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Hemin Utilization Is Related to Virulence of *Streptococcus pneumoniae*

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***Streptococcus pneumoniae* is a causative agent for bacterial pneumonia, otitis media, meningitis, and bacteremia. Mechanisms for acquisition of iron by this organism under low-iron conditions were investigated. Siderophore production was not detected by either chemical or biological methods. Its utilization of iron-containing compounds found in human hosts was tested. Both hemin and hemoglobin supported the full growth of *S. pneumoniae* in a culture lacking other iron sources, while lactoferrin and transferrin failed to do so. A mutant defective in hemin utilization was isolated and was less virulent than wild-type *S. pneumoniae* in experimental animals.**

Streptococcus pneumoniae (pneumococcus) is a common inhabitant of the human upper respiratory tract and a major pathogen for bacterial pneumonia, otitis media, bacteremia, and meningitis. It causes considerable morbidity and mortality throughout the world, especially among children, the elderly, and immunocompromised individuals (18). The mechanisms for pneumococcal disease are not fully understood. Production of a capsule, pneumolysin, neuraminidase, and immunoglobulin A protease contributes to the virulence of *S. pneumoniae* (for a recent review, see reference 13). The capsule is a layer of polysaccharides surrounding the bacterium and provides protection for pneumococci against phagocytosis by macrophages and polymorphonuclear leukocytes (40). Pneumolysin exhibits a wide range of activities in vitro. In addition to hemolytic activity, it inhibits the migration and bactericidal activities of phagocytic cells; represses the lymphokine and antibody production ability of lymphocytes; activates the classical complement pathway, resulting in depletion of serum opsonic activity; and causes cell vacuolation and disruption of the respiratory epithelium structure that lead to cell death (12, 27, 29, 37). Immunoglobulin A protease degrades human immunoglobulin A1, and neuraminidase cleaves the glycoproteins and glycolipids of mammalian cell membranes (21, 36). *S. pneumoniae* may use these virulence factors to damage host tissues and evade the host defense system. However, it is not clear how this organism survives in infected humans, where growth conditions are far from optimal, especially in the supply of iron.

Iron is an essential nutrient for the growth of all organisms and has very low solubility under physiological conditions. In humans, iron molecules are associated with high-affinity iron-binding proteins, such as transferrin, lactoferrin, hemoglobin, and myoglobin. The ability to acquire iron under low-iron conditions is related to the virulence of a variety of bacterial pathogens (30). Our knowledge about iron transport is based on studies of gram-negative bacteria. In response to iron limitation, bacterial cells produce outer membrane proteins that are capable of scavenging iron from iron-containing compounds either by direct contact or through mediation with siderophores. Siderophores are low-molecular-weight and high-affinity iron chelators. The mechanism for the siderophore-mediated iron transport system has been extensively studied in *Escherichia coli* (for recent reviews, see references 3 and 6). Under iron-limiting conditions, *E. coli* induces the synthesis and secretion of siderophores, such as aerobactin or enterochelin. After association with ferric iron, ferric siderophores are transported inside the cell by the orchestrated activities of specific outer membrane, periplasmic, and cytoplasmic membrane proteins. In contrast, non-siderophore-producing bacteria, such as *Neisseria gonorrhoeae* and *Haemophilus influenzae*, acquire iron by directly binding with transferrin, lactoferrin, or hemoglobin molecules (7, 14, 17, 25). Information about the iron transport of gram-positive bacteria is limited. Siderophores have been detected in the culture supernatant of *Bacillus subtilis* (19), *Streptomyces* sp. (20), *Mycobacterium smegmatis* (23, 35), and *Corynebacterium diphtheriae* (32) but not in that of *Listeria monocytogenes* (1) and *Streptococcus mutans* (11). The molecular basis for the iron uptake system of *B. subtilis* has been described recently (33). The biochemistry and genetics of the non-siderophore-mediate iron uptake system in gram-positive bacteria remain unclear. In this study, we investigated the iron transport system of *S. pneumoniae* and its relationship with pneumococcal infection.

The *S. pneumoniae* strains used in this study were virulent serotype 2 (Pn-2) and nonencapsulated mutant Rx-1. These organisms were stored at −70°C in 10% glycerol and routinely grown in either Todd-Hewitt Broth (THB) or THB supplemented with 0.5% yeast extract (THB-Y) at 37°C in an atmosphere of 5% CO_2. To restrict the growth of *S. pneumoniae* by iron limitation, low-iron THB or THB-Y medium was prepared either by treatment with 2% Chelex-100 (Bio-Rad, Hercules, Calif.) for 4 h before autoclaving or by addition of ethylenediamine di-*o*-hydroxyphenylacetic acid (EDDA; Sigma, St. Louis, Mo.). Chelex 100-treated medium was supplemented with 100 μM calcium chloride and 1 mM magnesium sulfate before use. The concentration of EDDA used in low-iron THB medium was established in a preliminary study. The growth of Pn-2 was slow in Chelex-treated

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medium and was totally inhibited in medium containing EDDA at a concentration higher than 700 μM.

Studies with *E. coli* and other gram-negative bacteria indicate that siderophore synthesis is negatively regulated by iron (15, 16). To characterize the iron transport of *S. pneumoniae*, we employed bioassays and chemical tests to determine whether Pn-2 cells are capable of producing extracellular iron-chelating compounds under low-iron conditions. The culture supernatant of Pn-2 grown in Chelex 100-treated THB-Y medium was used in this study. A siderophore bioassay was performed on a THB-Y agar plate containing EDDA and seeded with approximately 10^5 CFU of Pn-2 cells. Various amounts of culture supernatant were spotted into the precut wells on the plates. The growth of Pn-2 cells was examined after incubation for 48 h. No zone of growth surrounding the well was observed. The Arnow and Csaky reactions were used as chemical methods to test the presence of phenolate- and hydroxamate-type siderophores, respectively (2, 8). The Pn-2 culture supernatant was negative for both reactions. In a control group, the culture supernatant of *E. coli* DH5α grown in Chelex 100-treated THB-Y medium was positive for phenolate. The ability of *S. pneumoniae* to produce iron-chelating compounds was also assessed by the universal siderophore detection assay (34). However, the growth requirements of *S. pneumoniae* are complex; ingredients in THB medium interfere with the chrome azurol S reaction. To circumvent this difficulty, Pn-2 cells were cultured in Chelex 100-treated modified PGT medium, which has been successfully used to detect siderophore production by *C. diphtheriae* (39). PGT medium was composed of Casamino Acids, pantothenic acid, glutamic acid, tryptophan, maltose, and inorganic salts (4). Modified PGT medium was prepared by reducing the concentration of Casamino Acids from 3 to 0.5%. The growth of Pn-2 in modified PGT medium was comparable to that in THB medium. The culture supernatant of late-log-phase Pn-2 cells in deferrated, modified PGT medium was collected after centrifugation. It was negative by the universal siderophore detection assay. In contrast, under similar growth conditions, the culture supernatant of either *E. coli* DH5α or *C. diphtheriae* was positive by this assay. These results suggest that *S. pneumoniae* does not produce extracellular siderophores or iron-chelating growth factors under low-iron conditions.

Since both chemical and biological assays failed to reveal the presence of siderophores in the culture supernatant of *S. pneumoniae*, iron uptake by this organism may rely on other mechanisms, such as direct contact of cells with iron-containing proteins. To test this possibility, we employed a bioassay to determine whether iron-containing proteins can support the growth of *S. pneumoniae* under low-iron conditions. Results are shown in Fig. 1. The growth of Pn-2 cells was inhibited by EDDA. Iron-saturated human transferrin and lactoferrin (Sigma) had little stimulating effect on cell growth. In contrast, the growth of *S. pneumoniae* in EDDA-containing THB medium was fully restored by addition of either hemin or hemoglobin (Sigma). The inhibitory effect of EDDA was also reversed by addition of ferric sulfate at a concentration of 700 μM (data not shown). Pn-2 cells grown in deferrated THB medium supplemented with hemin had approximately the same doubling time (1 h) as cells grown in untreated medium. However, excess hemin inhibited cell growth; maximal growth of Pn-2 was supported by 8 μM hemin. A large number of bacteria, such as *Bacteroides fragilis, E. coli, H. influenzae, N. gonorrhoeae, Plesiomonas shigelloides, Porphyromonas gingivalis, Vibrio cholerae, Yersinia pestis*, and *Y. enterocolitica*, have the ability to use heme-containing compounds as iron sources for growth under low-iron conditions (7, 9, 22, 24–26, 31, 38). *S. pneumoniae* is the first gram-positive bacterium demonstrated to have this activity.

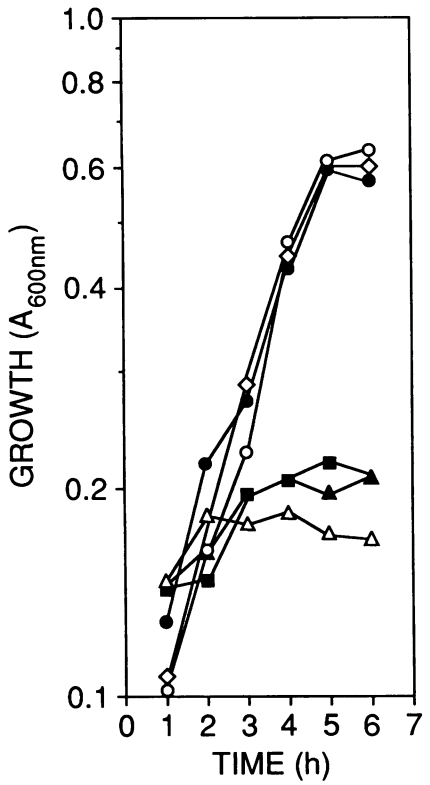

FIG. 1. Growth of wild-type *S. pneumoniae* Pn-2 in THB. Symbols: ○, no addition; △, addition of EDDA (700 μM); ●, addition of EDDA plus hemin (8 μM); ◇, addition of EDDA plus hemoglobin (2 μM); ▲, addition of EDDA plus transferrin (60 μg); ■, addition of EDDA plus lactoferrin (60 μg). Cell growth was monitored by measuring A_{600}.

The ingredients of growth medium for *S. pneumoniae*, THB, are complex and are anticipated to contain free ferric iron and small amounts of heme-containing compounds. The iron chelator EDDA is unable to remove covalently bound iron from hemin. The growth of Pn-2 cells in untreated THB, but not in the same medium containing EDDA, indicates that the concentration of hemin in the growth medium is too low to support cell growth, and *S. pneumoniae* may possess mechanisms to transport ferric iron in untreated THB. Studies of other gram-positive non-siderophore-producing bacteria, namely, *S. mutans* and *L. monocytogenes*, have suggested a reductive iron assimilation system for iron acquisition (1, 11). In this model, ferric iron is reduced to the ferrous form by either a membrane-associated ferric reductase or secreted reducing agents before being transported. We did not investigate whether *S. pneumoniae* has such an iron uptake system.

S. pneumoniae is one of the bacteria most frequently isolated from bacteremic patients. Its ability to utilize hemin may play a role in the survival of pneumococci in infected humans. To evaluate the significance of hemin utilization in the pathogenesis of pneumococcal infection, we isolated mutants defective in hemin utilization by chemical mutagen-

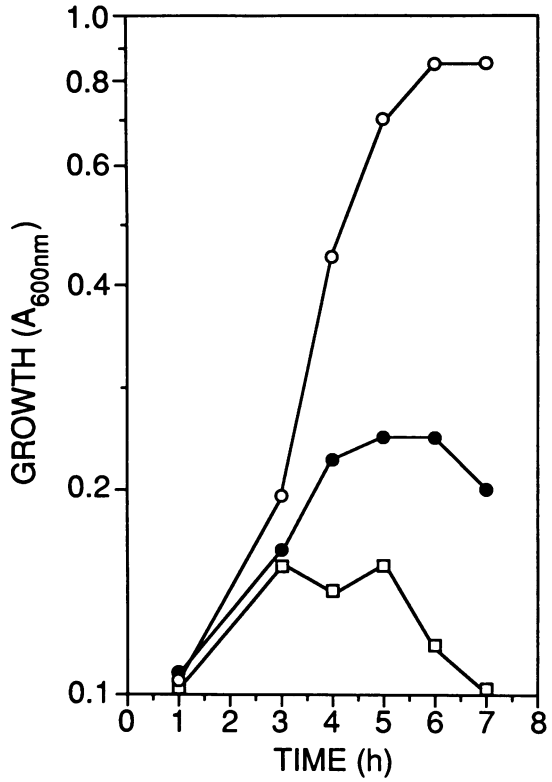

FIG. 2. Growth of the hemin utilization mutant *S. pneumoniae* ST330 in THB-Y. Symbols: ○, no addition; ●, addition of EDDA; □, addition of EDDA plus hemin (8 μM). Growth of cultures were monitored by measuring A_{600}.

esis. Briefly, 100 ml of log-phase Pn-2 cells (A_{600} = 0.5) grown in THB-Y medium were harvested by centrifugation at 12,000 × *g* for 15 min at 4°C, washed twice with 10 ml of 50 mM Tris–50 mM maleate buffer (pH 6.0), and suspended in 0.5 ml of the same buffer. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (Sigma) was added to the cell suspension to a final concentration of 670 μg/ml. After incubation at 37°C for 15 min without shaking, cells were washed twice with 10 ml of Tris-maleate buffer and suspended in 10 ml of THB-Y medium. Less than 1% of the Pn-2 cells survived after *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine treatment. To facilitate screening of iron uptake mutants, we took advantage of the bactericidal effect of streptonigrin, an antibiotic that is toxic to cells having active iron uptake activity (41). This method has been used in the isolation of iron uptake mutants of *E. coli* (43), *Serratia marcescens* (42), and *Neisseria meningitidis* (10). An aliquot (0.5 ml) of mutagenized Pn-2 cells was transferred to 5 ml of THB-Y medium and incubated at 37°C for 4 h. Streptonigrin was added to the culture to a final concentration of 5 μg/ml, the MIC for wild-type Pn-2 cells determined in preliminary studies. After incubation for another 5 h, cells were mixed with top agar containing 5 μg of streptonigrin per ml, poured onto THB-Y plates, and incubated at 37°C overnight. Streptonigrin-resistant colonies were collected and tested for hemin utilization in THB-Y medium containing EDDA. After screening 20 streptonigrin-resistant colonies, we identified one mutant, ST330, which was persistently resistant to streptonigrin and failed to grow as vigorously as wild-type *S. pneumoniae* cells when cultured in THB-Y medium containing EDDA and hemin (Fig. 2). Mutant ST330 was hemolytic and encapsulated, comparable to wild-type cells. Hemolytic activity was measured with 2% washed goat erythrocytes (27). The presence of a capsule in *S. pneumoniae* was examined microscopically after staining with India ink.

The virulence of the putative hemin utilization mutant of *S. pneumoniae* for BALB/c mice was evaluated. Cells of wild-type Pn-2 and mutant ST330 were grown to the mid-log phase, harvested, washed twice, and suspended in phosphate-buffered saline to the volume of the original cell culture. Aliquots (100 μl) of serial dilutions of cells were intraperitoneally injected into mice. A group of 10 mice was used for every dilution. The survival time of mice was recorded, and the results are shown in Fig. 3. At the maximum dose tested (10^5 CFU), all of the mice challenged with wild-type cells died within 24 h. Mice challenged with mutant ST330 died after 4 days. As the dose was reduced, the survival time increased. At the lowest dose tested (10^3 CFU), the average survival time for mice challenged with Pn-2 cells was 1.4 days while that for mice challenged with mutant ST330 was longer than 7 days. These results suggest that the putative hemin utilization mutant was less virulent than wild-type cells and the ability to compete for heme with host heme-carrying proteins in vivo may be an important determinant for the pathogenesis of *S. pneumoniae* in infected animals.

Hemin utilization by *S. pneumoniae* may be related to its hemolytic activity. The role of pneumolysin in pneumococcal infection has been studied in vivo. Mice injected with a sublethal dose of pneumolysin are protected from pneumococcal challenges (28). The 50% lethal dose of a defined pneumolysin-negative mutant for mice is much higher than

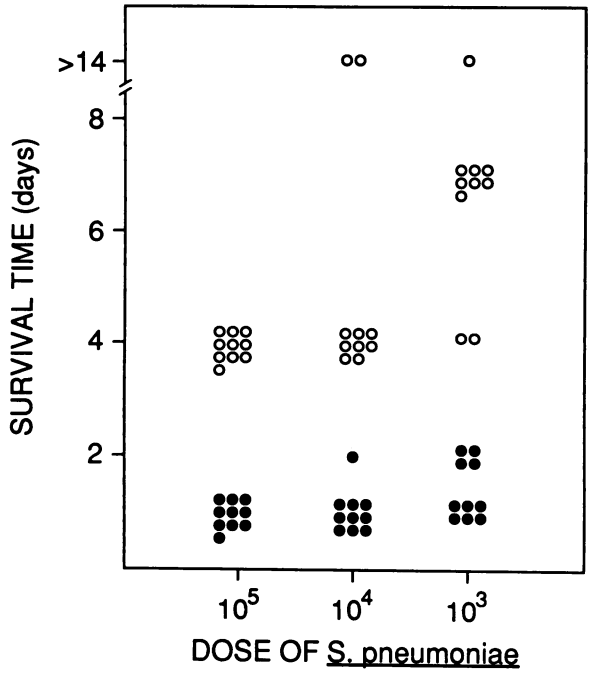

FIG. 3. Virulence comparison of the wild type and the putative hemin utilization mutant of *S. pneumoniae*. Groups of mice were challenged through intraperitoneal injection with various doses of Pn-2 (●) and mutant ST330 (○). Survival time was recorded after injection. The data were analyzed by the Fisher exact test ($P < 0.0001$).

that of isogenic parental cells (5). The rate of clearance of the nonhemolytic mutant from the blood is faster than that of hemolytic cells. When this information is combined with our findings, it appears possible that the low virulence of a nonhemolytic mutant may be due partly to the limited supply of hemin for the growth of *S. pneumoniae* in mammalian hosts. Pneumolysin production and hemin utilization may work in concert to establish the infection by *S. pneumoniae*. Lysis of erythrocytes or other host cells by pneumolysin results in release of heme-containing proteins and creates an environment more favorable for the growth and multiplication of *S. pneumoniae*.

In conclusion, we have shown that *S. pneumoniae* does not produce siderophores under low-iron conditions and can use either hemin or hemoglobin as a sole source for required iron. A mutant defective in hemin utilization was less virulent than wild-type cells. Characterization of the hemin utilization system may help us understand how *S. pneumoniae* survives in infected mammalian hosts and define the role of iron uptake in pneumococcal infections.

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