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Siderophore Production by *Pseudomonas pseudomallei*

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Eighty-four strains of *Pseudomonas pseudomallei* isolated from patients with melioidosis were examined for siderophore production. All the strains were shown to produce siderophore both on chrome azurol S agar plates and in liquid medium under iron-deficient conditions. Chemical assays indicated that the siderophore belongs to the hydroxamate class. Addition of iron to the culture medium resulted in increased culture growth with markedly decreased yield of siderophore. Siderophore produced by strain U7 was purified by gel filtration chromatography, and the molecular weight was estimated to be 1,000. When this partially purified siderophore was added to culture medium, it promoted iron uptake by *P. pseudomallei* in the presence of EDTA and enhanced growth of the organism in the presence of transferrin. We have given this siderophore the trivial name malleobactin.

Pseudomonas pseudomallei has been associated with melioidosis, which is endemic in Southeast Asia, northern Australia, and Central America (5, 17, 24). *P. pseudomallei* is a major cause of community-acquired septicemia in northeast Thailand (5). Some cases have been reported in veterans of Southeast Asia conflicts and in visitors and residents of endemic areas (3, 13, 18, 28). *P. pseudomallei* is an environmental organism widely found in water and soil in the tropics (10, 17). Infection is thought to be acquired by inoculation, inhalation, or aspiration and possibly ingestion of the organism (13, 16). *P. pseudomallei* can cause acute infections, characterized by a progressive septicemia, which may be rapidly fatal even with antibiotics and supportive therapy (5). The mortality rate in patients with overwhelming septicemia is 70% (5). The infection can also occur in chronic or subclinical forms in endemic areas.

Pulmonary melioidosis was a frequent occurrence in U.S. soldiers during the Vietnam War (29). Little is known about the pathogenic mechanisms of *P. pseudomallei* infection. A heat-labile exotoxin and a proteolytic enzyme have been described (6, 12, 16). Polypeptides extracted from certain strains of this organism were shown to increase mortality when introduced intraperitoneally into mice infected with the organism (19). Dannenberg and Scott (9) demonstrated that avirulent strains of *P. pseudomallei* were unable to multiply or accumulate in host tissue.

Iron is essential for bacterial growth; the ability to acquire iron from the host is a prerequisite for the establishment and maintenance of infection (2, 20, 22, 30). Most iron in mammalian hosts is bound to globular proteins such as transferrin and lactoferrin and is not readily available for use by microorganisms. In response, many microorganisms synthesize and excrete low-molecular-weight specific iron chelators or siderophores to compete for iron with the host (20, 30). Siderophores have been implicated in the virulence of bacteria including *Escherichia coli*, *Pseudomonas aeruginosa*, *Pseudomonas cepacia*, and *Vibrio anguillarum* (4, 7, 8, 26, 27). It has been suggested that the inhibitory effect of human serum on several bacteria is associated with iron deprivation (30). A recent study by Ismail et al. (15) suggested that the ability of *P. pseudomallei* to resist elimination from the host and cause systemic diseases in humans is partly due to its serum-resistant properties. The present study was designed to determine whether *P. pseudomallei* produces siderophores and to describe some of the properties of any siderophore produced by this organism.

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MATERIALS AND METHODS

Strains. *P. pseudomallei* isolates from patients with melioidosis were generously provided by D. Dance, Wellcome-Mahidol University Oxford Tropical Medicine Research Program, Bangkok, Thailand.

Culture conditions. Cultures were maintained on M9 agar with 0.5% glucose. For production of siderophores, cultures were grown in 0.5% Casamino Acids (CAA medium; Difco Laboratories, Detroit, Mich.) (26). Cultures were routinely grown for 24 h at 30°C with maximum aeration. To minimize iron contamination, all glassware was acid washed. All reagents and media were prepared with water purified by the Milli-Q system (Millipore Corp., Bedford, Mass.). For growth promotion experiments, cultures were grown in M9 medium with 0.5% glucose. To some cultures transferrin (human, substantially iron free; Sigma Chemical Co., St. Louis, Mo.) was added to 100 μg/ml.

Detection of siderophore. The method to detect siderophore production was based on that described by Schwyn and Neilands (25). Chrome azurol S (CAS) plates were used to screen 84 strains of the organism for production of siderophores. Orange halos around the colonies are indicative of siderophore activity. CAS solution was also used for quantitation of siderophore production in culture supernatants (25). Cultures were centrifuged at 10,000 × *g* for 10 min, and 50 μl of supernatant was added to 0.95 ml of CAS solution. After reaching equilibrium, the A_{630} was measured.

The CAS siderophore assay was also used on aqueous and ethyl acetate extracts of culture supernatants. Culture supernatant (50 ml) was adjusted to pH 2.0 with HCl and extracted with ethyl acetate (2 volumes of ethyl acetate:5 volumes of supernatant). The pH of the aqueous fraction was adjusted to 7.2 with NaOH. The ethyl acetate fraction was rotary evaporated to dryness and then dissolved in 1.0 ml of methanol. Both fractions were assayed for siderophores.

Detection of catechol and hydroxamic acid. The assay used to detect catechols was that described by Arnow (1). Hy-

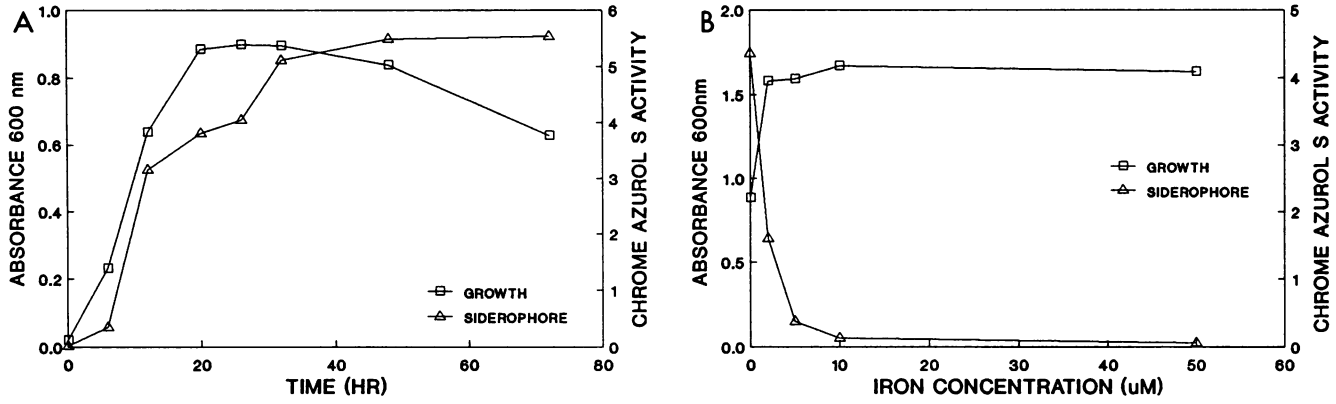

FIG. 1. (A) Effect of growth on siderophore production by *P. pseudomallei* U7. (B) Effect of iron concentration on siderophore production. Cultures were grown in CAA medium at 30°C. Growth was measured by reading the A_{600}. Siderophore yield is expressed as CAS activity/A_{600} unit.

droxamic acid was determined by the method described by Emery and Neilands (11) in which oxidation of hydroxamic acid yields a material with a very strong absorption at 264 nm.

Purification of siderophore. Culture supernatant (1 liter) from strain U7 was lyophilized; the residue was dissolved in 5 ml of deionized water and centrifuged to remove the undissolved material. A 2-ml sample of this material was applied to a Sephadex G-25 (Pharmacia) gel filtration column (1.5 by 70 cm) equilibrated with water-methanol (10:1, vol/vol) as a solvent. Fractions of 3 ml were collected and assayed for siderophores by CAS assay. The positive fractions were pooled, lyophilized, and resuspended in 1.0 ml of water. Approximately 5 mg of partially purified siderophore was obtained from 1 liter of culture supernatant. For growth enhancement experiments, this partially purified siderophore was filter sterilized.

The molecular weight of the siderophore produced by *P. pseudomallei* was estimated by gel filtration on a P-2 acrylamide column (1.5 by 80 cm; exclusion limit, 100 to 1,800; Bio-Rad) with 2,3-dihydroxybenzonic acid (M_r = 154), chloragenic acid (M_r = 354), ferrozine (M_r = 470), flavin adenine dinucleotide (M_r = 785), and streptomycin sulfate (M_r = 1457) as molecular weight standards. A mixture of 100 μg of siderophore and 100 μg of each compound in a 2-ml total volume was applied to the P-2 column. Fractions of 3 ml were collected (flow rate, 1 ml/min), and the A_{280} was determined. Fractions were also assayed for siderophore activity with CAS solution.

Iron binding activity of the siderophore. The iron binding activity of the siderophore was confirmed by the method of Hu et al. (14). Two columns were packed with Sephadex G-25 and equilibrated with deionized water. A mixture of 5 μl of $^{59}FeCl_3$ (100 μCi/ml; Amersham Corp.) and 30 μg of $FeCl_3$ was passed over one column. The other column was loaded with a mixture which contained a 5 μl of $^{59}FeCl_3$ (100 μCi/ml), 30 μg of $FeCl_3$, and 500 μg of partially purified siderophore. The columns were eluted with deionized water. Fractions of 1 ml were collected, and the amount of $^{59}FeCl_3$ was determined in an LKB Compugamma counter. Column fractions were also assayed for siderophore activity by the CAS assay.

Iron uptake assay. Cultures were grown in M9 medium extracted with 8-hydroxyquinoline and supplemented with 0.5% glucose (23) to a density of 10^8 CFU/ml. The cells were harvested, washed twice with medium, and resuspended in 10 ml of fresh medium to a final A_{600} of 0.2. Cell suspensions were preincubated in 250-ml Erlenmeyer flasks for 10 min at 37°C with shaking. Transport assay mixtures contained either 0.5 μCi of $^{59}FeCl_3$ and 10 μl of 0.1 M EDTA (Fisher Scientific Co., Fair Lawn, N.J.) or 5 μCi of $^{59}FeCl_3$, 75 μg of siderophore, and 10 μl of 0.1 M EDTA. The reaction mixtures were equilibrated for 15 min. The assay was initiated by the addition of the labeled Fe mixtures to the cell suspensions. Samples of 1 ml were removed at 5-min intervals, filtered through a 0.22-μm-pore-size Millipore filter, and washed with 5 ml of 10 mM Tris (pH 7.5)–0.9% NaCl. The amount of $^{59}FeCl_3$ accumulated on the filters were measured in an LKB Compugamma counter.

RESULTS

Detection of siderophore production. Eighty-four strains of *P. pseudomallei* isolated from patients with melioidosis were screened for siderophore production by the CAS assay of Schwyn and Neilands (25) both on CAS agar plates and in CAA medium. The CAS assay is a functional assay based on the high affinity of siderophores for iron and is independent of their structural classification. When a siderophore removes the iron from the Fe-CAS-hexadecyltrimethylammonium bromide (HDTMA) complex, its color turns from blue to orange. Orange halos around the colonies on CAS agar plates are indicative of siderophore excretion. Siderophores in culture supernatants can be detected by reacting supernatants with CAS solution and observing the color change from blue to orange. The activity can be quantitated by measuring A_{630}.

All 84 strains of *P. pseudomallei* were positive for siderophore production. The majority of strains showed a correlation between the CAS agar assay and production in the CAA medium, while some strains did not (data not shown). This might be due to the toxicity of HDTMA to certain strains, as they grew poorly on CAS agar. There were no significant differences among strains isolated from different sites of infection. The strains examined were isolated from blood, sputa, wounds, or urine. The reaction rate of CAS solution with the siderophores was also examined and found to reach equilibrium after 4 h.

The relationship between growth and siderophore production was examined in strain U7 (Fig. 1A). Siderophore

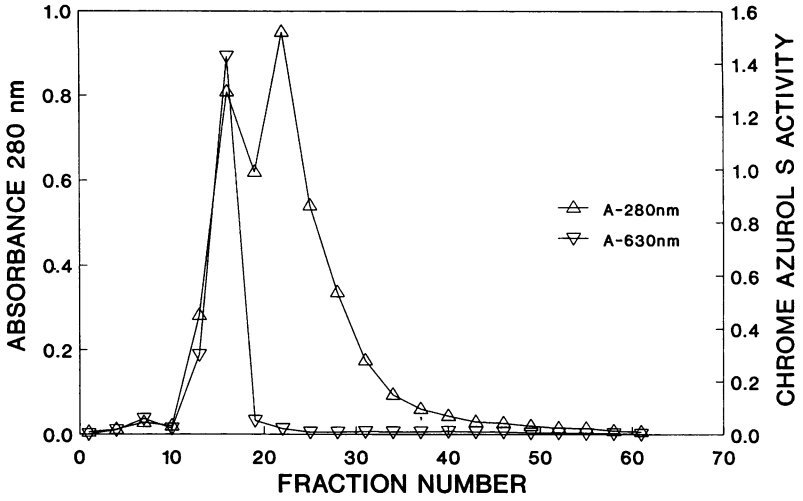

FIG. 2. Chromatographic profile on a Sephadex G-25 column of concentrated culture supernatant of strain U7. The column was eluted with H_2O-methanol (10:1) at a flow rate of 1 ml/min. Fractions (3 ml) were collected and assayed for siderophore activity by the CAS assay.

production was first evident in the late log phase. After the culture reached the stationary phase, the concentration of siderophore continued to increase slowly, and the activity remained stable throughout the assay period (72 h). The stability is suggestive of a hydroxamate structure, since catechol siderophores are more rapidly oxidized (21).

Effect of iron on siderophore production of *P. pseudomallei*. *P. pseudomallei* U7 was grown in CAA medium with increasing concentrations of iron at 30°C for 18 h with maximum aeration. The cell density increased when more than 2 μM iron was added, and there were only slight differences in cell growth with iron concentrations from 2 to 100 μM. Siderophore production was inversely proportional to the amount of iron added (Fig. 1B). This indicates that iron enhanced growth of *P. pseudomallei* U7 and repressed its siderophore production. Furthermore, higher yields of siderophore by *P. pseudomallei* U7 could be induced by growth in low-iron medium.

Siderophore purification and characterization. Culture supernatants from six strains isolated from different clinical sites were extracted with ethyl acetate, and the ethyl acetate and aqueous fractions were assayed for CAS activity. The aqueous fraction of the supernatants was positive in the CAS assay, and the ethyl acetate fractions were negative. This suggests that the siderophore is not of the catechol type, since the catechol siderophores are usually ethyl acetate extractable (21). Extracts from all six strains were also negative in the Arnow test (1), which detects phenolate compounds (data not shown). The method of Emery and Neilands (11) was used for the detection of hydroxamate compounds, and all six supernatants yielded an A_{264} when oxidized. There was a correlation between the level of hydroxamate activity and CAS activity. These findings suggest that the siderophore produced by *P. pseudomallei* is of the hydroxamate type, but we have not yet determined whether more than one type of hydroxamate is produced by these strains.

The siderophore was purified from culture supernatants of strain U7 for further characterization. The first peak from the Sephadex G-25 column was shown to contain CAS activity (Fig. 2). The molecular mass of the siderophore was estimated by gel filtration on P-2 to be 1,000 Da based on the average of two determinations.

Activity of the siderophore. Iron binding activity of the siderophore was demonstrated by its ability to mobilize iron within a gel filtration column (14). ^{59}Fe alone did not elute with water from the column, but a mixture of ^{59}Fe and siderophore yielded mobile ^{59}Fe from the column (Fig. 3). Almost all radioactivity was recovered from the column when both ^{59}Fe and siderophore were used. Siderophore could be detected by the CAS assay in the fractions with very high radioactivity. These results indicate that the siderophore does bind iron and that the activity remained stable after purification.

Iron uptake from ^{59}Fe-labeled ferric siderophore by iron-starved *P. pseudomallei* U7 was demonstrated (Fig. 4). When a mixture of partially purified siderophore and $^{59}FeCl_3$ was added to the medium, iron was taken up by strain U7 cells in the presence of EDTA. The cells continued to accumulate ^{59}Fe throughout the assay period. In contrast, no

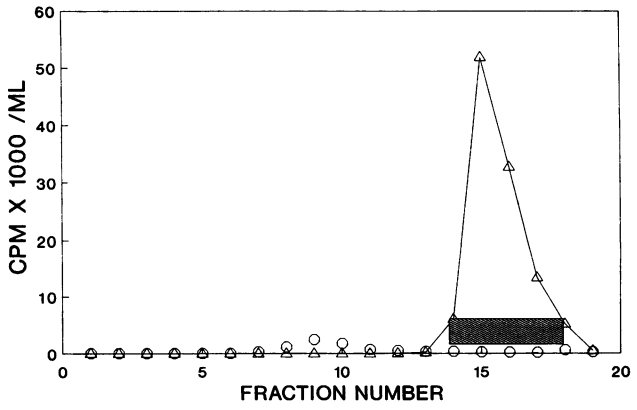

FIG. 3. Iron binding activity of *P. pseudomallei* siderophore determined on G-25 columns. The reaction mixtures were loaded on separate columns and eluted with water. Fractions of 1 ml were collected, and the amount of radioactivity was determined in an LKB Compugamma counter. Fractions were also assayed for siderophore activity by the CAS assay. Symbols: ○, $^{59}FeCl_3$; △, $^{59}FeCl_3$ plus siderophore; shaded area represents fractions positive by the CAS assay.

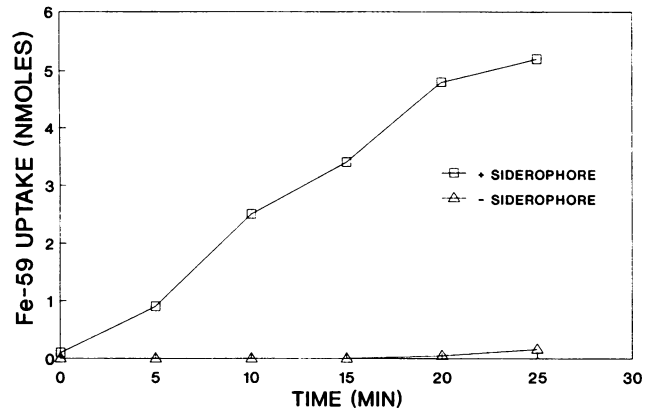

FIG. 4. Promotion of iron uptake by *P. pseudomallei* siderophore. Strain U7 was grown to a density of 10^8 CFU/ml. Reaction mixtures contained 0.5 μCi of $^{59}FeCl_3$, 75 μg of siderophore, and 10 μl of 0.1 M EDTA (□) or 0.5 μCi of $^{59}FeCl_3$ and 10 μl of 0.1 M EDTA (△). Samples of 1 ml were removed at the indicated intervals, and the amount of $^{59}FeCl_3$ accumulated was determined.

iron accumulation occurred when $^{59}FeCl_3$ alone was added to the cultures in the presence of EDTA.

Effect of *P. pseudomallei* siderophore on growth. Siderophores should also be able to stimulate growth in the presence of iron chelators such as transferrin or EDTA. To determine the effects of the siderophore on the growth of *P. pseudomallei* in the presence of transferrin, we grew strain 316a, which is a low siderophore producer, at 37°C in M9 medium, M9 medium with 100 μg of transferrin per ml, and M9 medium with transferrin plus siderophore. Samples were taken at selected time intervals, and the cell density was measured at 600 nm. The presence of transferrin in the medium resulted in a long lag phase. Addition of the siderophore reversed the growth inhibition by transferrin and increased the growth rate (Fig. 5). Similar results were also observed when EDTA was added to the growth medium (data not shown). When iron was added to the medium containing transferrin or EDTA, the inhibition of growth was reversed. These data suggest that this siderophore can remove iron from transferrin and EDTA.

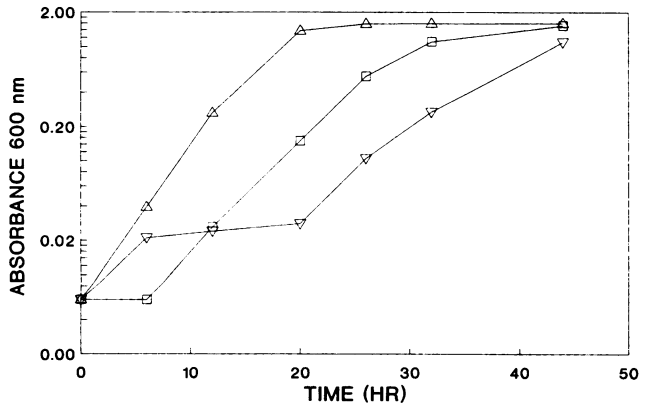

FIG. 5. Effect of siderophore on growth of *P. pseudomallei*. Strain 316a, a low siderophore producer, was grown in M9 medium supplemented with 100 μg of transferrin per ml (▽), no additives (□), or 100 μg of transferrin per ml and 10 μg of siderophore (△). Growth was measured by determining the A_{600} at selected intervals.

DISCUSSION

Iron is required for the growth of nearly all microorganisms (2, 20, 22). To acquire iron from a host, certain microorganisms have been found to produce specific high-affinity iron binding compounds, termed siderophores. In this study, we examined 84 strains of *P. pseudomallei* isolated from patients with melioidosis for production of siderophores. All strains examined produced siderophores detectable by CAS assay (25). Two methods were used to determine the chemical class of the siderophores. The results indicated that *P. pseudomallei* produced siderophores of the hydroxamate type. We found no indication that these strains produced any siderophores of the phenolate class. The siderophore of *P. pseudomallei* was water soluble without a yellow-green fluorescence, which is different from the siderophores found in fluorescent pseudomonads. The molecular weight of the siderophore was estimated to be approximately 1,000 by gel filtration chromatography. Siderophore production decreased markedly during growth in CAA medium with added iron, whereas addition of iron in medium increased growth. These results agree with previous studies that siderophore production is iron regulated (2, 20, 21, 30).

Siderophore produced by strain U7 of *P. pseudomallei* was partially purified by gel filtration chromatography. It enhanced the growth of strain 316a in the presence of transferrin, suggesting that it could remove iron from transferrin. This compound was shown to bind iron and promote iron uptake by *P. pseudomallei*. Thus, it has all the pertinent characteristics of a siderophore. This compound appears to be a previously undescribed siderophore. We suggest that this siderophore be given the trivial name of malleobactin. Studies are currently in progress to further characterize the structure of malleobactin.

Dannenberg and Scott (9) reported that the ability of *P. pseudomallei* to accumulate in host tissue might be related to its virulence. Since malleobactin enhances the growth of *P. pseudomallei*, it could play an important role in the pathogenesis of the infection by allowing the organism to multiply in host tissue or survive in the bloodstream. However, it remains to be determined whether malleobactin is produced in vivo and whether it contributes to the virulence of *P. pseudomallei*.

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