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Siderophore Presence in Sputa of Cystic Fibrosis Patients

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Sputum samples from the lungs of cystic fibrosis patients harboring *Pseudomonas aeruginosa* infections were collected and examined for the presence of the siderophore pyoverdine. Fluorescence quenching, due to the addition of ferric ion, as well as column and thin-layer chromatography results indicated that all samples contained the siderophore. Six samples furnished sufficient material after purification to allow us to obtain visible absorbance spectra. These spectra were characteristic of the ferrated analog of the *P. aeruginosa* pyoverdine, that is, ferripyoverdine, and in all cases they indicated a degree of ferration in excess of 50%. *P. aeruginosa* in the cystic fibrosis lung is thus iron stressed and responds by synthesizing pyoverdine, which subsequently binds ferric ion.

Iron is an essential nutrient for the vast majority of microbes examined. In environments where the metal is lacking, microorganisms secrete avid ferric ion chelators, termed siderophores, to sequester and deliver it to the organisms (9, 17).

Siderophores are thought to be functional during infections. Sokol (20) noted that *Pseudomonas aeruginosa* mutants which do not express the ferripyochelin (one of the two siderophores of the organism)-binding protein on their cell surfaces are cleared more rapidly from a mouse corneal-infection model. Sokol also observed that the lethality of the mutant in a burn infection model was far less than that of the parent strain. That siderophores function as virulence factors has been demonstrated (1, 10, 11, 16, 20, 22, 23). This conclusion is based on data which demonstrate that mutants incapable of either siderophore synthesis or siderophore transport are far less virulent than are their wild-type counterparts.

While a number of bacteria may colonize the cystic fibrosis (CF) lung, *P. aeruginosa* is the most common and most serious of the pathogens (21). Progressive destruction of lung tissue, secondary to the combined effects of viscous mucous and microbial infections, is the primary cause of morbidity and mortality in CF patients. Even though the bacterium may be responsive to antibiotic treatments, it is seldom, if ever, eliminated from the CF lung (3). How *P. aeruginosa* acquires the iron required for growth in the CF lung is an unanswered question and the subject of the investigation reported herein. We present spectral and chromatographic data which demonstrate that when present in the CF lung, the bacterium synthesizes one of the siderophores characteristic of the pseudomonads, that is, pyoverdine. In addition, pyoverdine isolated from CF sputa is ferrated to a noticeable degree, indicating that the bacterium is able to acquire ferric ion in the CF lung. (Preliminary findings were presented previously [6].)

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MATERIALS AND METHODS

Sputum collection and treatment. Sixteen male and female CF patients (ages 7 through 28), who were positive for the presence of mucoid *P. aeruginosa*, donated respiratory tract sputum samples which were collected in sterile, plastic receptacles at the CF center of the Lutheran General Hospital, Park Ridge, Ill. Samples were placed on ice and were processed either immediately after being transported to the laboratory or after being stored overnight on ice at 4°C. No differences were noted between the samples processed in either of these ways. Because of the low volume of some sputum samples, in some instances sputa were pooled, yielding 12 samples which were analyzed further. During the course of this investigation, CF patients who were not infected by mucoid *P. aeruginosa* were unavailable to us.

Isolation of mucoid *P. aeruginosa* from CF patients. Pure cultures of mucoid *P. aeruginosa* were isolated by diluting the sputa (approximately a 10^{-7} dilution) of the CF patients in this study. The dilution was made in sterile saline (0.85%), and an aliquot of the solution was then plated onto King B (13) plates. Colonies which had the proper morphology and which exuded a green, fluorescent compound into the medium were purified and identified as *P. aeruginosa* with the computer-assisted identification Microlog (Biolog, Hayward, Calif.) system.

Isolation of pyoverdine from in vitro cultures. All glassware was treated with HCl (19) or EDTA (14) to remove iron; water and media were deferrated (8) by the use of Chelex 100 (Bio-Rad, Richmond, Calif.) columns (1.5 by 30 cm). New, individual column materials were used for each sample analyzed.

Pyoverdine was isolated by the following method. In vitro pure cultures of mucoid *P. aeruginosa* were grown in liquid medium under iron-restricted conditions as previously described (12). The cultures were acidified with HCl to pH 3.85 and centrifuged at 10,000 × *g* to precipitate the alginate, and the supernatant containing the pyoverdine was recovered. The supernatant was applied to a column (1.5 by 30 cm) of octadecyl silane (ODS; in water at pH 3.85) (Prepex, Phenomenex, Inc., Torrance, Calif.) (7) in tandem with a column (1.5 by 30 cm) of CM Sephadex C-25 (Pharmacia, Piscataway, N.J.). The system was washed with 10 volumes of water (pH 3.85), after which no more fluorescent material

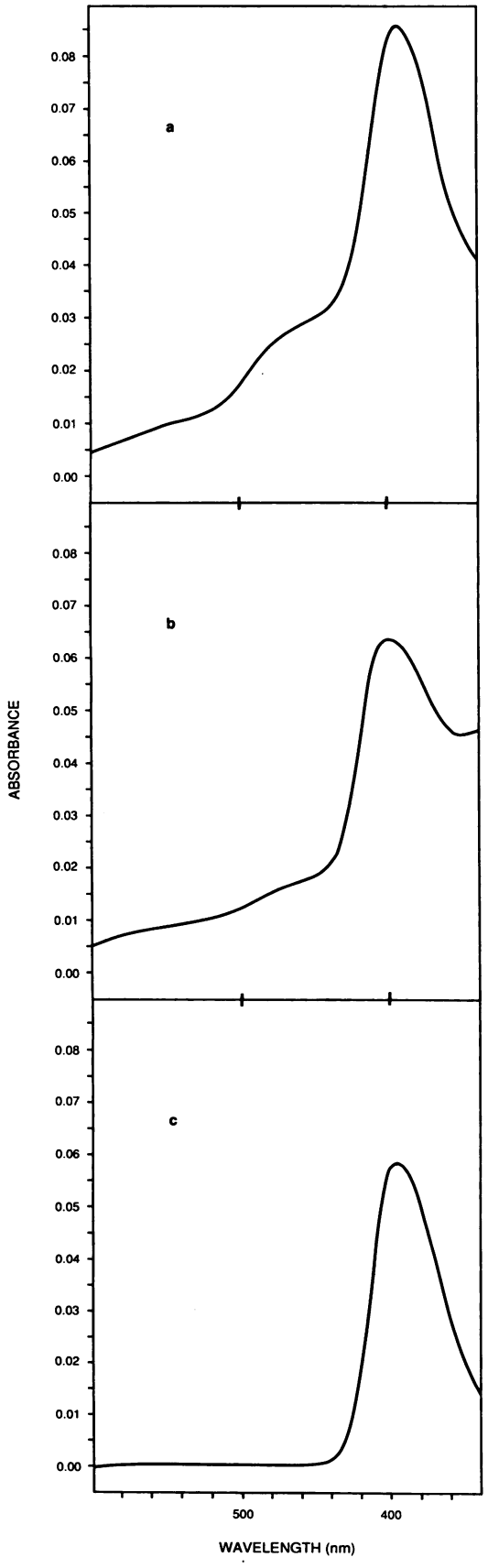

FIG. 1. Visible absorbance spectra of partially ferrated pyoverdines from the sputa of a female (a) and a male (b) CF patient and of pyoverdine from an in vitro culture of a mucoid *P. aeruginosa* strain isolated from the male CF patient (c).

was eluted from the ODS column onto the CM Sephadex C-25 column. The ODS column was removed, and the CM Sephadex C-25 column was washed with 10 volumes of water (pH 6.8) until no more fluorescent material was eluted. Pyoverdine (one of the two remaining fluorescent bands on the column) was then selectively eluted with either 0.05 or 0.1 M sodium acetate or NaCl (both at pH 6.5). An aliquot of this CM Sephadex C-25 eluant chromatographed on a Sephadex G-25 fine (Pharmacia) column in water as a single fluorescence peak with an elution volume consistent with a molecular weight reported for pyoverdine (15). The remainder of the CM Sephadex C-25 eluant was concentrated by rotary evaporation and was dialyzed against water (Spectra/Por mwco:500; Spectrum, Los Angeles, Calif.). The purified pyoverdine was again concentrated by rotary evaporation. Thin-layer chromatography with these concentrated samples on Silica Gel G (Analtech, Newark, Del.) plates, eluted with 70% ethanol as described previously (18), yielded single fluorescent spots with R_f values consistent with those for pyoverdine (12, 18). Absorbance spectra (measured with a Zeiss DMR 21 spectrophotometer) of the material thus isolated were characteristic of the nonferrated siderophore (15, 18), indicating that pyoverdine from iron-deficient cultures did not acquire ferric ion during the isolation procedure.

Isolation of pyoverdine from CF sputa. Each CF patient's sputum sample (0.5 to 4.0 ml) was transferred to a graduated test tube, and an aliquot was tested for fluorescence quenching (15) with 10 mM $FeClO_4$–0.1 M $HClO_4$ (2). The specimen cup was rinsed twice with water, and the washes were added to the tube. The sample was acidified to pH 3.5 with 0.2 M HCl and was centrifuged at 10,000 × *g* for 2.5 min to precipitate the mucus. The supernatant was removed and placed in another tube, the precipitate was rinsed with water (pH 3.85) and centrifuged again, and this supernatant was combined with the first. This mucus-free supernatant was subjected to the ODS-CM Sephadex C-25 procedure described above except that the volumes of the columns used were 2 and 1.5 ml, respectively. The CM Sephadex C-25 eluant, an aliquot of which was tested with ferric perchlorate, was concentrated by rotary evaporation, and the visible spectrum was recorded with 0.3-ml microcuvettes.

Estimations of pyoverdine concentrations and degrees of ferration. Pyoverdine concentrations were calculated with an extinction coefficient (15) of 1.65×10^4 M^{-1} cm^{-1} at 378 nm. A stock solution of 0.2 M $FeCl_3$–0.1 M HCl was diluted for use in pyoverdine titration experiments. Percent ferration was estimated from the visible absorbance spectrum of each sample as described below.

RESULTS

All of the sputum samples displayed the likelihood of containing a siderophore(s), as they fluoresced when illuminated with UV light and tested positive (i.e., showed fluorescence quenching and color change) for the presence of an iron chelator when ferric perchlorate was added (2, 15). Half of the 12 samples contained sufficient material for spectral analysis. The visible absorbance spectra of two such sam-

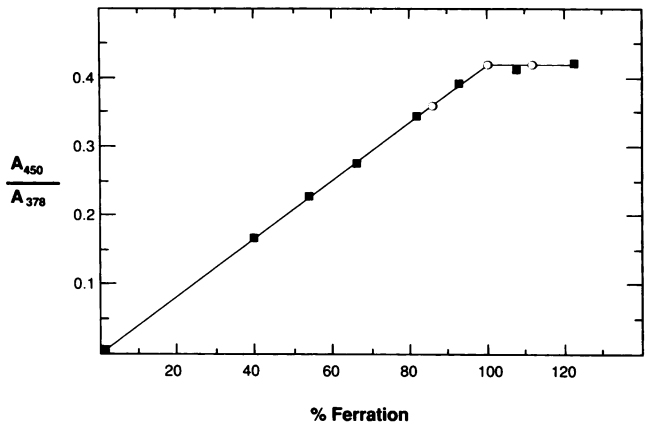

FIG. 2. Correlation of percent ferric ion chelation by pyoverdine with the A_{450}/A_{378} ratio. The percent ferration was obtained as described in the text for pyoverdine from mucoid CF *P. aeruginosa* isolates obtained from the sputa of a female (■) and a male (○) patient. Percent ferration points above 100% indicate a molar ratio of ferric ion to pyoverdine greater than 1:1, and thus the A_{450}/A_{378} values remain constant.

ples are displayed in Fig. 1a and b. In addition to the absorbance maximum typical of the compound, i.e., about 400 nm (15), the characteristic "iron shoulders" at 450 and 550 nm are also observed. These shoulders are indicative of ferripyoverdine, i.e., the ferric analog of the siderophore. That the presence of ferric ion in the pyoverdine from the sputa of CF patients is not an artifact of the isolation procedure is indicated by the bottom curve of Fig. 1. This spectrum is that of the pyoverdine obtained by the same method as that applied to the sputum samples but from an in vitro pure culture of mucoid *P. aeruginosa* isolated from the sputum sample used to generate the results in Fig. 1b. This spectrum is characteristic of nonferrated pyoverdine in that no iron shoulders are present and the absorbance maximum occurs at 390 nm.

That the plastic receptacles used to collect the sputa were not donating ferric ion to the sputum samples was determined by putting purified pyoverdine, isolated from laboratory cultures of mucoid *P. aeruginosa*, into identical sterile receptacles, storing them on ice at 4°C overnight, and then recording the spectra. None of the samples treated in this manner displayed a spectrum indicative of the ferrated siderophore, i.e., ferripyoverdine.

To determine to what degree the pyoverdines from CF sputa were ferrated, a standard curve was generated by using information from absorbance spectra. Pyoverdines from *P. aeruginosa* isolates from two CF patients were titrated with known amounts of ferric chloride. As described by others (15), the A_{550} and A_{450} (iron shoulders) increase until a 1:1 ratio of ferric ion to pyoverdine is attained, after which there is no further increase. An isosbestic point for the spectral shift that accompanies that transition occurs at 378 nm. Thus, for pyoverdine-ferripyoverdine mixtures, the A_{450}/A_{378} can be used to estimate the percent ferration independent of siderophore concentration with ratios ranging from 0 (no ferric ion chelation) to about 0.42 (100% ferric ion chelation) (Fig. 2). The maximal ratio we obtained (0.42) is quite similar to those which we estimated from the published spectra of others (15, 18) and those for pyoverdine from three previously isolated mucoid *P. aeruginosa* strains in our laboratory collection.

TABLE 1. Presence of ferrated pyoverdine in CF sputum samples[a]

Sample	Vol (ml)	Pyoverdine concn (μM)	% Ferration	R_f
1	1.7	0.64	54	0.045
2	4.0	0.48	58	0.045
3	1.5	1.55	71	0.040
4	3.0	1.39	88	0.045, 0.060
5	1.3	0.49	65	0.040
6	0.5	0.58	74	0.040
$\bar{x} \pm SD$		0.85 ± 0.48	68 ± 12	

[a] Six sputum samples were processed, and pyoverdine contents as well as degrees of ferration were determined from the visible absorbance spectra, as described in Materials and Methods and the legend to Fig. 2. Samples 1 and 2 were each pooled from two different female patients. Sample 3 was from an individual male patient, and samples 4, 5, and 6 were from individual female patients.

All of the sputum samples analyzed spectrophotometrically contained pyoverdine ferrated to some degree, as all six had ferripyoverdine spectra similar to those noted in Fig. 1a and b. In all cases, the degrees of ferration of the sputum samples exceeded 50% (Table 1). The pyoverdine content of the sputa averaged slightly less than 10^{-6} M, and in the samples analyzed, pyoverdine contents did not differ greatly with age or sex of the patient or sample size. When the samples were further concentrated and examined by thin-layer chromatography, all displayed fluorescent spots with R_f values consistent with those displayed by authentic pyoverdine. Pyoverdines isolated from in vitro cultures of mucoid CF *P. aeruginosa*, including some isolates obtained from the sputa of this study, had R_f values of 0.043, 0.065, 0.133, and 0.230. The multiplicity of R_f values reflects the presence of pyoverdine analogs differing in the composition of the side group of the pyoverdine chromophore (4).

While the six samples described above furnished enough material for spectral analysis, the other six did not. They did, however, meet other criteria indicative of pyoverdine presence. Besides the fluorescence quenching and the associated color change generated by the addition of ferric perchlorate, all contained fluorescent material with chromatographic behavior on ODS and CM Sephadex C-25 columns identical to that of the samples described in the legend to Fig. 1. Five of the six also produced sufficient material for thin-layer chromatography analysis and had R_f values similar to those presented in Table 1. The sixth sample contained too little material to provide conclusive results on thin-layer chromatograms.

DISCUSSION

Procaryotes which are present in iron-limited environments often invoke the synthesis and expulsion of siderophores to sequester ferric ion (9). In gram-negative bacteria, the resultant ferrisiderophore binds to an iron-repressible outer membrane protein to initiate the ferric ion assimilation of the cell. A previous study (5) has shown that *P. aeruginosa*, recovered without subculturing from the lungs of a CF patient, contains the cognate iron-repressible outer membrane proteins, indicating that the bacterium is iron stressed in this environment. Haas et al. (12) recently demonstrated that mucoid *P. aeruginosa* isolated from CF patients synthe-

sizes the siderophores typical of nonmucoid members of the species, that is, pyoverdine and pyochelin.

Previously, indirect evidence which indicates that siderophores function as virulence factors has accumulated (1, 10, 16, 20, 22, 23). One study (11), in which enterobactin was recovered from guinea pigs experimentally infected with *Escherichia coli*, demonstrated the presence of a siderophore during an infection. The present study is, to the best of our knowledge, the first to demonstrate that the siderophore of a pathogen is present in a tissue infected by a bacterium under nonlaboratory conditions. That *P. aeruginosa* invokes pyoverdine to overcome iron starvation in the CF lung is suggested by our isolation of ferrated pyoverdine from the sputa of CF patients and by the fact that the bacterium was recovered (without subculturing) from the lungs of a CF patient and shown to contain the cognate iron-repressible outer membrane proteins necessary for ferrisiderophore assimilation (5). The bacterium's need to sequester ferric ion may thus result in the development of strategies allowing better control of *P. aeruginosa* and therefore in the decrease of the corresponding morbidity and mortality that the organism causes CF patients.

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