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Siderophore Production by *Pasteurella multocida*

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Pasteurella multocida **grown under conditions of iron deprivation secreted into the culture medium a growth-enhancing factor which functioned as a siderophore. The siderophore was found to be neither a phenolate nor a hydroxamate by chemical tests and bioassays and was given the trivial name multocidin. Multocidin was partially purified and found to be a highly polar, nonaromatic, and dialyzable compound. This is the first report demonstrating the production of a siderophore by *P. multocida*.**

Pasteurella multocida capsular type A is the etiological agent of avian pasteurellosis (23). This is a highly contagious disease which occurs as a hyperacute, acute, or chronic form in domesticated and wild birds. The hyperacute and acute diseases are characterized by septicemia, irreversible lesions in the lung, liver, and spleen, and eventual death caused by an endotoxin (13). With the recognition of the involvement of this organism in the disease, studies have been conducted for over 35 years on the pathogenicity of *P. multocida* and immune mechanisms in relation to the control of the disease.

Studies in vitro have shown that the bacteriostatic and bactericidal action of immune serum on *P. multocida* was mediated by antibody and complement and reversed by iron compounds (10–12). A detailed biochemical study of the way antibody and complement exerted their antibacterial effects on *P. multocida* showed that they inhibited net RNA synthesis (12). This was followed by the inhibition of all macromolecular synthesis. The study also suggested that the prevention of bacterial death by iron was due to a greater availability of iron for macromolecular synthesis, cell multiplication, and growth of the bacteria. There was no indication, however, that a siderophore was involved in these reactions, as seen in other species of bacteria (21).

More recent studies showed that rabbits infected with *P. multocida* responded with a fever and that the concentration of iron in the plasma decreased (15). Since it had been shown that the ability to produce a siderophore was diminished by small elevations in temperature in other microorganisms (8, 16), these authors (15) hypothesized that a fever, coupled with a reduction in the plasma iron concentration, may be a part of a coordinated host defense against pasteurellosis. The implication of the above findings is that iron may play an important role in the pathogenesis of *P. multocida* infections.

Because iron has many physiological roles in *P. multocida*, an understanding of the means by which iron is transported into *P. multocida* is important. Recent studies (6; M. J. Corbett, L. Lelkes, M. Lynn, and M. Solotorovsky, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, K194, p. 204) established that *P. multocida* possesses an efficient iron transport system necessary for the accumulation of iron for growth. No phenolate- or hydroxamate-type siderophores were detected in the culture supernatant of iron-starved cells. In this paper we provide evidence that the supernatant from cultures of *P. multocida* grown under low-iron conditions contained significant amounts of a siderophore and that the siderophore was different from the traditional phenolate- and hydroxamate-type siderophores produced by other species of bacteria and may have a novel chemical structure.

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MATERIALS AND METHODS

Chemicals and glassware. Ethylenediamine-di(*o*-hydroxyphenylacetic acid) (EDDA) was purchased from Sigma Chemical Co., St. Louis, Mo., and was crystallized by the procedure of Rogers (24). 2,3-Dihydroxybenzoic acid and 2,2′-dipyridyl were also purchased from Sigma. Deferoxamine mesylate (Desferal) was obtained from CIBA-GEIGY Corp., Summit, N.J. Schizokinen was isolated from *Bacillus megaterium* ATCC 19213 and partially purified by the procedure of Mullis et al. (18). Radioactive iron was purchased from Amersham Corp., Arlington Heights, Ill., as $^{59}FeCl_3$ in 0.1 N HCl (specific activity, 12 μCi/μg). All solutions were made in Milli Q water (Millipore Corp., Bedford, Mass.) unless specified otherwise. To minimize iron contamination, we soaked all glassware overnight in 0.5% EDTA and rinsed it 10 times in deionized water.

Chemical assays. The Arnow test (1) was used to detect phenolate-type siderophores in the culture extracts. The presence of hydroxamates was determined by the Csaky test with both unhydrolyzed and hydrolyzed culture extracts (3, 9). The method of Emery and Neilands (4) was also used for the detection of hydroxamates. In this method, the formation of a nitrosodimer with a hydroxamate was monitored at A_{264}. The iron content of various media was determined with a Ferrozine [3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine] reagent by the procedure described by Holzberg and Artis (14).

Bacterial strains and growth conditions. Four strains of *P. multocida* (X73, P1059, Urbach, and L8503) were used throughout this study. Strains X73 and P1059 were obtained from K. L. Heddleston, NADC, Ames, Iowa. Strains Urbach and L8530 were isolated from turkeys that had died of avian pasteurellosis in Minnesota. The bacteria were maintained in freeze-dried form. Each strain was grown overnight on blood agar at 37°C. For siderophore production, the culture was transferred to a low-iron, chemically defined medium (CDM) (7), the composition of which is presented in Table 1. CDM was prepared as a 5× stock solution without aspartic acid, tyrosine, and $MgSO_4 \cdot 7H_2O$. The stock solution was then deferrated by passage through a Chelex-100 (Bio-Rad Laboratories, Richmond, Calif.) column. Subsequent experiments revealed that the deferration process was not necessary if chemicals of high purity were

TABLE 1. Composition of CDM[a]

Component	Concn (g/liter)
$Na_2HPO_4 \cdot 12H_2O$	32.3
KH_2PO_4	1.36
NaCl	1.19
$MgSO_4 \cdot 7H_2O$	0.25
Glucose	6.0
L-Arginine	0.2
L-Aspartic acid	1.6
L-Cysteine hydrochloride	0.12
L-Serine	0.2
L-Glutamic acid	0.15
L-Isoleucine	0.065
L-Leucine	0.065
L-Phenylalanine	0.095
L-Tyrosine	0.09
Calcium pantothenate	0.002
Nicotinamide	0.005
Thiamine hydrochloride	0.0001
Orotic acid	0.0015

[a] The medium was deferrated by passage through Chelex-100 resin. The final iron concentration, as determined by the ferrozine assay (14), was < 0.02 μg/ml.

used. The CDM 5× stock solution was filter sterilized and diluted appropriately with a sterile solution containing aspartic acid, tyrosine, and $MgSO_4 \cdot 7H_2O$ to obtain 1× CDM. The iron content of this medium was <0.02 μg/ml, as determined by the Ferrozine assay. The iron-rich medium referred to as CDM+Fe contained 25 μM ferric ammonium citrate. The iron-stressed medium contained CDM and 50 μM 2,2′-dipyridyl (CDM+DP).

Salmonella typhimurium enb-7, a phenolate auxotroph, was provided by J. B. Neilands, University of California, Berkeley. It was maintained in the laboratory on tryptose agar plates at 37°C. *Arthrobacter flavescens* JG-9, a hydroxamate auxotroph, was obtained from P. J. Szaniszlo, University of Texas, Austin. It was maintained in the laboratory on yeast extract-Casamino Acids-sucrose medium (5) supplemented with 100 μg of deferoxamine mesylate per liter at room temperature.

Bioassays for siderophores. *S. typhimurium enb-7* was used as the indicator strain for phenolate-type siderophores, with 2,3-dihydroxybenzoic acid as the positive control (22). *A. flavescens* JG-9 was used as the indicator strain for hydroxamate-type siderophores and was assayed in a medium without deferoxamine mesylate (29) by using schizokinen as the positive control. *P. multocida* Urbach was used as the indicator strain in both plate and photometric bioassays for the pasteurella siderophore. The plate assay was performed in a molten medium containing 0.9% agarose (LMP agarose; Bethesda Research Laboratories, Inc., Gaithersburg, Md.) suspended in CDM plus 4 μg of EDDA per ml. A 100-ml volume of the molten medium was seeded with a 0.5-ml volume of *P. multocida* Urbach containing 25 Klett units, and 60 ml of the mixture was poured into each of several petri plates (150 by 15 mm). After the seeded plates had solidified, 6-mm wells were cut, and up to 20 μl of the test solutions was added. Growth enhancement was monitored at 24 and 48 h after incubation at 37°C. Iron at a concentration of 500 ng in 10 μl of water (in the form of ferric ammonium citrate) was used as a positive control. In the photometric assay, 25 μl of the same indicator strain adjusted to 25 Klett units was added to 4.5 ml of CDM containing 1 μg of EDDA per ml. Different volumes of test solutions were added to the medium, and the tubes were incubated on a reciprocating shaker at 37°C. The ability of the test solutions to enhance the growth of the indicator strain was measured by an increase in turbidity estimated with a Klett-Summerson colorimeter.

Production of the pasteurella siderophore. A single colony of each strain of *P. multocida* propagated from overnight growth on blood agar was inoculated into a tube containing 3 ml of CDM and shaken overnight at 37°C. For large-scale siderophore production, 0.5% of this growth was inoculated into a flask containing 500 ml of CDM+DP, aerated by shaking, and incubated at 37°C. Cells were harvested during the stationary phase or at other times as indicated in the text. The culture filtrates obtained from the spent media after centrifugation (16,000 × *g*, 30 min, 4°C) were filter sterilized and concentrated by freeze-drying.

Extraction of the pasteurella siderophore. Three different extractions were used in an attempt to isolate the siderophore from the culture filtrates: ethyl-acetate, chloroform-phenol-ether, and methanol extractions. A 25-ml portion of 10×-concentrated culture filtrate was adjusted to pH 1.5 and extracted with 10 ml of ethyl acetate. The organic layer was rotary evaporated to dryness and then dissolved in 5 ml of water. Another 25-ml portion of 10× concentrated culture filtrate was acidified to pH 2 and extracted with 20 ml of chloroform-phenol (1:1, wt/wt). Diethyl ether (150 ml) was added to the organic layer, mixed, and extracted with 10 ml of water. The extract was washed with ether and then adjusted to pH 7.0. The solution was freeze-dried and suspended in 5 ml of water. A third extract was prepared by adding 75 ml of methanol to the residue obtained from freeze-drying 500 ml of the culture filtrate. The mixture was stirred at room temperature overnight and then centrifuged to remove the undissolved material. The yellow supernatant was rotary evaporated to dryness and then suspended in 5 ml of water. This fraction was referred to as the ME (methanol) extract. All extracts were filter sterilized before use in the bioassay.

Purification of the pasteurella siderophore. A 2-ml portion of the ME extract of culture filtrate from strain X73 containing the pasteurella siderophore was further purified by gel filtration chromatography on a Bio-Gel P-2 polyacrylamide (Bio-Rad) column (1.5 by 90 cm) equilibrated with water-methanol (10:1) solvent. The fractions were tested for siderophore activity by the photometric assay with *P. multocida*. The positive fractions were pooled, freeze-dried, and suspended in 2 ml of water. This material was then loaded onto a 10-ml Dowex AG2X8 (Bio-Rad) column which had been converted to the acetate form. After being washed with water, the column was eluted with 100 ml of NH_4Cl by using a linear concentration gradient of 50 mM to 1 M. The fractions were assayed for pasteurella siderophore activity. The positive fractions were pooled, freeze-dried, and suspended in 1 ml of water. A portion of this material was further purified by high-pressure liquid chromatography (HPLC) on a Hewlett-Packard 1090 chromatograph equipped with an autosampler, diode array UV detector, and model 3392 electronic integrator. A 100-μl sample was loaded onto a C18 column (4.6 mm inner diameter by 25 cm; particle size 5 μm; Rainin Instrument Co.) and eluted at 1 ml/min with the following gradient: 100% water (8 min), linear gradient from 100% water to 100% methanol (1 min), 100% methanol (6 min), and linear gradient from 100% methanol to 100% water (5 min). The A_{280} was monitored. The fractions (0.5 ml) were freeze-dried, dissolved in 100 μl of water, and tested for the presence of the pasteurella siderophore. A 50-μl portion of each positive fraction was

loaded onto a Partisil SAX 10 column (4.6-mm inner diameter by 25 cm; Whatman, Inc., Clifton, N.J.) and eluted at 1 ml/min with the following gradient: ammonium acetate (50 mM, pH 5.0) for 10 min, ammonium acetate (200 mM, pH 5.8) for 10 min, and 1:1 (vol/vol) methanol-ammonium acetate (200 mM, pH 5.8) for 10 min. The fractions (1 ml) were freeze-dried, dissolved in 100 μl of water, and tested for the presence of the pasteurella siderophore. The positive fractions were characterized by thin-layer chromatography (TLC).

Iron-binding activity of the pasteurella siderophore. The iron-binding role of the pasteurella siderophore was tested by the method described by Norrod and Williams (21). Either uninoculated media or Urbach culture filtrates were added to iron-free transferrin (Sigma) and incubated at 37°C for 1 h. The binding of iron by the transferrin in the absence or presence of the siderophore was measured at 470 nm after each addition of 20 μl of 1.0 mM ferric nitrilotriacetate, an iron compound that reacts stoichiometrically with transferrin (2).

The iron-binding role of the pasteurella siderophore was further confirmed by an additional method. Two 10-ml glass pipettes were packed with Sephadex G-10 (Pharmacia, Inc., Piscataway, N.J.). The columns were equilibrated with Milli Q water. One of the columns was loaded with an iron mixture which contained 5 μl of ^{59}Fe (1 μCi in 0.1 N HCl) and 50 μg of ferric chloride in water. The other column was loaded with the iron mixture plus 50 μl of the pasteurella siderophore, which had been partially purified on a Bio-Gel P-2 column. The columns were eluted with water, and fractions (1.5 ml) were counted for radioactivity in a Biogamma Counter II (Beckman Instruments, Inc., Fullerton, Calif.). Similar studies were done with columns packed with Bio-Gel P-2.

Detection of the iron-solubilizing activity of the pasteurella siderophore. The dialysis method of Russell et al. (25) was used, with modifications, to test the culture filtrates from spent media for their ability to solubilize iron. CDM (1 ml) was placed inside a dialysis sac (molecular weight exclusion limit, ca. 4,000), and 105 μl of a mixture containing 50 μl of test sample, 50 μg of cold Fe^{3+}, and 5 μl of ^{59}Fe (ca. 1 μCi) was added. The samples were dialyzed against 10 ml of 50 mM Tris hydrochloride buffer (pH 7.0) in 15-ml plastic centrifuge tubes. Dialysis was complete after 20 h at room temperature. Samples (500 μl) were withdrawn from the dialysate and counted for radioactivity in a Biogamma counter II.

TLC. TLC was carried out on Silica Gel F-254 plates (Merck & Co., Inc., Rahway, N.J.) or cellulose K2F plates (Whatman) in the following solvent systems: chloroform-methanol-water, 35:12:2; methanol-ammonium hydroxide, 100:1.5; and ethyl acetate-methanol-acetic acid, 80:1:1. The plates were sprayed with either 0.25% ninhydrin in acetone or 0.5% dipyridyl in 95% ethanol–0.2% $FeCl_3$ in 95% ethanol (1:1). In some instances, the Silica Gel F-254 was scraped from the plates after development, extracted with 50% methanol, and freeze-dried for the bioassay.

High-voltage paper electrophoresis. A mixture of $^{59}FeCl_3$ and partially purified pasteurella siderophore was spotted on the center of an 8-in. (20.32 cm) strip of Whatman 3MM paper and subjected to electrophoresis (ca. 40 V/cm, 30 to 40 min) by using a 0- to 2-kV power supply (LKB Instruments, Inc., Rockville, Md.). The following buffers were used: glacial acetic acid-formic acid-water, 25:72:903, pH 1.8; and 100 mM sodium phosphate, pH 6.6. After electrophoresis, the paper was sprayed with ninhydrin reagent and then cut into 0.25-in. (ca. 0.64 cm) pieces for counting the radioactivity.

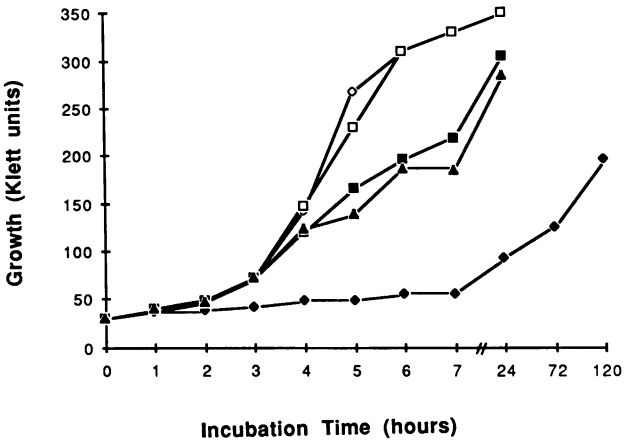

FIG. 1. Growth kinetics of *P. multocida* X73 in CDM (▲), CDM plus 0.1 μM $FeCl_3$ (■), CDM plus 1 μM $FeCl_3$ (□), CDM plus 10 μM $FeCl_3$ (◇), and CDM plus 100 2,2′-μM dipyridyl (◆). Samples of bacterial growth were taken at the indicated times, and growth was expressed as Klett units.

RESULTS

Detection of siderophore activity in spent culture media and their ME extracts. *P. multocida* Urbach, L8530, X73, and P1059 all grew well in CDM, which contained <0.02 μg of Fe per ml. The growth rate for each strain, however, was slightly different. Urbach grew faster than X73 both on the CDM agarose plate and in the CDM broth (data not shown). Figure 1 shows the growth kinetics of X73 in the presence or absence of different concentrations of iron or the iron chelator 2,2′-dipyridyl. The addition of ferric chloride to CDM increased the growth rate and the final cell density. A longer lag phase and slower growth rate were observed in the presence of 2,2′-dipyridyl, but eventually, after 120 h of incubation, the broth became turbid. We were also successful in serially transferring both X73 and Urbach into CDM+DP or CDM three times. This led us to speculate that some iron transport compound or siderophore(s) was produced by *P. multocida* when it was grown in CDM or CDM+DP. The culture filtrates of the Urbach strain grown in CDM or CDM+DP appeared to chelate iron, as measured by the decreased ability of iron-free transferrin to bind iron in the presence of the culture filtrates (Fig. 2). To confirm the presence of a siderophore, we tested the culture filtrates of all four strains (X73, P1059, Urbach, and L8530) of *P. multocida* grown in CDM or CDM+DP in a bioassay on EDDA-CDM-agarose plates with the Urbach strain. All of the culture filtrates reversed the inhibition by EDDA and enhanced the growth of the indicator strain. The supernatants from inoculated CDM+Fe cultures also enhanced the growth of the indicator strain. In contrast, the uninoculated CDM+Fe did not enhance growth. A mixture of CDM+Fe culture filtrate plus 25 μM EDDA also enhanced growth. This indicated to us that there was also some siderophore activity in the CDM+Fe culture filtrate. Furthermore, since the bioassays done with serial dilutions of CDM and CDM+Fe culture filtrates did not differentiate the titers of pasteurella siderophore activity, it was not clear whether the siderophore present in the various culture filtrates was the same compound. Tenfold-concentrated culture filtrates,

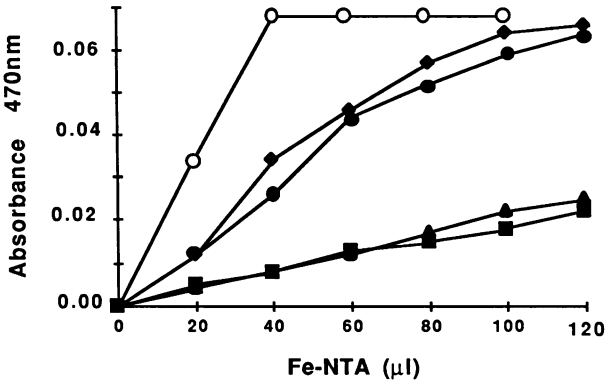

FIG. 2. Effects of culture filtrates from *P. multocida* Urbach on the binding of iron by transferrin. The culture filtrate and iron-free transferrin were incubated for 60 min at 37°C. The binding of iron by transferrin was measured at 470 nm after each addition of 20 μl of ferric nitrilotriacetate (Fe-NTA). Symbols: ○, water; ◆, CDM; ●, CDM+DP; ■, culture filtrate from CDM; ▲, culture filtrate from CDM+DP.

however, showed negative reactions for phenolates in the Arnow test and for hydroxamates in both the Csaky test and the periodate assay (4). These concentrated culture filtrates were also negative for phenolate- and hydroxamate-type siderophores, as determined by the bioassays with phenolate- and hydroxamate-dependent indicator organisms.

Several iron chelators, 2,3-dihydroxybenzoic acid, deferoxamine mesylate, or partially purified schizokinen, failed to enhance the growth of the Urbach indicator strain when tested in the bioassay. Instead, these chelators showed inhibition zones around the testing wells on the overseeded bioassay plates. It was thus speculated that a distinct type of iron transport compound existed in *P. multocida*.

Three types of extractions were carried out to concentrate the *P. multocida* siderophore: ethyl acetate, chloroform-phenol-ether, and methanol extractions. The ethyl acetate and chloroform-phenol-ether extractions were used by Simpson and Oliver (26) to isolate phenolate- and hydroxamate-type siderophores, respectively. All three extracts were negative in the Arnow and Csaky tests. A 2-μl quantity of each extract was sufficient to yield positive pasteurella siderophore activity. At least 20 μl of each extract was needed to yield positive results in the bioassay with the phenolate-dependent auxotroph *S. typhimurium enb-7*, while up to 40 μl of each extract yielded negative results in the bioassay with the hydroxamate-dependent auxotroph *A. flavescens* JG-9. Extraction with methanol resulted in the greatest recovery of siderophore activity and was used throughout the study.

Our studies indicated that the pasteurella siderophore stimulated the growth of *P. multocida* Urbach in the EDDA-CDM photometric bioassay. The indicator strain without EDDA reached the stationary phase after 50 h of incubation, while EDDA at 1 μg/ml completely inhibited growth. The addition of ferric ammonium citrate at various concentrations to EDDA-CDM enhanced growth and shortened the length of time needed to reach the stationary phase. It was therefore possible to estimate the relative titer of the pasteurella siderophore by using this photometric bioassay and determining the incubation time needed for the indicator strain to reach the stationary phase of growth. The ME extract of the CDM culture filtrate enhanced the growth of the indicator strain in a shorter time than did that of the CDM+Fe culture filtrate. These results indicated to us that a higher concentration of the pasteurella siderophore was present in the CDM filtrate than in the CDM+Fe filtrate. Further proof was obtained by comparing the iron-solubilizing activity of the ME extracts of these filtrates. Almost twice the amount of iron-solubilizing capacity was present in the CDM filtrate as in the CDM+Fe filtrate (Table 2). Furthermore, different strains of *P. multocida* had different iron requirements. It was found that the ME extract of the Urbach strain CDM filtrate had a higher siderophore content than that of the Urbach strain CDM+DP or CDM+Fe filtrate. The ME extract of the X73 strain CDM+DP filtrate had the highest siderophore content.

TABLE 2. Iron-solubilizing activity of ME extracts of spent media from *P. multocida* Urbach cultures[a]

Sample (50 μl)	Total cpm in dialysate
Water	1.4×10^4
ME extract from spent CDM	3.2×10^5
ME extract from spent CDM+DP	3.5×10^5
ME extract from spent CDM+Fe	1.4×10^5

[a] The samples and $^{59}FeCl_3$ were placed inside dialysis sacs and dialyzed against buffer as described in Materials and Methods.

Partial purification of the pasteurella siderophore. Six samples of ME extracts of the Urbach and X73 strain CDM, CDM+Fe, and CDM+DP filtrates were fractionated on a Bio-Gel P-2 column. The pasteurella siderophore was eluted from the column in the same fractions in all the ME samples (Fig. 3). This result indicated that the siderophore produced in each of the different media may be the same compound. We were also able to separate 2,2'-dipyridyl from the siderophore in the ME extract of the CDM+DP filtrate. Active fractions from the Bio-Gel P-2 column were also positive for iron-solubilizing activity in the dialysis assay method. Further proof of siderophore activity was obtained by demonstrating the ability of the siderophore to mobilize iron through a gel filtration column (Fig. 4). Although ^{59}Fe alone does not elute with water from a Sephadex G-10 or

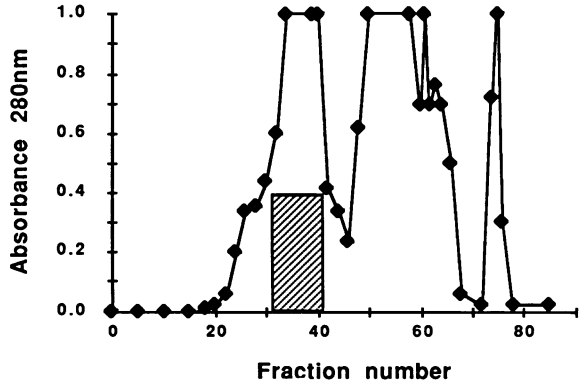

FIG. 3. Chromatographic profile on a Bio-Gel P-2 column of an ME extract of *P. multocida* X73 grown in CDM+DP. The column was eluted with water-methanol (10:1) solvent. The flow rate was 15 ml/h. Samples (3 ml) were collected for each fraction. Pasteurella siderophore activity was assayed in each fraction by a photometric bioassay with *P. multocida* Urbach as the indicator strain. The shaded area contained fractions that were positive for pasteurella siderophore activity.

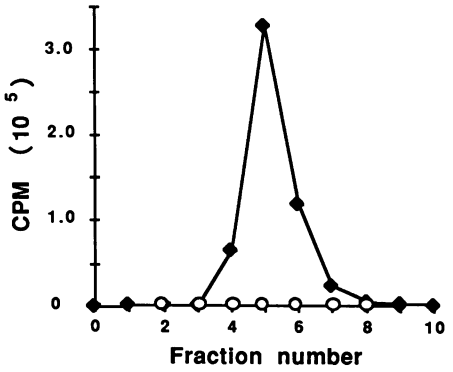

FIG. 4. Iron-binding activity of the pasteurella siderophore determined on Sephadex G-10 columns. The test samples were loaded on separate columns and eluted with water. Eluates (1.5 ml) were collected for each fraction and counted in a Biogamma Counter II. Symbols: ○, $^{59}FeCl_3$ only; ◆, mixture of $^{59}FeCl_3$ and the pasteurella siderophore.

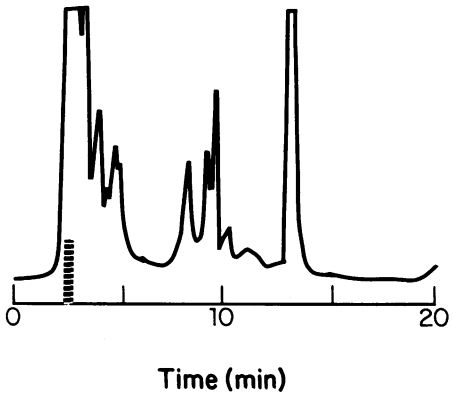

FIG. 6. HPLC profile on a C18 reverse-phase column of pasteurella siderophore peak material from the Dowex AG2X8 column (Fig. 5). See the text for details. The pasteurella siderophore was found in the fraction which eluted at 2.5 min (shaded area). The A_{280} was monitored.

Bio-Gel P-2 column, mixing ^{59}Fe with the siderophore-active fractions yielded mobile ^{59}Fe from both columns. When we used a mixture of ^{59}Fe and siderophore-active fractions, we recovered almost all the radioactivity from the Sephadex G-10 column. This phenomenon was not due to a reduction of the ferric ions by the siderophore-active fractions, because no apparent Fe^{2+} was detected with the Ferrozine reagent without prior reduction by ascorbic acid.

Siderophore-positive fractions of strain X73 from the Bio-Gel P-2 column were freeze-dried and further purified on a Dowex AG2X8 column. Siderophore-positive fractions were eluted from the column with a concentration gradient of NH_4Cl (Fig. 5). Further purification of the pasteurella siderophore was accomplished by C18 reverse-phase HPLC. Fractions (0.5 ml) were collected and assayed for siderophore activity by the bioassay. The majority of the siderophore was not retained by the C18 column and eluted at 2.5 min (Fig. 6). Similar results were also obtained when samples of the ME extracts of CDM and CDM+Fe filtrates were chromatographed on Bio-Gel P-2, freeze-dried, and then further purified by C18 reverse-phase HPLC. Active material isolated by C18 reverse-phase HPLC was rechromatographed on an HPLC anion-exchange column. The peak at 13.47 min (Fig. 7) contained the pasteurella siderophore. A sufficient quantity of the pasteurella siderophore for structure elucidation is currently being purified by HPLC.

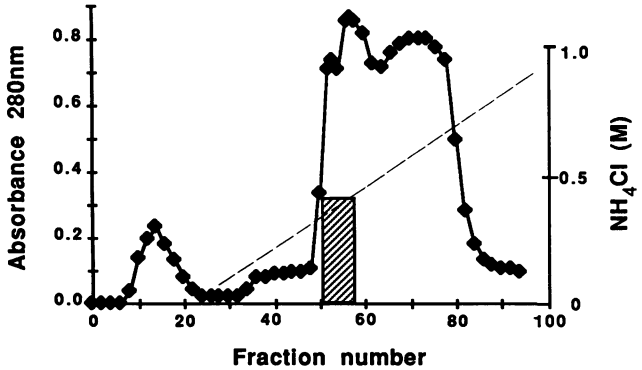

FIG. 5. Ion-exchange chromatography on Dowex AG2X8 of the pasteurella siderophore sample of strain X73 from the Bio-Gel P-2 column (Fig. 3). The column was washed with water and then developed with a linear gradient of 50 mM to 1 M ammonium chloride. The flow rate was 10 ml/h. Samples (1.5 ml) were collected for each fraction. Eluants were tested for the pasteurella siderophore by the bioassay. The shaded area contained the siderophore. The dotted line indicates the gradient.

Further characterization of the pasteurella siderophore. A mixture of a siderophore-positive fraction eluted from the C18 column and $^{59}FeCl_3$ did not migrate from the point of origin when subjected to high-voltage paper electrophoresis at pH 1.8. In contrast, the ferrated complex migrated as an anion at pH 6.6. When subjected to TLC on Silica Gel F-254 plates, samples of the mixture failed to migrate with three different solvent systems, and all the radioactivity remained at the origin.

A siderophore-positive fraction (1 mg) eluted from the C18 column was spotted across a TLC Silica Gel F-254 plate and developed in chloroform-methanol-water solvent. Bioassays done with the material scraped from the plates and extracted with 50% methanol revealed that the siderophore remained at the origin. This spot fluoresced when observed under a long-wavelength UV light and yielded negative reactions with ninhydrin and hydroquinone sprays.

Siderophore-positive material recovered from the HPLC Partisil SAX 10 column at 13.47 min had a very low extinction coefficient from 400 to 240 nm, suggesting that it was not an aromatic compound. Maximum absorption occurred at 215 nm.

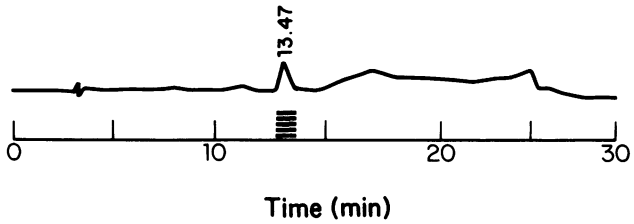

FIG. 7. HPLC profile on a Partisil SAX 10 anion-exchange column of pasteurella siderophore peak material from the C18 reverse-phase column (Fig. 6). See the text for details. The pasteurella siderophore was found in the fraction which eluted at 13.47 min (shaded area). The A_{280} was monitored.

DISCUSSION

In this study the presence of a growth-enhancing factor for *P. multocida* was detected by bioassays. This factor was present in culture filtrates from *P. multocida* grown in a low-iron medium (CDM). The iron-binding property of this growth-enhancing factor was demonstrated by its ability to significantly reduce iron binding to transferrin, to mobilize ^{59}Fe through Bio-Gel P-2 and Sephadex G-10 columns, and to solubilize iron in an equilibrium dialysis system. Since this growth-enhancing factor has fulfilled most of the criteria recently described by Neilands and Ratledge (20) for a compound to be classified as a siderophore, it can be considered a pasteurella siderophore.

The behavior of the pasteurella siderophore on a C18 reverse-phase HPLC column and on Silica Gel F-254 TLC plates indicated a highly polar, water-soluble compound. Negative results in the Arnow and Csaky tests and the absence of a charge transfer band between 420 and 500 nm indicated a structure of the pasteurella siderophore that is not related to those of other, well-characterized catechol- and hydroxamate-type siderophores. We hypothesize that the pasteurella siderophore has a novel chemical structure and should be termed multocidin. The occurrence of a siderophore which is neither a phenolate nor a hydroxamate is not unique. Recently, Smith and co-workers (27, 28) isolated a structurally novel siderophore from *Rhizobium meliloti* which utilizes ethylenediaminedicarboxyl and α-hydroxycarbonyl functional groups for iron binding.

A common characteristic feature of many well-defined bacterial iron transport systems is the inhibition of siderophore production during growth in high-iron medium (19). We found that multocidin was present in higher concentrations during growth in low-iron media (CDM and CDM+DP) than during growth in high-iron medium (CDM+Fe). This result was detected in our photometric bioassay and in our iron solubilization assay (Table 2) and demonstrated that very little multocidin was produced by *P. multocida* grown under high-iron conditions.

Another finding in our studies was that highly concentrated ME extracts and partially purified (C18 reversed-phase HPLC, 2.5-min eluate) multocidin enhanced the growth of the phenolate-dependent auxotroph *S. typhimurium enb-7* in the bioassay. This was not an unusual finding because others (17) have reported that the bioassay with *S. typhimurium enb-7* was not specific for phenolate.

In summary, these data clearly show for the first time the existence and nature of a pasteurella siderophore called multocidin. We are currently purifying the multocidin to study its chemical structure and its role in the pathogenesis of avian pasteurellosis.

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