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A Siderophore Production Mutant of *Bordetella bronchiseptica* Cannot Use Lactoferrin as an Iron Source

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Bordetella bronchiseptica **secreted a hydroxamate siderophore when grown in Fe-depleted medium. A Tn*5lac* insertion mutant of *B. bronchiseptica*, DBB22, did not produce this hydroxamate siderophore and was incapable of using lactoferrin as an Fe source. Our data suggest that *B. bronchiseptica* uses a siderophore for removal of Fe from lactoferrin and transferrin rather than relying upon a receptor for these host Fe-binding proteins.**

With one exception (2), iron (Fe) has been shown to be an essential nutrient for all organisms examined. Fe is important for a variety of biochemical oxidation-reduction reactions, such as oxidative phosphorylation and synthesis of DNA precursors (18). Most Fe in mammalian hosts is found intracellularly in ferritin, hemosiderin, and hemoproteins (28). Extracellular Fe^{3+} is bound to lactoferrin (LF) in exocrine secretions, such as saliva, tears, and mucosal secretions, and to transferrin (TF) in plasma. One consequence of extracellular Fe sequestration is that LF and TF bind Fe^{3+} with such high avidity (K_D, $\geq 10^{-20}$ M) that many microbes cannot remove the ion for microbial metabolism (27). Thus, Fe sequestration is a nonspecific mechanism that prevents or suppresses microbial growth in mucosal secretions and tissue fluids, termed nutritional immunity (27).

Obviously, bacterial pathogens have evolved ways to remove Fe from LF and TF and allow growth of the pathogen despite nutritional immunity. *Bordetella bronchiseptica* is a mucosal pathogen which is commonly associated with respiratory diseases in domesticated animals (7). *B. bronchiseptica* is thought to initiate atrophic rhinitis (atrophy of the nasal turbinates) in confinement-reared swine (26), a syndrome that commonly results from a mixed infection with *Pasteurella multocida* (6). Atrophic rhinitis can also predispose swine to further respiratory infections, including pneumonia, and such infections are of considerable economic importance (7). *B. bronchiseptica* infects dogs, causing kennel cough, a severe respiratory infection similar in some respects to human pertussis. *B. bronchiseptica* has been reported to infect humans, but this is rare (31).

Since it is believed that LF is the primary Fe source on the mucosal surface (13) (with uncertain contributions by TF, heme, and other Fe sources), it is likely that *B. bronchiseptica* evolved a specialized mechanism involved in the acquisition of Fe from this Fe-binding protein. Redhead et al. (22) published the first report on Fe uptake by *Bordetella* spp. This report concluded that *B. pertussis* does not produce a siderophore and utilizes a TF-LF receptor-mediated mechanism of Fe removal from TF and LF. We, and others, had reported that *B. pertussis* secretes a hydroxamate siderophore under conditions of Fe stress (1, 9). Other reports in the literature have suggested that *B. pertussis* and *B. bronchiseptica* possess two Fe transport systems. Redhead and Hill (21) reported that *B. pertussis* internalized much less Fe from TF when physical contact between the cell and the protein was prevented. They concluded that *B. pertussis* possesses a receptor for TF which enables the bacterium to acquire Fe from the protein relatively efficiently and that siderophore-mediated delivery of Fe to the bacterium is not very efficient. Menozzi et al. (14) identified a TF-LF-binding protein in both *B. pertussis* and *B. bronchiseptica*. They found that growth of *B. pertussis* and *B. bronchiseptica* with TF and LF reached a higher cell density when the bacteria were able to contact the protein physically. However, the kinetics of growth was not examined in these experiments, as Menozzi et al. (14) reported growth density at only one time point. Again, these researchers suggested that *Bordetella* spp. possess two separate mechanisms for acquiring Fe from TF and LF and that the receptor is more efficient than the siderophore at removing Fe from the proteins. These conclusions were in contrast to our previous findings. We had demonstrated that *B. pertussis* grew with the same doubling time in the log phase and reached the same final cell density whether the LF was free in the medium or sequestered inside a dialysis membrane (blocking physical contact between the cell and the Fe carrier) (1).

To resolve these conflicting data, we isolated a Tn*5lac* insertion in *B. bronchiseptica* that blocked siderophore production. This allowed us to test whether siderophore production is essential for removal of Fe from TF and LF.

Iron-limited growth of *B. bronchiseptica*. By using conditions previously described for *B. pertussis* (1), we were able to Fe limit the growth of *B. bronchiseptica* MBORD 846 (obtained from James Musser) in Chelex-treated Stainer-Scholte medium (CSSM; 1, 25) and in Chelex-treated defined medium (CDM; 30). Fe-limited growth in CDM was reversed by addition of 10 μM $FeSO_4$, 30% saturated LF (33 μM protein, 10 μM Fe), 10 μM heme, and Fe-dicitrate (data not shown). In these experiments, we used bovine LF (Sigma Chemical Co., St. Louis, Mo.) as a nonhuman source of the Fe-binding protein, as we were unable to locate a commercial source of porcine LF in the United States. When human LF or TF was used as the sole source of Fe in CDM, MBORD 846 grew at the same rate and to the same final extent in the presence of the human proteins as with the bovine LF, indicating that the organism does not discriminate between these mammalian Fe-binding proteins.

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MBORD 846 was also capable of growth with bovine LF when physical contact between the cell and the protein was prevented by a dialysis membrane (data not shown).

Siderophore production by *B. bronchiseptica*. Filter-sterilized culture supernatant from Fe-limited MBORD 846 reacted positively in the Csaky assay (4), which detects hydroxamates. In a representative experiment, Fe-starved MBORD 846 culture supernatants contained 78 μM hydroxamate (compared with a hydroxylamine standard), while culture supernatants from Fe-replete cells did not react in the Csaky assay. This indicated that the hydroxamate material in MBORD 846 culture supernatants was produced in response to Fe limitation, a characteristic common to microbial siderophores (19). No phenolic compound was detected in the culture supernatants as determined by the Arnow assay (3).

Tn*5lac* mutagenesis. Tn*5lac* was delivered to MBORD 846 on pDLA1, a derivative of suicide vector pGP704 (16). SF800 (P1::Tn*5lac*) (obtained from D. Kaiser; 10) was the source of Tn*5lac*. The bacteriophage lysate was used to transduce SY327[λpir](pGP704) (16). Plasmid DNA was isolated from pooled transductants by an alkali lysis procedure (12) and transferred to *Escherichia coli* SY327[λpir] by electroporation by using a Gene Pulser (Bio-Rad, Richmond, Calif.) and the manufacturer's protocol. Plasmid DNA was isolated from each transformant and electroporated into SM10[λpir] (16). One of these transformants, SM10 (pDLA1), mobilized the plasmid at a higher frequency than the others, as determined by spot matings, and was used in subsequent experiments.

pDLA1 was conjugally mobilized into MBORD 846 essentially as previously described (29). Transconjugants were replica plated onto CSSM–10 μM $FeSO_4$–5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) at 40 μg/ml; CSSM–50 μM 2,2′-dipyridil–X-Gal; and CSSM–chrome azurol S dye (CAS; which detects all types of Fe-chelating compounds [24]) and a streptomycin- and kanamycin-containing Bordet-Gengou agar plate and incubated at 37°C for 24 h. Residual Fe in the CSSM agar plates was reduced by using Bacto Agar (Difco, Detroit, Mich.) washed extensively with methanol (30). Colonies were examined for differences in β-galactosidase expression on high- and low-Fe plates and for differences in the sizes of the yellow halos surrounding the colonies on CAS plates.

A total of 13,000 transconjugants were screened in plate assays. We did not detect significant differences in β-galactosidase activity in any Tn*5lac* insertion in response to Fe availability. We did isolate several insertions that behaved atypically on CAS plates compared with the wild-type parent, which typically produced yellow halos about 0.5 cm in diameter. These insertion mutants either produced very large halos or grew poorly on CAS plates. These mutants were further examined in broth culture for growth rate, siderophore production, and β-galactosidase production by the method of Miller (15).

We identified one insertion mutant, designated DBB22, which showed no siderophore production. This insertion mutant grew at the same rate and to the same final extent as MBORD 846 in minimal medium, suggesting that the transposon insertion did not create auxotrophy. No hydroxamate was detected when the organism was grown in CSSM or CDM, which contains all amino acids and several vitamins (30). No Fe-binding material was detected in the culture supernatant from Fe-starved DBB22 with the CAS assay (24) or by examination of the difference absorption spectrum of filter-sterilized culture supernatant (Fig. 1) as previously described (1). Disruption of Fe-starved DBB22 cells by treatment in a French press also did not release any intracellular hydroxamate (data not shown).

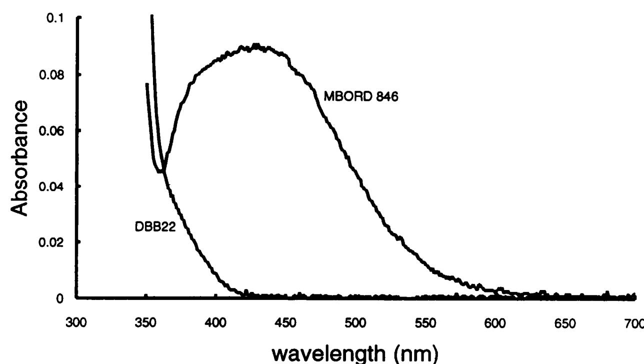

FIG. 1. Comparison of the difference absorption spectra of culture supernatants from Fe-starved MBORD 846 and DBB22, indicating that DBB22 excretes no Fe-binding material into the culture supernatant.

Outer membrane protein components of bacterial Fe transport systems are often synthesized in greater quantities in response to Fe starvation (19). Therefore, we prepared Triton X-100-insoluble membranes from MBORD 846 and DBB22 grown in CSSM with or without 10 μM $FeSO_4$ as described by Redhead and Hill (20). The Triton X-100-insoluble fractions were subjected to sodium dodecyl sulfate (SDS)–7.5 and 15% polyacrylamide gel electrophoresis (PAGE) (11), and the proteins were visualized by silver staining (32). MBORD 846 and DBB22 both expressed several proteins that were consistently regulated by the Fe levels in the medium (Fig. 2). Fe-repressible proteins (expressed only during low Fe availability) migrated at 104, 98, 88, 77, 63, 34, and 23 kDa in SDS-PAGE. It is possible that the Fe-repressible protein we observed at 34 kDa is the TF-LF-binding protein reported by Menozzi et al. (14). Conversely, Fe-inducible proteins (expressed during high Fe availability) migrated at 101, 70, and 67 kDa in both wild-type and mutant strains. Thus, we concluded that the transposon insertion in DBB22 had minimally affected the metabolism and cell surface components of the organism and had only blocked siderophore production.

Southern blot analysis of DBB22. Genomic DNA from DBB22 was examined on Southern blots as previously described (12), to determine whether lack of siderophore production was the result of a single transposon insertion. *Bordetella* genomic DNA was isolated (5), digested to completion with *Eco*RI (Promega, Madison, Wis.), and electrophoresed on a 0.6% agarose gel at 25 V for 18 h. The DNA was transferred to a NitroPlus membrane (Micron Separations, Inc., Westboro, Mass.) as previously described (12). Prehybridization, hybridization, and washing were performed as previously described (12). The filters were hybridized with pGP704, the parent plasmid of pDLA1, or a 1.2-kb *Bgl*II-*Pst*I fragment containing the Kan^r-encoding gene of Tn*5*. We also probed genomic DNA with an 800-bp fragment homologous with the IS*50* elements of Tn*5*. Probes were labelled with [α-^{32}P]dCTP (New England Nuclear) by using a random primer labelling kit (Bethesda Research Laboratories, Gaithersburg, Md.) in accordance with the manufacturer's specifications. After hybridization, the filter was washed (12), dried, wrapped in Saran Wrap, and exposed to AR film (Kodak, Rochester, N.Y.).

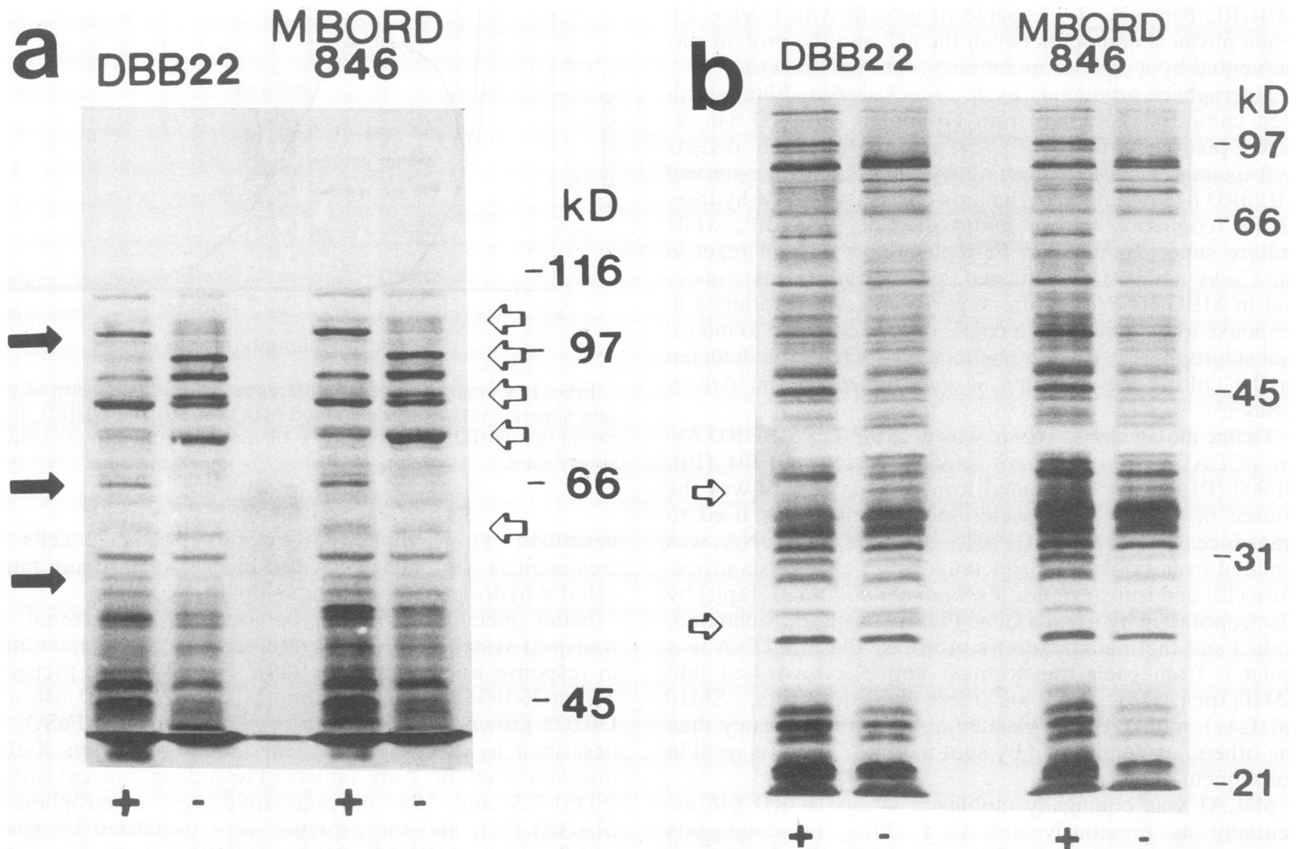

FIG. 2. SDS-PAGE of Triton-insoluble membrane fractions isolated from MBORD 846 and DBB22 grown with 10 μM $FeSO_4$ (+) or without Fe (−). The proteins were separated on 7.5% (a) and 15% (b) gels and stained with silver. Open arrows indicate proteins observed to be consistently Fe repressible in several different experiments. Closed arrows point to Fe-inducible proteins consistently observed in several experiments. Other proteins that appear to be responsive to Fe levels in this figure were not observed in all experiments. kD, kilodaltons.

The probe specific for the Kan^r-encoding gene hybridized to a single 11.2-kb fragment (Fig. 3), indicating that the Tn*5lac* element was present at a single site in DBB22. When DBB22 genomic DNA was probed with vector plasmid pGP704, two fragments, of 8.4 and 5.9 kb, hybridized to the plasmid-specific probe, indicating that plasmid DNA was still present in DBB22 (Fig. 3). We could not isolate plasmid DNA from DBB22 by SDS lysis followed by a CsCl-ethidium bromide gradient purification procedure (12). When the IS*50*-specific probe was used, these same three fragments, (11.2, 8.4, and 5.9 kb) hybridized to the probe (Fig. 3). These results suggested that pDLA1 integrated into the chromosome in its entirety and was present at a single site as a plasmid cointegrate. Cointegration of the plasmid was presumably mediated by the intact IS50_R element on Tn*5lac* (10). We did not detect differences in the levels of β-galactosidase produced in different high- and low-Fe environments, as would be expected if the transposon had inserted itself into genes involved in the synthesis of an Fe-regulated compound. This was not surprising, since the insertion left the *lacZ* gene of Tn*5lac* downstream of promoters on the plasmid rather than downstream of *B. bronchiseptica* genomic sequences.

Siderophore production is essential for growth of *B. bronchiseptica* with LF. We compared the abilities of DBB22 and MBORD 846 to use bovine LF as the sole Fe source in CDM (Fig. 4). MBORD 846 was capable of using bovine LF as an

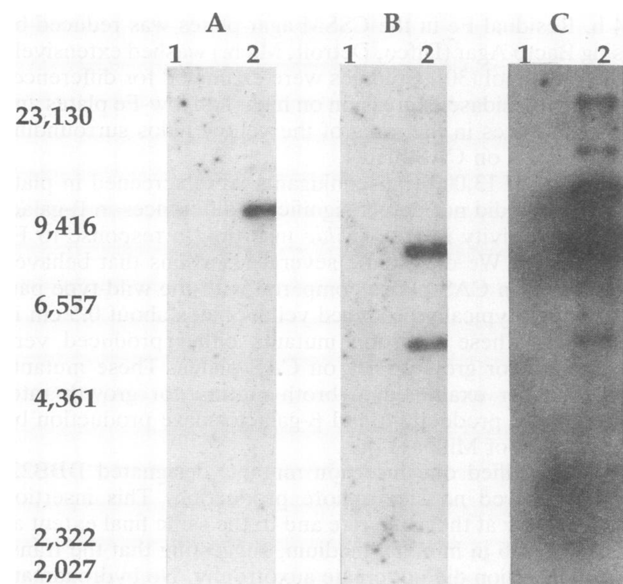

FIG. 3. Autoradiograph of Southern blots probed with a 1.2-kb *Bgl*II-*Pst*I fragment of the kanamycin resistance gene of Tn*5* (A), pGP704 (B), and the IS*50*-specific probe (C). The lanes contained *Eco*RI-digested genomic DNAs from DBB1 (lane 1) and DBB22 (lane 2). Lambda *Hin*dIII molecular weight standards are indicated at the left.

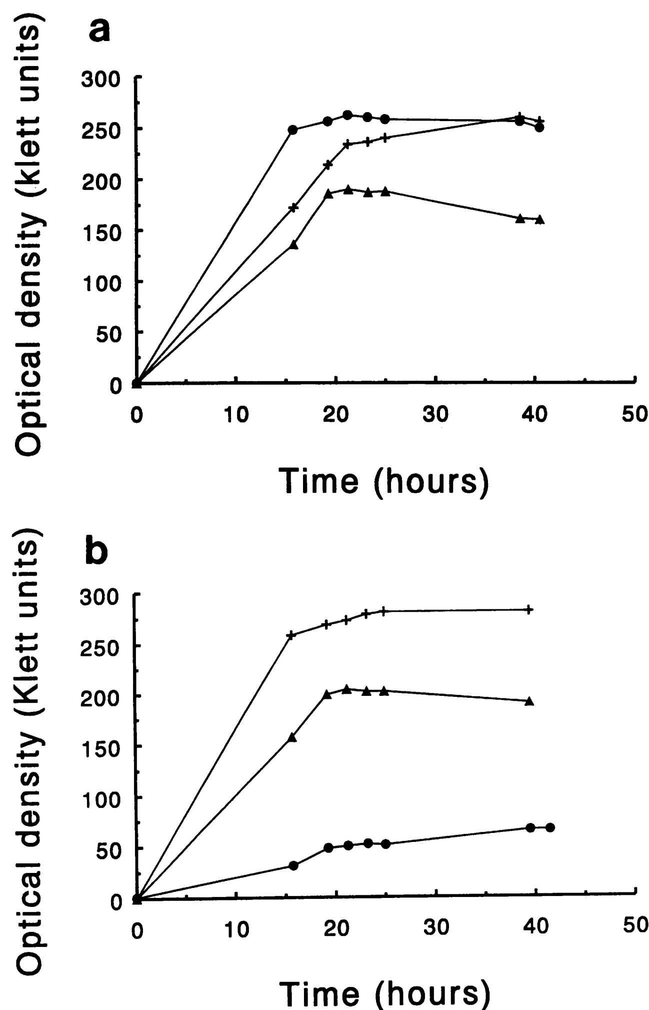

FIG. 4. Growth of MBORD 846 (a) and DBB22 (b) in CDM containing 5 μM Fe-citrate (+), bovine LF (●), or no added Fe (▲).

Fe source (Fig. 4A), but DBB22 was unable to utilize the Fe bound to bovine LF (Fig. 4B). Further, LF severely inhibited the growth of DBB22, far below the growth of DBB22 in CDM (no Fe). We interpret this to indicate that LF bound the residual Fe in CDM and blocked the ability of DBB22 to obtain this modest amount of Fe. DBB22 was capable of using heme and Fe-dicitrate as sole Fe sources (data not shown).

The conclusion that *B. bronchiseptica* may use only a siderophore-mediated Fe transport system is also consistent with what is known about the biology and genetic relationships between *Bordetella* spp. Although *B. pertussis* is thought to be an exclusively human pathogen (8), the *Bordetella* spp. that infect mammalian hosts (*B. pertussis*, *B. parapertussis*, and *B. bronchiseptica*) are highly related at the genetic level. On the basis of the results of multilocus enzyme electrophoretic comparison of these three species, Musser et al. (17) suggested that these should actually be considered variants of a single species. Thus, it is likely that the Fe transport systems of these organisms are closely related, if not identical. Further, *B. bronchiseptica* often infects a variety of mammalian hosts (7, 31) and does not have the restricted host range of *B. pertussis* and *B. parapertussis*. This suggests that the Fe transport system of *B. bronchiseptica* (and, by inference, those of *B. pertussis* and *B. parapertussis*) evolved to utilize Fe sources from a variety of different hosts. By contrast, the TF and LF receptor-mediated Fe transport systems produced by a variety of pathogens all depend upon a receptor that is highly specific for the TF and/or LF from the host species that the pathogen infects (23). Thus, we suggest that it is unlikely that *Bordetella* spp. depend upon a TF-LF receptor for removal of Fe from these host proteins and instead uses a siderophore to obtain Fe during infection.

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REFERENCES

1. **Agiato, L.-A., and D. W. Dyer.** 1992. Siderophore production and membrane alterations by *Bordetella pertussis* in response to iron starvation. Infect. Immun. **60:**117–123.
2. **Archibald, F.** 1983. *Lactobacillus planarum*, an organism not requiring iron. FEMS Microbiol Lett. **19:**29–32.
3. **Arnow, E.** 1937. Colorimetric determination of the components of 3,4-dihydroxylphenylalanine–tyrosine mixtures. J. Biol. Chem. **118:**531–537.
4. **Csaky, T. Z.** 1948. On the estimation of bound hydroxylamine in biological samples. Acta Chem. Scand. **2:**450–454.
5. **Dyer, D. W., and J. J. Iandolo.** 1983. Rapid isolation of DNA from *Staphylococcus aureus*. Appl. Environ. Microbiol. **46:** 283–285.
6. **Elling, F., and K. B. Pedersen.** 1985. The pathogenesis of persistent turbinate atrophy induced by toxigenic *Pasteurella multocida* in pigs. Vet. Pathol. **22:**469–474.
7. **Goodnow, R. A.** 1980. Biology of *Bordetella bronchiseptica*. Microbiol. Rev. **44:**722–738.
8. **Gordon, J. E., and R. I. Hood.** 1951. Whooping cough and its epidemiological anomalies. Am. J. Med. Sci. **222:**333–361.
9. **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.
10. **Kroos, L., and D. Kaiser.** 1984. Construction of Tn*5lac*, a transposon that fuses *lacZ* expression to exogenous promoters, and its introduction into *Myxococcus xanthus*. Proc. Natl. Acad. Sci. USA **81:**5816–5820.
11. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
12. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
13. **Masson, P. L., J. F. Heremans, and C. H. Dive.** 1966. An iron-binding protein common to many external secretions. Clin. Chim. Acta **14:**729–734.
14. **Menozzi, F. D., C. Gantiez, and C. Locht.** 1991. Identification and purification of transferrin- and lactoferrin-binding proteins of *Bordetella pertussis* and *Bordetella bronchiseptica*. Infect. Immun. **59:**3982–3988.
15. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 352–355. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
16. **Miller, V. I., and J. J. Mekalanos.** 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. J. Bacteriol. **170:**2575–2583.
17. **Musser, J. M., E. L. Hewlett, and M. S. Peppler.** 1986. Genetic diversity and relationships in populations of *Bordetella* spp. J. Bacteriol. **166:**230–237.
18. **Neilands, J. B.** 1981. Iron absorption and transport in microorganisms. Annu. Rev. Nutr. **1:**27–45.
19. **Neilands, J. B.** 1982. Microbial envelope proteins related to iron. Annu. Rev. Microbiol. **36:**285–309.
20. **Redhead, K., and T. Hill.** 1983. Variability in the surface exposure of the outer membrane of *Bordetella pertussis*. J. Gen.

Microbiol. **17**:35–39.

21. **Redhead, K., and T. Hill.** 1991. Acquisition of iron from transferrin by *Bordetella pertussis*. FEMS Microbiol. Lett. **77**:303–308.
22. **Redhead, K., T. Hill, and H. Chart.** 1987. Interaction of lactoferrin and transferrins with the outer membrane of *Bordetella pertussis*. J. Gen. Microbiol. **133**:891–898.
23. **Schryvers, A. B., and G. C. Gonzalez.** 1990. Receptors for transferrin in pathogenic bacteria are specific for the host's protein. Can. J. Microbiol. **36**:145–147.
24. **Schwyn, B., and J. B. Neilands.** 1987. Universal chemical detection and determination of siderophores. Anal. Biochem. **160**:47–56.
25. **Stainer, D. W., and M. J. Scholte.** 1971. A simple chemically defined medium for the production of phase I *Bordetella pertussis*. J. Gen. Microbiol. **63**:211–220.
26. **Switzer, W. P.** 1956. Studies on infectious atrophic rhinitis. Am. J. Vet. Res. **17**:478–484.
27. **Weinberg, E. D.** 1978. Iron and infection. Microbiol. Rev. **42**:45–66.
28. **Weinberg, E. D.** 1990. Cellular iron metabolism in health and disease. Drug Metab. Rev. **22**:531–579.
29. **Weiss, A. A., A. R. Melton, K. E. Walker, C. Andraos-Selim, and J. J. Meidl.** 1989. Use of promoter fusion transposon Tn*5* *lac* to identify mutations in *Bordetella pertussis vir*-regulated genes. Infect. Immun. **57**:2674–2682.
30. **West, S. E. H., and P. F. Sparling.** 1987. Aerobactin utilization by *Neisseria gonorrhoeae* and the cloning of a genomic DNA fragment that complements *Escherichia coli fhuB* mutations. J. Bacteriol. **169**:3414–3421.
31. **Woolfrey, B. F., and J. A. Moody.** 1991. Human infections associated with *Bordetella bronchiseptica*. Clin. Microbiol. Rev. **4**:243–255.
32. **Wray, W., T. Boulikas, V. P. Wray, and R. Hancock.** 1981. Silver staining of proteins in polyacrylamide gels. Anal. Biochem. **118**:197–203.