Growth of *Actinobacillus pleuropneumoniae* Is Promoted by Exogenous Hydroxamate and Catechol Siderophores

MOUSSA S. DIARRA,¹ JULIA A. DOLENCE,² E. KURT DOLENCE,² IHAB DARWISH,²
MARVIN J. MILLER,² FRANÇOIS MALOUIN,³ AND MARIO JACQUES^{4*}

Département de Microbiologie, Faculté de Médecine, Université Laval, Sainte-Foy, Québec, Canada G1V 7P4¹;
Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, Indiana 46556²;
Microcide Pharmaceuticals Inc., Mountain View, California 94043³; and Département de Pathologie
et Microbiologie, Faculté de Médecine Vétérinaire, Université de Montréal,
St-Hyacinthe, Québec, Canada J2S 7C6⁴

Received 7 September 1995/Accepted 15 December 1995

Siderophores bind ferric ions and are involved in receptor-specific iron transport into bacteria. Six types of siderophores were tested against strains representing the 12 different serotypes of *Actinobacillus pleuropneumoniae*. Ferrichrome and bis-catechol-based siderophores showed strong growth-promoting activities for *A. pleuropneumoniae* in a disk diffusion assay. Most strains of *A. pleuropneumoniae* tested were able to use ferrichrome (21 of 22 or 95%), ferrichrome A (20 of 22 or 90%), and lysine-based bis-catechol (20 of 22 or 90%), while growth of 36% (8 of 22) was promoted by a synthetic hydroxamate, *N*⁵-acetyl-*N*⁵-hydroxy-L-ornithine tripeptide. *A. pleuropneumoniae* serotype 1 (strain FMV 87-682) and serotype 5 (strain 2245) exhibited a distinct yellow halo around colonies on Chrome Azurol S agar plates, suggesting that both strains can produce an iron chelator (siderophore) in response to iron stress. The siderophore was found to be neither a phenolate nor a hydroxamate by the chemical tests of Arnow and Csaky, respectively. This is the first report demonstrating the production of an iron chelator and the use of exogenous siderophores by *A. pleuropneumoniae*. A spermidine-based bis-catechol siderophore conjugated to a carbacephalosporin was shown to inhibit growth of *A. pleuropneumoniae*. A siderophore-antibiotic-resistant strain was isolated and shown to have lost the ability to use ferrichrome, synthetic hydroxamate, or catechol-based siderophores when grown under conditions of iron restriction. This observation indicated that a common iron uptake pathway, or a common intermediate, for hydroxamate- and catechol-based siderophores may exist in *A. pleuropneumoniae*.

Actinobacillus pleuropneumoniae is the causative agent of porcine fibrinohemorrhagic necrotizing pleuropneumonia, a severe disease causing large economic losses in industrialized swine production (37). Twelve capsular serotypes are described; serotypes 1 and 5 are predominant in Québec and in the United States, while serotype 2 is important in most European countries (31, 38). The mechanism by which the bacterium invades and colonizes the host has been the subject of a large body of research. Several secreted products, outer membrane components (outer membrane proteins [OMPs]) and lipopolysaccharides, and capsules have been implicated as virulence factors (3, 4, 11, 20, 41). In addition, three pore-forming RTX toxins (ApxI and ApxII, which are hemolytic, and ApxIII) have been described and characterized (13, 21).

Pathogenic bacteria have a strict nutritional requirement for iron, but in mammalian tissues, most iron is complexed with other molecules, notably transferrin in plasma, lactoferrin in mucous secretions and in polymorphonuclear leukocyte granules, and hemoglobin (1, 24). To obtain iron, pathogenic bacteria possess high-affinity iron uptake systems which consist in part of OMPs expressed under conditions of iron limitation. Most aerobic, facultative anaerobic, and saprophytic microorganisms have the ability to produce or to use high-affinity iron-binding compounds, termed siderophores, that are capable of chelating ferric iron and allow its assimilation through

cell surface receptors (16, 27, 34). Therefore, the ability to produce and utilize siderophores has been frequently linked to the virulence of certain pathogenic bacteria (27). Siderophores are broadly grouped into two classes, namely, hydroxamates and catecholates, according to the chemical group that is involved in forming the iron ligands (35). In addition, restricted availability of iron in a host functions as an important signal leading to the enhanced expression of a wide variety of bacterial toxins and other virulence determinants (24, 27).

Little is known about the iron acquisition mechanisms of *A. pleuropneumoniae*, but the presence of iron uptake systems might represent an important virulence mechanism for this bacterium. Under iron-restricted growth conditions, *A. pleuropneumoniae* can use porcine transferrin, hemoglobin, and various porphyrin compounds as sources of iron but it cannot utilize bovine or human transferrin (3, 10, 14). Analysis of the serological response to outer membrane antigens during *A. pleuropneumoniae* infection in pigs has identified a number of OMPs that are reactive only with convalescent serum (10). Two of the iron-repressible proteins have been shown to bind transferrin in an in vitro binding assay (15, 39, 42). One of the *A. pleuropneumoniae* transferrin-binding proteins (*M*_r of 60,000) has been cloned (14). Recently, *A. pleuropneumoniae* lipopolysaccharide was shown, by Bélanger et al., to bind pig hemoglobin (3). Until now, no siderophores have been detected in *A. pleuropneumoniae*.

The aim of the present study was to investigate the capacity of *A. pleuropneumoniae* strains of various serotypes to obtain iron from hydroxamate or catechol siderophores. We report that *A. pleuropneumoniae* can utilize these siderophores for growth and show that a carbacephalosporin covalently linked

* Corresponding author. Mailing address: Département de Pathologie et Microbiologie, Faculté de Médecine Vétérinaire, Université de Montréal, 3200 rue Sicotte, St-Hyacinthe, Québec, Canada J2S 7C6. Phone: (514) 773-8521 ext. 8348. Fax: (514) 778-8108. Electronic mail address: jacqum@ERE.UMontreal.CA.

FIG. 1. Structures of lysine-based bis-catechol ISD-I-207 (A), spermidine-based bis-catechol ISD-I-201 (B), and tripeptide-based hydroxamate ISD-I-204 (C) siderophores, which were evaluated for their potential to promote growth of *A. pleuropneumoniae*, and structures of siderophore-carbacephalosporin conjugate JAM-3-089 (D) and EKD-5-273 (E), which were evaluated for their antibacterial activity. Ar or Ph is a phenyl group.

to a catechol-based siderophore exhibits activity against this microorganism, which is dependent on iron uptake systems for both catechol and hydroxamate type siderophores.

MATERIALS AND METHODS

Siderophores and siderophore-antibiotic conjugates. The chemical structures of the synthetic siderophores and siderophore-antibiotic conjugates used in this study are shown in Fig. 1. The iron-chelating portion of the hydroxamate ISD-I-204 contained a tripeptide sequence (N^5 -acetyl- N^5 -hydroxy-L-ornithine) similar to that of ferrichrome (Porphyrin Products, Logan, Utah) and ferrichrome A (Sigma Chemicals, St. Louis, Mo.) (28), also used in this study. Ferrichrome is a cyclic hexapeptide produced by many fungal species, including *Ustilago sphaerogena*, some *Aspergillus* species, and all *Penicillium* species, and contains three contiguous δ -N-hydroxy-L-ornithine residues and three glycine residues (18, 35). In ferrichrome A, the triglycyl peptide of the ferrichrome is replaced by the sequence seryl-seryl-glycyl and the acyl part of the hydroxamic acid bound is *trans*- β -methyl glutaconic rather than acetic acid (35). Desferrioxamine B (Desferal), composed of 1-amino- ω -hydroxylamino alkanes coupled by succinates, was also used in growth promotion tests and was kindly provided by Ciba Geigy. The catechol ISD-I-201 is derived from hydroxybenzoyl-based spermidine and contains N^1, N^{10} -bis(2,3-dihydroxybenzoyl)- N^5 -succinoylspermidine, and catechol ISD-I-207 containing bis(2,3-dihydroxybenzoyl)-L-lysine is also isolated from

Azotobacter vinelandii (22, 29). Their iron-chelating group is therefore similar to that of agrobactin and parabactin (28). The antibiotic conjugated to siderophores was a carbacephalosporin (loracarbef; Eli Lilly and Co., Indianapolis, Ind.). The siderophores and siderophore-antibiotic conjugates were synthesized at M. Miller's laboratory (University of Notre Dame, Notre Dame, Ind.). The synthesis, purification, and full characterization of all of the compounds tested have been described in detail in earlier publications (28–30). Compounds were stored as 10 mM solutions at -20°C in *N,N*-dimethyl sulfoxide or in methanol.

Bacterial strains and growth conditions. *A. pleuropneumoniae* reference strains representing serotypes 1 to 12 were used in the present study. In addition, a total of nine field isolates of *A. pleuropneumoniae* representing serotypes 1 and 5 were obtained from the Bacteriology Diagnostic Laboratory, Faculté de Médecine Vétérinaire, Université de Montréal, St-Hyacinthe, Québec, Canada. Bacteria from frozen stock were streaked onto chocolate agar plates prepared with Bacto GC Medium Base (Difco, Detroit, Mich.), Bacto hemoglobin (Difco), and 0.25% IsoVitalX (BBL, Montreal, Quebec, Canada). Plates were then incubated for 16 to 20 h at 37°C in 5% CO_2 . For most experiments, the strains were subcultured onto Mueller Hinton agar (MHA) or broth (MHB) (Difco) plates supplemented with NAD at 15 $\mu\text{g}/\text{ml}$ for an additional 16 to 20 h. Conditions of iron restriction were obtained after addition of 50 μg of deferrated EDDHA [ethylenediamine di-(*O*-hydroxyphenylacetic acid); Sigma] per ml or 100 μM 2,2'-dipyridyl (Sigma). Iron-rich media were obtained by adding 5 μM FeCl_3 (Sigma). Aqueous solutions of the test siderophores and/or ferric iron

chelator (EDDHA) were added by sterile filtration through a sterile filter assembly (pore size, 0.2 µm; Fisher).

Growth curves. Two-milliliter volumes of overnight cultures in MHB were used to inoculate 50 ml of fresh MHB containing EDDHA. Synthetic siderophores were added at 50 µM, and ferrichrome was added at 24 µM. All flasks were incubated at 37°C with agitation (300 rpm) for 8 h. Aliquots were removed every hour to determine the culture turbidity (optical density at 540 nm).

Growth promotion assay and antibiotic diffusion test. The bacteria were tested for their ability to use different sources of iron by using a growth promotion test (40). Susceptibilities to different siderophore-antibiotic conjugates were determined by a growth inhibition test. The plates, with or without EDDHA, were inoculated with a sterile cotton swab dipped in a bacterial suspension in saline (approximately 10⁸ CFU/ml). Disks (diameter, 6 mm) containing 0.04 µmol of test compounds were placed on the surfaces of agar plates to allow growth promotion (by siderophores) or inhibition (by siderophore-antibiotic conjugates). Plates were incubated at 37°C in 5% CO₂ for 24 h, and then growth promotion or inhibition zones around the disks were measured. Disks containing diluted dimethyl sulfoxide were used as controls. The isolation of bacteria resistant to siderophore-β-lactam conjugates was done by subculturing on MHA a colony present in the inhibition zone around the disk containing a siderophore-β-lactam conjugate.

Siderophore production assay. The production of a siderophore was evaluated by a qualitative chromogenic assay using chrome azurol S (CAS; Sigma) in the culture medium (44). This is a highly sensitive chemical method for the detection of siderophores. It is based on their affinity for iron(III), and its effectiveness is therefore independent of their chemical structure. When a strong chelator (i.e., siderophore) removes iron from the dye, its color turns from blue to orange. Agar plates were supplemented with 100 µM 2,2'-dipyridyl in addition to CAS. One colony was used to inoculate blue agar CAS plates. *Escherichia coli* H455, kindly provided by K. Hantke, Universität Tübingen (Tübingen, Germany), and *Pasteurella haemolytica*, kindly provided by C. Rioux, Veterinary Infectious Disease Organization (Saskatoon, Saskatchewan, Canada), were used as positive and negative controls, respectively.

Extraction of siderophores and chemical assays. The extraction of siderophores from bacteria was performed as described by Hu et al. (19). Cells from overnight cultures were used to inoculate 150 ml of MHB with EDDHA and incubated with agitation at 37°C. Cells were harvested during the stationary phase, and the supernatant obtained after centrifugation (12,000 × g for 30 min at 4°C) was filter sterilized and concentrated by freeze-drying. Methanol was added, and the mixture was stirred at room temperature overnight and then centrifuged to remove the undissolved material. The yellow supernatant was evaporated to dryness and then suspended in 2 ml of water. The Arnow test (2) was used to detect catechol type siderophores, while the presence of hydroxamate was determined by the Csaky test (9).

Outer membrane preparation. Cells from two chocolate agar plates were used to inoculate 1 liter of MHB containing NAD at 15 µg/ml. After incubation for 6 h, EDDHA at 50 µg/ml was added and growth was continued for an additional 10 h (10). The extraction of outer membrane from bacteria was performed as described by Hamel et al. (17). Bacteria were harvested by centrifugation at 12,000 × g for 15 min, and whole cells were then suspended in lithium chloride buffer (200 mM lithium chloride, 100 mM lithium acetate [pH 6.0]). Next, the bacteria were shaken with 6-mm-diameter glass beads at 300 rpm for 2 h at 45°C. The resulting spheroplasts were removed by centrifugation at 10,000 × g for 20 min, and the supernatant was collected and centrifuged at 55,000 × g for 2 h. The pelleted OMP preparation was washed once and then resuspended in phosphate-buffered saline and stored frozen (−20°C). The protein content was determined by the method of Lowry et al. (26) with bovine serum albumin as a standard. The membrane samples were suspended in electrophoresis sample buffer containing 1% sodium dodecyl sulfate (SDS) and 5% 2-mercaptoethanol. The samples were heated to 100°C for 5 min before being loaded for electrophoresis in discontinuous 0.1% SDS–10% polyacrylamide gels (23). Gels were stained with Coomassie brilliant blue.

Immunoblotting and search for FhuA-like OMP. Electrophoretic transfer of SDS-polyacrylamide gel electrophoresis-separated proteins to nitrocellulose membranes and immunoblotting were performed essentially as described by Towbin et al. (46). Nonspecific binding sites were blocked by incubating the membranes for 1 h at room temperature in Tris-saline buffer (TBS) (10 mM Tris, 150 mM NaCl [pH 7.4]) containing 2% casein. All other incubations were followed by 3-min washes with TBS. Membrane was next incubated first overnight at 4°C with either monoclonal antibody FhuA6.9 (reactive against the C terminus) or monoclonal antibody FhuA6.14 (reactive against the N terminus) directed against *E. coli* OMP FhuA (8) and then for 1 h at room temperature with a goat anti-mouse immunoglobulin G (heavy plus light chains)–horseradish peroxidase conjugate (Bio-Rad Laboratories, Richmond, Calif.). Reaction was revealed by addition of 4-chloro-1-naphthol and hydrogen peroxide (Sigma). *E. coli* K-12 strain SG303/*fhuA* and strain SG303/*fhuA* containing plasmid pGC01 with the *fhuA* gene were used as controls. Monoclonal antibodies and control *E. coli* strains were kindly provided by James W. Coulton, Department of Microbiology and Immunology, McGill University, Montréal, Québec, Canada.

RESULTS

Growth promotion by siderophores. To determine whether *A. pleuropneumoniae* can utilize exogenous siderophores for growth, the ability of hydroxamate tripeptides and bis-catechols to reverse the growth inhibition caused by EDDHA was evaluated by using a growth promotion assay (Table 1). Results indicate that *A. pleuropneumoniae* can obtain iron from both hydroxamate and catechol siderophores. The natural hydroxamate siderophore ferrichrome and the lysine-based bis-catechol siderophore ISD-I-207 exhibited the best growth promotion of all compounds tested. All strains of *A. pleuropneumoniae* tested were able to use ferrichrome and ferrichrome A, except one field strain of serotype 5 (86-31-1774), which was not able to use ferrichrome and ferrichrome A, and the reference strain of serotype 10, which was not able to use ferrichrome A. Growth of the reference strains of serotypes 3 and 8 and all serotype 5 strains, except strain 86-31-1774, were stimulated by the synthetic hydroxamate N⁵-acetyl-N⁵-hydroxy-L-ornithine tripeptide (ISD-I-204). Desferrioxamine B and ferric chloride (also 0.04 µmol on disks) were inactive (not shown). Except for the reference strains of serotypes 7 and 10, all of the tested strains of *A. pleuropneumoniae* were able to use the lysine-based bis-catechol (ISD-I-207) for growth, while the slightly different spermidine-based bis-catechol ISD-I-201 exhibited some activity only with reference strains of serotypes 3 and 9. Control disks containing diluted dimethyl sulfoxide did not inhibit or promote bacterial growth. Because most strains of *A. pleuropneumoniae* used ferrichrome for growth, we determined whether a membrane receptor for ferrichrome similar to *E. coli* FhuA was present in *A. pleuropneumoniae*. The results indicated that OMPs of *A. pleuropneumoniae* did not cross-react on immunoblotting with monoclonal antibodies directed against *E. coli* FhuA protein (data not shown).

Growth curves. Ferrichrome, synthetic hydroxamate ISD-I-204, and lysine-based bis-catechol ISD-I-207 were also tested for growth promotion activity in liquid culture deferrated by the addition of 50 µg of EDDHA per ml. As shown in Fig. 2, control cells of *A. pleuropneumoniae* serotype 5 strain 2245 grew very poorly in MHB with EDDHA, while addition of ferrichrome (24 µM) promoted strong growth. Trihydroxamate ISD-I-204 and bis-catechol ISD-I-207 were also able to promote growth of *A. pleuropneumoniae*, but to a lesser extent than ferrichrome did.

Detection of siderophore production. CAS agar plates were used to determine whether *A. pleuropneumoniae* serotype 1 (strain FMV 87-682) and serotype 5 (strain 2245) produce siderophores in response to iron stress. Both strains of *A. pleuropneumoniae* exhibited a distinct yellow halo around the colonies, indicative of the presence of a chelator of iron. Culture supernatants of these two strains grown in MHB supplemented with EDDHA were analyzed by the tests of Arnow and Csaky. These assays failed to detect the presence of catechol and hydroxamate compounds in the culture supernatant of organisms grown under conditions of iron limitation.

Siderophore-antibiotic conjugate activity. Hydroxamate- and catechol-carbacephalosporin conjugates were evaluated for antibacterial activities against *A. pleuropneumoniae* serotype 1 (strain FMV 87-682) and serotype 5 (strain 2245) (Table 2). Although siderophore-antibiotic conjugates have been shown to use iron uptake systems for entry into bacteria (7), the activity of the conjugates did not exactly correlate with the ability of the bacteria to use the siderophore portion of the molecules for growth. Even though both ferrichrome and the trihydroxamate ISD-I-204, having the N⁵-acetyl-N⁵-hydroxy-L-ornithine chelating components, and the bis-catechol-based

TABLE 1. Promotion of growth of *A. pleuropneumoniae* strains by various siderophores in disc diffusion tests^a

Serotype and strain	Diam (mm) of zone of growth promotion by indicated siderophore				
	Bis-catechol		Hydroxamate ^b		
	ISD-I-207	ISD-I-201	Ferrichrome	Ferrichrome A	ISD-I-204
Serotype 1					
4074 ^c	29 (28–30) ^d	0	31 (30–32)	14 (13–15)	0
Q87-586	29 (28–30)	0	30 (29–31)	15 (14–16)	0
FMV 87-586	31 (30–32)	0	30 (29–31)	14 (13–15)	0
FMV 87-682	33 (32–34)	0	31 (30–32)	15 (14–16)	0
87-41-1888	32 (31–33)	0	31 (29–33)	15 (14–16)	0
Serotype 2, 4226 ^c	28 (26–30)	0	31 (29–33)	15 (14–16)	0
Serotype 3, 1421 ^c	28 (27–29)	16 (14–18)	29 (28–30)	16 (15–17)	17 (17–18)
Serotype 4, 1462 ^c	31 (29–33)	0	30 (27–31)	12 (11–12)	0
Serotype 5					
750	31 (29–33)	0	32 (30–34)	16 (15–17)	18 (16–20)
L20 ^c	28 (27–29)	0	31 (29–33)	18 (17–19)	15 (14–16)
K17 ^c	29 (25–33)	0	26 (24–28)	16 (15–17)	15 (14–16)
SH-86-5163	29 (27–31)	0	28 (24–32)	25 (25–26)	15 (13–17)
2245	29 (27–31)	0	33 (32–34)	15 (14–16)	21 (20–23)
86-31-1774	28 (26–30)	0	0	0	0
86-4780	31 (29–33)	0	28 (27–29)	22 (21–23)	16 (15–17)
Serotype 6, FEMO ^c	24 (20–28)	0	27 (25–29)	12 (12–13)	0
Serotype 7, WF83 ^c	0	0	27 (25–29)	17 (16–18)	0
Serotype 8, 405 ^c	35 (33–37)	0	33 (32–34)	16 (15–17)	18 (16–20)
Serotype 9, 13261 ^c	28 (27–29)	18 (16–20)	26 (24–29)	16 (15–17)	0
Serotype 10, 13039 ^c	0	0	12 (11–13)	0	0
Serotype 11, 56153 ^c	28 (27–29)	0	36 (36–37)	16 (16–17)	0
Serotype 12, 8329/85 ^c	33 (32–34)	0	32 (32–33)	19 (18–20)	0

^a Disc diffusion tests were performed on MHA plates supplemented with EDDHA (50 µg/ml).^b No growth promotion was obtained with desferrioxamine B.^c Reference strain for serotype.^d Mean (range) for two different experiments. FIG. 2. Growth of *A. pleuropneumoniae* serotype 5 strain 2245 in the presence of either natural hydroxamate (ferrichrome), synthetic hydroxamate (ISD-I-204), or catechol (ISD-I-207) siderophores. Growth was evaluated in MHB (□), MHB deferred with 50 µg of EDDHA per ml (◇), MHB deferred with EDDHA and supplemented with 24 µM ferrichrome (△), 50 µM synthetic hydroxamate ISD-I-204 (+), or 50 µM synthetic catechol ISD-I-207 (○).

siderophore ISD-I-207 showed strong growth-promoting activities for *A. pleuropneumoniae* 2245 under iron-restricted conditions (Tables 1 and 2), only the bis-catechol siderophore-β-lactam conjugate JAM-3-089 presented an inhibitory activity (Table 2). The growth-promoting activity of the hydroxamate ISD-I-204 was overall less potent than was that of the catechol ISD-I-207 (Table 1 and Fig. 2), and this may explain the difference in the antibacterial activities of the two conjugated antibiotics. Interestingly, loracarbef showed no activity against strain FMV 87-682 unless it was associated with the bis-catechol siderophore (Table 2).

Bis-catechol-carbacephalosporin conjugate JAM-3-089 was also the sole conjugate with which resistant colonies of *A. pleuropneumoniae* serotype 5 (strain 2245) arose. Such a resistant strain of *A. pleuropneumoniae* serotype 5 (strain 2245) was isolated and named 2245^R. The mutant strain was tested for growth promotion by various siderophores. As shown in Table 2, ferrichrome, synthetic trihydroxamate ISD-I-204, and bis-catechol ISD-I-207 promoted the growth of the wild-type strain while only ferrichrome demonstrated a weak promoting activity with the mutant strain. Strain 2245^R also failed to use trihydroxamate ISD-I-204 to overcome the effect of EDDHA in the medium (Fig. 3). The mutant strain also was tested for growth inhibition by the same conjugate, JAM-3-089, and hydroxamate-β-lactam conjugate EKD-5-273. Resistance to JAM-3-089 was acquired by strain 2245^R, while the hydroxamate-β-lactam conjugate EKD-5-273 remained without effect. The OMP profiles of serotype 5 strain 2245 and mutant 2245^R were compared. As expected, several OMPs were expressed when strains were grown under conditions of iron restriction, but no significant differences between the strains were noted (data not shown).

TABLE 2. Growth inhibition obtained with trihydroxamate-loracarbef conjugate EKD-5-273 and bis-catechol-loracarbef conjugate JAM-3-089 and growth promotion obtained with hydroxamate (ferrichrome and ISD-I-204) and bis-catechol (ISD-I-207) siderophores for *A. pleuropneumoniae* serotype 1 (FMV 87-682), serotype 5 (2245), and the strain 2245 mutant (2245^R) resistant to JAM-3-089 in disc diffusion tests^a

Siderophore portion	Drug portion	Identification	Diam (mm) of zone of growth inhibition or promotion ^b					
			FMV 87-682		2245		2245 ^R	
			Fe ⁺	Fe ⁻	Fe ⁺	Fe ⁻	Fe ⁺	Fe ⁻
None	Phenylglycyl-carbacephalosporin	Loracarbef	0	0	-24 (-22--26)	0	-21 (-20--22)	0
Spermidine-based bis-catechol	D-Phenylglycyl-carbacephalosporin	JAM-3-089	-20 (-18--22)	0	-21 (-20--22)	0	-9	0
Tri- δ -N-OH- δ -N-acetyl-L-ornithine	D-Phenylglycyl-carbacephalosporin	EKD-5-273	0	0	0	0	0	0
	None	Ferrichrome	0	+31 (+30--33)	0	+33 (+32--34)	0	+13
	None	ISD-I-204	0	0	0	+21 (+19--23)	0	0
Lysine-based bis-catechol	None	ISD-I-207	0	+33 (+32--34)	0	+29 (+26--31)	0	0

^a Disc diffusion tests were performed on MHA plates supplemented with 50 μ g of EDDHA per ml (Fe⁻) to evaluate the growth ability of siderophore portions or on MHA supplemented with 5 μ M FeCl₃ (Fe⁺) to evaluate the inhibitory activity of antibiotics.

^b -, inhibition; +, promotion. Results are presented as the mean (range) for three different experiments.

DISCUSSION

Our results with the hydroxamate compounds (ferrichrome and trihydroxamate) revealed that all serotypes of *A. pleuropneumoniae* except serotype 10 and one field strain of serotype 5 were capable of using ferrichrome as a growth-promoting agent under iron-limited conditions. This could be important

FIG. 3. Growth of *A. pleuropneumoniae* serotype 5 strain 2245 resistant to JAM-3-089 (2245^R) in the presence of either natural hydroxamate (ferrichrome), synthetic hydroxamate (ISD-I-204), or catechol (ISD-I-207) siderophores. Growth was evaluated in MHB (□), MHB deferrated with 50 μ g of EDDHA per ml (◇), MHB deferrated with EDDHA and supplemented with 24 μ M ferrichrome (△), 50 μ M synthetic hydroxamate ISD-I-204 (+), or 50 μ M synthetic catechol ISD-I-207 (○).

in situations of coexistence with ferrichrome-producing microorganisms in their habitat niches. None of the synthetic peptides tested in the present study were as potent as the natural siderophore ferrichrome in the bioassay. The weaker activity of the synthetic tri- δ -N-acetyl- δ -N-hydroxy-L-ornithine tripeptide, ISD-I-204, may be due to its zwitterionic charge (29); ferrichrome, in contrast, is an uncharged compound. Desferrioxamine B is used for the treatment of iron overload. This siderophore did not promote growth of any of the 22 strains of *A. pleuropneumoniae* tested, indicating that none of them was able to use this compound as an iron chelator under iron-restricted conditions. The lysine-based bis-catechol ISD-I-207 was the sole catechol that showed significant growth-promoting activity for most strains of *A. pleuropneumoniae* under iron-deficient conditions. Nevertheless, our data suggest that *A. pleuropneumoniae* can acquire iron from both types of siderophores (hydroxamate and catechol based). Acquisition of iron from siderophores produced by other microbial species has already been described for *E. coli* and *Salmonella typhimurium* (27). This ability is due to the fact that these bacteria possess systems of transport that include outer membrane receptors for siderophores that they do not produce. The putative *A. pleuropneumoniae* receptor for hydroxamates is apparently different from *E. coli* FhuA, as determined by the lack of reactivity with the FhuA-specific monoclonal antibodies used in this study. Some of the iron-repressive OMPs previously observed to be present in *A. pleuropneumoniae* (10, 15, 39, 42) might be implicated in the siderophore-mediated iron acquisition.

Nieven et al. (39) were not able to detect siderophores in the culture medium of one strain of *A. pleuropneumoniae* (ATCC 27088) grown under iron-restricted conditions. Our data demonstrated that *A. pleuropneumoniae* serotype 1 (strain 87-682) and serotype 5 (strain 2245) secrete into the culture medium an iron chelator (siderophore) in response to iron stress. Results obtained with the Arnow and Csaky tests indicate that the *A. pleuropneumoniae* siderophore has a structure that is not

related to well-characterized catechol and hydroxamate siderophores. The occurrence of a siderophore which is neither a phenolate nor a hydroxamate is not unique, and Hu et al. (19) reported the occurrence of such a siderophore in *Pasteurella multocida*. Smith and Neilands (45) isolated a structurally novel siderophore from *Rhizobium meliloti* which utilizes ethylenediaminedicarboxyl and α -hydroxycarbonyl functional groups for iron binding.

Several natural iron-chelating antibiotics have been described (36, 43). The antibiotic albomycin has been shown to be a linear iron-binding peptide attached to a toxic thioribosyl unit. The iron-binding portion of albomycin is similar to that of ferrichrome, and both are actively carried into *E. coli* cells by normal iron transport processes by the FhuA OMP receptor (6). Several studies also have shown that the addition of a catechol moiety to the acyl group of cephalosporins enhanced the antimicrobial activity of these drugs under iron-restricted conditions (32, 33, 47). Loracarbef is a potent new carbacephalosporin (5). Cephalosporins target penicillin-binding proteins and generally enter gram-negative bacterial cells through porins OmpC and OmpF (25). Indications that both synthetic trihydroxamate and bis-catechol could deliver loracarbef to bacteria via iron transport pathways were presented by Brochu et al. (7). *A. pleuropneumoniae* is particularly susceptible to β -lactam antibiotics, and MICs for this organism are low (37). Our results showed that bis-catechol-based, not hydroxamate-based, siderophores inhibited growth of *A. pleuropneumoniae* when conjugated to loracarbef in iron-rich medium. This activity was also noticeably superior to that of the unconjugated loracarbef against strain FMV 87-682 but not against strain 2245 (Table 2). Since we have shown that growth of *A. pleuropneumoniae* 2245 was promoted by hydroxamate siderophores, the lack of activity from the hydroxamate conjugate EKD-5-273 may be due either to inefficient transport through the cell outer membrane or to a lower affinity of the antibiotic conjugate for its cellular target, the penicillin-binding proteins, in comparison with that of JAM-3-089 or the unconjugated drug.

It was shown that the mechanisms of resistance to siderophore-antibiotic conjugates similar to those used in this study included the presence of nonfunctional receptors or the absence of specific outer membrane receptors of the ferric siderophore in *E. coli* (7). Siderophore growth promotion studies with *A. pleuropneumoniae* mutant 2245^R were useful in partially explaining their resistance to the catechol conjugate. The phenotype exhibited by mutant 2245^R was completely different from that of the parental wild-type strain. Resistance to the catechol conjugate and the inability of any siderophores to stimulate the growth of this mutant would suggest that all the tested siderophore types were funneled through the same pathway during passage into the cell. TonB is a periplasmic protein which is essential for assimilation of both catechol and hydroxamate siderophores. A disabled TonB allows resistance to antibiotic conjugates and prevents growth promotion by related siderophores (12). It seems that a TonB-like protein exists in *A. pleuropneumoniae* and that the dysfunction of this protein may explain the phenotype of mutant 2245^R.

The inability of *A. pleuropneumoniae* mutant 2245^R to adequately use both hydroxamates (ferrichrome and ISD-I-204) and catechol (ISD-I-207) to stimulate its growth under iron-restricted conditions (Table 2 and Fig. 3) strongly suggests that the mechanism of resistance of the 2245^R mutant is linked to iron transport processes.

It is known that *A. pleuropneumoniae* cells grown under iron-limited conditions can bind and use specifically porcine transferrin, hemin, and hemoglobin (3, 10, 14, 15, 42). The present work is the first study which demonstrates that *A.*

pleuropneumoniae can produce an iron chelator and can make use of exogenous microbial siderophores (hydroxamate and catechol) to obtain iron for growth under iron-restricted conditions. Our results suggest that *A. pleuropneumoniae* has at least one siderophore uptake pathway, or pathways that have a common intermediate, for hydroxamates, catechols, and catechol-antibiotic conjugates.

ACKNOWLEDGMENTS

We thank J. W. Coulton, Department of Microbiology and Immunology, McGill University, for the generous gift of monoclonal antibodies and bacterial strains; C. Rioux, Veterinary Infectious Disease Organization, for a bacterial strain; and M. C. Lavoie, GREB, École de Médecine Dentaire, Université Laval, Sainte-Foy, Québec, Canada, for advice.

This study was partly supported by grants from the Natural Sciences and Engineering Research Council of Canada to F.M. and M.J., and F.M. was also the recipient of a full-time scholarship award from the Medical Research Council of Canada. Research by M.J.M.'s group at the University of Notre Dame was supported by the NIH.

REFERENCES

1. Aisen, R., and A. Leiman. 1972. Lactoferrin and transferrin, a comparative study. *Biochim. Biophys. Acta* **257**:313-323.
2. Arnow, L. E. 1937. Colorimetric determination of the compounds of 3,4-dihydroxyphenylalanine-tyrosine mixture. *J. Biol. Chem.* **118**:531-537.
3. Bélanger, M., C. Bégin, and M. Jacques. 1995. Lipopolysaccharides of *Actinobacillus pleuropneumoniae* bind pig hemoglobin. *Infect. Immun.* **63**:656-662.
4. Bertram, T. A. 1990. *Actinobacillus pleuropneumoniae*: molecular aspects of virulence and pulmonary injury. *Can. J. Vet. Res.* **54**:S53-S56.
5. Bodurow, C. C., B. D. Boyer, J. Brennan, C. A. Bunnell, J. E. Burks, M. A. Carr, C. W. Doecke, T. M. Eckrich, J. W. Fisher, J. P. Gardner, B. J. Graves, P. Hines, R. C. Hoving, B. G. Jackson, M. D. Kinnick, C. D. Kohert, J. S. Lewis, W. D. Luke, L. L. Moore, J. M. Morin, Jr., R. L. Nist, D. E. Prather, D. L. Sparks, and W. C. Vladuchik. 1989. An enantioselective synthesis of loracarbef (LY163892/KT3777). *Tetrahedron Lett.* **30**:2321-2324.
6. Braun, V., K. Gunthner, K. Hantke, and L. Zimmermann. 1983. Intracellular activation of albomycin in *Escherichia coli* and *Salmonella typhimurium*. *J. Bacteriol.* **156**:308-315.
7. Brochu, A., N. Brochu, T. I. Nicas, T. R. Parr, Jr., A. A. Minnick, Jr., E. K. Dolence, J. A. McKee, M. J. Miller, M. C. Lavoie, and F. Malouin. 1992. Modes of action and inhibitory activities of new siderophore- β -lactam conjugates that use specific iron uptake pathways for entry into bacteria. *Antimicrob. Agents Chemother.* **36**:2166-2175.
8. Carmel, G., and J. W. Coulton. 1991. Internal deletions in the FhuA receptor of *Escherichia coli* K-12 define domains of ligand interactions. *J. Bacteriol.* **173**:4394-4403.
9. Csaky, T. Z. 1948. On the estimation of bound hydroxylamine in biological materials. *Acta Chem. Scand.* **2**:450-454.
10. Deneer, H. G., and A. A. Potter. 1989. Effect of iron restriction on the outer membrane proteins of *Actinobacillus (Haemophilus) pleuropneumoniae*. *Infect. Immun.* **57**:798-804.
11. Fenwick, B. W., B. I. Osburn, and H. J. Olander. 1986. Isolation and biochemical characterization of two lipopolysaccharides and a capsular-enriched polysaccharide preparation from *Haemophilus pleuropneumoniae*. *Am. J. Vet. Res.* **47**:1433-1441.
12. Fischer, E., K. Günter, and V. Braun. 1989. Involvement of ExbB and TonB in the transport across the outer membrane of *Escherichia coli*: phenotypic complementation of *exbB* mutants by overexpressed *tonB* and physical stabilization of TonB by ExbB. *J. Bacteriol.* **171**:5127-5134.
13. Frey, J., J. T. Bossé, Y.-F. Chang, J. M. Cullen, B. Fenwick, G. F. Gerlach, D. Gygi, F. Haesebrouck, T. J. Inzana, R. Jansen, E. M. Kamp, J. Macdonald, J. I. MacInnes, K. R. Mittal, J. Nicolet, A. N. Rycroft, R. P. A. M. Segers, M. A. Smits, E. Stenbaek, D. K. Struck, J. F. van den Bosch, P. J. Willson, and R. Young. 1993. *Actinobacillus pleuropneumoniae* RTX-toxins: uniform designation of haemolysins, cytotoxins, pleurotoxin and their genes. *J. Gen. Microbiol.* **139**:1723-1728.
14. Gerlach, G. F., C. Anderson, A. A. Potter, A. Klashinsky, and P. J. Willson. 1992. Cloning and expression of a transferrin-binding protein from *Actinobacillus pleuropneumoniae*. *Infect. Immun.* **60**:892-898.
15. Gonzalez, G. C., D. L. Caamano, and A. B. Schryvers. 1990. Identification and characterization of a porcine-specific transferrin receptor in *Actinobacillus pleuropneumoniae*. *Mol. Microbiol.* **4**:1173-1179.
16. Griffiths, E. 1987. Iron in biological systems, p. 1-25. In J. J. Bullen and E. Griffiths (ed.), *Iron and infection: molecular, clinical and physiological aspects*. Wiley, Chichester, England.
17. Hamel, J., B. R. Brodeur, Y. Larose, P. S. Tsang, A. Belmazaaza, and S.

- Montplaisir.** 1987. A monoclonal antibody directed against a serotype-specific, outer-membrane protein of *Haemophilus influenzae* type b. *J. Med. Microbiol.* **23**:163–170.
18. **Hider, R. C.** 1984. Siderophores mediate absorption of iron. *Struct. Bonding* **58**:25–87.
 19. **Hu, S.-P., L. J. Felice, V. Sivanandan, and S. K. Maheswaran.** 1986. Siderophore production by *Pasteurella multocida*. *Infect. Immun.* **54**:804–810.
 20. **Inzana, T. J.** 1991. Virulence properties of *Actinobacillus pleuropneumoniae*. *Microb. Pathog.* **11**:305–316.
 21. **Jansen, R., J. Briaire, A. M. V. Geel, E. M. Kamp, A. L. J. Gielkens, and M. A. Smits.** 1994. Genetic map of *Actinobacillus pleuropneumoniae* RTX-toxin (Apx) operons: characterization of the ApxIII operons. *Infect. Immun.* **62**:4411–4418.
 22. **Knosp, O., M. von Tigerstrom, and W. J. Page.** 1984. Siderophore-mediated uptake of iron in *Azotobacter vinelandii*. *J. Bacteriol.* **159**:341–347.
 23. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680–685.
 24. **Litwin, C. M., and S. B. Calderwood.** 1993. Role of iron in regulation of virulence genes. *Clin. Microbiol. Rev.* **6**:137–149.
 25. **Livermore, D. M.** 1991. Antibiotic uptake and transport by bacteria. *Scand. J. Infect. Dis.* **74**(Suppl.):15–22.
 26. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275.
 27. **Martinez, J. L., A. Delgado-Iribarren, and F. Baquero.** 1990. Mechanisms of iron acquisition and bacterial virulence. *FEMS Microbiol. Rev.* **75**:45–56.
 28. **Miller, M. J., and F. Malouin.** 1993. Microbial iron chelators as drug delivery agents: the rational design and synthesis of siderophore-drug conjugates. *Acc. Chem. Res.* **26**:241–249.
 29. **Miller, M. J., F. Malouin, E. K. Dolence, C. M. Gasparski, M. Ghosh, P. R. Guzzo, B. T. Lotz, J. A. McKee, A. A. Minnick, and M. Teng.** 1993. Iron transport-mediated drug delivery, p. 135–159. *In* P. H. Bentley and R. Ponsford (ed.), Recent advances in the chemistry of anti-infective agents. Royal Society of Chemistry, Cambridge.
 30. **Minnick, A. A., J. A. McKee, E. K. Dolence, and M. J. Miller.** 1992. Iron transport-mediated antibacterial activity and development of resistance to hydroxamate and catechol siderophore-carbacephalosporin conjugates. *Antimicrob. Agents Chemother.* **36**:840–850.
 31. **Mittal, K. R., R. Higgins, S. Larivière, and M. Nadeau.** 1992. Serological characterization of *Actinobacillus pleuropneumoniae* strains isolated from pigs in Québec. *Vet. Microbiol.* **32**:135–148.
 32. **Mochizuki, H., H. Yamada, Y. Oikawa, H. Murakami, J. Ishiguro, H. Kosuzume, N. Aizawa, and E. Mochida.** 1988. Bactericidal activity of M13659 enhanced in low-iron environments. *Antimicrob. Agents Chemother.* **32**:1648–1654.
 33. **Nakagawa, S., M. Sanada, K. Marsuda, N. Hazumi, and N. Tanaka.** 1987. Biological activity of BO-1236, a new antipseudomonal cephalosporin. *Antimicrob. Agents Chemother.* **31**:1100–1115.
 34. **Neilands, J. B.** 1982. Microbial envelope proteins related to iron. *Annu. Rev. Microbiol.* **36**:285–309.
 35. **Neilands, J. B., and K. Nakamura.** 1991. Detection, determination, isolation, characterization and regulation of microbial iron chelates, p. 1–14. *In* G. Winkelmann (ed.), Handbook of microbial iron chelates. CRC Press, Boca Raton, Fla.
 36. **Neilands, J. B., and J. R. Valenta.** 1985. Antibiotics and their complexes, p. 313–333. *In* H. Segel (ed.), Metal ions in biological systems. Marcel Dekker, New York.
 37. **Nicolet, J.** 1992. *Actinobacillus pleuropneumoniae*, p. 401–408. *In* A. D. Leman, B. E. Straw, W. L. Mengeling, S. Dallaire, and D. J. Taylor (ed.), Diseases of swine, 7th ed. Iowa State University Press, Ames.
 38. **Nielsen, R.** 1986. Serological characterization of *Actinobacillus pleuropneumoniae* strains and proposal of new serotype 12. *Acta Vet. Scand.* **27**:453–455.
 39. **Nieven, D. F., J. Donga, and F. S. Archibald.** 1989. Response of *Haemophilus pleuropneumoniae* to iron restriction: changes in the outer membrane protein profile and the removal of iron from porcine transferrin. *Mol. Microbiol.* **3**:1083–1089.
 40. **Ong, S. A., T. Peterson, and J. B. Neilands.** 1976. Agrobactine, a siderophore from *Agrobacterium tumefaciens*. *J. Biol. Chem.* **254**:1860–1865.
 41. **Paradis, S. E., D. Dubreuil, S. Rioux, M. Gottschalk, and M. Jacques.** 1994. High-molecular-mass lipopolysaccharides are involved in *Actinobacillus pleuropneumoniae* adherence to porcine respiratory tract cells. *Infect. Immun.* **62**:3311–3319.
 42. **Ricard, M. A., F. S. Archibald, and D. F. Nieven.** 1991. Isolation and identification of putative porcine transferrin receptor from *Actinobacillus pleuropneumoniae* biotype 1. *J. Gen. Microbiol.* **137**:2733–2740.
 43. **Rogers, H. J.** 1987. Bacterial iron transport as a target for antibacterial agents, p. 223–233. *In* G. Winkelmann, D. van der Helm, and J. B. Neilands (ed.), Iron transport in microbes, plants and animals. VCH, Weinheim, Germany.
 44. **Schwyn, B., and J. B. Neilands.** 1987. Universal chemical assay for the detection and determination of siderophores. *Anal. Biochem.* **160**:47–56.
 45. **Smith, M. J., and J. B. Neilands.** 1984. Rhizobactin, a siderophore from *Rhizobium meliloti*. *J. Plant Nutr.* **7**:449–458.
 46. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**:4350–4354.
 47. **Watanabe, N.-A., T. Nagasu, K. Katsu, and K. Kitoh.** 1987. E-0702, a new cephalosporin, is incorporated into *Escherichia coli* cells via the *tonB*-dependent iron transport system. *Antimicrob. Agents Chemother.* **31**:497–504.