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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. [$^{55}Fe^{3+}$]ferrichrome and [$^{55}Fe^{3+}$]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 antibiotically active thioribosyl pyrimidine derivative is bound to a trihydroxamate iron carrier formed by three linked N^5-acetyl-N^5-hydroxy-ornithine residues. Two serine residues form the bridge between the antibiotic and the iron carrier (Fig. 1).

In a tRNA 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 tRNA 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 Fe^{3+} 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, Fe^{3+} is reduced to Fe^{2+}, 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 Fe^{3+}, 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: *fhuA*, which encodes an outer membrane transport protein; *fhuB*, which encodes a cytoplasmic membrane transport protein; *fhuC*, which encodes an ATPase at the inner side of the cytoplasmic membrane; and *fhuD*, 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

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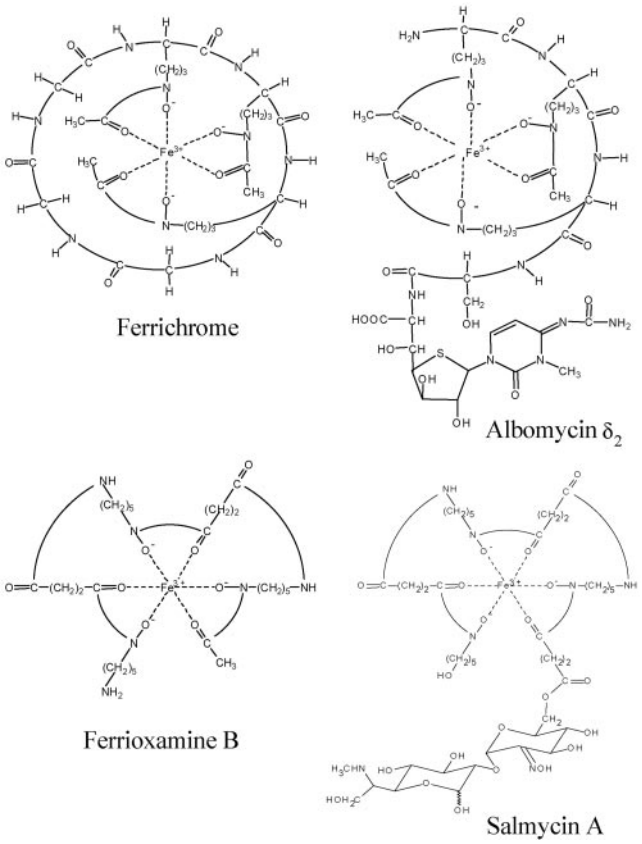

FIG. 1. Chemical structures of the ferric hydroxamates used in the present study. Ferrichrome and ferrioxamine B are growth-promoting ferric siderophores; albomycin and salmycin are growth-inhibiting antibiotics.

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%) plates supplemented with 4% defibrinated sheep blood at 37°C in an incubator under 5% CO_2 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

Strain or plasmid	Relevant genotype[a]	Reference or source
Strains		
S. pneumoniae R6	Unencapsulated wild type	R. Hakenbeck
S. pneumoniae API1	*fhuD* (spr0934) mutant of R6 (*fhuB fhuG fhuC*)[a]	This study
S. pneumoniae API2	spr1687 mutant of R6	This study
S. pneumoniae APD1	Δ*fhuD*[b] of R6	This study
S. pneumoniae APT1	*fhuB*::Tn*5* (spr0935::Tn*5*) of R6 (*fhuG fhuC*)[a]	This study
S. pneumoniae APT2	*fhuG*::Tn*5* (spr0936::Tn*5*) of R6 (*fhuC*)[a]	This study
S. pneumoniae APD1CI	*fhuD* downstream of *malM* in APD1	This study
Streptomyces sp. strain ATCC 700974	Albomycin production strain	ATCC
E. coli DH5α		This institute
E. coli BL21(DE3)		This institute
E. coli SIP401	MC4100 *fur-28 zbf-15*::Tn*10*	S. Patzer
Plasmids		
pJDC9	*ermB* Erm^r	R. Hakenbeck
pET-28a	Kan^r	Merck Biosciences
pMOD3	Amp^r	Epicentre Biotechnologies
pAPBP	*fhuD* cloned in pET-28a	This study
pAPT	Amp^r Erm^r; *ermB* of pJDC9 cloned in pMOD3	This study
pAPID1	Internal fragment of *fhuD* cloned in pJDC9	This study
pAPID2	Internal fragment of spr1687 cloned in pJDC9	This study
pAPIC	*fhuD* downstream of *malM* of pJDC9	This study

[a] Parentheses indicate polar effects on the transcription of genes downstream of the mutated *fhu* genes. Erm^r, Kan^r, and Amp^r 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 (Sarstedt, Nuembrecht, Germany), which were incubated at 37°C without shaking, and the optical density at 578 nm (OD_{578}) was measured.

Albomycin was purified as described previously (17), salmycin was obtained from L. Vertesy, former Hoechst AG, Frankfurt, Germany. Ferrichrome, ferrioxamine B, streptonigrin, and hemin chloride were purchased from Sigma-Aldrich, Schnelldorf, Germany.

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 lysozyme prior to the addition of proteinase K. PCRs were performed with High-Fidelity Phusion Polymerase (Finnzyme, Espoo, Finland). Restriction enzymes were from Roche Biochemicals (Mannheim, Germany). Single and double digestions were performed according to the manufacturer's guidelines. Ez-Tn*5* 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 pJDC9 (11) according to the standard method. An internal fragment of *fhuD* was PCR amplified from the *S. pneumoniae* R6 genomic DNA with the primer pair AATACT*TCTAGA*GAGCATGCGCCTG and GTTG*GAATTC*ATGAGGCTGCTAACG, each having one unique restriction enzyme recognition site (marked in italics). Digested PCR product was cloned between the unique EcoRI-XbaI sites of pJDC9 in *E. coli* DH5α. A plasmid with the right insert was confirmed by sequencing and designated pAPID1. Plasmid pAPID1 was purified from *E. coli* and transformed in *S. pneumoniae* R6. Erythromycin-resistant colonies were picked, and the insertion site was confirmed by PCR and by direct sequencing with outward primers specific to the plasmid sequence flanking the cloning site. The mutant with the desired insertion in *fhuD* was designated API1. Similarly, an internal fragment of spr1687 was PCR amplified from the *S. pneumoniae* R6 genomic DNA with the primer pair AGCTC*GGATCC*AACAGAGATAACC and TGATT*GAATTC*CGCCTCCGCTTAG and cloned between BamHI-EcoRI of pJDC9 to produce the disruption plasmid pAPID2. This pAPID2 was transformed into *S. pneumoniae* R6 to create API2 with an insertion in spr1687.

Construction of the transposon. To perform genomewide random in vitro mutagenesis of *S. pneumoniae*, a transposon was made by using the transposon construction vector pMOD3, purchased from Epicenter. The full *ermB* operon was PCR amplified from pJDC9 with the primer pair AGCA*AAGCTT*GGCGGAAACGTAAAAG and TCCTT*GGATCC*TGTCAGTAGTATACC (unpublished plasmid sequence obtained from D. A. Morrison, University of Illinois, Chicago) and cloned in pMOD3 such that *ermB* remained within the mosaic end (ME) repeat sequence recognized by the Ez-Tn*5* transposase. Plasmid isolated from ampicillin and erythromycin double-resistant *E. coli* transformants was sequence verified for *ermB* insert and designated pAPT. Transposon containing the *ermB* gene (1.5 kb) was PCR amplified from pAPT by using the primer pair complementary to pMOD3 covering the ME region, as described by Epicenter.

Generation of Tn*5* *ermB* insertion library and mutant selection. Transposition was performed with Ez-Tn*5* transposase as recommended by the supplier. In short, purified PCR-amplified transposon and *S. pneumoniae* R6 genomic DNA were incubated with the Ez-Tn*5* transposase in the presence of Mg^{2+} at 37°C for 4 h. The reaction was stopped, and the DNA was ethanol precipitated. Transposition by Ez-Tn*5* transposase introduces a 9-bp sequence gap on either strand adjacent to the insertion (19, 20). Since transfer of the insertion into *S. pneumoniae* chromosome by homologous recombination requires intact strand flanking heterologous inserted sequence (1), the gap was repaired by simultaneous treatment with T4 DNA ligase and T4 DNA polynucleotide kinase in the presence of deoxynucleoside triphosphates (250 μM) and ATP (1 mM) for 30 min at room temperature. The reaction was continued for another 30 min after 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-resistant transformants were pooled to yield a Tn*5* insertion library and saved as a glycerol stock. The library was screened for albomycin-resistant mutants. To confirm that the albomycin resistance derived form the transposon insertion and not from spontaneous mutations, genomic DNA was isolated from colonies that were both albomycin and erythromycin resistant and transformed back into wild-type R6. Equal amounts of each transformation mixture were examined for erythromycin resistance and erythromycin-albomycin double resistance. Similar numbers of transformants on the single and double antibiotic plates indicated coselection of the resistance traits confirming albomycin resistance by Tn*5* *ermB* insertion. Two such mutants, APT1 and APT2, were isolated and Tn*5* insertion site was determined by sequencing with transposon-specific outward primers.

Construction of a *fhuD* deletion mutant. A 4.5-kb region covering the *fhuD* sequence was PCR amplified from genomic DNA with the primers TGCTTGAACTTGCTTGTTGG and TTCAACATTGGCCTTAACCA. In silico restriction analysis of the *fhu* region sequence of *S. pneumoniae* R6 revealed two native SphI restriction sites. The fragment was digested with SphI, which cleaves 105 bp downstream of the start codon and 16 bp upstream of the stop codon of *fhuD* 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 904-bp deletion in *fhuD*. This ligation mixture served as the template for nested PCR with the primers AGCTATGGCAGGACTTACAAC and GACCACGGCTTACAAGATCAG to amplify a 3-kb region of the *fhu* region with the *fhuD* deletion. The PCR product was gel purified and transformed into *S. pneumoniae* R6. Transformants were selected with streptonigrin in the presence of ferrichrome. Individual colonies were checked by PCR for the deletion. A colony designated APD1 showing a 0.9-kb shorter sequence than the wild type in the *fhu* region was selected for further study.

Complementation of the *fhuD* mutant by insertion of *fhuD* in the chromosomal *mal* region. APD1 Δ*fhuD* was complemented by inserting *fhuD* into the chromosomal *malMP* region. The C-terminal fragment of *malM* was PCR amplified with the primer pair CTT*GAGCTC*TTTGCTGAGTATA and GATAA*CATATG*TAGTTGTCTCCTG and *fhuD* with the primer pair GTTTAAGGAGTTC*ATATG*AAGAACAA and TGTG*TCTAGA*CCGAGTATACCTGGA. The *malM* product was digested with SacI and NdeI, and the *fhuD* product was digested with NdeI and XbaI. Both products were gel purified, and a three-way ligation was performed with a SacI-XbaI fragment of pJDC9. The resulting plasmid pAPIC was purified from *E. coli*. Insertion duplication at the *malM* region introduced *fhuD* downstream of *malM*, resulting in strain APD1CI.

All genetic constructs were confirmed by DNA sequencing.

Albomycin and salmycin sensitivity assays. Aliquots (50 μl) of an *S. pneumoniae* culture grown in THY medium for 12 to 14 h were mixed with 4 ml of molten THY agar supplemented with 5% sheep blood and overlaid on a THY blood agar plate. Filter paper disks (6 mm in diameter) containing 1 μg of albomycin or salmycin were placed on the plates, and growth inhibition was examined after 24 h of incubation at 37°C. Inhibition of albomycin and salmycin activity by ferrichrome and ferrioxamine B was tested on THY agar plates onto which paper strips containing 0.5 μg of either albomycin, salmycin, ferrichrome, ferrioxamine B, or hemin chloride were placed at right angles to the antibiotic strips.

Albomycin activity in the culture medium of the production strain was determined with *E. coli* SIP401, which was grown overnight in TY medium. A 20-μl aliquot was mixed with 3 ml of TY soft agar (7.5 g of agar/liter) and overlaid on TY solid agar plates (15 g of agar/liter). Antibiotic solutions (5 μl) were spotted, and growth examined after incubation at 37°C for 16 to 18 h.

[$^{55}Fe^{3+}$]ferrichrome and [$^{55}Fe^{3+}$]ferrioxamine B transport into *S. pneumoniae*. *S. pneumoniae* was grown to an OD_{578} of 0.6 in THY broth supplemented with 5 μM ferrichrome. Cells were pelleted, washed with THY broth, and suspended to an OD_{578} of 0.6 in THY broth supplemented with 0.4 mM nitrilotriacetate. To load deferri-ferrichrome and deferri-ferrioxamine B with $^{55}Fe^{3+}$, 4 μl of a 10 mM solution of the deferrated hydroxamates, 32 μl of 0.255 mM $^{55}Fe^{3+}Cl_3$ (specific activity, 2,642 mBq/mg; Perkin-Elmer, Boston, MA), and 4 μl of 0.2 N HCl were added to 40 μl of water. Transport was started by adding 18 μl of this mixture to 1.8 ml of the cell suspension. Samples of 0.2 ml were taken after 0, 6, 12, 15, 18, 24, and 30 min; filtered through cellulose nitrate filters (pore size, 0.45 μm; Pall Life Sciences, Ann Arbor, MI); and washed twice with 5 ml of 0.1 M LiCl. The filters were dried, and the radioactivity was determined in a liquid scintillation counter.

Isolation of His_6-FhuD. Complete *fhuD* was PCR amplified from genomic DNA with the primer pair GTTTAAGGAGTTC*ATATG*AAGAACAA and TAAGACT*GGATCC*TGTGTTTATACCGA. The PCR product was digested with NdeI and BamHI and ligated into pET28a digested with NdeI and BamHI. The

resulting plasmid pAPBP 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-nitrilotriacetic acid (Ni-NTA) agarose column. The *fhuD* derivative was initially cloned in *E coli* DH5α and then transformed into *E. coli* BL21(DE3). *E. coli* BL21(DE3)/pAPBP was grown with vigorous shaking at 37°C in TY medium supplemented with kanamycin (30 μg/ml) until an OD_{578} 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. His_6-FhuD was eluted with 1-ml aliquots of 100 mM Tris-HCl (pH 8)–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 His_6-FhuD in 50 mM Tris-HCl–250 mM NaCl (pH 7.8) was incubated with 1 μM 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 was tested 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, ferrioxamine B, also inhibited the action of albomycin (Fig. 2A). The structure of ferrioxamine differs from that of ferrichrome (Fig. 1). Ferrioxamine 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 ferrioxamine B, ferrichrome, and albomycin, the coordination of Fe^{3+} 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 ferrioxamine B analogue danoxamine linked to an antibiotically active amino disaccharide (Fig. 1) (39). Results similar to those seen with albomycin were obtained (Fig. 2B).

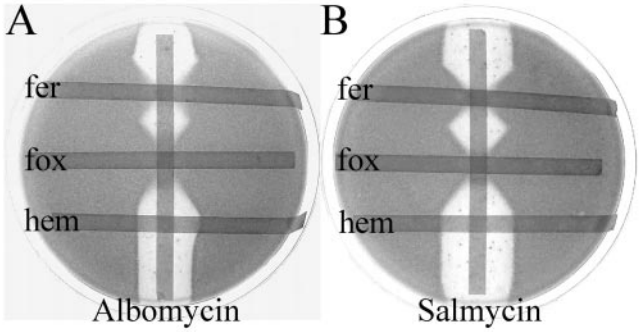

FIG. 2. Effect of ferrichrome (fer), ferrioxamine B (fox), and heme (hem) on the activity of albomycin (A) and salmycin (B). Blood agar plates were seeded with *S. pneumoniae* R6. A paper strip containing one of the antibiotics was placed on each plate. Paper strips each containing one of the ferric hydroxamates or heme were then placed on the plates at right angles to the antibiotic paper strips as shown. The white areas show growth inhibition by the antibiotics, which does not occur when the ferric siderophores counteract the antibiotics.

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

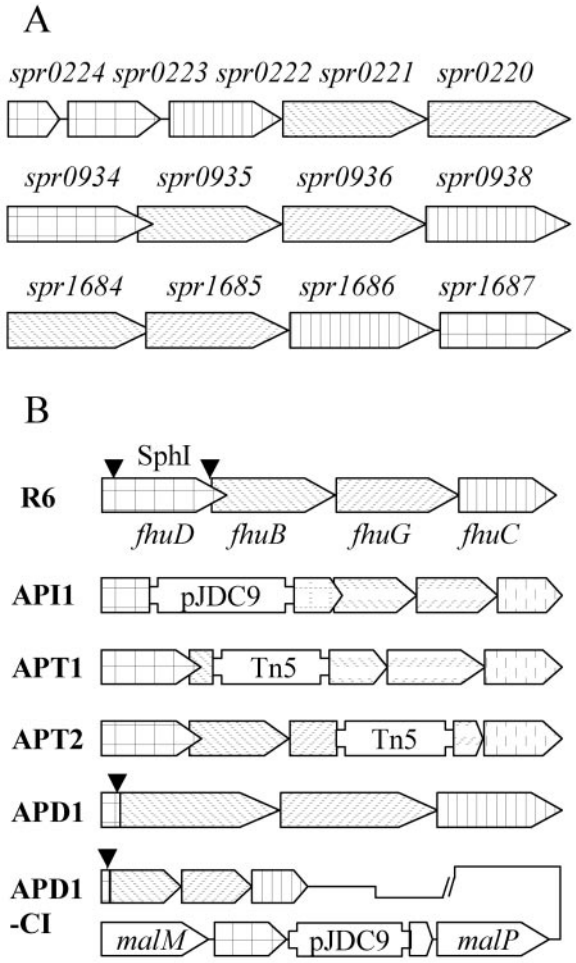

FIG. 3. (A) Sections from the genome of *S. pneumoniae* R6 with the predicted iron transport genes. The identical hatchings indicate related gene functions. (B) Arrangement of the *fhu* genes (spr0934–0936 and spr0938 of panel A) of *S. pneumoniae* R6 and mutants constructed in the present study. Mutants APT1 *fhuB* and APT2 *fhuG* were constructed by in vitro Tn*5* mutagenesis of the entire genome, and mutant API1 *fhuD* was constructed by insertion duplication mutagenesis of the chromosome with a derivative of plasmid pJDC9 encoding an internal *fhuD* fragment. APD1 Δ*fhuD* contains an internal deleton in *fhuD*, and APD1CI is a derivative of APD1 in which *fhuD* was cloned downstream of *malM*. The discontinuous hatchings of the *fhu* genes downstream of the mutated gene indicate predicted polar effects on the downstream gene transcription.

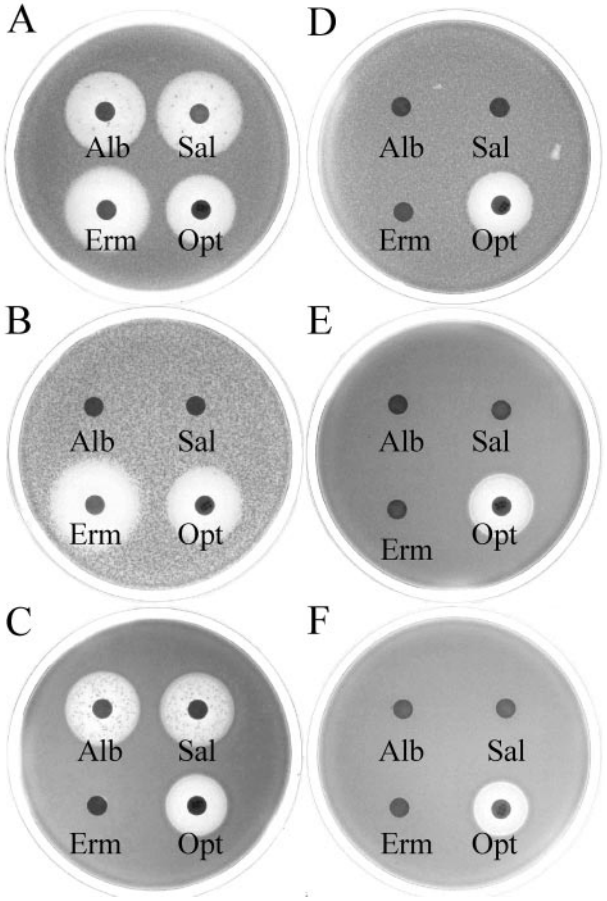

FIG. 4. Sensitivity of *S. pneumoniae* R6 (A) and mutants APD1 Δ*fhuD* (B), APD1CI $fhuD^+B^+G^+C^+$ (C), API1 *fhuD* (D), APT1 *fhuB* (E), and APT2 *fhuG* (F) to the antibiotics albomycin (alb), salmycin (sal), erythromycin (erm), and optochin (opt). Antibiotics were spotted onto filter paper disks and applied to the blood agar plates. The plates were incubated at 37°C for 24 h.

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 *fhu* DNA fragment of the isolated APD1 deletion mutant was 904 bp shorter than the wild-type DNA fragment (Fig. 5). 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 Δ*fhuD* mutant APD1 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

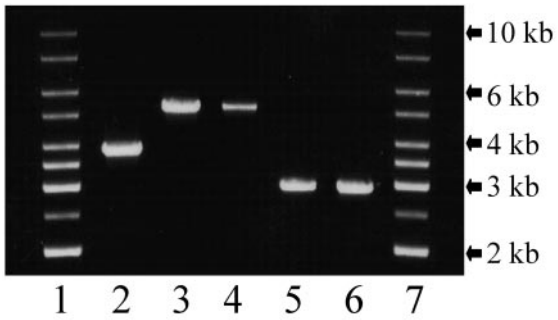

FIG. 5. DNA fragments obtained by PCR of the entire *fhu* operon of *S. pneumoniae* R6 (lane 2), and mutants APT1 *fhuB*::Tn*5* (lane 3), APT2 *fhuG*::Tn*5* (lane 4), APD1 Δ*fhuD* (lane 5), and APD1CI Δ*fhuD* in the *fhu* operon but *fhuD*$^+$ downstream of *malM* (lane 6). Lanes 1 and 7, nucleotide size markers. The primers GACCACGGCTTACAAGATCAG and AGCTATGGCAGGACTTACAAC were used for PCR.

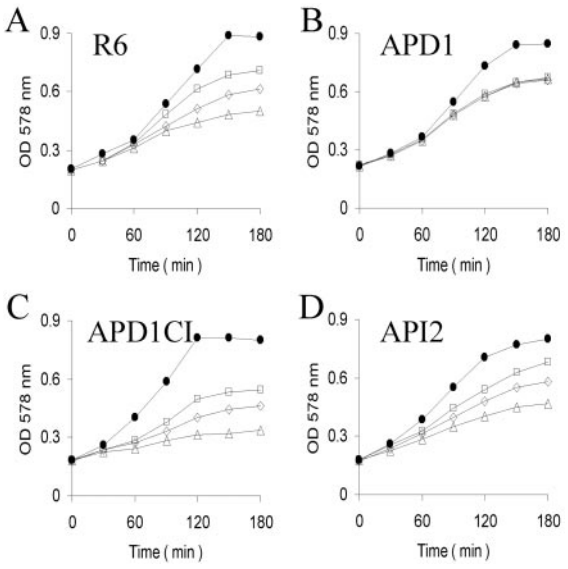

FIG. 6. Sensitivity to streptonigrin (1 μg/ml) of *S. pneumoniae* R6 and the mutants APD1 Δ*fhuD*, APD1CI *fhuD*$^+$ of APD1, and API2 mutated in spr1687. Symbols: □, absence of ferrichrome and ferrioxamine B; △, presence of 2.5 μM ferrichrome; ◇, presence of 2.5 μM ferrioxamine B; ●, no addition.

the wild-type strain R6 and strain APD1CI was enhanced. Strain APD1CI is derived from strain APD1 in which wild-type *fhuD* was cloned in the *mal* locus to test transcomplementation of the lacking transport activity in contrast to a possible *cis*-regulatory activity of *fhuD* on the expression of the downstream *fhuBGC* 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).

***fhuB and fhuG* are necessary for albomycin and salmycin sensitivity.** The *fhuD* gene is not always linked to the genes that encode the transport proteins across the cytoplasmic membrane. For example, *S. aureus* encodes two *fhuD* genes that are both not linked to *fhuCBG* (36).To exclude that the *S. pneumoniae* R6 *fhuD* complements the spr0220–spr0224 gene cluster, which lacks a functional *fhuD* (Fig. 3A) or another unidentified gene cluster, isolated *S. pneumoniae* DNA was randomly mutagenized in vitro with EZ-Tn*5*. EZ-Tn*5* encodes a mutated Tn*5* transposase with a 1,000-fold greater in vitro transposition frequency than the wild-type Tn*5* (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-Tn*5* transposon. Two mutants, APT1 and APT2, were isolated which contained EZ-Tn*5* insertions in *fhuB* and *fhuG* (Fig. 5), respectively, which encode polypeptides forming the predicted transmembrane transporter adjacent to *fhuD*. The *fhu* fragment of mutants APT1 and of APT2 was 1,391 bp longer than the *fhu* 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 FhuD is part of the FhuBGC 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 [$^{55}Fe^{3+}$]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 Δ*fhuD* mutant APD1 and was transported better into the *fhuD*-complemented APD1CI than into the wild type (Fig. 7A). In the latter case, the *malM* promoter might be stronger than the *fhuD* promoter, thereby resulting in more FhuD and consequently more transport if the FhuD step is rate limiting. Transport of ferrioxamine B was only seen in the *fhuD*-complemented APD1CI strain (Fig. 7B).

Binding of albomycin and ferrichrome to the isolated FhuD protein. Primarily, binding proteins determine the substrate specificity of bacterial ABC importers (2). To examine whether FhuD functions as a binding protein, FhuD was isolated and purified. The *fhuD* 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-FhuD 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 ferric hydroxamates and heme was examined by protection of His_6-FhuD against proteolytic digestion by added proteinase K. This assay demonstrated substrate binding to the *E. coli* FhuD protein, whose proteolysis is inhibited by cognate substrates (29). This was also the case with FhuD of *S. pneumoniae*, which was completely degraded by proteinase 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 proteinase 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-

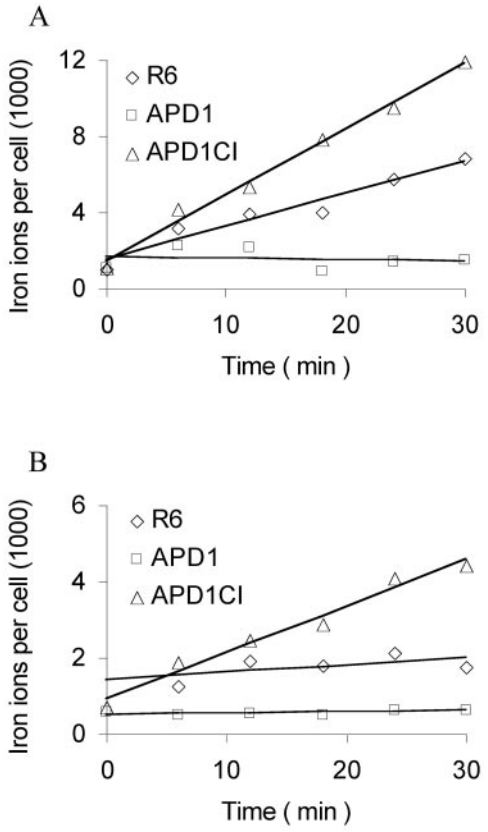

FIG. 7. Transport of [$^{55}Fe^{3+}$]ferrichrome (A) and [$^{55}Fe^{3+}$]ferrioxamine B (B) into *Streptococcus pneumoniae* R6, APD1 Δ*fhuD*, and APD1CI *fhuD*$^{+}$ of APD1.

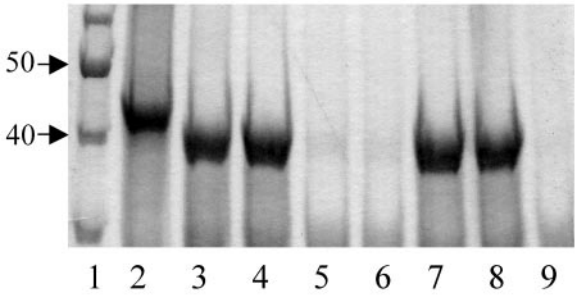

FIG. 8. Proteolysis of His_6-FhuD by proteinase K in the absence (lane 9) or presence of ferrichrome (lane 3), ferrioxamine B (lane 4), heme (lane 6), albomycin (lane 7), salmycin (lane 8), $FeCl_3$ (lane 5), untreated His_6-FhuD (lane 2), and molecular size markers (lane 1).

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 the antibiotics. 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 nitrilotriacetate 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 proteinase 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 com-

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 fhuC* 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 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* 0100993. This system was first designated *pit2* (9) and then *pia* (10) since the transport substrate was not identified. $^{55}Fe^{3+}Cl_3$ uptake is not lower in a *pit2A* mutant (*fhuD*) and requires a second mutation in *pit1B* (spr1684 of Fig. 3) to be 73% lower than that of the wild type after a 15- to 30-min incubation (9). Growth of the *pit2A* mutant is more strongly reduced than that of the wild type in THY medium treated with Chelex-100 to remove iron and is restored by addition of $FeCl_2$. The sensitivity of the *pit2A* mutant to streptonigrin is reduced. Unfortunately, a nomenclature other than *fhu* was also used in a recent study of a ferrichrome uptake system in *S. pyogenes* (21). The *ftsA ftsB ftsC ftsD* genes, as organized on the chromosome, correspond to the *fhuC*, *fhuD*, *fhuB*, and *fhuG* genes, respectively. The clinical *S. pyogenes* strains we examined were resistant to albomycin and salmycin (unpublished results). Either the described Fts system is much more specific than the hitherto-studied ferrichrome transport systems or the antibiotic moieties are not released from the iron carriers. We do not expect to find a ferrichrome transport system that does not transport albomycin in these strains.

Among the functionally characterized *fhuD* genes, *fhuD* of *S. pneumoniae* is more closely related to *E. coli fhuD* than to the *fhuD* genes of *B. subtilis* and *S. aureus*. Therefore, the *fhu* operon of *S. pneumoniae* probably did not evolve within the gram-positive bacteria but was rather acquired by horizontal gene transfer. This is further evidenced by the presence of a 27-kb region containing the *fhu* operon in the *S. pneumoniae* 0100993 genome that displays features of a gram-negative bacterial pathogenicity island (9). In the *S. pneumoniae* R6 genome 40 open reading frames are predicted to be derived from gram-negative bacteria (24), and this might be a consequence of competence. The finding that spr0224/0223 is disrupted agrees with the observation that many open reading frames for transporters are disrupted in *S. pneumoniae* R6 and suggests that truncated foreign genes are acquired or that *S. pneumoniae* genes not required for growth in the fastidious *S. pneumoniae* environment are mutated (24).

Heme was shown to be another iron source for *S. pneumoniae* (9). PiuA encoded by spr1687 bound to hemin-agarose and more weakly to hemoglobin-agarose and isolated PiuA bound heme (38).

The use of the antibiotics albomycin and salmycin facilitated the assignment of the *fhu* locus to a ferric hydroxamate transport system. The ferric hydroxamate transport system in *S. pneumoniae* offers means to develop antibiotics with ferric hydroxamates as carriers. Albomycin and salmycin are promising examples of how the structures of such chemically synthesized antibiotics might appear. The ferric hydroxamate transport-system tolerates chemically different hydroxamates with different ligands. The need for iron acquisition makes transport-negative mutants less virulent (9). In addition, the surface-exposed iron transport lipoproteins are strong antigens, and their use for active and passive immunization protects mice against invasive *S. pneumoniae* disease (25).

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