Albomycin Uptake via a Ferric Hydroxamate Transport System of *Streptococcus pneumoniae* R6

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The antibiotic albomycin is highly effective against *Streptococcus pneumoniae*, with an MIC of 10 ng/ml. The reason for the high efficacy was studied by measuring the uptake of albomycin into *S. pneumoniae*. Albomycin was transported via the system that transports the ferric hydroxamates ferrichrome and ferrioxamine B. These two ferric hydroxamates antagonized the growth inhibition by albomycin and salmycin. Cross-inhibition of the structurally different ferric hydroxamates to both antibiotics can be explained by the similar iron coordination centers of the four compounds. [55Fe3+]ferrichrome and [55Fe3+]ferrioxamine B were taken up by the same transport system into *S. pneumoniae*. Mutants in the adjacent *fhuD, fhuB*, and *fhuG* genes were transport inactive and resistant to the antibiotics. Albomycin, ferrichrome, ferrioxamine B, and salmycin bound to the isolated FhuD protein and prevented degradation by proteinase K. The *fhu* locus consisting of the *fhuD, fhuB, fhuG*, and *fhuC* genes determines a predicted ABC transporter composed of the FhuD binding lipoprotein, the FhuB and FhuG transport proteins, and the FhuC ATPase. It is concluded that active transport of albomycin mediates the high antibiotic efficacy in *S. pneumoniae*.

The human pathogen *Streptococcus pneumoniae* causes pneumonia, meningitis, bacteremia, and otitis media. Although the bacterium is sensitive to many antibiotics and a vaccine is available, it is still a major cause of death in developed countries and even more so in developing countries. Therefore, it is important to test the efficacy of antibiotics on *S. pneumoniae* and their mechanisms of action and entry into cells in order to design new antibiotics against this pathogen.

Albomycin is produced by streptomycetes as a mixture of structurally closely related compounds and is highly effective against many gram-negative and some gram-positive bacteria. The MIC of albomycin (5 ng/ml) against *Escherichia coli* is much lower than that of ampicillin (100 ng/ml). Albomycin was once used in the Soviet Union to treat human bacterial infections (18). Although the antibiotic was identified in 1951, the correct chemical structure was not determined until 1982 (2). Albomycin belongs to the group of sideromycins that consist of antibiotic moieties linked to iron carriers termed siderophores (4, 28, 40). In albomycin the antibiologically active thioribosyl pyrimidine derivative is bound to a trihydroxamate iron carrier formed by three linked N5-acetyl-N5-hydroxy-ornithine residues. Two serine residues form the bridge between the antibiotic and the iron carrier (Fig. 1).

In a RNA synthetase inhibition assay used to isolate new antibiotics, the seryl-thioribosyl pyrimidine moiety of albomycin, designated SB-217452, was isolated from the culture supernatant of *Streptomyces* sp. strain ATCC 700974 and shown to inhibit in vitro seryl-tRNA synthetases (37). SB-217452 is highly active against isolated RNA synthetases (50% inhibitory concentration value of 8 nM) but poorly active against bacteria, e.g., MIC of 256 µg/ml (0.4 mM) for *Staphylococcus aureus*, presumably because of its low permeation into the bacterial cells (37). In contrast, complete albomycin is actively transported in energy coupled steps across the outer and the cytoplasmic membranes of *E. coli* and other gram-negative bacteria, provided they contain the cognate transport system (22). This transport system takes up ferrichrome (Fig. 1), which provides cells with iron. Deferri-ferrichrome is synthesized by fungi, secreted, complexes Fe3+ in the medium with an extremely high specificity and affinity, and is then transported into fungi and bacteria by ferrichrome-specific transport systems. Inside cells, Fe3+ is reduced to Fe2+, which has a much lower affinity to deferri-ferrichrome and is then preferentially incorporated into redox-enzymes, iron sulfur proteins, and cytochromes of the intermediary metabolism and respiratory chains. Albomycin binds Fe3+, and its iron complex is identical to that of ferrichrome (Fig. 1).

Ferrichrome and albomycin transport has been studied in most detail with *Escherichia coli* K-12. Albomycin-resistant mutants were used to characterize the albomycin and ferrichrome transport systems. Both transport systems were shown to be identical and encoded by four genes: *fluA*, which encodes an outer membrane transport protein; *fluB*, which encodes a cytoplasmic membrane transport protein; *fluC*, which encodes an ATPase at the inner side of the cytoplasmic membrane; and *fluD*, which encodes a periplasmic binding protein. Proteins encoded by three additional genes—*tonB, exbB*, and *exbD*—are involved in energization of transport across the outer membrane; Transport across the cytoplasmic membrane is energized by the ATP hydrolysis of an ABC transporter (5, 8).

The crystal structures of ferrichrome and albomycin bound to FhuA (15, 16, 31) and FhuD (13, 14) reveal identical binding sites for the iron complexes. Once inside the cells, the antibiotic must be released from the iron carrier to be active (22) by cleavage through peptidase N (7). Most of the antibiotic part remains inside the cells, whereas the iron carrier is secreted. In
peptidase N mutant cells albomycin is not cleaved and instead serves as an iron carrier.

Most antibiotics enter cells by diffusion. The results obtained with E. coli and albomycin demonstrate that coupling of antibiotics with low permeation rates to actively transported molecules strongly increases the efficacy of the antibiotics. Cephalosporins coupled to catecholate iron carriers increased their efficiency more than 100-fold compared to the unsubstituted cephalosporins (as summarized in reference 4). Such compounds act as Trojan horses through which the antibiotic is smuggled into cells by a substrate transport system.

In a study aimed at testing the use of albomycin as an antibiotic against the highly sensitive S. pneumoniae (MIC, 10 ng per ml), we determined the uptake of albomycin. The use of albomycin and salmycin as reagents enabled us to characterize the genetic determinants of the ferric hydroxamate transport system and its specificity, which otherwise would have been difficult to achieve due to the weak growth promotion of S. pneumoniae to the ferric hydroxamates at iron-limiting conditions and poor iron transport rates. We show that ferric hydroxamates are transported via a single system that is determined by four genes encoding a putative ABC transporter.

### MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** The strains and plasmids used in the present study are listed in Table 1. Avirulent, highly transformable, unencapsulated Streptococcus pneumoniae R6 strain was used as a reference strain and as a parental strain for the construction of the mutants. S. pneumoniae was routinely grown in THY broth (Todd-Hewitt broth supplemented with 5% yeast extract; Roth, Karlsruhe, Germany) in screw-cap culture tubes with minimum headspace and without shaking or on THY agar (1.5%) supplemented with 4% defibrinated sheep blood at 37°C in an incubator under 5% CO2 and 95% relative humidity. Unless otherwise stated, E. coli was grown in TY medium (0.8% tryptone, 0.5% yeast extract [Difco Laboratories], 0.5% NaCl) at 37°C with shaking at 200 rpm. Solid medium consisted of 1.5% agar in the TY medium. If required, antibiotics were included in the media as follows: for E. coli

### TABLE 1. Strains and plasmids used in this study

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<th>Strain or plasmid</th>
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*a Parentheses indicate polar effects on the transcription of genes downstream of the mutated fhu genes. Erm', Kan', and Amp' indicate erythromycin, kanamycin, and ampicillin resistance, respectively.

*b In this mutant, fhuB, fhuG, and fhuC are transcribed under the control of the putative fhu promoter upstream of fhuD.
ampicillin at 100 μg/ml, erythromycin at 500 μg/ml, and kanamycin at 30 μg/ml and for S. pneumoniae erythromycin at 1 μg/ml. For the transformation of S. pneumoniae R6, competent cells and derivatives were prepared as described previously (23) using the synthetic 17-residue competence-stimulating peptide CSP1 (500 ng/ml). S. pneumoniae growth curves were determined by growing cells in 4 ml of liquid medium in push-capped disposable cuvettes (Fremstad, Nuembrecht, Germany), which were incubated at 37°C without shaking, and the optical density at 578 nm (OD578) was measured.

Albomycin was purified as described previously (17), salmycin was obtained from L. Vertesy, former Hoechst AG, Frankfurt, Germany. Ferrichrome, ferri-

DNA manipulations and sequence analysis. Standard methods (34) were used for the isolation of chromosomal DNA and plasmid DNA except for the isolation of pneumococcal DNA, where lysis was induced by the addition of 0.1% sodium deoxycholate instead of lysosome prior to the addition of proteinase K. PCR reactions were performed with High-Fidelity Phusion Polymerase (Finzyme, Espoo, Finland). Restriction enzymes were from Roche Biochemicals (Mannheim, Germany). Single and double digestions were performed according to the manufacturer’s guidelines. Ez-Tn5 transposase, T4 DNA polymerase, T4 DNA kinase, and T4 DNA ligase were purchased from Epicenter Biotechnologies (Madison, WI). Gel extraction of DNA and PCR product purification was regularly done by using a QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany).

Sequence analysis software. For complete genome sequence analyses of S. pneumoniae R6 and TIGR4, the NCBI database (http://ncbi.nlm.nih.gov) was used. Nucleotide and protein sequences were analyzed by BLAST (http://ncbi.nlm.nih.gov) and CLUSTAL W (http://align.genome.jp). Restriction analysis was done by using Webcutter 2.0 (http://rna.lundberg.gu.se/cutter2/), and lipoprotein signal sequences were identified by using SignalP (http://www.cbs.dtu.dk/services/SignalP/).

Construction of insertion duplication mutants. In S. pneumoniae insertion duplication mutants of targeted genes by single crossover were generated with plasmid pD90 (11) according to the standard method. An internal fragment of fluA was PCR amplified from the S. pneumoniae R6 genomic DNA with the primers pair AGCTCGAGAGGATCCGGAATTC and TCTAGAGGGATCC. The PCR product was gel purified and ligated into pET28a digested with NdeI and BamHI. The resulting plasmid was transformed into E. coli and selected on ampicillin.

Isolation of His6-FhuD. Complete fluA deletion mutant. A 4.5-kb region covering the fluA sequence was PCR amplified from genomic DNA with the primers TCTAGTGCTTGGTGG and TCTACACATTGGCCTATTACCA. In silica restriction analysis of the fluA region sequence of S. pneumoniae R6 revealed two native SpII restriction sites. The fragment was digested with SpIHI, which cleaves 105 bp downstream of the start codon and 16 bp upstream of the stop codon of fluA to yield three fragments of 0.9, 1.2, and 2.4 kb. The 1.2- and 2.4-kb fragment was gel purified and ligated, resulting in a 0.94-bp deletion in fluA. This ligation mixture served as the template for nested PCR with the primers AGCTTGGCAGAGGTTTACAC and GACCACGGGCTAAGCAG to amplify a 3-kb region of the fluA region with the fluA deletion. The PCR product was gel purified and transformed into S. pneumoniae R6. Transformants were selected with strepta-

Albomycin and salmycin sensitivity assays. Aliquots (50 μl) of an S. pneu-

Acclimation of the fhuD mutant by insertion of fluA in the chromosomal mal region. APD1 Sβha was complemented by inserting fluA into the chromosomal malIP region. The C-terminal fragment of malM was PCR amplified with the primer pair CTGGGCCTTGGTGG and TATAAC ATATGTAGTGTGCTCCTG and fluA with the primer pair GCTTGGCAGAGGTTTACAC and GACCACGGGCTAAGCAG to amplify a 3-kb region of the fluA region with the fluA deletion. The PCR product was gel purified and transformed into S. pneumoniae R6. Transformants were selected with strepto-

Isolation of His6-FhuD. Complete fluA deletion mutant. A 4.5-kb region covering the fluA sequence was PCR amplified from genomic DNA with the primers TCTAGTGCTTGGTGG and TCTACACATTGGCCTATTACCA. The PCR product was digested with NdeI and BamHI and ligated into pET28a digested with NdeI and BamHI. The addition of T4 DNA ligase (fast) and additional ATP (200 μM) to close the nick. The reaction was stopped by heating at 70°C for 15 min. Aliquots of the reaction mixture were used to transform competent S. pneumoniae R6. Erythromycin-re-
resulting plasmid pABP BP encoded FhuD fused at the N-terminal end to a 20-residue peptide which contains a sequence of six histidine residues for purification on a nickel-nitritotriacetic acid (Ni-NTA) agarose column. The fhuD derivative was initially cloned in E. coli DH5λ and then transformed into E. coli BL21(DE3). E. coli BL21(DE3)pABP was grown with vigorous shaking at 37°C in TY medium supplemented with kanamycin (30 μg/ml) until an OD600 of 0.6 was reached. Overexpression of fhuD was induced by 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). After further cultivation for 4 h, cells were harvested by centrifugation and frozen at −70°C. They were thawed for 30 min on ice and then lysed with 10 μg of hen egg white lysozyme/ml in 10 ml of 50 mM Tris-HCl/100 mM NaCl/10% glycerol/1 mM phenylmethanesulfonyl fluoride (pH 8).

After 30 min incubation on ice, the suspension was sonicated to reduce the viscosity, after which the cell debris was removed by centrifugation for 30 min at 14,000 × g at 4°C Ni-NTA-agarose (1 ml; QIAGEN, Hilden, Germany) was added to the supernatant, and the mixture was weakly shaken for 1 h at 4°C and then poured into a column. The column was washed three times with 10 ml of the buffer described above supplemented with 500 mM NaCl and 50 mM imidazole. Hins-FhuD was eluted with 1 ml aliquots of 100 mM Tris-HCl/500 mM NaCl and a gradient of 250 to 500 mM imidazole. Samples were taken at each step and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Proteolytic digestion of liganded and unliganded FhuD. Equal amounts (5 μg) of purified recombinant Hins-FhuD in 50 mM Tris-HCl/250 mM NaCl (pH 7.8) was incubated with 1 μg test substrate in a 100-μl volume at room temperature for 15 min. To each tube, 2 μg of proteinase K was added, and incubation continued for another 30 min at 37°C. Reaction was stopped by adding phenylmethylsulfonyl fluoride (1 mM final concentration). Trichloroacetic acid was added (10% final concentration), and the precipitate was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

RESULTS

Characterization of the ferrichrome and albomycin transport system of S. pneumoniae. In a study of the activity of albomycin against the most common bacterial pathogens, a broad spectrum of gram-negative bacteria but a rather narrow spectrum of gram-positive bacteria were sensitive (A. Pramanik et al., unpublished data). Among the sensitive gram-positive strains, the unencapsulated S. pneumoniae R6 was particularly sensitive. It was inhibited by 10 ng of albomycin/ml in contrast to S. aureus, which required 100 ng of albomycin/ml for complete growth inhibition. To explain the very high sensitivity of S. pneumoniae R6, we set out to identify the genes that conferred albomycin sensitivity to S. pneumoniae R6.

If albomycin is taken up by the same transport system as ferrichrome, as in E. coli, ferrichrome should compete with albomycin uptake. This test was performed by placing paper strips containing albomycin on a plate seeded with S. pneumoniae R6 and placing paper strips containing ferrichrome at right angles to the albomycin strips. As shown in Fig. 2A, ferrichrome antagonized the antibiotic action of albomycin. Heme, on the other hand, had no effect. However, another ferric hydroxamate, ferroxamine B, also inhibited the action of albomycin (Fig. 2A). The structure of ferroxamine differs from that of ferrichrome (Fig. 1). Ferroxamine B is a linear molecule consisting of three 1-amino-5-hydroxylaminopentane units linked by two succinic acid residues and an acetyl group at the free N-hydroxyl group. Despite the different structures of ferroxamine B, ferrichrome, and albomycin, the coordination of Fe3+ is very similar in all three compounds. This part of the structures may form the major recognition site for the S. pneumoniae transport proteins, as was determined in the crystal structures of the E. coli FhuA and FhuD proteins loaded with albomycin and ferrichrome (14–16, 31). Another antibiotic, salmycin, was also tested. Salmycin consists of the ferroxamine B analogue danoxamine linked to an antibiologically active amino disaccharide (Fig. 1) (39). Results similar to those seen with albomycin were obtained (Fig. 2B).

To relate the high albomycin sensitivity of S. pneumoniae R6 to uptake by active transport, the ferric hydroxamate carrier was released from the thioribosyl pyrimidine by treatment with proteinase K and pronase E, which cleave the seryl linkage between the iron carrier and the antibiotic moiety (22). The activity of the proteinase-treated samples against S. pneumoniae R6 was strongly reduced to one-third of the inhibition zone size of the untreated sample. Reduction of the activity was even stronger when iron-free albomycin was treated with the proteinases, in which case nearly no inhibition zones were observed (data not shown).

fhuD is necessary for albomycin and salmycin sensitivity.

The genome of S. pneumoniae R6 (24) predicts open reading frames for three putative iron transport systems: spr0224–0220, spr0934–0938, and spr1684–1687 (Fig. 3A). The percentages of identity between the related genes were as follows: compared to spr0934, 10% for spr0224/0223 and 24% for spr1687; compared to spr0935, 12% for spr0221 and 28% for spr1684; compared to spr0936, 21% for spr0220 and 21% for spr1685; and compared to spr0938, 17% for spr0222 and 32% for spr1686. Since the predicted binding protein in the first cassette, spr0224–0220, contains a stop codon resulting in the two open reading frames spr233 and spr224 of 65 and 123 amino acid residues, respectively, this DNA region was not studied further. In the following, spr0934 will be designated fhuD, spr0935 fhuB, spr0936 fhuG, and spr0938 fhuC (Fig. 3B) according to the nomenclature introduced by Clancy et al. (12), who studied siderophore-dependent iron acquisition of a group B streptococcus. We refrained from using the pia gene nomenclature introduced by Brown et al. (9) because the fhu designation specifically refers to ferric hydroxamate uptake, which we introduced for E. coli (26) and has been used since then for gram-negative and gram-positive bacteria (5, 8, 30, 31). spr0937 is an open reading frame on the cDNA strand and is probably not related to ferrichrome transport.

fhuD and spr1687 were chosen for insertion-duplication mutagenesis since the encoded proteins contain a predicted signal...
peptide cleavage site and a lipid attachment site for lipoproteins. In gram-positive bacteria the lipoproteins are attached to the outer surface of the cytoplasmic membrane and are constituents of ABC substrate transporters. Insertional inactivation of \( fhuD \) in mutant API1 rendered cells albomycin and salmycin resistant (Fig. 4), but inactivation of spr1687 did not. Therefore, the \( fhuD, fhuB, \) and \( fhuG \) genes were studied further. An export resistance mechanism for structurally unrelated antibiotics was ruled out by the lack of change in the sensitivity to optochin (Fig. 4). Sensitivity to erythromycin was used as a control for the genetic constructs in the insertion duplication and transposon mutants in which the erythromycin resistance gene \( ermB \) was received along with the foreign DNA.

To confirm the FhuD activity in antibiotic sensitivity and to examine its role in ferric hydroxamate transport, an internal SphI fragment in \( fhuD \) was deleted, leaving transcription of the other \( fhu \) genes from the native promoter unaffected. A mutant carrying this chromosomal \( fhuD \) deletion was selected on plates containing ferrichrome and streptonigrin. The \( fhuD \) deletion mutant was expected to show an increase in streptonigrin resistance since in \( E. coli \) sensitivity to streptonigrin depends on the intracellular iron concentration and has been used to isolate iron supply mutants that are streptonigrin resistant (7). Cross application of filter papers soaked with ferrichrome and streptonigrin, respectively, on blood agar plates seeded with \( S. pneumoniae \) R6 resulted in a pronounced streptonigrin inhibition zone. The \( fhuD \) deletion mutant was albomycin and salmycin resistant (Fig. 4). In liquid culture the streptonigrin sensitivity of mutant APD1 was not enhanced by ferrichrome and ferrioxamine B (Fig. 6), but the sensitivity of...
the wild-type strain R6 and strain APD1CI was enhanced. Strain APD1CI is derived from strain APD1 in which wild-type fluD was cloned in the mal locus to test transcomplementation of the lacking transport activity in contrast to a possible cis-regulatory activity of fluD on the expression of the downstream fluBGC genes (Fig. 3). The recombinant APD1CI was sensitive to albomycin and salmycin (Fig. 4). Growth in the presence of maltose increased sensitivity to albomycin, presumably because maltose positively regulates transcription of the malM gene (data not shown). Ferrichrome increased the sensitivity to streptonigrin more strongly than ferrioxamine B, a finding that agrees with the higher ferrichrome transport rate compared to the ferrioxamine transport rate (see below).

fluB and fluG are necessary for albomycin and salmycin sensitivity. The fluD gene is not always linked to the genes that encode the transport proteins across the cytoplasmic membrane. For example, S. aureus encodes two fluD genes that are both not linked to fluCBG (36). To exclude that the S. pneumoniae R6 fluD complements the spr0220–spr0224 gene cluster, which lacks a functional fluD (Fig. 3A) or another unidentified gene cluster, isolated S. pneumoniae DNA was randomly mutagenized in vitro with EZ-Tn5. EZ-Tn5 encodes a mutated Tn5 transposase with a 1,000-fold greater in vitro transposition frequency than the wild-type Tn5 (19, 20). S. pneumoniae R6 was transformed with the in vitro mutagenized DNA, and recombinants resistant to both albomycin and erythromycin were selected. The mutated loci were sequenced by using primers complementary to the EZ-Tn5. Two mutants, APT1 and APT2, were isolated which contained EZ-Tn5 insertions in fluB and fluG (Fig. 5), respectively, which encode polypeptides forming the predicted transmembrane transporter adjacent to fluD. The flu fragment of mutants APT1 and of APT2 was 1,391 bp longer than the flu fragment of the wild-type strain R6 (Fig. 5). Both mutants were resistant to albomycin and salmycin (Fig. 4). Since these were the only selected mutants, it is likely that FluD is part of the FluBGC transporter.

Transport of ferrichrome and ferrioxamine B into S. pneumoniae. We did not find appropriate conditions in a minimal medium to determine the transport kinetics of $^{55}$Fe$^{3+}$]ferrichrome and $[^{59}$Fe$^{3+}$]ferrioxamine B into S. pneumoniae R6. Therefore, transport was determined in THY broth supplemented with 0.4 mM nitrilotriacetate to reduce the available iron. Ferrichrome was transported into the wild-type strain R6 but not into the ΔfluD mutant APD1 and was transported better into the fluD-complemented APD1CI than into the wild type (Fig. 7A). In the latter case, the malM promoter might be stronger than the fluD promoter, thereby resulting in more FluD and consequently more transport if the FluD step is rate limiting. Transport of ferrioxamine B was only seen in the fluD-complemented APD1CI strain (Fig. 7B).

Binding of albomycin and ferrichrome to the isolated FluD protein. Primarily, binding proteins determine the substrate specificity of bacterial ABC importers (2). To examine whether FluD functions as a binding protein, FluD was isolated and purified. The fluD gene was cloned in plasmid pET-28a, which resulted in a protein with six histidine residues at the N-terminal end. Synthesis of His$_6$-FluD in E. coli was induced by 1 mM IPTG, and the protein was purified by affinity chromatography on a Ni-NTA agarose column. Binding of the ferrichromates and heme was examined by protection of His$_6$-FluD against proteolytic digestion by added proteasine K. This assay demonstrated substrate binding to the E. coli FluD protein, whose proteolysis is inhibited by cognate substrates (29). This was also the case with FluD of S. pneumoniae, which was completely degraded by proteasine K in the absence of substrate (Fig. 8, lane 9) and truncated to a smaller, stable product in the presence of ferrichrome, ferrioxamine B, albomycin, and salmycin (Fig. 8, lanes 3, 4, 7, and 8) but not in the presence of heme (Fig. 8, lane 6), another possible iron source for S. pneumoniae, or FeCl$_3$ (Fig. 8, lane 5). The assay depended on the resistance of ferrichrome and albomycin to proteasine K. Cyclic ferrichrome is not degraded (29), and the resistance of albomycin was tested. Iron-loaded albomycin used in the assay was resistant since the activity was not de-
creased but iron-free albomycin was degraded (data not shown). Iron coordination renders albomycin protease resistant even in the seryl bridge between the iron center and the antibiotic which is not involved in iron binding.

In another assay, isolated FhuD was incubated with albomycin, and the antibiotic activity of albomycin was tested on plates. FhuD reduced albomycin activity (data not shown), which suggests that binding to FhuD decreases the free albomycin concentration available for entering the cells. It also shows that added substrate-loaded FhuD cannot functionally contact FhuB and FhuG and deliver albomycin to the transport system.

**DISCUSSION**

Of the three putative iron transport systems of *S. pneumoniae* R6, genes spr0934–0936, and spr0938 encoded a ferric hydroxamate transport system through which ferrichrome, ferrioxamine B, albomycin, and salmycin were taken up into cells. Ferrichrome and ferrioxamine B interfered with the activity of albomycin. The two antibiotics inhibit protein synthesis: albomycin interferes with serine loading of the seryl-tRNA, and salmycin inhibits at an unknown target (V. Braun, unpublished results). Therefore, the ferric hydroxamates did not inhibit at the antibiotics’ target sites but interfered with their transport. This conclusion is supported by the phenotype of the transport-negative mutants in the ferric hydroxamate transport genes which were resistant to the antibiotics. The definite antibiotic resistance phenotype of the mutants indicates a single ferric hydroxamate transport system. This finding is supported by the low sequence identity between the *fhu* genes and the related genes of the two other putative iron transport systems of *S. pneumoniae* R6 which range from 10 to 32%.

Although the iron transport rate by the hydroxamates in *S. pneumoniae* was low (less than 10% of the transport rates with *E. coli*), the rate was sufficient to render cells highly sensitive to albomycin. The concentration of albomycin that inhibited the synthetase was comparable to the MIC. The low transport rate in *S. pneumoniae* might be caused by a sufficient iron supply in the rich medium in which the bacteria were grown. The amount of nitrotitrionatate added to the medium to complex the iron was probably not sufficient to reduce the iron level to a growth-limiting concentration. The situation is further compounded by the probable low iron requirement of *S. pneumoniae*, which does not contain membrane-bound electron transport chains or have a tricarboxylic acid cycle in which most of the iron of respiratory bacteria is used.

Where do the hydroxamates come from in the primarily human environment of *S. pneumoniae*? Compounds that coordinate iron similarly to the way hydroxamates coordinate iron might be present. The ferric hydroxamate transport proteins, primarily FhuD, recognize the immediate iron coordination center and tolerate a variety of ligands, as the four hydroxamates used in the present study demonstrate. Ferric siderophores not synthesized by a particular strain are nevertheless commonly taken up by the strain, e.g., ferrichrome synthesized by the fungus *Ustilago sphaerogena* is actively transported by *E. coli*. *E. coli* discriminates more strongly between the hydroxamate structures than *S. pneumoniae* in that it transports ferrichrome and albomycin but not ferrioxamine B (27, 33) and is resistant to salmycin. Binding of ferrichrome and albomycin to FhuD of *S. pneumoniae* was shown by inhibition of FhuD degradation by protease K. The *E. coli* FhuD discriminates between substrates such as ferrichrome, aerobactin, and albomycin and other ferric hydroxamates that are not transported (29, 33). The structure of *E. coli* FhuD with loaded substrate has been resolved at the atomic level and reveals recognition of the ferric hydroxamate center. Distinct structures are observed in substrate-loaded FhuD proteins. 

![FIG. 7. Transport of [55Fe3+]ferrichrome (A) and [55Fe3+]ferrioxamine B (B) into Streptococcus pneumoniae R6, APD1 ΔfhuD, and APD1CI ΔfhuD of APD1.](http://jb.asm.org/)

![FIG. 8. Proteolysis of His6-FhuD by proteinase K in the absence (lane 9) or presence of ferrichrome (lane 3), ferrioxamine B (lane 4), heme (lane 6), albomycin (lane 7), salmycin (lane 8), FeCl3 (lane 5), untreated His6-FhuD (lane 2), and molecular size markers (lane 1).](http://jb.asm.org/)
pared to unloaded FhuD. Molecular dynamic simulations of the *E. coli* FhuD (30) and small angle crystal scattering of the *S. aureus* FhuD (36) revealed small changes upon substrate binding.

We designated the genes involved in ferric hydroxamate transport as *fhuD* (encodes binding lipoprotein), *fhuB* and *fhuG* (encode transmembrane transport proteins), and *fhuC* (encodes ATPase). This nomenclature agrees with that of *Bacillus subtilis*, from which the first ferric hydroxamate transport system of gram-positive bacteria was partially characterized (35), *S. aureus* (36), and a group B streptococcus (12). The *fhuD* *fhuB* *fhuG* genes of *S. pneumoniae* are transcribed in the same direction and most likely form an operon. *B. subtilis* has the same gene order, but *fhuD* is transcribed in the opposite direction (8, 35). In *S. aureus* the gene order is *fhuC* *fhuB* *fhuD* *fhuG*, whereas *fhuD1* and *fhuD2* are located at other sites on the chromosome (36). In the group B streptococcus all four genes have the same transcription polarity but are arranged *fhuC* *fhuD* *fhuB* *fhuG* (12). An iron transport system was studied in a clinical isolate of *S. pneumoniae* 01000993. This system was first designated *pit2* (9) and then *pia* (10) since the transport substrate was not identified. The *pit2A* gene encodes a small protein that is less than one-third of the size of its *S. aureus* counterpart. The *pit2A* gene is located on the chromosome (36), and the *pit1B* gene (spr1684 of Fig. 3) is next to *pit2A* on the chromosome. The *pit1B* gene is likely to be a member of the same operon as *pit2A*.

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