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Identification of Alcaligin as the Siderophore Produced by *Bordetella pertussis* and *B. bronchiseptica*

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The siderophores produced by iron-starved *Bordetella pertussis* and *B. bronchiseptica* were purified and were found to be identical. Using mass spectrometry and proton nuclear magnetic resonance, we determined that the siderophore produced by these organisms was identical to alcaligin, a siderophore produced by *Alcaligenes denitrificans*.

Organisms of the genus *Bordetella* are obligate pathogens of the upper respiratory tract of a variety of animal hosts. *Bordetella pertussis* and *Bordetella parapertussis* are obligate human pathogens (15). Although occasionally seen in infected humans (15), *Bordetella bronchiseptica* is a common pathogen of several other mammalian hosts (7). *Bordetella avium* infects the upper respiratory tract of domestic fowl (10). These organisms cause similar syndromes characterized by infection of the respiratory mucosal surface, without subsequent bloodstream dissemination (15). Our studies (1, 6) and others (2, 8) suggested that *B. pertussis* and *B. bronchiseptica* removed Fe from lactoferrin (LF) and transferrin by producing a hydroxamate siderophore. We have now purified the siderophores produced by these organisms and found that these siderophores were identical. Although we previously suggested the term "bordetellin" to denote the *Bordetella* siderophore (1), the siderophores produced by *B. pertussis* and *B. bronchiseptica* were identical to alcaligin, the Fe chelator produced by *Alcaligenes denitrificans* (13).

B. pertussis DBP2 (a streptomycin-resistant derivative of strain Tohama I) and *B. bronchiseptica* MBORD846 were used in these studies; growth conditions have been described previously (1, 6). To prepare desferri-siderophore, 2 g of lyophilized culture supernatant from a logarithmic-phase iron-starved culture was dissolved in 10 ml of water and passed through two C_{18} Sep-Pak cartridges (Waters Associates), connected in series. The cartridges were washed with 20 ml of water, and the siderophore was eluted with 50% methanol. The methanol was removed by rotary evaporation, and the material was dissolved in 0.1% trifluoroacetic acid (TFA) and applied to a Vydac C_{18} high-pressure liquid chromatography (HPLC) column (4.6 mm by 25 cm). The column was washed with 0.1% TFA (solvent A) for 10 min, and then a linear gradient of 0.08% TFA in 70% acetonitrile (solvent B) was applied at 1% B per min (flow rate, 1 ml/min). Purification of the ferri-siderophore complex was similar, except that this material required 80% methanol–1% acetic acid for elution from the C_{18} Sep-Pak cartridges. Siderophores were purified to homogeneity by this protocol (Fig. 1). *B. pertussis* and *B. bronchiseptica* culture supernatants yielded desferri- and ferri-siderophores that behaved identically during purification, suggesting that these were the same iron chelator (data not shown).

We previously showed that a Tn*5lac* insertion mutation in *B. bronchiseptica* DBB22 blocked siderophore production, while other Fe transport components were intact (6). Strain DBB22 was also unable to use LF as an iron source for growth (6). We were unable to purify siderophore from culture supernatants of strain DBB22, indicating that this compound was uniquely found in culture supernatants from siderophore-proficient organisms (data not shown). To ensure that the purified *Bordetella* siderophore was biologically functional, we added purified desferri-siderophore to DBB22 cultures grown with 30% Fe-saturated bovine LF (6). While DBB22 was growth limited by LF (Fig. 2), the addition of purified desferri-siderophore to these cultures restored growth. This suggested that the purified siderophore transferred Fe from LF to DBB22. We inferred that the purified siderophores were biologically active and not structurally altered during purification.

HPLC-purified siderophores from *B. pertussis* and *B. bronchiseptica* were analyzed by liquid secondary ion mass spec-

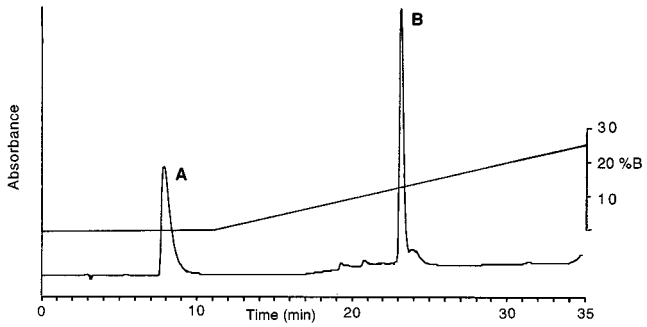

FIG. 1. HPLC chromatogram showing the elution of siderophore and desferri-siderophore from *B. pertussis*. Peak A shows the migration of the ferri-siderophore complex, while peak B shows the migration of the desferri-siderophore. Iron-free alcaligin coeluted with the *Bordetella* desferri-siderophore (peak B) at ≈23 min. The HPLC chromatogram for the siderophore material isolated from *B. bronchiseptica* was identical to that from *B. pertussis*.

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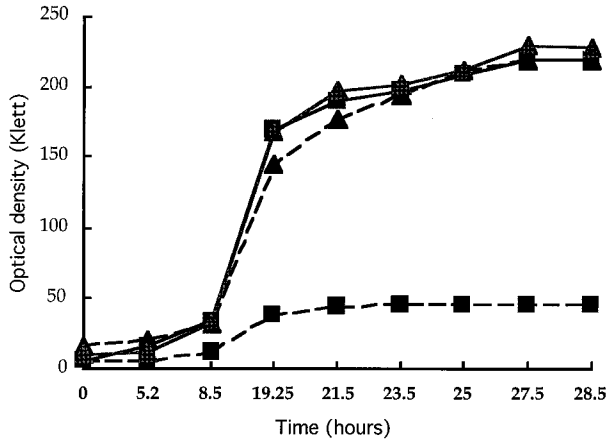

FIG. 2. Purified alcaligin supports growth of strain DBB22 with LF. *B. bronchiseptica* MBORD846 (▲) and DBB22 (■) were grown in Chelex-treated Stainer-Scholte medium (1, 6) with LF in the presence (solid lines) or absence (dashed lines) of purified alcaligin.

trometry. Samples were dissolved in water, applied to the liquid secondary ion mass spectrometry probe with 1 to 2 μl of thioglycerol-glycerol (1:1, vol/vol), and analyzed by using a Kratos MS-50S mass spectrometer operating with a Cs^+ ion source (5). Scans were taken at 300 s/decade, and spectra were recorded on a Gould electrostatic recorder. Accurate mass measurements of the two major peaks observed from the *B. pertussis* preparation at *m/z* 389 and 405 were carried out at a mass resolution of ≈8,000 (M/ΔM). The C-12 monoisotopic ion for the glycerol protonated tetramer, i.e., $(glycerol_4 + H)^+$ at *m/z* 369.1972, was used as the reference mass for peak matching.

For fragment ion analysis, tandem mass spectrometry was used with a four-sector mass spectrometer (Kratos Concept IIHH) in the positive-ion mode. The isotopically pure 12-C component of the protonated molecular ion $(M + H)^+$ at *m/z* 405.2 was selected and collisionally activated. The resulting fragment ions were separated and detected in MS2 as previously described (3). Data obtained for the siderophores produced by *B. pertussis* DBP2 and *B. bronchiseptica* MBORD846 were identical. For the iron-containing preparation, the two most abundant peaks were seen at *m/z* 862 and 884, with two additional peaks of much lower abundance at *m/z* 1319 and 1341. In contrast, the iron-free preparation contained abundant peaks at lower mass, i.e., *m/z* 389, 405, and 427, with a second cluster of lower-abundance peaks at *m/z* 809, 831, and 862. These latter peaks were interpreted as arising from a compound with a mass of 404 Da, i.e., *m/z* 389 $(M - O + H)^+$, *m/z* 405 $(M + H)^+$, *m/z* 427 $(M + Na)^+$, *m/z* 809 $(M_2 + H)^+$, *m/z* 831 $(M_2 + Na)^+$, and *m/z* 862 $(M_2 - 2H + Fe^{III})^+$. The iron-containing preparation did not contain peaks arising from monomer, but instead showed peaks for dimers and trimers: *m/z* 862 $(M_2 - 2H + Fe^{III})^+$, *m/z* 884 $(M_2 - 3H + Na + Fe^{III})$, *m/z* 1319 $(M_3 - 5H + 2\,Fe^{III})^+$, and *m/z* 1341 $(M_3 - 6H + Na + 2\,Fe^{III})^+$. The presence of such a small siderophore with a molecular weight of 404 suggested a simple structure, and a search was carried out against previously identified compounds of this class. A match was obtained for alcaligin, a symmetrical cyclic hydroxamate-containing siderophore from *A. denitrificans* (13). To ascertain whether the *Bordetella* siderophore was identical, its elemental composition was determined by high-resolution liquid secondary ion mass spectrometry. The accurate mass measurement yielded an *m/z* value of 405.200 for the singly protonated siderophore and an *m/z* value of 389.204 for the peak presumably formed by loss of oxygen, i.e., $(M - O + H)^+$. These masses agree very well with the elemental compositions of $C_{16}H_{29}N_4O_8$ (MH^+ calculated 405.1985) and $C_{16}H_{29}N_4O_7$ (MH^+ calculated 389.2036), the elemental compositions expected from singly protonated alcaligin and its deoxy fragment, respectively.

A tandem mass spectrometry experiment was carried out to better define the *Bordetella* siderophore and confirm its identity with alcaligin. The major fragment ions generated from the parent molecular ion at *m/z* 405 could be readily rationalized based on the alcaligin structure (Fig. 3). In particular, the major fragment ion at *m/z* 203 corresponded to a symmetric

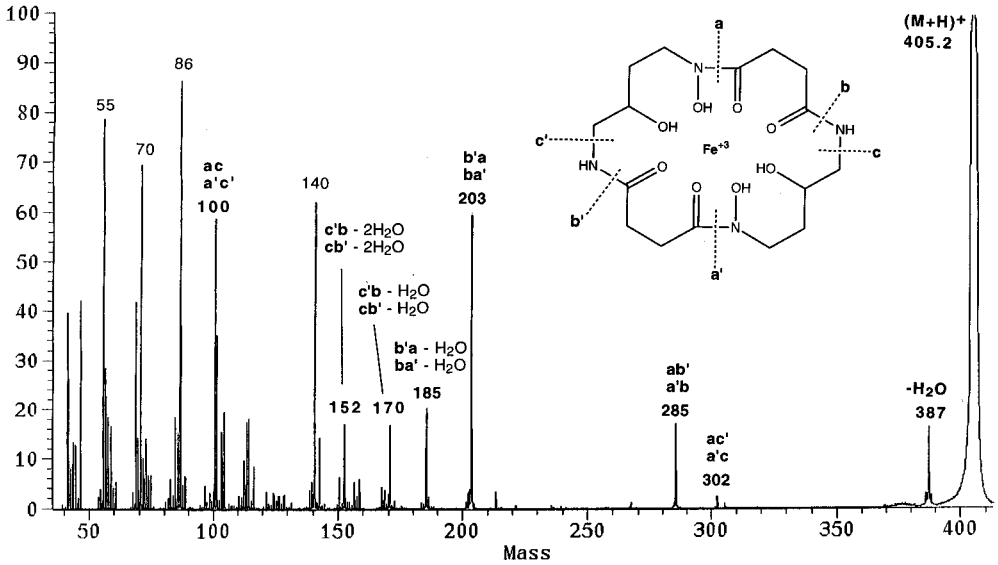

FIG. 3. Positive-ion tandem mass spectrum of the *B. pertussis* desferri-siderophore. The major fragment ions from the parent desferri-siderophore peak, $(M + H)^+$ at *m/z* 405.2, are assigned as originating from two-bond cleavages at various combinations of the symmetrical bonds a and a′, b and b′, and/or c and c′. For simplicity, fragment ions are labeled according to the fragment generated between the bonds cleaved in a clockwise fashion, i.e., a(bca′)b′ is listed as ab′, etc.

cleavage of the molecule between the two internal amide and hydroxamate moieties, i.e., b′(c′)a or b(c)a′ (Fig. 3, inset). These half-molecule fragments could undergo elimination of water to yield the peak at *m*/*z* 185. The peaks at *m*/*z* 302 and 285 are unique fragmentation products arising from cleavage at the hydroxamate linkage and either side of the amide nitrogens, a(bca′)b′ and a′(b′c′a)b and a(bca′b′)c′ and a′(b′c′ab)c, respectively. The identities of several low-mass fragment ions were less certain because of the degeneracy of possibilities (see, for example, *m*/*z* 140, 86, 70, and 55 in Fig. 3).

We obtained a sample of purified alcaligin from Kenneth Raymond (University of California, Berkeley). Under similar HPLC conditions, authentic alcaligin eluted exactly with the *Bordetella* siderophore at 22 min (≈13% solvent B; data not shown). The alcaligin standard produced a tandem mass spectrometry spectrum essentially identical to that obtained for the *Bordetella* siderophore (data not shown). We also compared the proton nuclear magnetic resonance (^{1}H-NMR) spectra of the two siderophores. ^{1}H-NMR spectra were recorded on a GE GN-500 spectrometer at 15°C. HPLC-purified *B. pertussis* siderophore (M_r, 404) was lyophilized several times from 99.96% D_2O (Aldrich) and dissolved in 0.3 ml of 99.996% CD_3OD (Cambridge Isotope Laboratories). A trace of acetone was added as an internal reference (δ 2.225). Data were analyzed as previously described (14). The ^{1}H-NMR spectrum of the *Bordetella* siderophore (data not shown) was, within experimental variability, identical to that reported for alcaligin by Nishio et al. (13).

Although *A. denitrificans* is a marine microorganism (11), *Bordetella* spp. and *A. denitrificans* are taxonomically close (11, 12). Thus, the observation that *B. pertussis*, *B. bronchiseptica*, and *A. denitrificans* produce an identical siderophore may not be surprising. *A. denitrificans* has occasionally been observed to cause disease in immunocompromised patients (4, 9), although the organism is normally noninfectious (4, 9). Current studies in our laboratory concern whether alcaligin production is important for supporting *B. bronchiseptica* growth in vivo. These data might also indicate whether *A. denitrificans* depends upon alcaligin-mediated Fe transport while growing in an immunocompromised host. Alternatively, disease-associated *A. denitrificans* strains may possess a unique Fe transport system.

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REFERENCES

1. **Agiato, L. A., and D. W. Dyer.** 1991. Siderophore production and membrane protein alterations by *Bordetella pertussis* in response to iron starvation. Infect. Immun. **60:**117–123.
2. **Armstrong, S. K., and M. O. Clements.** 1993. Isolation and characterization of *Bordetella bronchiseptica* mutants deficient in siderophore activity. J. Bacteriol. **175:**1144–1152.
3. **Basus, V. J., M. Billeter, R. A. Love, R. M. Stroud, and I. D. Kuntz.** 1988. Structural studies of α-bungarotoxin. 1. Sequence-specific ^{1}H NMR resonance assignments. Biochemistry **27:**2763–2771.
4. **Decre, D., G. Arlet, C. Danglot, J. C. Lucet, G. Fournier, E. Bergogne-Berezin, and A. Philippon.** 1992. A β-lactamase-overproducing strain of *Alcaligenes denitrificans* subsp. *xylosoxydans* isolated from a case of meningitis. J. Antimicrob. Chemother. **30:**769–779.
5. **Falick, A. M., G. H. Wang, and F. C. Walls.** 1986. Ion source for liquid matrix secondary ionization mass spectrometry. Anal. Chem. **58:**1308–1311.
6. **Foster, L. A., and D. W. Dyer.** 1993. A siderophore production mutant of *Bordetella bronchiseptica* does not grow with lactoferrin as an iron source. Infect. Immun. **61:**2698–2702.
7. **Goodnow, R. A.** 1980. Biology of *Bordetella bronchiseptica*. Microbiol. Rev. **44:**722–738.
8. **Gorringe, A. R., G. Woods, and A. Robinson.** 1990. Growth and siderophore production by *Bordetella pertussis* under iron-restricted conditions. FEMS Microbiol. Lett. **66:**101–106.
9. **Hearn, Y. R., and R. M. Gander.** 1991. *Achromobacter xylosoxydans*. An unusual neonatal pathogen. Am. J. Clin. Pathol. **96:**211–214.
10. **Hinz, K.-H., G. Glunder, and H. Luders.** 1978. Acute respiratory disease in turkey poults caused by *Bordetella bronchiseptica*-like bacteria. Vet. Rec. **103:**262–263.
11. **Krieg, N. R., and J. G. Holt (ed.).** 1984. Bergey's manual of systematic bacteriology, vol. 1. Williams & Wilkins, Baltimore.
12. **Musser, J. M., E. L. Hewlett, M. S. Peppler, and R. K. Selander.** 1987. Genetic diversity and relationships in populations of *Bordetella* spp. J. Bacteriol. **166:**230–237.
13. **Nishio, T., N. Tanaka, J. Hiratake, Y. Katsube, Y. Ishida, and J. Oda.** 1988. Isolation and structure of the novel dihydroxamate siderophore alcaligin. J. Am. Chem. Soc. **110:**8733–8734.
14. **Walls, F. C., et al.** 1990. Experience with multichannel array detection in tandem mass spectrometric characterization of biopolymers at the picomole level, p. 197–216. *In* A. L. Burlingame and J. A. McCloskey (ed.), Biological mass spectrometry. Elsevier, Amsterdam.
15. **Wardlaw, A. C., and R. Parton.** 1988. The host-parasite relationship in pertussis, p. 327–352. *In* A. C. Wardlaw and R. Parton (ed.), Pathogenesis and immunity in pertussis. John Wiley & Sons, New York.