INFECTION AND IMMUNITY, Dec. 1978, p. 878-890
0019-9567/78/0022-0878$02.00/0

Vol. 22, No. 3
Printed in U.S.A.

Biological Activities of Pyochelins: Iron-Chelating Agents of *Pseudomonas aeruginosa*

PINGHUI V. LIU AND FATEMEH SHOKRANI

Department of Microbiology and Immunology, University of Louisville School of Medicine, Health Sciences Center, Louisville, Kentucky 40232

Received for publication 25 September 1978

Strains of *Pseudomonas aeruginosa* able to grow readily in serum (serum resistant) produce siderophores in large quantity, enabling them to extract iron from transferrins. The term pyochelin has been proposed for this group of compounds. Pyochelin extractable with ethyl acetate and designated pyochelin A appears to be a mixture of catechols and other phenolates. The structures of water-soluble siderophores, designated pyochelin B, have not been determined. Pyochelins enabled growth in serum of strains of serum-sensitive *P. aeruginosa* and other gram-negative bacilli. Serum-resistant strains of *P. aeruginosa* tended to be more virulent than equally toxigenic strains of the serum-sensitive group. However, incorporation of pyochelins into the inocula of serum-sensitive strains could reduce, rather than enhance, their virulence. Utilization of pyochelins by serum-sensitive strains of *P. aeruginosa* rendered some of these organisms resistant to pyocins which were otherwise lethal to them.

Because host iron is bound to transferrins and other iron-binding proteins, most microbes are unable to obtain sufficient iron for growth in tissue fluids (6, 13, 23, 27). Furthermore, hosts usually respond to microbial infections by reducing the iron content of their blood (27). This type of host resistance has been referred to as nutritional immunity (13). Some microbes, however, produce chelating agents that enable them to grow in vivo. These chelating agents are generally referred to as siderophores, and those produced by mycobacteria (24), *Bacillus* species (3, 4), and enteric bacilli (7, 14, 19, 20, 22) have been studied extensively. Generally, these siderophores contain either hydroxamate or phenolate groups which are capable of coordination with a central ferric ion (20), and in the case of mycobactin both groups appear to be found on the same molecule (24).

It has been known for some time that strains of *Pseudomonas aeruginosa* vary greatly in ability to initiate growth in animal sera with small inocula. Those able to grow readily are referred to as serum resistant, wherease those unable to do so are referred to as serum sensitive. The mechanism of serum resistance probably involves production of siderophores, because addition of iron to sera enables serum-sensitive strains to grow, and furthermore, the injection of iron into animals enhances the virulence of these organisms (6).

Siderophores produced by *P. aeruginosa* have never been characterized, and no name has even been proposed for this group of compounds. Therefore, we propose the term pyochelin for this group of compounds. "Pyo" is derived from the specific epithet "pyocyanea" of this organism, whereas chelin indicates the chelating nature of the group. The term "aeruginochelin" is cumbersome. Furthermore, in the description of the bacteriocin produced by this species, the term pyocin is more commonly used than aeruginocin. Pyochelin is a more convenient term than aeruginochelin. The present communication describes the general biological activities of these compounds.

(Part of this work was taken from a thesis submitted by F.S. to the University of Louisville School of Medicine in partial fulfillment of the requirements for the M.S. degree.)

MATERIALS AND METHODS

Organisms used. *P. aeruginosa* strains used included 150 clinical isolates and an additional 50 standard strains previously used in serotyping and phagetyping studies. Other organisms included *P. pseudomallei*, *P. reptilivora*, *P. aureofaciens*, *P. chlororaphis*, *P. fluorescens*, *P. stutzeri*, several species of the genera *Escherichia*, *Klebsiella*, *Salmonella*, and *Shigella*, *Vibrio cholerae*, and aeromonads. Sources of these organisms have been described (12, 15, 16).

Screening for ability to grow in serum. Organisms were inoculated into 2 ml of Trypticase soy broth (TSB; Baltimore Biological Laboratory) and incubated at either 32 or 22°C (depending on their

temperature requirements) for 24 to 48 h to achieve maximum growth. One loopful of culture, usually containing about 4×10^9 cells per ml, was diluted into 1 ml of saline. This process achieves an approximately 1/200 dilution, and the resulting cell suspension contains about 2×10^7 cells per ml. One loopful of this saline suspension contained about 10^5 cells, and this was used as the standard inoculum. The standard inoculum was spot inoculated onto a mixture of equal volumes of Seitz-filtered animal sera (beef, horse, human, and rabbit) and 2.5% autoclaved agar (Difco). Ten to 12 organisms were inoculated on one plate, and the plates were incubated at either 32 or 22°C according to the temperature requirements of the organism. After 24 h those organisms that showed good growth were designated serum resistant; those that required more then 48 h to give good growth were regarded as serum sensitive. Some organisms failed to grow completely even after a prolonged incubation, and these were designated nongrowers in serum.

Screening for ability to utilize siderophores of other bacteria. Serum-resistant organisms were usually rapid producers of some siderophores. These organisms were first grown on a blood agar with brain heart infusion base (Difco) at the appropriate temperature. One loopful of the inoculum was taken from the growth and streaked heavily in the center of a serum agar plate across the whole diameter. After incubation for 24 to 48 h, the serum-sensitive organisms were appropriately diluted as described in the preceding section, and one loopful of diluted inoculum was streaked perpendicularly to the colony of the serum-resistant organism to a distance of about 1 cm. Four organisms could be streaked on one side of the colony and another four could be streaked on the other side. The plates were incubated further for 24 h at the appropriate temperature for the growth of the test organism. Those organisms able to utilize siderophores formed visible colonies around the colony of the serum-resistant organism, whereas the part of the inoculum far away from the latter showed no growth. The phenomenon is a sort of satellite formation like that usually seen with a *Haemophilus influenzae* growing around the colony of a staphylococcus on a blood agar plate. The only difference was that the agar used here contained sera as the sole source of nutriment.

Testing the effect of pyochelin on the activity of pyocin. The procedure used was essentially that of Govan and Gillies (8). A preliminary selection was made of serum-resistant strains of *P. aeruginosa* that also produced pyocin. These organisms were streaked in the center of the agar plates; after incubation for 20 h at 32°C, the colonies were scraped off with sterile cotton swabs, and the remaining organisms were killed by inverting the agar on the top part of a petri dish containing chloroform and leaving it for about 20 min.

One loopful of the broth culture of the organism to be tested without dilution in saline was streaked perpendicularly across the tract of the serum-resistant strain, and the plate was incubated further at 32°C for 20 h. The media used for this purpose were Trypticase soy agar (TSA), rabbit serum agar (RSA), and RSA enriched with 0.01% (wt/vol) $FeCl_3$.

Production of pyochelin in synthetic medium. To facilitate purification and characterization of pyochelin, a synthetic medium must be as simple as possible in composition. It has to support good growth of the organism, with the final pH of growth in the range of 7.0 to 7.4. The medium developed for this purpose contained: saturated ammonium sulfate, 5 ml; 20% glucose, 50 ml; 20% sodium succinate, 10 ml; 10% Na_2HPO_4, 10 ml; 1% $CaCl_2$, 1 ml; 1% $MgSO_4$, 1 ml; and distilled water, 900 ml. The ingredients were made up in concentrated form, Seitz-filtered, and added aseptically to autoclaved, distilled water. This medium was distributed to six 1-liter shaking flasks with baffles and shaken at 32°C for about 24 h.

After centrifugation and filtration of the supernatant fluid through a membrane filter (Millipore Corp.), the titer of pyochelin was estimated by making a serial twofold dilution in saline and placing 0.1 ml of each dilution into a hole, about 1 cm in diameter, that was cut in an RSA plate (about 12 ml of agar per plate). A serum-sensitive strain of *P. aeruginosa*, designated PA-1, was suspended in saline to make about 2×10^7 cells per ml, and one loopful of this suspension was streaked near the well to a distance of about 0.5 cm. The reciprocal of the highest dilution that gave a barely visible growth of PA-1 after 24 h of incubation at 32°C was taken as units of pyochelin. Most serum-resistant strains of *P. aeruginosa* produced pyochelin at 128 to 1,024 U/ml, whereas serum-sensitive strains produced from 4 to 32 U of activity. The difference between the serum-resistant group and the serum-sensitive group in their production of pyochelin appeared to be quantitative rather than qualitative.

The strain of *P. aeruginosa* selected for routine production of pyochelin was designated PA-46. It was selected because it has all three of the qualifications needed for this study, i.e., serum resistance, rapid growth in the synthetic medium with high yield of pyochelin, and production of pyocin.

Production of pyochelins of PA-46 was also done on a somewhat larger scale of 250 liters in a fermentor (Grain Processing Co., Muscatine, Iowa) with the above-mentioned synthetic medium. The supernatant was acidified to pH 3.0 with the addition of HCl and concentrated to about 18 liters by vacuum evaporation before delivery to this laboratory. This material has been the source of pyochelin in most of our study described here.

Characterization of pyochelins. One hundred milliliters of the crude, concentrated pyochelin preparation of PA-46 was extracted with an equal volume of ethyl acetate five times, and the extracts were combined and evaporated in a Swissco evaporator to about 10 ml. Pyochelin activity extracted with ethyl acetate was only 5 to 10% of the original preparation, and the bulk of the activity remained with the residue in water-soluble form. The type of pyochelin extractable with ethyl acetate was designated group A, and those that remained in water-soluble form were designated group B. Group A pyochelins were further characterized with thin-layer chromatography on a Polygram silica gel (5 by 20 cm), 0.25-mm-layer prepared plate (Brinkmann), using cyclohexane and ethyl acetate (1:1) as solvent, in an ascending chromatography (21).

Chromatograms were examined in UV light, and the fluorescent zones were outlined with pencil. Each

of the zones was scraped off into a vial and extracted with ethyl acetate for testing the pyochelin activities with the above-mentioned technique.

The chromatograms were also stained with ferric chloride ferrocyanide reagent according to Hathway (11) and compared with the product of *Escherichia coli* made with the same medium and extracted similarly.

RESULTS

Growth of *P. aeruginosa* in sera. One hundred twenty-eight of 150 stock *P. aeruginosa* strains (85%) were able to grow readily with beef serum, but only 82 strains (55%) were able to grow with rabbit serum as the sole nutriment (Table 1). The results with horse and human sera were somewhat intermediate between those with beef and rabbit sera. Most strains kept in our laboratory for about 20 years by monthly subcultures on TSA retained the ability to grow in serum. About 30 (60%) of 50 strains of fresh clinical isolates were able to grow on RSA. It appeared that there was no significant difference in the percentage of serum resistance among old laboratory strains and fresh clinical isolates.

Growth of serum-sensitive strains on RSA could be enhanced by the addition of $FeCl_3$ (Fig. 1). This observation indicated that the factor that was limiting the growth of these organisms in serum was the non-availability of iron. The serum-resistant strains were able to produce a sufficient quantity of siderophores to enable them to grow on serum agar, and these siderophores diffused out to a considerable distance from their colonies. When serum-sensitive strains were streaked near the colonies of serum-resistant strains, the former grew well by using the siderophores of the latter (Fig. 2).

By using different combinations of serum-resistant and serum-sensitive strains of *P. aeruginosa*, it was found that there were many different patterns of satellite formation that could be differentiated. In Table 2 are shown examples of these patterns, using 5 serum-resistant strains as producers of pyochelins and 32 serum-sensitive strains as indicators. If these differences in the abilities of serum-resistant strains to help the growth of serum-sensitive strains on serum agar were really due to differences in the type of pyochelins produced by these organisms, the number of pyochelins that could be produced by this species would be fairly large. In the case of mycobactin, at least nine groups with many subtypes within each group are known (24), and therefore it is not surprising that a metabolically versatile species such as *P. aeruginosa* can produce many types of siderophores.

TABLE 1. *Variations in the degrees of growth of 150 strains of P. aeruginosa in different sera*[a]

Serum	No. (%) of:	
	Serum-sensitive strains	Serum-resistant strains
Beef	22 (15)	128 (85)
Human	40 (27)	110 (73)
Horse	31 (21)	119 (79)
Rabbit	68 (45)	82 (55)

[a] Serum sensitive means the failure of the organisms to initiate growth within 24 h on serum agar with about 10^5 cells per loopful of inoculum. Serum-resistant means the ability to start growth and show visible colonies with the same inoculum.

Utilization of siderophores of other bacteria by *P. aeruginosa*. The ability of *P. aeruginosa* to utilize siderophores of other bacteria, such as enteric bacilli, *Vibrio*, *Aeromonas*, and even *Bacillus* species, was tested on RSA. It was found that practically all strains of *P. aeruginosa* were able to utilize enterochelin produced by *E. coli* readily, in spite of the fact that their utilization of pyochelins produced by other strains of *P. aeruginosa* was rather inconsistent. The failure of some strains of *P. aeruginosa* to grow around the colonies of other strains of the same species may have been due to inhibition by pyocins rather than to inability to utilize pyochelins.

In Fig. 3, 4, and 5 are shown some experiments which indicate the ability of a strain of *E. coli*, no. 6, to help a serum-sensitive strain of *P. aeruginosa*, PA-88, to grow in serum. A saline suspension of the *E. coli*, containing about 10^9 cells per ml, was made, and 10-fold serial dilutions of this suspension were streaked with a cotton swab in parallel lines on an RSA plate.

A similar 10-fold serial dilution of *P. aeruginosa* suspension was made, and the dilutions were spot inoculated on the lines of *E. coli*. The plate was incubated at 32°C for 20 h. The *E. coli* grew only up to a dilution of 10^{-3}, or a suspension containing 10^6 cells per ml (Fig. 3). The *P. aeruginosa* grew only up to a dilution of 10^{-2}, or a suspension containing 10^7 cells per ml. However, when the higher dilution of *P. aeruginosa* was spot inoculated on the lines of *E. coli* that grew, even a small inoculum was able to initiate growth. In Fig. 4 and 5, this observation was extended by streaking the *E. coli* lines with a 10^{-2} dilution of cell suspension, and a high dilution of *P. aeruginosa* was spot inoculated on these lines. The same inocula of *P. aeruginosa* were also placed on TSA as a control to compare the number of colonies developing on both media. It was found that the number of colonies of *P. aeruginosa* that developed on the lawn of *E. coli* on serum agar was essentially the same as

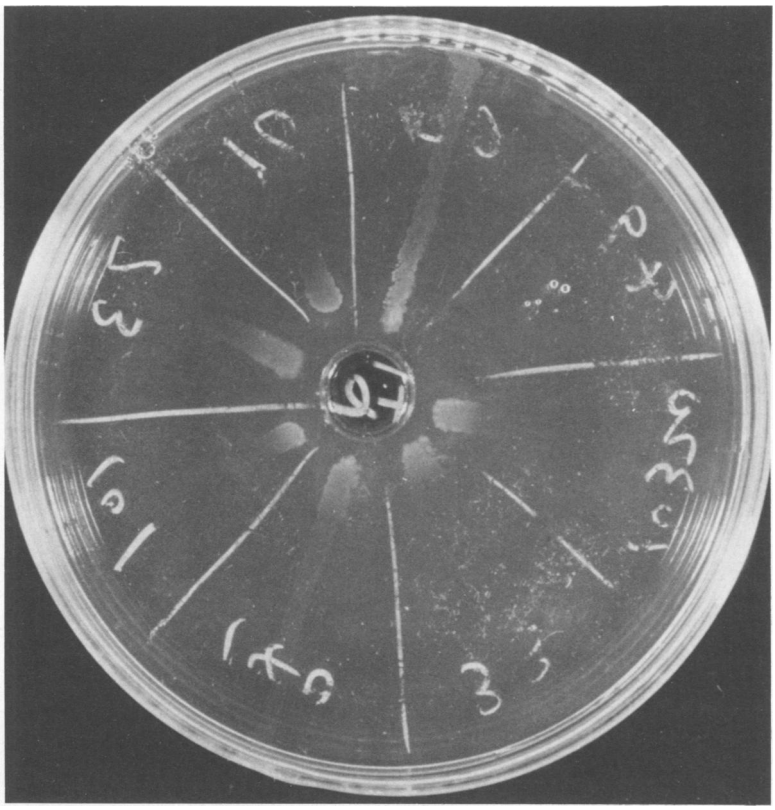

FIG. 1. *Growth of serum-sensitive strains of P. aeruginosa on RSA around a well containing a 0.01% solution of $FeCl_3$.*

the number that developed on TSA, indicating that practically a single cell was able to initiate growth on the lawn of *E. coli*. The same organism was able to grow on serum with a minimum of one loopful of cell suspension containing about 10^7 cells per ml, or about 10^5 organisms.

Most strains of *P. aeruginosa* were able to utilize siderophores of *V. cholerae*, *Aeromonas liquefaciens*, *Aeromonas formicans*, and *Bacillus megaterium*. The ability to utilize siderophores of *Aeromonas salmonicida* was limited to about 70% of the organisms tested.

We also tested several strains each of *P. reptilivora*, *P. fluorescens*, *P. aureofaciens*, *P. chlororaphis*, and *P. stutzeri* for their abilities to utilize siderophores of the above-mentioned gram-negative bacilli, and they were all able to do so.

Utilization of pyochelin by other bacteria. Utilization of pyochelins by other gram-negative bacilli was found to occur to different degrees. Of the 50 strains of *E. coli* tested, 30 were able to utilize pyochelin A and 40 were able to use pyochelin B of PA-46. Some strains of *Salmonella*, *Shigella*, and even *Alcaligenes faecalis* were found to be able to use pyochelin. One strain each of *A. liquefaciens* and *A. formicans* were found to be able to use pyochelin B but not pyochelin A. Most other species of pseudomonads, such as *P. fluorescens*, *P. reptilivora*, *P. aureofaciens*, *P. chlororaphis*, and *P. stutzeri*, were found to be able to use pyochelins to varying degrees.

Preliminary characterization of pyochelins of PA-46. One hundred milliliters of the concentrated material received from the Grain Processing Co. was extracted five times with an equal amount of ethyl acetate, and each extract was concentrated to 10 ml by evaporation. Activities of these extracts were tested with a strain of serum-sensitive *P. aeruginosa*, PA-1. The five extracts showed pyochelin activity at 1:1,280, 1:160, 1:40, 1:20, and 1:10 dilutions, respectively.

It was estimated that only about 5 to 10% of the chelating activities were extracted with ethyl acetate, and the bulk of activities remained in the water residue. The pyochelins extracted with ethyl acetate were designated group A, and those that remained in the water residue were designated group B. The group A pyochelins were

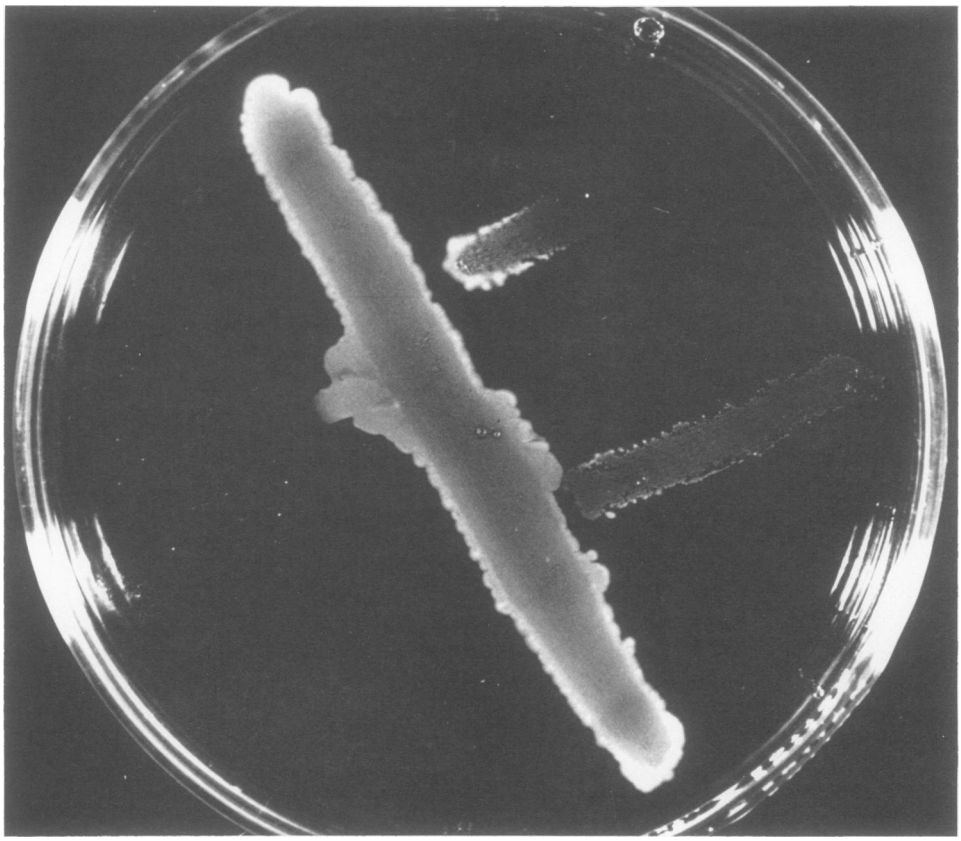

FIG. 2. *Satellite formation of a serum-sensitive strain of P. aeruginosa around the colony of a serum-resistant strain. The center colony is the serum-resistant strain, and the upper right side of the center colony is the serum-sensitive strain. On the lower right side is a serum-resistant organism whose growth was not enhanced by the center colony. On the left side is an organism that was unable to grow on its own; its growth was not helped by the center colony.*

TABLE 2. *Different patterns of growth of serum-sensitive P. aeruginosa around the colonies of five serum-resistant strains of the same species*

Pattern type	Serum-resistant strains[a]					No.[b] of serum-sensitive strains showing this pattern
	PA-46	PA-78	PA-86	PA-87	PA-138	
1	−	−	−	−	−	11
2	+	+	+	+	+	3
3	+	−	+	+	+	4
4	−	+	−	−	−	7
5	+	+	+	+	−	1
6	−	−	+	+	+	6

[a] +, Growth within 24 h at 37°C; −, no growth at the end of 24 h at 37°C.

[b] Thirty-two strains tested.

analyzed by thin-layer chromatography in comparison with a sample of enterochelin produced in our laboratory with *E. coli* strain 6 in the same medium. An example of this chromatography is shown in Fig. 6. The *P. aeruginosa* extract separated into 12 spots and enterochelin separated into five spots detectable by UV light. Seven spots of *P. aeruginosa* extract were able to enhance the growth of PA-1 on RSA, with three that were quite strong and four that were weak. After spraying the chromatograms with ferric chloride ferrocyanide reagent, enterochelin yielded five distinct blue spots with R_f values of 0.62, 0.50, 0.42, 0.31, and 0.20, respectively. Of the three major spots of pyochelin, one stained blue and two stained violet; they had R_f values of 0.47, 0.41, and 0.26, respectively. The blue color was characteristic of the enterochelin type of catechol compounds, whereas the violet color was characteristic of phenols, such as phloracetophenone and salicylic acid, which chelate preferentially with Fe^{3+} ion (11). It has been known for some time that fluorescein of *P. aeruginosa* is produced only in iron-poor me-

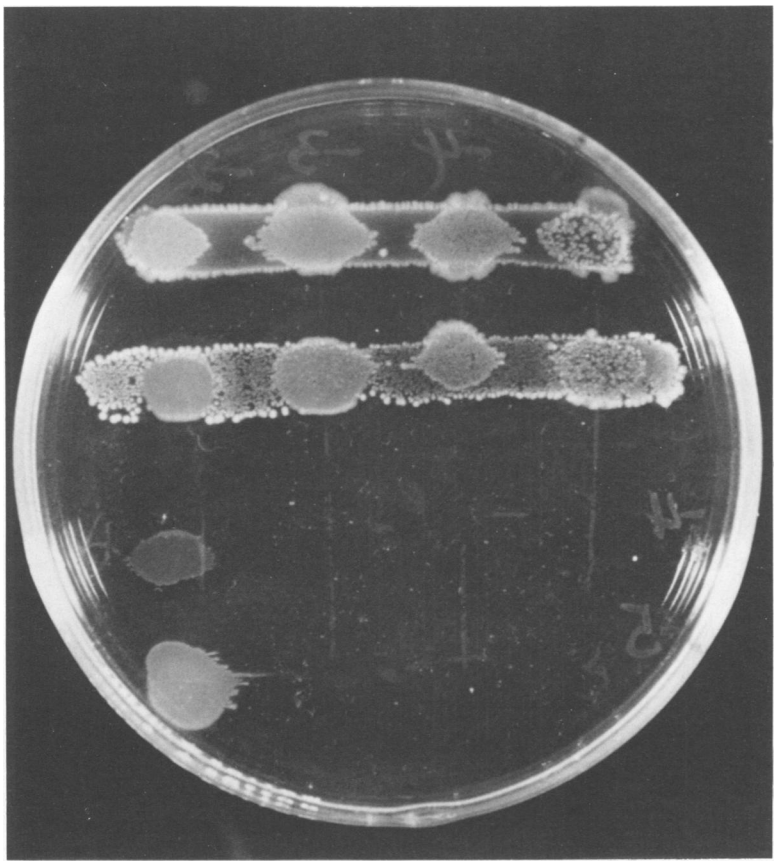

FIG. 3. *Growth of serum-sensitive P. aeruginosa PA-88 that was helped by a serum-resistant E. coli. Tenfold serial dilutions of a cell suspension of E. coli containing 10^9 cells per ml were made, and each dilution was streaked in parallel lines on an RSA plate. A similar dilution of P. aeruginosa was made and spot inoculated on the lines of E. coli. After incubation at 32°C for 24 h, the colonies of E. coli grew only at 10^{-2} and 10^{-3} dilutions. The colonies of P. aeruginosa grew only at a 10^{-2} dilution when they were alone. However, the P. aeruginosa grew up to a 10^{-5} dilution when they were spot inoculated on the lines of the E. coli colonies that grew.*

dium (25), and, therefore, we suspected from the beginning of this study that this pigment, and possibly others, may have pyochelin activity. However, there is no evidence so far to indicate that any of the pigments produced by this species can function as a siderophore.

Effect of pyochelin on pyocin activity. During the study of pyochelin it was found that some pyocin-sensitive strains of *P. aeruginosa* that were also serum-sensitive became resistant to pyocin when they were utilizing pyochelin to grow on serum agar. Interaction between the pyochelins and the pyocins could occur in several different ways, and examples of these are shown in Fig. 7 and 8. The serum-resistant strain PA-46 was a producer of pyocins which killed many other strains of *P. aeruginosa*. This strain was streaked perpendicularly on TSA and RSA. After the growth and removal of PA-46 colonies, four strains of *P. aeruginosa*, designated PA-88, PA-101, PA-103, and 117, were cross-streaked on the tract of PA-46. After incubation for another 20 h at 32°C, the results shown in Fig. 7 were obtained. PA-88 was killed on the tract of PA-46 when it was grown on TSA. The result on RSA, however, was exactly the opposite. This organism failed to grow alone on serum agar, but it grew on the tract of PA-46. This observation indicates that the pyochelin of PA-46 enabled PA-88 to grow on serum agar and, in addition, the utilization of pyochelin made PA-88 resistant to the action of pyocin. Strain PA-101 was also killed on TSA, but it grew on the tract of PA-46 on serum agar. The growth of PA-101 was different from that of PA-88 in that the former extended beyond the tract of PA-46 and was,

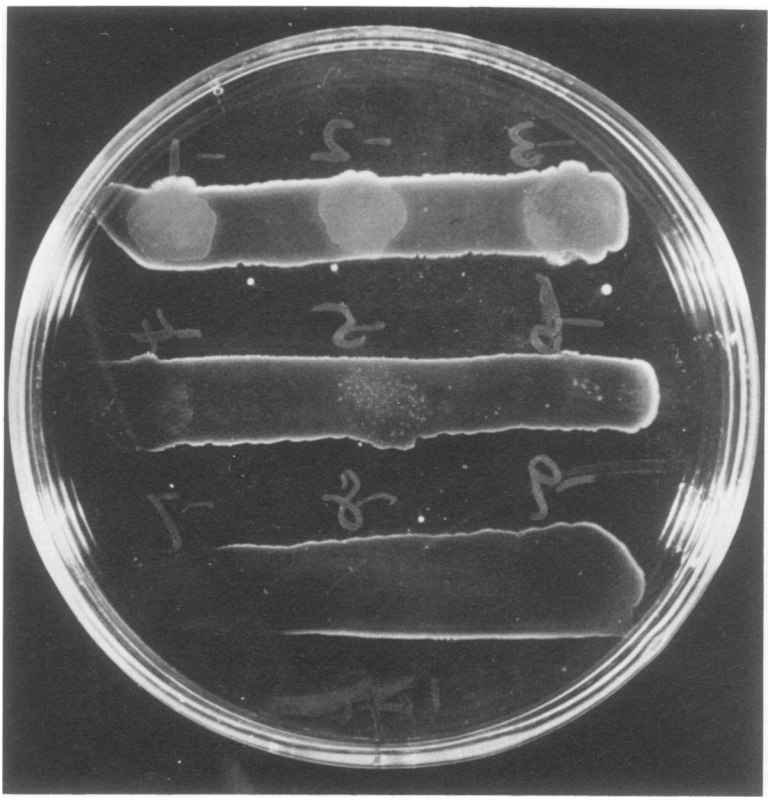

FIG. 4. *In an experiment similar to that shown in Fig. 3, a 10^{-2} dilution of E. coli was streaked in three lines on an RSA plate. Ten-fold serial dilutions of P. aeruginosa (PA-88) were spot inoculated on these lines. After incubation at 32°C for 24 h, it was found that P. aeruginosa grew in dilutions up to 10^{-7}. The number of the colonies of P. aeruginosa that grew on RSA within the colonies of E. coli were about the same as those that grew on nutrient agar, indicating that a single cell of P. aeruginosa was able to initiate growth.*

therefore, wider than the growth of PA-88. This observation indicated that there were probably more than one type of pyochelin produced by PA-46, and one type diffused far beyond the tract of the producing strain whereas the other type remained within the tract. PA-88 was able to use only the type that remained within the tract, but PA-101 used both for growth.

The results obtained with the third strain, PA-103, appeared to corroborate this idea because the organisms growing on the tract of PA-46 were mostly killed by pyocin, with only a few surviving colonies, although the growth occurred on both sides of the tract of PA-46. The fourth strain, 117, was a rough, serum-resistant strain; its growth on TSA was resistant to pyocin and was also unaffected by PA-46 on the serum agar.

The observations on the differences of pyocin activities on TSA and RSA did not really confirm the suspicion that resistance of strains PA-88, PA-101, and PA-103 to pyocin was due to utilization of pyochelins, because the compositions of TSA and RSA were quite different and the differences in the behaviors of the organisms could have been due to many other nutritional factors. Survival of a few colonies of PA-103 on the tract of PA-46 on serum agar also raised the question of whether there are variants within each strain regarding their abilities to use pyochelin. It was decided, therefore, to repeat the experiment with only RSA. We used one RSA enriched with $FeCl_3$ (0.01%) and one plain RSA as the control. We selected several variants of PA-103 for this purpose. When this strain was streaked on TSA, two colonial forms, one large and the other small, were usually obtained. We also picked a colony of PA-103 that survived within the tract of PA-46 on serum agar and a subculture of the organism that grew just outside the tract of PA-46 on serum agar. These four variants of PA-103 were tested on the two types of RSA; the results are shown in Fig. 8.

Of the two colonial forms selected on TSA the small colony (S) actually grew better on serum

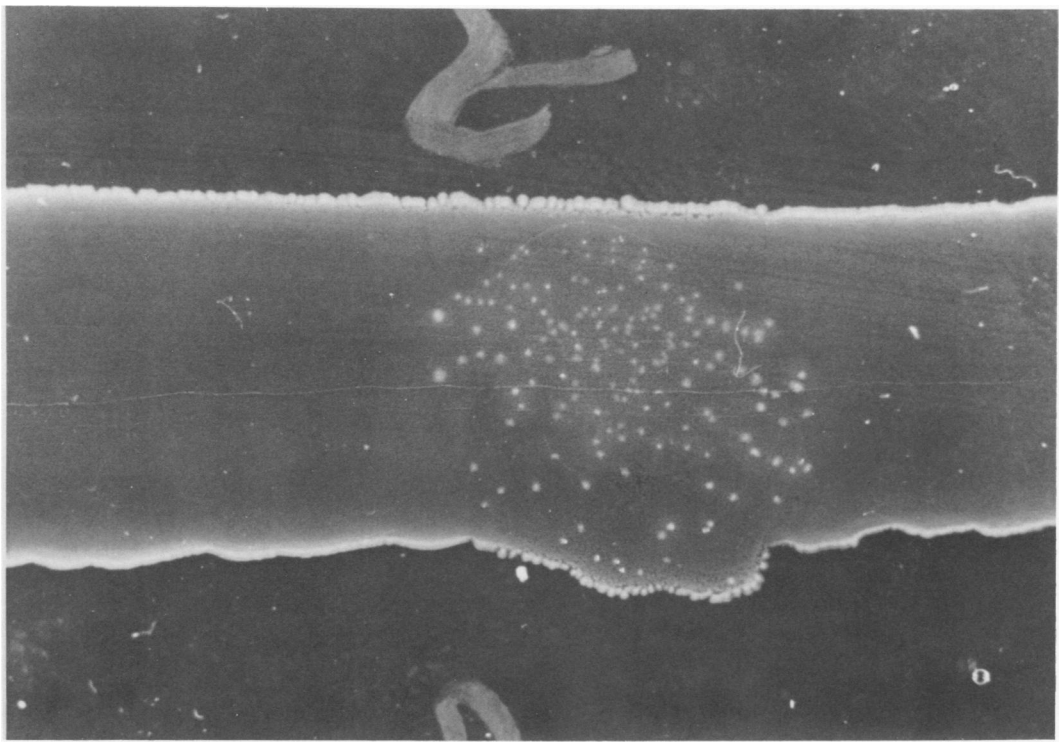

FIG. 5. *Part of Fig. 4 enlarged to show the individual colonies of P. aeruginosa (PA-88) growing in the colony of E. coli no. 6.*

agar than the large colony (L), and both forms were resistant to pyocin of PA-46 when they were growing on serum agar. Both forms were susceptible to pyocin when they were growing on serum agar enriched with $FeCl_3$; PA-88 and PA-101 behaved in the same manner. These observations confirmed that resistance of these organisms to pyocin when growing on the tract of PA-46 on serum agar was due to their utilization of pyochelin, because in the presence of $FeCl_3$ either PA-46 did not produce pyochelins or the serum-sensitive strain did not use them and they were just as susceptible to pyocin as when they were growing on TSA.

A variant of PA-103 picked from the tract of PA-46 on serum agar was found to be resistant to pyocin even on the serum agar enriched with $FeCl_3$ (colony A, Fig. 8). The fourth variant (colony B, Fig. 8), however, picked from a colony of PA-103 growing just outside of the tract of PA-46, was as susceptible to pyocin as the original strain.

The mechanism of inhibition of pyocin activity by pyochelin is not known at present. It can be due either to the blocking of the site of attachment of pyocin by pyochelin or to some changes in the surface receptors of the organisms when they are utilizing pyochelin. Both of these mechanisms are known to occur in the case of enterochelin and colicin (9, 10, 26).

Correlation and noncorrelation of pyochelin activity with virulence of *P. aeruginosa*. Virulence of serum-resistant and serum-sensitive strains of *P. aeruginosa* were compared by intraperitoneal injection into mice of serial 10-fold dilutions of the cell suspensions. We already had the data on the in vitro toxigenicities of these organisms regarding their production of protease, phospholipase C, and exotoxin A (14, 16). Serum-resistant and serum-sensitive strains of about equal toxigenicities were matched in the comparison. It was indeed found that serum-resistant strains tended to show higher mortality by intraperitoneal injection than the serum-sensitive group. This observation confirmed the prevailing concept that production of siderophores is correlated with virulence of bacteria.

We then proceeded to see if pyochelin produced by serum-resistant strains would enhance the virulence of serum-sensitive strains. The results, however, were not what we expected. The effect of pyochelin A of PA-46 was tested by evaporating the ethyl acetate-extracted prepa-

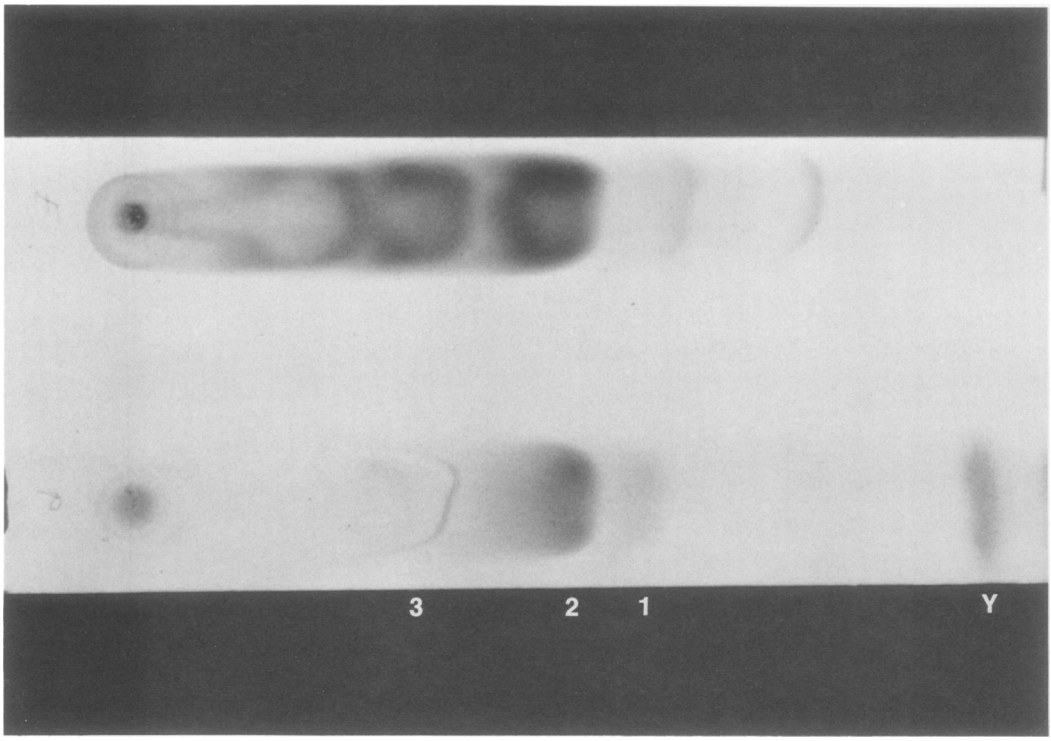

FIG. 6. *Comparison of group A pyochelin (lower part) with enterochelin (upper part) in thin-layer chromatography. Enterochelin separated into five spots that all stained blue with ferric chloride ferrocyanide reagents. Staining of pyochelins with this reagent varied. Of the three major spots of activity, one (1) stained blue and the other two (2 and 3) stained violet. The band indicated by the letter Y was a yellowish-orange pigment that did not stain with the ferrocyanide reagent and did not have pyochelin activity.*

ration and redissolving the dry, oily material in TSB. The use of TSB was necessary because this material did not dissolve well in water or saline. This solution was further diluted with TSB to give about 1,000 U of pyochelin A activity per ml. Strain PA-1, which was a strain of relatively low virulence, was used to determine if pyochelin A would enhance its virulence as compared with the same cell suspended in the plain TSB. The mortality of mice injected with cells of PA-1 suspended in TSB, with or without the pyochelin A, was exactly the same (Table 3). The same experiment was repeated with pyochelin B that had been made by dialyzing the crude water residue in distilled water after ethyl acetate extraction of the original material. The low-molecular-weight materials that came out of the dialysis bags were concentrated by lyophilization and passed through a column of diethylaminoethyl-cellulose. Pyochelin activity did not adsorb to the column and was eluted in the first fraction following the void volume. The material had about 1,200 U of activity per ml. Cells of PA-1 were suspended in this pyochelin B solution, and its virulence to mice was compared with the same cells suspended in saline. The suspension of cells of PA-1 in pyochelin B solution reduced, rather than enhanced, its virulence (Table 4). Since the same preparation increased the growth of the organism in serum, the one probable explanation for the reduction in virulence was that the pyochelin preparation inhibited the toxin production. This possibility was tested with strain PA-103, which has been the standard strain used in the production of exotoxin A (17). However, no significant difference in the toxin production in vitro was noted by incorporation of pyochelin B into the medium. Increased availability of iron is known to decrease production of exotoxin A of *P. aeruginosa* (1), and this mechanism might possibly operate in vivo, but it is not likely that the level of iron available in vivo will be as high as that in TSA medium used in the toxin production in vitro even with incorporation of pyochelin B in the inocula.

DISCUSSION

The ability of *P. aeruginosa* strains to grow in serum appeared to be due to their production

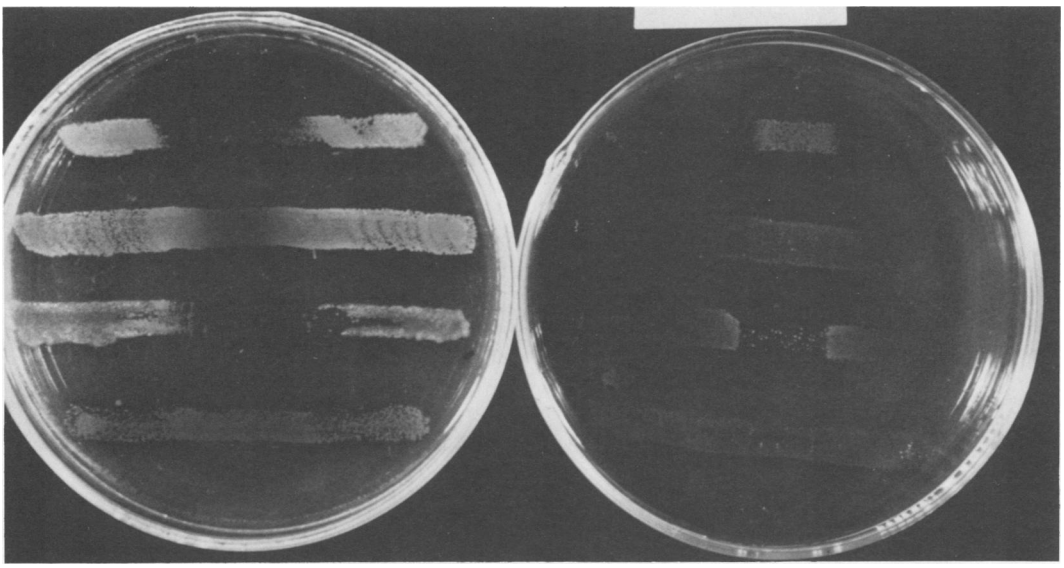

FIG. 7. *Effect of pyochelin utilization of P. aeruginosa on their sensitivity to pyocins. In the center of these TSA (left) and RSA (right) plates was streaked a serum-resistant and pyocin-producing strain of P. aeruginosa (PA-46). After removal of the colonies and sterilization of PA-46, four other strains of P. aeruginosa were streaked perpendicularly to the tract of this organism. The growth patterns of the three serum-sensitive P. aeruginosa strains on RSA were the opposite of those observed on TSA. See text for details.*

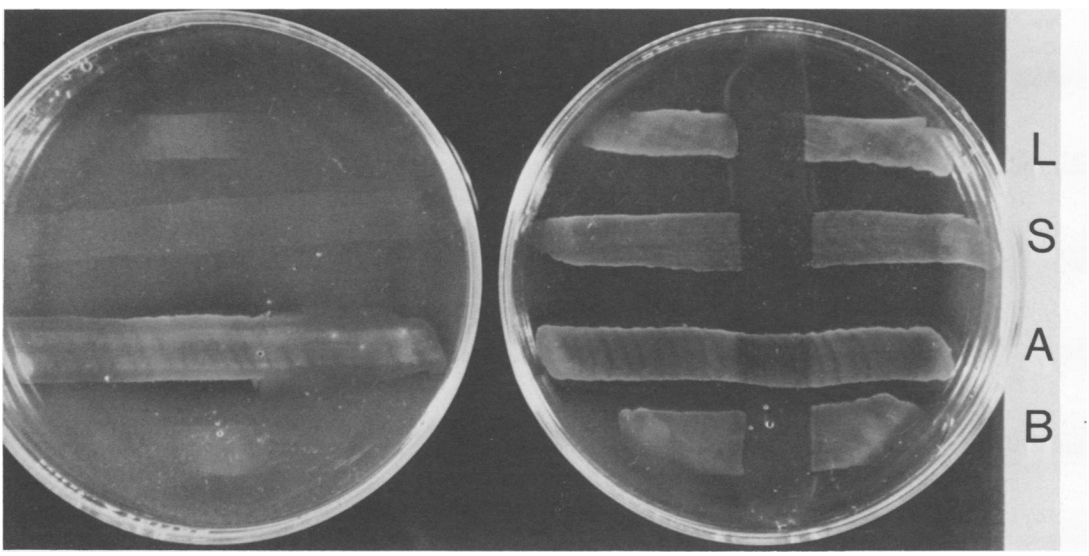

FIG. 8. *Effect of pyochelin utilization on the pyocin sensitivity of P. aeruginosa was tested again on RSA with (right) and without (left) incorporation of 0.01% $FeCl_3$. On RSA with added iron the sensitivity of the organisms to pyocins was the same as that on TSA. See text for details.*

of some siderophores that enable the organisms to utilize iron bound to transferrins. We have been using the term pyochelins for these compounds. The term indicates the name of the species that produced it and the nature of the compounds as chelating agents. We are aware of the term ferribactin used by Maurer et al. (18) for a siderochrome produced by *P. fluorescens*, but this term does not indicate the name of the organism that produced it and, also, the metals that are chelated by it may not be limited to iron.

TABLE 3. *Lack of influence of pyochelin A on the virulence of a serum-sensitive P. aeruginosa (PA-1) injected intraperitoneally into mice*

Cells suspended in:[a]	Cells/ml	Mortality of mice (no. dead/total) at day:				
		1	2	3	4	5
TSB with pyochelin A (1,000 U/ml)	5×10^8	5/5				
	5×10^7	3/5	4/5	5/5		
	5×10^6	1/5	3/5	3/5	3/5	3/5
	5×10^5	0/5	0/5	0/5	0/5	0/5
TSB	5×10^8	5/5				
	5×10^7	4/5	5/5			
	5×10^6	0/5	1/5	3/5	3/5	3/5
	5×10^5	0/5	0/5	0/5	0/5	0/5

[a] One milliliter of cells suspended in TSB with pyochelin A or in TSB alone was injected.

TABLE 4. *Reduction of virulence of a serum-sensitive P. aeruginosa strain (PA-1) by incorporation of pyochelin B into the inoculum*

Cells suspended in:[a]	Cells/ml	Mortality of mice (no. dead/total) at day:				
		1	2	3	4	5
Pyochelin B (1,200 U/ml)	5×10^9	3/5	4/5	4/5	4/5	4/5
	5×10^8	0/5	0/5	0/5	0/5	0/5
	5×10^7	0/5	0/5	0/5	0/5	0/5
Saline	5×10^9	5/5				
	5×10^8	4/5	4/5	4/5	5/5	
	5×10^7	0/5	1/5	1/5	1/5	1/5

[a] One milliliter of cell suspension was injected intraperitoneally.

There appeared to be many different types of pyochelins, some extractable with ethyl acetate and some not extractable. Those extractable with ethyl acetate have been designated group A pyochelins and those remaining in water residues have been designated group B pyochelins. The group A pyochelins appeared to be phenolates, and in the supernatants of the culture fluids of strain PA-46 in the synthetic medium described here, this group constituted only a minor portion (5 to 10%) of the total pyochelin activities. The structures of the compounds in this group are probably different from those of enterochelins produced by *E. coli* (19) because the former stained violet with ferrocyanide reagents whereas the latter stained blue. The structures of the group B pyochelins are not known at present. Whether they are hydroxamate compounds such as aerobactin (7) remains to be seen. It is likely that both of these groups contain a large variety of pyochelins because the ethyl acetate extract of PA-46, upon thin-layer chromatography, yielded no less than seven active spots that were observed by UV fluorescence. Three of these spots were strong enough to be stained with ferrocyanide reagent; one stained blue and two stained violet. The violet spots were much more intensive than the blue one. It is also likely that different strains of *P. aeruginosa* will produce different pyochelins in different proportions, and these differences probably accounted for the differences in the patterns of satellite formation of serum-sensitive strains around the colonies of serum-resistant strains.

Most strains of *P. aeruginosa* appeared to be able to utilize enterochelin, aerobactin, and the siderophores of many other gram-negative bacilli, such as *V. cholerae*, *A. liquefaciens*, *A. formicans*, and *A. salmonicida*. They were even able to utilize siderophores of gram-positive bacteria, such as *Bacillus* species. Most of the gram-negative bacilli mentioned above appeared to be able to utilize pyochelins, but they failed to grow around the colonies of *P. aeruginosa* on serum agar. This was probably due to production of inhibitory substances by the latter. This observation indicated the advantages of *P. aeruginosa* in their competition with other bacteria to grow in vivo, and this probably accounts for, at least in part, the common clinical observations that infections with enteric bacilli or other bacteria are often superceded by those with pseudomonads. It was interesting, and almost scary, to note that a strain of serum-sensitive *P. aeruginosa* that required at least 100,000 cells

to initiate growth on serum agar when it was alone was able to initiate growth from a single cell when it was grown together with a strain of *E. coli* in a large inoculum. The outcome of this mixed culture depended on the abilities of each organism to overcome the other, but it was not uncommon to see that a few hundred cells of *P. aeruginosa* grown with 10^7 cells of *E. coli* per ml in serum eventually overcame the latter and the serum culture became a pure culture of *P. aeruginosa*.

Stimulation of growth of one bacterial species by products of other species is a phenomenon that is opposite to the antibiosis that has been studied extensively in the last 40 years in the rush to produce antibiotics. In 1935, Duliscouet (5) reported that staphylococci produced substances that either inhibited or enhanced the growth of *Corynebacterium diphtheriae*. He proposed the term probiotics for those staphylococcal products that enhanced the growth of diphtheria bacilli. This term appears to be quite appropriate to describe the siderophores of various bacteria that enhance the growth of other species. These compounds may be useful in clinical microbiology because they enhance and accelerate growth of fastidious organisms, and incorporation of them in special media may facilitate isolation of certain pathogens. These siderophores can also indicate the design of antibiotics that would inhibit bacterial growth by blocking the uptake of metals.

Utilization of pyochelins not only enabled serum-sensitive *P. aeruginosa* to grow in serum, but also some of them became resistant to pyocins that ordinarily would have killed them when they were growing in TSA. The mechanism for this resistance is not known at present.

One of the most important aspects of pyochelins, and the one that is probably the most important from the viewpoint of medical bacteriology, is their effect on the virulence of *P. aeruginosa*. It appeared that serum-resistant strains were usually more virulent than serum-sensitive strains if they were equally toxigenic. However, there were some serum-resistant strains that were not toxigenic, and these organisms were not particularly virulent. An unexpected finding during this study was that the exogenous pyochelins did not enhance the virulence of *P. aeruginosa* when this characteristic was measured by intraperitoneal injection of the organism into mice. Pyochelin A had no effect at all in this model, and pyochelin B actually reduced the virulence of a serum-sensitive strain of *P. aeruginosa*. The mechanism for this reduction in virulence is unknown at present, but it did not seem to involve inhibition of the production of exotoxin A.

It seems quite premature even to speculate on the mechanism by which these pyochelins affect the virulence of *P. aeruginosa* because the preparations used in our study were not pure and were mixtures of many different substances. Many more studies in the future are needed to understand the roles played by pyochelins in the pathogenesis of *P. aeruginosa*.

ACKNOWLEDGMENTS

This work was supported by a Public Health Service grant from the National Institute of Allergy and Infectious Diseases (AI05283) and a grant from the Ephraim Roseman Foundation.

We wish to express our gratitude to George Harding and K. Fok for technical assistance.

LITERATURE CITED

1. **Bjorn, M. J., B. H. Iglewski, S. K. Ives, J. C. Sadoff, and M. L. Vasil.** 1978. Effect of iron on yields on exotoxin A in cultures of *Pseudomonas aeruginosa*. Infect. Immun. **19**:785-791.
2. **Bullen, J. J., C. G. Ward, and S. N. Wallis.** 1974. Virulence and the role of iron in *Pseudomonas aeruginosa* infections. Infect. Immun. **10**:443-450.
3. **Byers, B. R., and J. E. L. Arceneaux.** 1977. Microbial transport and utilization of iron, p. 215-249. *In* E. D. Weinberg (ed.), Microorganisms and minerals. Marcell Dekker, Inc., New York.
4. **Byers, B. R., and C. E. Lankford.** 1968. Regulation of synthesis of 2,3-dihydroxybenzoic acid in *Bacillus subtilis* by iron and a biological secondary hydroxamate. Biochim. Biophys. Acta **165**:563-566.
5. **Duliscouet, R.** 1935. Action probiotique et antibiotique des staphylocoques chez les porteurs de germes diphtheriques. C. R. Seances Soc. Biol. Paris **118**:1277-1280.
6. **Forsberg, C. M., and J. J. Bullen.** 1972. The effect of passage and iron on the virulence of *Pseudomonas aeruginosa*. J. Clin. Pathol. **25**:65-68.
7. **Gibson, F., and D. E. Magrath.** 1969. The isolation and characterization of a hydroxamic acid (aerobactin) formed by *Aerobacter aerogenes* 62-1. Biochim. Biophys. Acta **192**:175-184.
8. **Govan, J. R. W., and R. R. Gillies.** 1969. Further studies in the pyocine typing of *Pseudomonas pyocyanea*. J. Med. Microbiol. **2**:17-25.
9. **Guterman, S. K.** 1971. Inhibition of colicin B by enterochelin. Biochem. Biophys. Res. Commun. **44**:1149-1155.
10. **Guterman, S. K.** 1973. Colicin B: mode of action and inhibition of enterochelin. J. Bacteriol. **114**:1217-1224.
11. **Hathway, D. E.** 1960. Plant phenols and tannins, p. 308-354. *In* I. Smith (ed.), Chromatographic and electrophoretic techniques. Interscience Publishers, Inc., New York.
12. **Hsieh, H., and P. V. Liu.** 1970. Serological identities of proteases and alkaline phosphatases of the so-called non-agglutinable (NAG) vibrios and those of *Vibrio cholerae*. J. Infect. Dis. **121**:251-259.
13. **Kochan, I.** 1973. The role of iron in bacterial infections with special consideration of host tubercle bacillus interaction. Curr. Top. Microbiol. Immunol. **60**:1-30.
14. **Kochan, I., J. T. Kvach, and T. I. Wiles.** 1977. Virulence-associated acquisition of iron in mammalian serum by *Escherichia coli*. J. Infect. Dis. **135**:623-632.
15. **Liu, P. V.** 1957. Survey of hemolysin production among species of pseudomonads. J. Bacteriol. **74**:718-727.

16. **Liu, P. V.** 1961. Identification of pathogenic pseudomonads by extracellular antigens. J. Bacteriol. **81**:28–35.
17. **Liu, P. V.** 1973. Exotoxins of *Pseudomonas aeruginosa*. I Factors that influence the production of exotoxin A. J. Infect. Dis. **128**:506–513.
18. **Maurer, B. A., W. Mueller, W. Keller-Schierlein, and H. Zaehn.** 1968. Stoffwechselprodukte von Mikroorganisms. 61 Mitt. Ferribactin, ein Siderochrom aus *Pseudomonas fluorescens* Migula. Arch. Mikrobiol. **60**:326–339.
19. **O'Brien, I. G., and F. Gibson.** 1970. The structure of enterochelin and related 2,3-dihydroxy-N-benzoylserine conjugates from *Escherichia coli*. Biochim. Biophys. Acta **215**:393–402.
20. **Pollack, J. R., and J. B. Neilands.** 1970. Enterobactin, an iron transport compound from *Salmonella typhimurium*. Biochem. Biophys. Res. Commun. **38**:989–992.
21. **Randerath, K.** 1966. Thin-layer chromatography. Academic Press Inc., New York.
22. **Rogers, H. J.** 1973. Iron-binding catechols and virulence in *Escherichia coli*. Infect. Immunol. **7**:455–456.
23. **Schade, A. L., and L. Caroline.** 1944. Raw hen egg white and the role of iron in growth inhibition of *Shigella dysenteriae*, *Staphylococcus aereus*, *Escherichia coli*, and *Saccharomyces cerevisiae*. Science **100**:14–15.
24. **Snow, G. A.** 1970. Mycobactins: iron-chelating growth factors from mycobacteria. Bacteriol. Rev. **34**:99–125.
25. **Totter, J. R., and F. T. Mosely.** 1953. Influence of the concentration of iron on the production of fluorescein by *Pseudomonas aeruginosa*. J. Bacteriol. **65**:45–47.
26. **Wayne, R., K. Frick, and J. B. Neilands.** 1976. Siderophore protection against colicins M.B.V. and Ia in *Escherichia coli*. J. Bacteriol. **126**:7–12.
27. **Weinberg, E. D.** 1974. Iron and susceptibility to infectious diseases. Science **184**:952–956.