1.1 Hz, 1H, H-18), 3.603 (ddd, $J = 13.4$, 7.8, 3.8 Hz, 1H, H-8), 3.704 (dd, $J = 12.7$, 3.4 Hz, 1H, H-17), 3.984 (ddd, $J = 14.7$, 12.4, 1.5 Hz, 1H, H-20), 4.140 (ddd, $J = 14.4$, 9.8, 4.8 Hz, 1H, H-10) (H-3–H-10 represent the major conformer, H-13–H-20 the minor conformer, about 70% of the major) perfectly reproduced the reported spectra of alcaligin (Nishio *et al.* 1988, supplementary material; Bergeron and McManis, personal communication), confirming the identity and high purity of the isolated *Bordetella* siderophores. Minor differences in ^{1}H-NMR spectra in these reports are attributed to different spectrometer frequencies and recording temperatures. The improved spectral dispersion in Figure 4 is due to the higher magnetic field strength of the instrument used in our studies. The strong singlet at 4.8 p.p.m. is caused by residual water (*H*DO) and the tall multiplet at 3.3 p.p.m. by incompletely deuterated solvent (CD_2HOD). The weak triplet at 1.18 p.p.m. is assigned to a trace of ethanol (CH_3CH_2OH; the corresponding quartet of CH_3CH_2OH is obscured by the multiplet at 3.6 p.p.m.).

Homonuclear shift correlation spectroscopy (COSY) in CD_3OD (Figure 5) revealed the presence of two sets of signals (see above) which are not related by coupling. Integration of the regular spectrum (Figure 6) showed the signals of one set to be less intensive (about 70%) than the corresponding signals of the other set [*cf.* the multiplets at 4.14 and 3.98 p.p.m. or at 3.60 and 3.49 p.p.m.: they were assigned (Nishio *et al.* 1988) to identical protons 'on the other side of the ring']. Close inspection of the spectrum provided by Bergeron reveals the same phenomenon whereas Nishio's spectrum integrates properly. Assuming the presence of two different, symmetric conformations of alcaligin present in CD_3OD solution rather than a single totally symmetrical

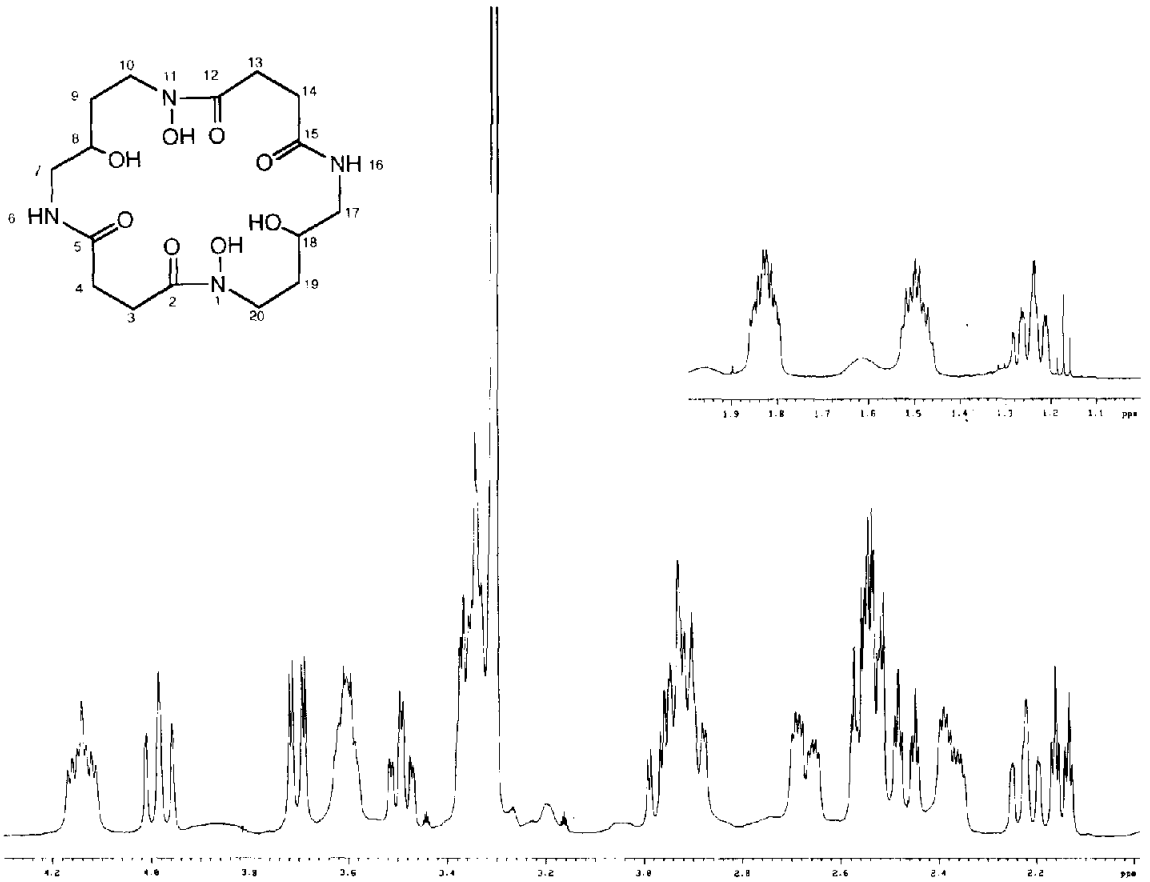

Figure 4. ^{1}H-NMR spectrum of purified *Bordetella* siderophore in CD_3OD. Inset, the molecular structure of alcaligin.

conformation a two-dimensional nuclear Overhauser spectroscopy (NOESY) experiment was performed (Figure 7). The NOESY showed extensive correlation between the two sets of signals due to chemical exchange of equivalent protons during the interconversion of the two conformations.

A significantly different proton spectrum of alcaligin was obtained in D_2O (Figure 8): ^{1}H-NMR (500 MHz, D_2O, 20°C) δ 1.592 (ddt, $J = 14.3$, 9.6, 4.8 Hz, 2H, H-9/19), 1.877 (dddd, $J = 14.8$, 8.9, 4.7, 3.6 Hz, 2H, H-9/19), 2.485 (ddd, $J = 15.6$, 7.3, 3.5 Hz, 2H, H-4), 2.595 (ddd, $J = 15.15$, 10.3, 3.4 Hz, 2H, H-4), 2.715 (ddd, $J = 17.3$, 7.2, 3.3 Hz, 2H, H-3), 2.962 (ddd, $J = 17.2$, 10.4, 3.5 Hz, 2H, H-3), 3.035 (dd, $J = 14.0$, 8.2 Hz, 2H, H-7/17), 3.352 (dd, $J = 14.0$, 4.2 Hz, 2H, H-7/17), 3.420 (dt, $J = 14.3$, 5.1 Hz, 2H, H-10/20), 3.678 (tt, $J = 8.2$, 4.1 Hz, 2H, H-8/18), 4.080 (ddd, $J = 14.4$, 9.6, 4.8 Hz, 2H, H-10/20) (major conformer). The assignments (numbering according to the alcaligin structural formula in Figure 4) of the 11 signals were made using COSY data (Figure 9) and are consistent with a predominant C_2-symmetric conformation of alcaligin in aqueous solution. The signal group at 4.8 p.p.m. is a remnant of a strong water peak (*H*DO) after suppression by presaturation. At 0.78 and 2.17 p.p.m. weak multiplets of sodium 3-(trimethylsilyl)-propionate, the internal standard, are observed. Since the purity of the sample was established by the CD_3OD spectra, the presence of weak signals in the D_2O spectrum is not caused by contaminants but most likely by less abundant conformers of alcaligin.

The structural symmetry of alcaligin revealed by the proton spectrum in D_2O was confirmed by carbon NMR spectroscopy (Figure 10). Eight strong signals were observed corresponding to eight chemically different carbon atoms in a symmetric alcaligin molecule: ^{13}C-NMR (125 MHz, D_2O, 30°C) δ 30.23 (C-9/19), 32.99 and 33.50 (C-3/13 and 4/14), 47.58 and 48.02 (C-7/17 and 10/20), 70.00 (C-8/18), 176.89 and 178.31 (C-2/12 and 5/15) (major conformer). A number of weaker signals cannot be assigned but is again most likely due to the presence of other alcaligin conformers.

The stoichiometry of ferric alcaligin was determined by analysis of plots of Job's method of continuous variation at two different pH values, using alcaligin purified from *Bordetella* species. Absorption maxima were observed at a calculated 0.5 molar fraction of iron for solutions of constant total 0.5 mM [Fe + alcaligin] at pH 2.0 (Figure 11A) and at 0.4 molar fraction iron at pH 6.0 (Figure 11B). These data are in agreement with the previously reported 1 iron:1 alcaligin stoichiometry at pH 2.0, and a 2 iron:3 alcaligin stoichiometry in the ferric chelator complex at pH 6.0 (Nishio *et al.* 1988).

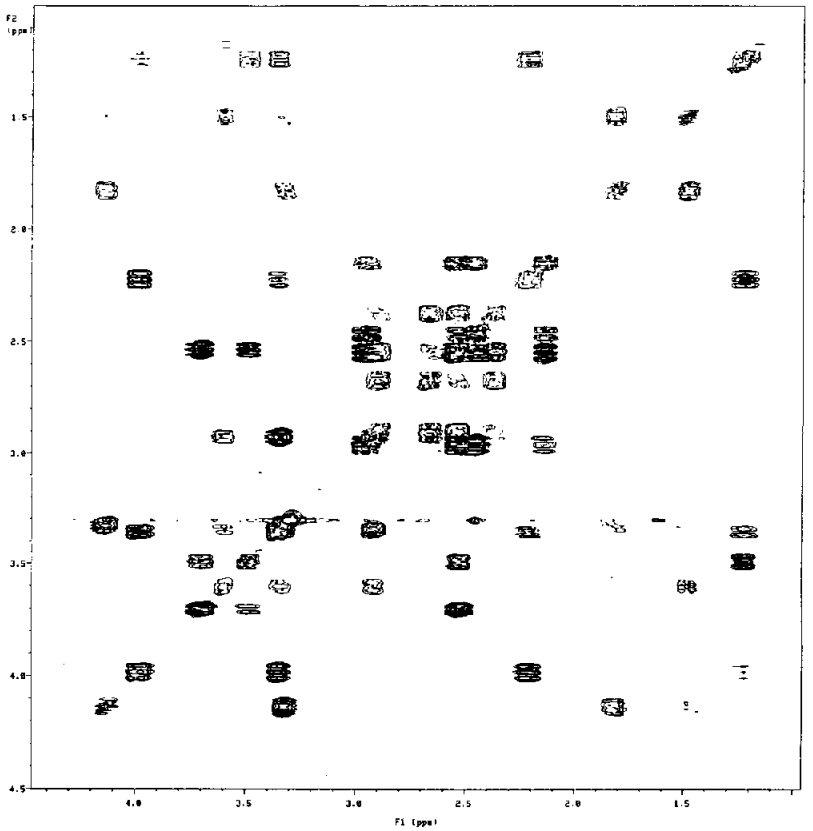

Figure 5. Two dimensional homonuclear shift correlation spectroscopy (COSY) of purified *Bordetella* siderophore in CD_3OD.

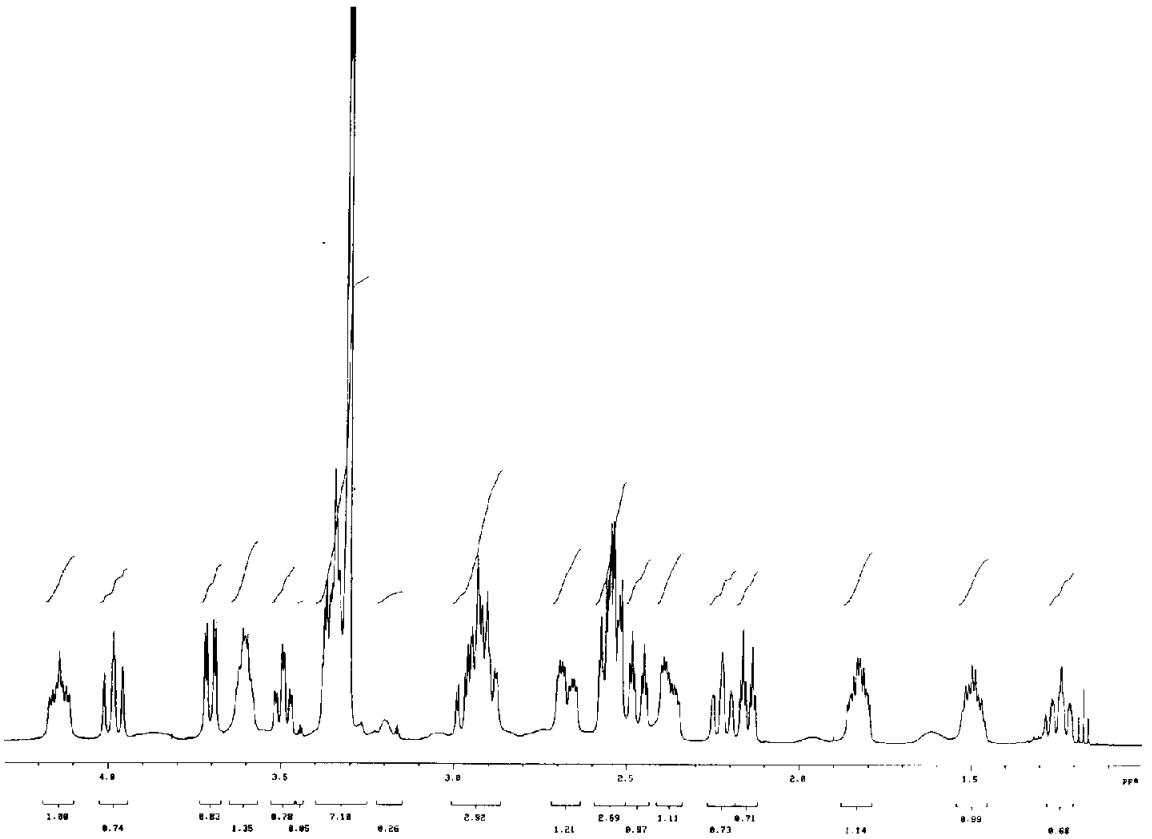

Figure 6. Integration of ^{1}H-NMR spectrum of purified *Bordetella* siderophore in CD_3OD.

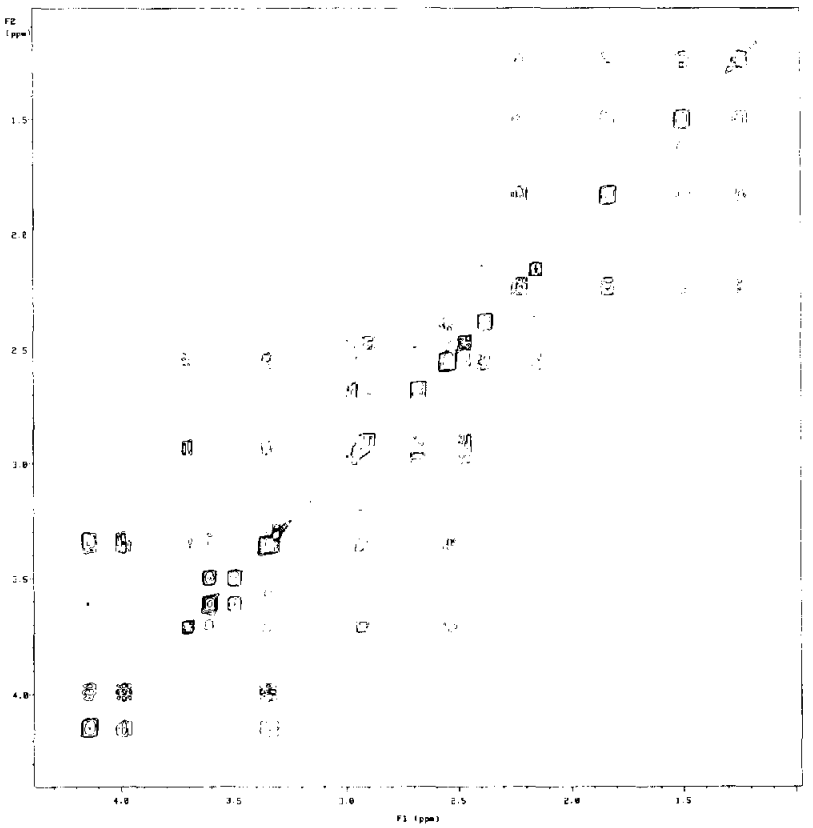

Figure 7. Two-dimensional nuclear Overhauser spectroscopy (NOESY) of purified *Bordetella* siderophore in CD_3OD.

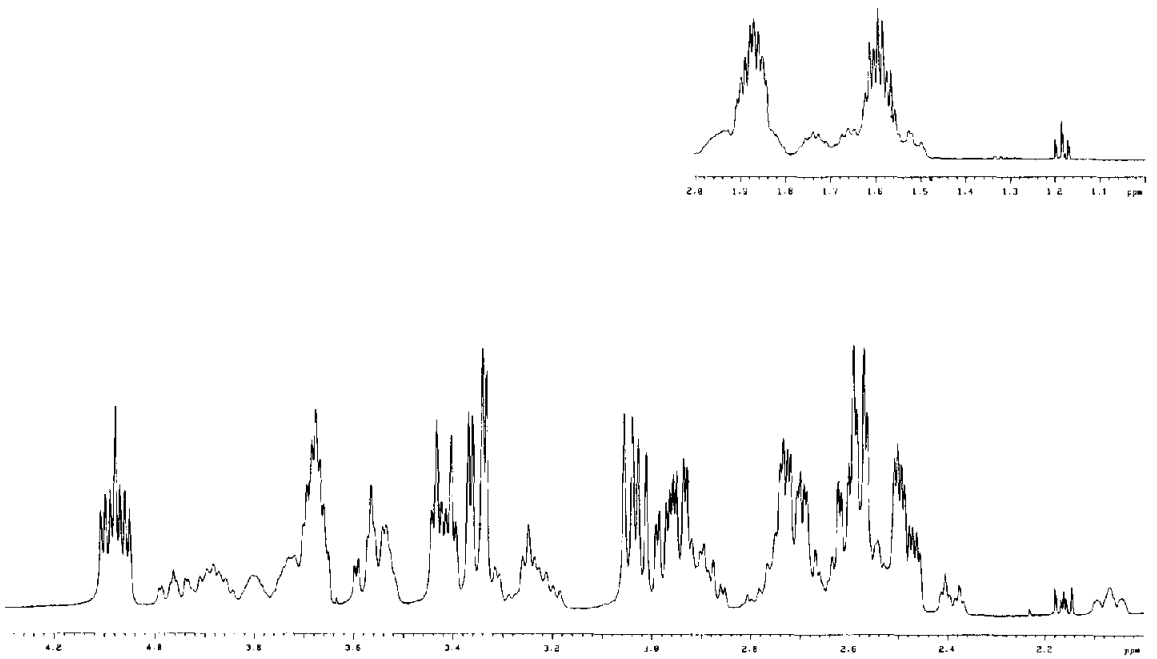

Figure 8. ^{1}H-NMR spectrum of purified *Bordetella* siderophore in D_2O (HDO peak at 4.82 p.p.m. suppressed).

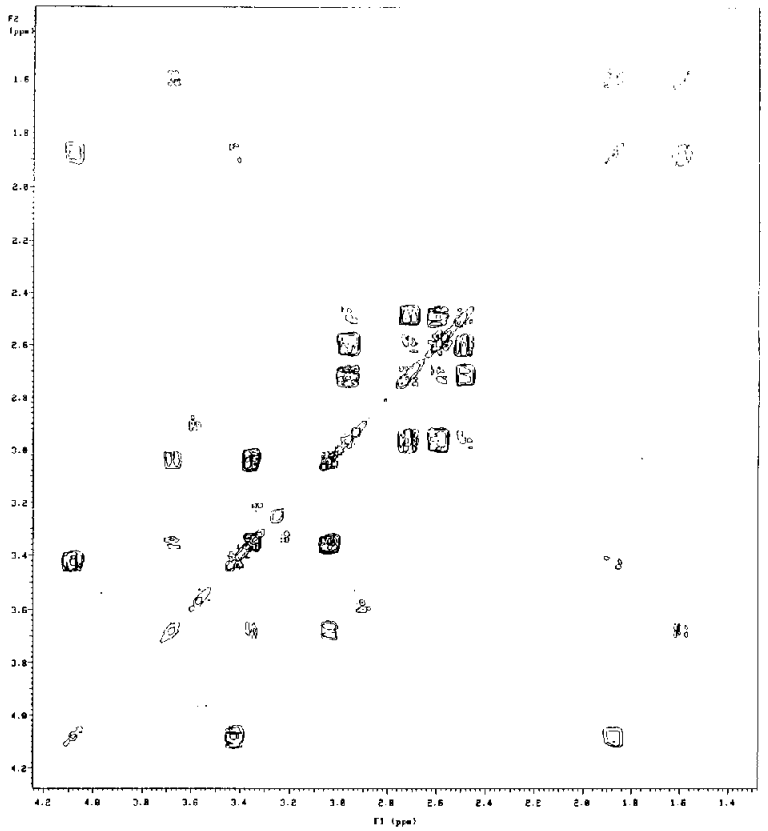

Figure 9. Two-dimensional homonuclear shift correlation spectroscopy (COSY) of purified *Bordetella* siderophore in D_2O.

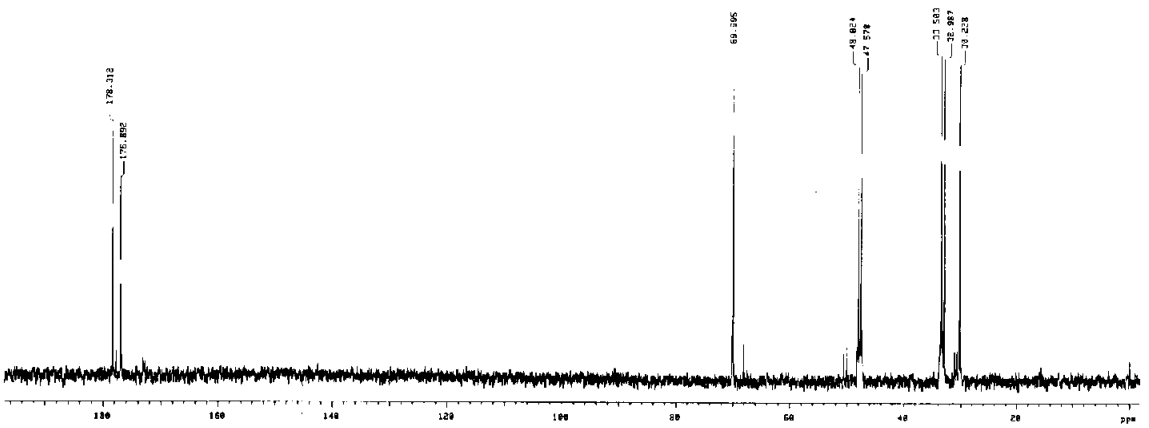

Figure 10. ^{13}C-NMR spectrum of purified *Bordetella* siderophore (D_2O temperature, 30°C).

Titration of purified alcaligin by the highly sensitive CAS universal siderophore assay revealed a linear relationship ($R^2 = 0.998$) between alcaligin concentration and relative absorbance $A/A_{reference}$ of the CAS dye complex at 630 nm wavelength at pH 6.0 for alcaligin concentrations up to 10 μM (Figure 12). With behavior typical of pure hydroxamate siderophores (Schwyn & Neilands 1987), the exchange rates for iron from the CAS dye complex to the purified siderophores were slow, achieving equilibrium in hours in the presence of the accelerating shuttle compound 5-sulfosalicyclic acid and in about 1 day without shuttle compound.

As an initial examination of *Bordetella* transport of iron supplied as ferric alcaligin, ^{55}Fe uptake assays were performed using purified alcaligin and suspensions of iron-starved bacteria. [^{55}Fe]Ferric alcaligin uptake assays demonstrated that the ^{55}Fe label of [^{55}Fe]ferric alcaligin was rapidly taken up by *Bordetella* cells grown in

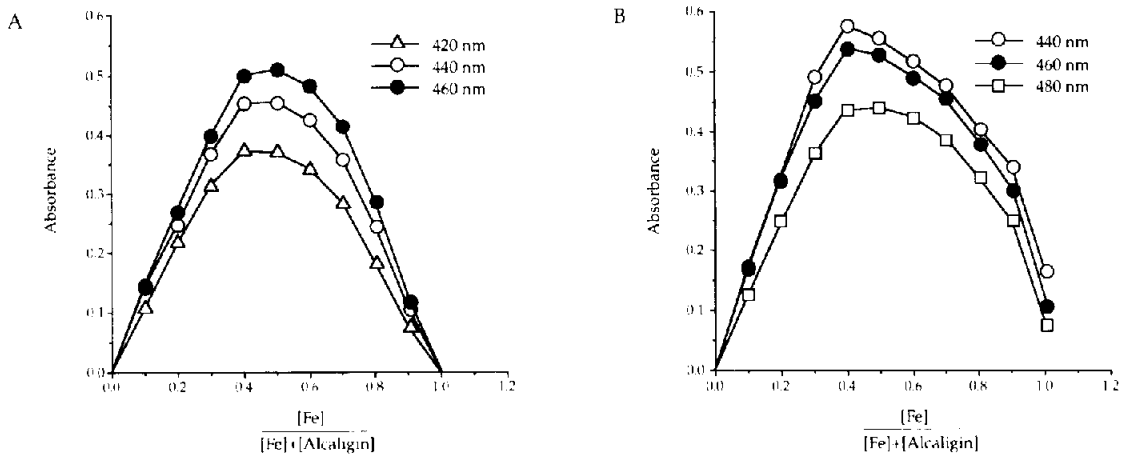

Figure 11. Stoichiometry of ferric alcaligin by Job's method of continuous variation. The molar fraction of iron in a constant total 0.5 mM [Fe + alcaligin] mixture in 20 mM KCl buffer, pH 2.0, or in 20 mM MES buffer, pH 6.0, was varied from 0.00 to 1.00. After 18 h at room temperature, absorbance values of the resulting solutions at pH 2.0 (**A**) were recorded at 420, 440 and 460 nm wavelength, or at 440, 460 and 480 nm wavelength at pH 6.0 (**B**), and plotted as a function of the molar fraction of iron.

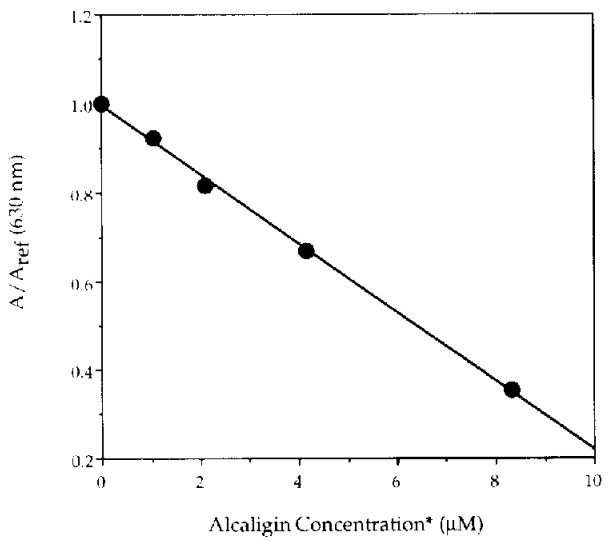

Figure 12. Linear relationship between relative absorbance $A/A_{reference}$ at 630 nm of CAS reaction and alcaligin concentration (*concentration is 2/3 the molar concentration since alcaligin forms 3:2 molar complexes with ferric iron at pH 6.0).

iron-depleted medium. Plots of mean ^{55}Fe d.p.m. values ± SD ($n = 3$) versus time, shown in Figure 13(A), indicated rapid and reproducible cell-association of ^{55}Fe supplied as [^{55}Fe]ferric alcaligin for washed cell suspensions of iron-starved *B. bronchiseptica* B013N. The initial uptake rates between zero time and 1 min were maximal, and rates were linear between 1 and 5 min time. Siderophore-deficient *B. bronchiseptica* mutant BRM3 exhibited virtually identical uptake rates as the wild-type parent strain B013N (data not shown). Furthermore, ^{55}Fe uptake from [^{55}Fe]ferric alcaligin was saturable, reaching a maximum at ferric alcaligin concentrations equivalent to 40 μM alcaligin when examined at 10 min time (Figure 13B).

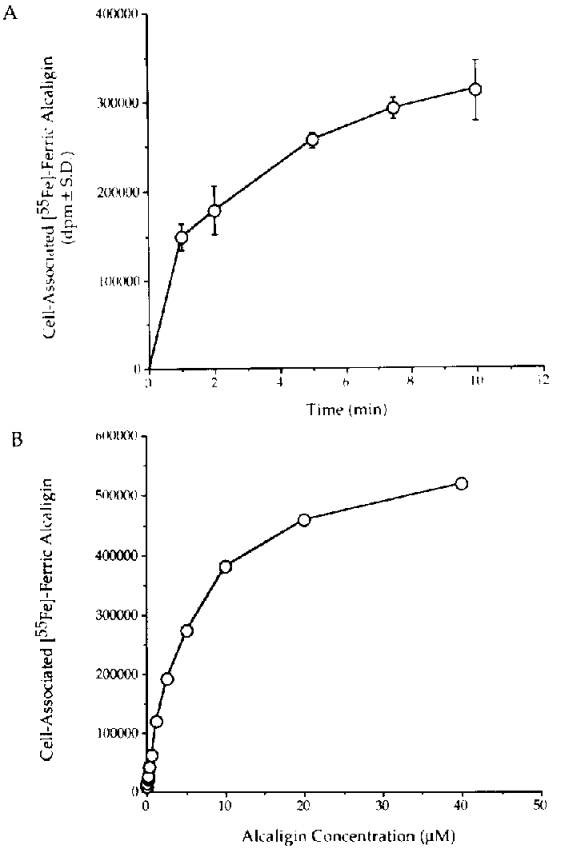

Figure 13. *Bordetella* [^{55}Fe]ferric alcaligin transport assays. The rate of uptake of the ^{55}Fe label of [^{55}Fe]ferric alcaligin by iron-starved *B. bronchiseptica* B013N using an alcaligin concentration of 5 μM, together with 3.3 μM ^{55}Fe(III) is shown (**A**) over 10 min time. Mean ^{55}Fe d.p.m. values ± SD ($n = 3$) are plotted as a function of time. Saturability of uptake (**B**) of the ^{55}Fe label of [^{55}Fe]ferric alcaligin for iron-starved *B. bronchiseptica* B013N was determined using a range of [^{55}Fe]ferric alcaligin concentrations equivalent to 0.04–40 μM alcaligin at a constant 3 alcaligin:2 ^{55}Fe(III) molar ratio and measured at 10 min time.

Discussion

We report the purification and structural analysis of hydroxamate siderophores produced by virulent *B. pertussis* UT25, *B. bronchiseptica* B013N and the *fur* mutant derivative B013N Mn^{r}4, and their identification as the macrocylic dihydroxamate alcaligin. The simple large-scale alcaligin purification procedure described in this report allowed the rapid and reproducible recovery of large quantities of highly purified deferrisiderophores. The purity of the recrystallized siderophore preparation was demonstrated by perfectly reproducing published proton NMR spectra. Evidence for biological activity of the purified siderophores was obtained using growth stimulation and ^{55}Fe transport assays in *Bordetella* cells. Uptake rates and saturability of iron uptake observed in the ^{55}Fe transport assays suggest the existence of a high-affinity ferric alcaligin transport system in *Bordetella* species, and provide the first direct demonstration, beyond simple growth stimulation, of alcaligin-mediated iron supply to *Bordetella* species. More detailed analysis of the ferric alcaligin transport process at the molecular level is required.

Our identification of alcaligin as the hydroxamate siderophore produced by *Bordetella* species is in agreement with the recent report of Moore *et al.* (1995). No direct comparison of efficiency of alcaligin recovery or of growth-stimulating activity for alcaligin purified as we describe versus the method of Moore *et al.* is possible using the available data.

Alcaligin was first purified from *A. denitrificans* subsp. *xylosoxydans* KN3-1, isolated from the sediment of Komatsunuma Lagoon by Lake Biwa, Japan (Nishio *et al.* 1988, Nishio & Ishida 1990). Structural analysis by Nishio *et al.* (1988) revealed alcaligin to be a 20-membered ring with two hydroxamate and two secondary amide functional groups in addition to two alcoholic hydroxyls, having the molecular formula $C_{16}H_{28}N_4O_8$ (MW 404). The molecule is thus a cyclic dimer of two repeating units consisting of succinic acid and 1-amino-4-(*N*-hydroxylamino)-2(*S*)-butanol. Consistent with this dimeric structure, it was determined in this study using high resolution proton and carbon NMR spectroscopy in D_2O that the molecule exists primarily in a 2-fold symmetric conformation in aqueous solution, although evidence suggests that several C_2-symmetric conformations of alcaligin are present at the same time in both methanolic and aqueous solution; published assignments of proton resonances might need to be revised.

Alcaligin is structurally similar to the cyclic dihydroxamate siderophore produced by the marine bacterium *Alteromonas haloplanktis*, bisucaberin (Kameyama *et al.* 1987, Takahashi *et al.* 1987); however, bisucaberin includes two residues of *N*-hydroxycadaverine instead of the hydroxyl-substituted *N*-hydroxyputrescine residues of alcaligin. Alcaligin binds ferric iron at a 3:2 molar ratio at pH 6.0 and 1:1 at pH 2.0 based on analysis of Job's plots of the continuous variation method (Nishio *et al.* 1988 and this study), and at pH 6.0 the ferric alcaligin complex exhibits a stability constant calculated as 10^{37} M^{-1} based on EDTA displacement studies (Nishio *et al.* 1988). The total synthesis of alcaligin has been accomplished by Bergeron *et al.* (1991).

Our preliminary attempts to produce ferric alcaligin crystals resulted in the formation of small red–orange needle crystals, putatively crystalline ferric alcaligin complexes, in concentrated aqueous siderophore solutions in the presence of ferric iron. Systematic refinement of crystallization conditions should provide crystals suitable for unambiguous elucidation of coordinating atoms in the ferric alcaligin complex by X-ray crystallography. Full details of crystallographic analysis of the ferric alcaligin complex will be reported at that time.

It has been proposed that members of the genus *Bordetella* be reclassified as subspecies of a single bacterial species based on shared phenotypic and serological characteristics, and DNA and rRNA hybridization analysis (Kloos *et al.* 1981, Kersters *et al.* 1984). Other taxonomic studies of *A. denitrificans* indicated a high degree of relatedness with *B. bronchiseptica* based on rRNA similarly (Kersters & De Ley 1984). In addition, the mol % G+C of *B. bronchiseptica* and *A. denitrificans* is similar, and the numerical phenetic correlation between the two species exceeds 0.90. *Alcaligenes* species generally occur in water and soil, but may occasionally cause opportunistic infections in humans. *B. bronchiseptica*, although primarily a pathogen of non-human mammals that may also occasionally infect humans (Woolfrey & Moody 1991), has been shown capable of growth in natural fresh water and seawater (Porter *et al.* 1991). These evolutionarily-related bacterial species share the ability to produce the potent hydroxamate siderophore alcaligin. Yet *B. pertussis*, a related pathogen that is not known to survive outside its human host, retains the capacity to produce alcaligin. Conservation of this activity, which may have evolved in response to the need to acquire growth-essential iron in an iron-restricted aquatic environment, suggests an advantageous role for the siderophore in iron acquisition by this obligate pathogen in the human host.

Acknowledgments

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References

Agiato LA, Dyer DW. 1992 Siderophore and membrane alterations by *Bordetella pertussis* in response to iron starvation. *Infect Immun* **60,** 117–123.

Armstrong SK, Clements MO. 1993 Isolation and characterization of *Bordetella bronchiseptica* mutants deficient in siderophore activity. *J Bacteriol* **174,** 1144–1152.

Armstrong SK, Parker CD. 1986 Surface proteins of *Bordetella pertussis*: comparison of virulent and avirulent strains and effects of phenotypic modulation. *Infect Immun* **54,** 308–314.

Bergeron RJ, McManis JS, Perumal PT, Algee SE. 1991 The total synthesis of alcaligin. *J Org Chem* **56,** 5560–5563.

Bordet J, Gengou O. 1906 Le microbe de la coqueluche. *Ann Inst Pasteur (Paris)* **20,** 731–741.

Brickman TJ, Armstrong SK. 1995 *Bordetella pertussis fur* gene restores iron-repressibility of siderophore and protein expression to deregulated *Bordetella bronchiseptica* mutants. *J Bacteriol* **177,** 268–270.

Csaky TZ. 1948 On the estimation of bound hydroxylamine in biological materials. *Acta Chem Scand* **2,** 450 454.

Derome, AD. 1987 *Modern NMR Techniques for Chemistry Research*. Oxford: Pergamon Press.

Field LH, Parker CD. 1979 Differences observed between fresh isolates of *Bordetella pertussis* and their laboratory passaged derivatives. In: Manclark CR, Hill JC, eds. *Proc. Int. Symp. on Pertussis*. Washington, DC: US Department of Health, Education, and Welfare; 124–132.

Gorringe AR, Woods G, Robinson A. 1990 Growth and siderophore production by *Bordetella pertussis* under iron-restricted conditions. *FEMS Microbiol Lett* **66,** 101–106.

Kameyama T, Takahashi A, Kurusawa S, *et al.* 1987 Bisucaberin, a new siderophore, sensitizing tumor cells to macrophage-mediated cytolysis. I. Taxonomy of the producing organism, isolation and biological properties. *J Antibiot* **40,** 1664–1670.

Kersters K, De Ley J. 1984 Genus *Alcaligenes* Castellani and Chalmers 1919, 936. In: Krieg NR, Holt JG, eds. *Bergey's Manual of Systematic Bacteriology, Vol. I*. Baltimore, MD: Williams & Wilkins; 361–373.

Kersters K, Hinz KH, Hertle A, *et al.* 1984 *Bordetella avium* sp. nov., isolated from the respiratory tracts of turkeys and other birds. *Int J Syst Bacteriol* **34,** 56–70.

Kloos WE, Mohapatra N, Dobrogosz WJ, Ezzell JW, Manclark CR. 1981 Deoxyribonucleotide sequence relationships among *Bordetella* species. *Int J Syst Bacteriol* **31,** 173–176.

Lankford CE. 1973 Bacterial assimilation of iron. *Crit Rev Microbiol* **2,** 273–331.

Moore CH, Foster LA, Gerbig JG, Dyer DW, Gibson BW. 1995 Identification of alcaligin as the siderophore produced by *Bordetella pertussis* and *Bordetella bronchiseptica*. *J Bacteriol* **177,** 1116–1118.

Neilands JB. 1952 A crystalline organo-iron pigment from a rust fungus (*Ustilago sphaerogena*). *J Am Chem Soc* **74,** 4846–4847.

Nishio T, Ishida Y. 1990 Production of dihydroxamate siderophore alcaligin by *Alcaligenes denitrificans* subsp. *xylosoxydans*. *Agric Biol Chem* **54,** 1837–1839.

Nishio T, Tanaka N, Hiratake J, Katsube Y, Ishida Y, Oda J. 1988 Isolation and structure of the novel dihydroxamate siderophore alcaligin. *J Am Chem Soc* **110,** 8733–8734.

Porter JF, Parton R, Wardlaw AC. 1991 Growth and survival of *Bordetella bronchiseptica* in natural waters and in buffered saline without added nutrients. *Appl Environ Microbiol* **57,** 1202–1206.

Redhead K, Hill T, Chart H. 1987 Interaction of lactoferrin and transferrins with the outer membrane of *Bordetella pertussis*. *J Gen Microbiol* **133,** 891–898.

Rogers HJ. 1973 Iron binding catechols and virulence in *Escherichia coli*. *Infect Immun* **7,** 445–456.

Schneider DR, Parker CD. 1982 Effect of pyridines on phenotypic properties of *Bordetella pertussis*. *Infect Immun* **38,** 548–553.

Schwyn B, Neilands JB. 1987 Universal chemical assay for the detection and determination of siderophores. *Anal Biochem* **160,** 47–56.

Stainer DW, Scholte MJ. 1970 A simple chemically defined medium for the production of phase I *Bordetella pertussis*. *J Gen Microbiol* **63,** 211–220.

Takahashi A, Nakamura H, Kameyama T, *et al.* 1987 Bisucaberin, a new siderophore, sensitizing tumor cells to macrophage-mediated cytolysis. II. Physico-chemical properties and structure determination. *J Antibiot* **40,** 1671–1676.

Weinberg ED. 1978 Iron and infection. *Microbiol Rev* **42,** 45–66.

Weinberg ED. 1984 Iron withholding: a defense against infection and neoplasia. *Physiol Rev* **64,** 65–102.

Woolfrey BF, Moody JA. 1991 Human infections associated with *Bordetella bronchiseptica*. *Clin Microbiol Rev* **4,** 243–255.