INFECTION AND IMMUNITY, Aug. 2002, p. 4389–4398 Vol. 70, No. 8
0019-9567/02/$04.00+0 DOI: 10.1128/IAI.70.8.4389–4398.2002

Characterization of Pit, a *Streptococcus pneumoniae* Iron Uptake ABC Transporter

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Received 14 January 2002/Returned for modification 20 March 2002/Accepted 22 May 2002

Bacteria frequently have multiple mechanisms for acquiring iron, an essential micronutrient, from the environment. We have identified a four-gene *Streptococcus pneumoniae* operon, named *pit*, encoding proteins with similarity to components of a putative *Brachyspira hyodysenteriae* iron uptake ABC transporter, Bit. An *S. pneumoniae* strain containing a defined mutation in *pit* has impaired growth in medium containing the iron chelator ethylenediamine di-*o*-hydroxyphenylacetic acid, reduced sensitivity to the iron-dependent antibiotic streptonigrin, and impaired virulence in a mouse model of *S. pneumoniae* systemic infection. Furthermore, addition of a mutation in *pit* to a strain containing mutations in the two previously described *S. pneumoniae* iron uptake ABC transporters, *piu* and *pia*, resulted in a strain with impaired growth in two types of iron-deficient medium, a high degree of resistance to streptonigrin, and a reduced rate of iron uptake. Comparison of the susceptibilities to streptonigrin of the individual *pit*, *piu*, and *pia* mutant strains and comparison of the growth in iron-deficient medium and virulence of single and double mutant strains suggest that *pia* is the dominant iron transporter during in vitro and in vivo growth.

Recent genetic screens for virulence determinants of bacterial pathogens have emphasized the importance of nutrient acquisition during bacterial growth in vivo. Many of the genes identified by these screens encode components of nutrient transporters or biosynthetic pathways of general metabolism, indicating that essential biosynthetic components and nutrients are often restricted in availability to microorganisms in mammals (25, 29, 33). A detailed understanding of the nutritional requirements of bacterial pathogens during growth in vivo is providing a better understanding of the physiological stresses placed upon them during the course of infection and should identify potential targets for novel antibiotic treatments or vaccines (6). Iron is one such nutrient which is essential for the growth of most bacteria but whose restricted availability within the host forms a nutritional barrier to infection (47). As a consequence many bacterial pathogens contain specialized iron uptake mechanisms to acquire iron from iron-containing mammalian proteins such as transferrin, hemin, and ferritin, either by direct binding of the iron source to the bacterial surface or through secreted low-molecular-weight, high-affinity iron scavengers called siderophores (9, 37, 46). Multiple and often partially redundant iron acquisition mechanisms are frequently present within a single pathogen, emphasizing the importance of iron acquisition for bacterial growth (1, 2, 5, 16, 37). Although pathogenic bacteria utilize a variety of environmental iron sources, frequently specific iron uptake ABC transporters transport the iron moiety into the cytosol across the membrane of gram-positive bacteria (7, 12) and the inner membrane of gram-negative bacteria (15, 48). A role during in vivo growth has been confirmed for numerous iron transporters of gram-negative pathogenic bacteria, including *Escherichia coli* (42), *Salmonella enterica* serovar Typhimurium (22), *Legionella pneumophila* (44), *Helicobacter pylori* (43), *Yersinia pestis* (1, 2), and *Neisseria* species (37). However, only a small number of iron transporters of gram-positive pathogens have been described previously, and there is limited information on their role in virulence (8, 12, 23, 36, 38).

Streptococcus pneumoniae is the commonest cause of bacterial pneumonia and a frequent cause of septicemia and meningitis (26). Growth of *S. pneumoniae* in iron-restricted medium is known to be supported by Fe^{2+}, Fe^{3+}, and heme-containing compounds but not by transferrin, lactoferrin, or ferritin (5, 40). There is no biochemical or genetic evidence that *S. pneumoniae* produces siderophores (40, 41). Recently we have described two *S. pneumoniae* operons, *piuBCDA* and *piaABCD*, encoding proteins which have similarity to iron uptake ABC transporters from both gram-negative and gram-positive bacteria (*piuBCDA* was previously called *pit1BCDA*, and *piaABCD* was previously called *pit2ABCD*) (5, 6). The phenotypes of the *piu* and *pia* mutant strains confirmed that they encode iron uptake systems, possibly utilizing hemoglobin as an iron source. Single mutation of *piu* or *pia* resulted in a mild or moderate reduction in virulence, respectively. However, a strain containing mutations in both *piu* and *pia* was severely attenuated in both pulmonary and systemic models of infection (5), suggesting that the function of at least one protein, Piu or Pia, is required for in vivo growth. To date *piu* and *pia* products are the only *S. pneumoniae* iron transporters which have been characterized genetically. The recently published *S. pneumoniae* genome (40) revealed the existence of a third ABC transporter operon encoding proteins with similarity to iron uptake transporters. In this paper we characterize this operon, termed *pit*, and investigate the in vitro and in vivo phenotypes of strains containing a defined *pit* mutation. By comparing the in vitro and in vivo phenotypes of strains con-

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TABLE 1. Strains, plasmids, and primers constructed or used in this study

Strain plasmid, or primer	Description or sequence	Reference
Strains		
0100993	Serotype III clinical isolate	25
pitA mutant	0100993 containing an insertion in *pitA* made with plasmid pPC16; Cm^r	This study
piaA mutant	0100993 containing an insertion in *piaA*; Cm^r	5
piuB mutant	0100993 containing an insertion in *piuB*; Cm^r	5
piuB/*piaA* mutant	0100993 containing an insertion in *piuB* and in *piaA*; Ery^r Cm^r	5
piuB/*piaA*/*pitA* mutant	*piuB*/*piaA* containing an insertion in *pitA* made with plasmid pPC32; Ery^r Cm^r Kan^r	This study
Plasmids		
pID701	Disruption vector for *S. pneumoniae* derived from pEVP3; Amp^r Cm^r	25
pucMUT	Disruption vector for *S. pneumoniae* derived from pUC18 (gift from S. Sriskandan); Kan^r	5
pPC16	pID701 with IT3.1/IT3.2 PCR product ligated into the *Xba*I site; Amp^r Cm^r	This study
pPC32	pucMUT with IT3.5/IT3.6 PCR product ligated into the *Xba*I site; Amp^r Cm^r	This study
Primers (5′ to 3′)		
IT3.1	GCT CTA GAC TCC TTA TAC ACT AGA TGG	
IT3.2	CGC TCT AGA CCT TAA TGT TAG CTC CGT C	
IT3.5	GGG GTA CCT CCT TAT ACA CTA GAT GG	
IT3.6	CGG GGT ACC TTA ATG TTA GCT CCG TC	
IT3.10	GGA ATT CCA TAT GAA AAA GAA ATG GAT GTA TTA TG	
IT3.11	GGA ATT CCG TAG TTA CCG AGA GCT AGG AC	
Sit3.1	TGA CGG AGC TAA CAT TAA GG	
Sit3.2	AAC GTT GTC TCG GAC AGT CA	
Sit3.5	ATG TCA GCT CCT TTC GTA GG	
Sit3.6	CAA TTG GAT GGA GCA GAT TC	
Sit3.7	TGA TTA CAG GGA CTG CTT TC	
Sit3.8	GGA AGA TCA AGG TGG AGG TA	
Sit3.9	GGA TGG AAT CAA CGC ACT TC	
Sit3.10	TGC TCC AAT TAA GCC CTC TG	
Sit3.14	GAG ATA GCG TCT ATC TTG G	
Sit3.15	CCC AGG AGT AGG CTC CTA C	

taining mutations in each *S. pneumoniae* iron uptake ABC transporter individually or in combination, we demonstrate that the *pia* product is probably the dominant *S. pneumoniae* iron transporter during both in vitro and in vivo growth.

MATERIALS AND METHODS

Bacterial strains, media, and culture conditions. *S. pneumoniae* strains used for this work are listed in Table 1. All mutant strains are derived from a capsular serotype 3 *S. pneumoniae* strain, 0100993, isolated from a patient with pneumonia and obtained from SmithKline Beecham plc. Ten *S. pneumoniae* clinical isolates (representing serotypes 2, 4, 7F, 17, 18C, 19A, 19F, 20, and 22) were obtained from J. Paton for PCR analysis of the distribution of *pitA*. *S. pneumoniae* strains were cultured at 37°C and 5% CO_2 on Columbia agar supplemented with 5% horse blood, in Todd-Hewitt broth supplemented with 0.5% yeast extract (THY), or with a previously described modified version of RPMI medium, RPMIm (5, 8). THY medium was depleted of cations by continuously agitating THY containing 2% Chelex-100 (Bio-Rad) for 8 h, followed by filter sterilization to remove the Chelex-100 and supplementation with 100 μM $CaCl_2$ and 2 mM $MgSO_4$. The iron content of 1 ml of medium was measured in parts per million with flame atomic absorption spectroscopy (performed by Stephen Bowyer, University of North London, using a Varian SpectrAA 220 spectrometer). When necessary, the following supplements were added to medium: chloramphenicol at 4 μg ml^{-1}, erythromycin at 0.4 μg ml^{-1}, kanamycin at 200 μg ml^{-1}, 10 to 50 μM $FeCl_3$, and the cation chelators 200 μM ethylenediamine di-*o*-hydroxyphenylacetic acid (EDDA; Sigma) and 400 μM 2,2′-dipyridyl (DIP; Sigma). Data for growth curves were collected either by measuring optical density at 580 nm (OD_{580}) of 1-ml cultures grown in sterilized 1.5-ml cuvettes at 1-h intervals or by using 96-well microtiter dishes (200 μl of culture per well) incubated at 37°C with no added CO_2 in a Multiskan Ascent instrument (Labsystems) which had been programmed to measure the OD_{540} at 1-h intervals. To minimize Fe contamination, stock solutions were made with MilliQ-purified water and disposable plasticware was used for all culture conditions. Strains were stored at −70°C as aliquots of THY broth culture (OD_{580} of 0.3 to 0.4) containing 10% glycerol. Plasmids were amplified in *E. coli* strain DH5α, grown at 37°C on Luria-Bertani medium with appropriate selection (35).

Nucleic acid isolation manipulations and analysis. Nucleic acids were isolated with the indicated kits: *S. pneumoniae* chromosomal DNA, Wizard genomic DNA isolation kits (Promega); plasmid DNA from *E. coli*, Qiagen plasmid kits (Qiagen); and *S. pneumoniae* RNA, SV Total RNA Isolation System (Promega). Prepared RNA samples were protected from degradation by addition of 0.5% RNasin (Promega) and storage as single-use aliquots at −70°C. The Access RT-PCR System (Promega) and target-specific primers were used to derive and amplify cDNA from RNA. The primer concentration for reverse transcription-PCR (RT-PCR) used for assessing operon structure was 400 pmol, and that for assessing the relative abundance of gene transcripts was 80 pmol. Cloning, transformation, restriction digests, and ligations of plasmid DNA were performed according to standard protocols (35). Nylon membranes for Southern hybridizations were prepared and probed with [^{32}P]dCTP-labeled probes made with the RediPrime random primer labeling kit (Amersham International Ltd.) according to standard protocols. *S. pneumoniae* sequence data were obtained from The Institute for Genomic Research (TIGR) website (http://www.tigr.org) and analyzed and manipulated with the programs MacVector (International Biotechnologies, Inc.) and Artemis3 (Genome Research Ltd.). Sequence similarity searches of the available nucleotide and protein databases (including unfinished microbial genomes) were performed with the BLAST program, available at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/blast/), and alignments of pairs of sequences were performed with the BLAST 2 service available on the World Wide Web at www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html. *S. pneumoniae* loci involved in iron transport were identified by using the Pedant website (http://pedant.mips.biochem.mpg.de/) and the search item "iron." RNA secondary structure was analyzed with the program Mfold 3.1 (http://bioinfo.math.rpi.edu/~mfold/rna/form1.cgi) (28). Clustal X was used for sequence alignment and phylogenetic analysis applying default parameters (altered gap penalties were not applied), except for the pairwise and multiple alignments where protein weight matrices of the Blosum series were used. Gaps in the alignment were not omitted. The cladogram was built by neighbor joining with the distance matrix generated by Clustal X and was represented with the program TreeViewPPC 1.6.2 available at http://taxonomy.zoology.gla.ac.uk/rod/rod.html. The reliability of each node was established by bootstrap methods. Nucleotide sequences were obtained with Applied Biosystems Dye Terminator Chemistry, and cycle sequencing was performed by the

Medical Research Council DNA Sequencing Service, Hammersmith Hospital, London, United Kingdom.

Construction of *pitA* strains. Plasmids, primers, and *S. pneumoniae* strains constructed and used for this work are described in Table 1. The construction of *piuB*, *piaA*, and *piuB*/*piaA* mutant strains has been described previously (5, 6). An internal portion of *pitA* (bp 358 to 697) was amplified by PCR and ligated into the suicide vector pID701 (primers IT3.1 and IT3.2, *Xba*I site) or pucMUT (primers IT3.5 and IT3.6, *Kpn*I site) to make the *pitA* disruption vectors pPC16 (chloramphenicol resistance) and pPC32 (kanamycin resistance), respectively. Plasmid insert identities were confirmed by DNA sequencing. *S. pneumoniae* mutant strains containing disrupted copies of *pitA* were constructed by insertion-duplication mutagenesis with a previously described transformation protocol and competence-stimulating peptide 1 (19, 25). The *pitA* single mutant strain was made by transforming *S. pneumoniae* strain 0100993 with pPC16. Transformation with pPC32 of the *piuB*, *piaA*, and *piuB*/*piaA* mutant strains created the *piuB*/*pitA* and *piaA*/*pitA* double disruption mutant strains and the *piuB*/*piaA*/*pitA* triple disruption mutant strain, respectively. Disruption of *pitA* was confirmed by PCR and Southern hybridization. All mutations were stable after two 8-h growth cycles (each representing approximately 10 rounds of cell division) in THY broth without antibiotic selection.

Streptonigrin sensitivity assays and ^{55}Fe transport assays. Sensitivity to streptonigrin disks was assessed as previously described (5). The data presented are the means from four different disks and are representative of similar results from at least two experiments. ^{55}Fe transport assays were modified from our previously described protocol (5). Quantities of 6×10^7 CFU from −70°C stocks of *S. pneumoniae* strains were subjected to iron stress by incubation for 1 h at 37°C in 1 ml of RPMIm. From this bacterial suspension, 300-μl aliquots were added to 400 μl of RPMIm containing 1.0 μCi of $^{55}FeCl_3$(NEN) ml^{-1} and incubated at 37°C. After 5, 15, and 30 min 200-μl aliquots were removed and immediately washed by addition of 800 μl of ice-cold RPMI containing 200 μM EDDA and centrifugation at 20,000 × *g* at 4°C. The bacterial pellet was washed with a further 1 ml of ice-cold RPMI containing 200 μM EDDA and centrifugation and then resuspended in RPMI and added to 5 ml of Optisafe scintillation fluid (Wallac). The radioactivity was counted with a Beckman LS 1801 scintillation counter. The results presented represent the mean for three separate assays for each strain investigated.

In vivo studies using mouse models of *S. pneumoniae* infection. Outbred male white mice (strain CD1; Charles River Breeders) weighing from 18 to 22 g were inoculated with defrosted and appropriately diluted (in 0.9% saline) stocks of *S. pneumoniae* strains. For mixed infections, inocula consisted of approximately equivalent numbers of cells of two strains. For the pneumonia model, mice were anesthetized by inhalation of halothane (Zeneca) and a 40-μl inoculum containing between 5×10^5 and 5×10^6 bacterial CFU was administered intranasally (i.n.). For the systemic model, mice were given a 100-μl inoculum containing 10^3 bacterial CFU by intraperitoneal (i.p.) injection. Mice were sacrificed after 24 h (i.p. inoculations) or 48 h (i.n. inoculations), and target organs were recovered and homogenized in 0.5 ml of 0.9% saline. Dilutions of the homogenized organs were plated on nonselective and selective medium for calculation of the competitive index (CI), defined as the ratio of mutant to wild-type strain recovered from the mice divided by the ratio of mutant to wild-type strain in the inoculum (3).

Statistical analysis. Results for ODs, streptonigrin sensitivity, and ^{55}Fe uptake were compared by two-sample unpaired Student's *t* tests. CIs were compared by two-sided Student's *t* test.

RESULTS

Identification and sequence analysis of *pitABCD*. Using the genome analysis and annotation website Pedant (http://pedant.mips.biochem.mpg.de/), we identified three operons, each comprising four genes, encoding probable iron uptake ABC transporters in the genome of the type 4 capsular serotype *S. pneumoniae* strain sequenced by TIGR. This genome sequence has recently been published, and the annotation describes these three operons as the only genetic loci encoding proteins similar to known bacterial iron transporters (41). Two of these operons, *pia* (SP1869-1872) and *piu* (SP1032-1035), encode the two previously described *S. pneumoniae* iron transporters (5). The third operon we have designated *pit* (pneumococcal iron transporter), and it is 3,551 bp in length, corresponding to ORFs SP0243 (*pitA*), SP0242 (*pitD*), and SP0241 (*pitB* and *pitC*) of the TIGR serotype 4 annotated genome (Fig. 1A).

The published amino acid sequence for the TIGR serotype 4 *S. pneumoniae* ORF SP0241 is interrupted by a stop codon (TGA) after 141 residues (41), and yet the BLAST alignment of PitB to BitE continues 3′ to this stop codon for a further 47 residues. Hence, in the TIGR strain PitB appears to be translated as a shortened protein, or the stop codon could have been introduced due to a sequencing error. To analyze this further, we determined the nucleotide sequence of a PCR fragment (amplified with primers Sit3.5 and Sit3.6) containing the 3′ end of *pitB* from the capsular serotype 2 *S. pneumoniae* strain D39. This sequence contains a G instead of a T as the initial base of the stop codon at residue 141, converting it to a glycine codon. As a result the two smaller ORFs are fused to form a single *pitB* ORF of 208 amino acid residues. Confirming our result, the recently published sequence of a laboratory strain originally derived from strain D39, R6, also encodes a glycine at this position and has a full-length *pitB* ORF (21). Finally, PCR with cDNA derived from the serotype 3 *S. pneumoniae* strain 0100993 used in this work as the template and the primers Sit3.5-Sit3.6 amplified a product spanning the position of the TIGR serotype 4 stop codon at residue 142, confirming that this region is transcribed in strain 0100993 (Fig. 1B).

Sequence similarity searches with BLAST of the derived amino acid sequences of the *pit* operon genes predict that one gene encodes a lipoprotein iron receptor (PitA, 339 residues), one gene encodes an ATPase (PitD, 363 residues), and two genes encode transmembrane permease proteins (PitB, 208 residues, and PitC, 274 residues). The closest homologs for these proteins are putative iron transporter proteins encoded by the *bit* locus of *Brachyspira hyodysenteriae* (13) and the product of an ORF present in the recently published *Streptococcus pyogenes* genome (14) (Table 2). PitA may contain an atypical lipoprotein peptidase cleavage site signal sequence (residues 8 to 12), but this is not recognized by the SignalP bioinformatics program (39); PitD contains Walker A (residues 36 to 47) and B (residues 154 to 161) motifs and the ABC signature characteristic of ATPases (residues 134 to 137) (27), and PitB and PitC both contain sequences matching the permease EAA motif (residues 175 to 194 and 241 to 260, respectively) (24). The *pitADBC* operon is flanked at the 5′ end by an ORF encoding a protein of unknown function and at the 3′ end by an ORF encoding a protein with 34% identity to a probable pyruvate formate-lyase-activating enzyme of *Archaeoglobus fulgidus*. In contrast to the *pia* operon, which is contained within a pathogenicity island (5), the G+C content of the *pit* operon is 38.2%, similar to the overall G+C content of the *S. pneumoniae* chromosome (38.9%) (5, 41), and searches of the region adjacent to the *pit* genes did not identify any genetic mobility genes. PCR with primers IT3.1 and IT3.2 amplified an internal portion of *pitA* from all *S. pneumoniae* strains investigated (11 strains representing 10 different serotypes) (data not shown), demonstrating that *pit* is widely distributed within *S. pneumoniae* strains. PitA and the two previously described lipoprotein iron receptors of *S. pneumoniae*, PiuA and PiaA, have no significant similarity when aligned with the BLAST 2 sequence program. Comparison of PiuA, PiaA, and PitA with closely related amino acid sequences

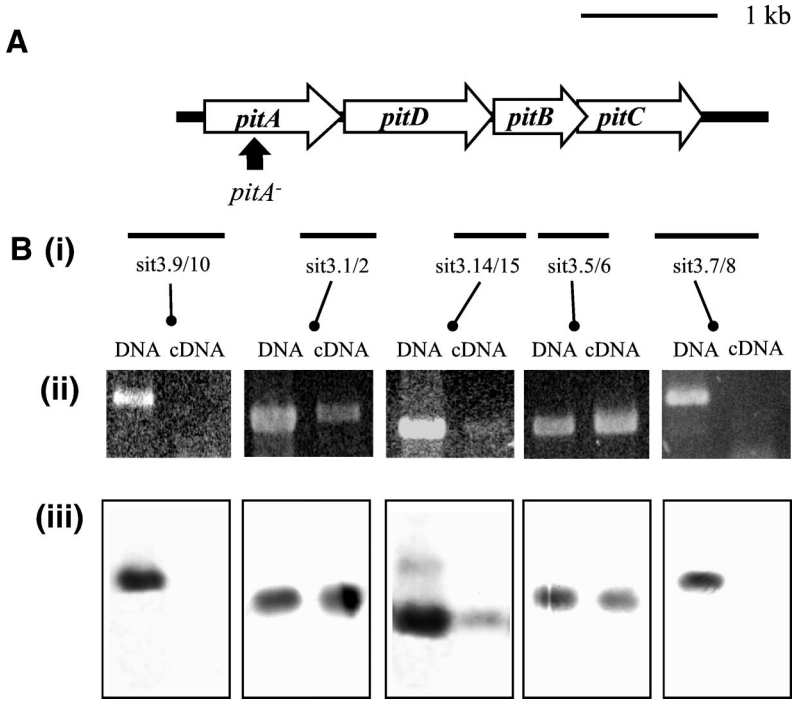

FIG. 1. (A) Genetic organization of the *pit* locus. Thick black line, chromosomal DNA; open boxes, *pit* ORFs (A, putative iron-binding lipoprotein receptor gene; B and C, putative transmembrane genes; D, putative ATPase gene); arrows, site of insertion in mutant strains. (B) Transcriptional analysis of the *pit* locus. Ethidium bromide-stained agarose gels containing products with the same primer pairs for PCR with *S. pneumoniae* chromosomal DNA as a template (on the left) and RT-PCR with *S. pneumoniae* RNA as the template (on the right) are shown in subpanel ii. RT-PCR mixtures containing no reverse transcriptase generated no products. Bars marked in subpanel i represent the corresponding target products for each pair of primers used. Boxes in subpanel iii show the results of Southern hybridizations of the corresponding PCR products when probed with a product representing the whole *pit* locus.

(identified by BLAST searches of finished and unfinished microbial genomes) indicates that these three proteins belong to distinct subgroups of lipoprotein iron receptors, as shown by the cladogram presented in Fig. 2. PitA is clustered with proteins predicted from the *Streptococcus equi* and *S. pyogenes* genome sequences, as well as BitA of *B. hyodysenteriae*.

In order to analyze the function of Pit, strains containing disrupted copies of *pitA* were constructed by insertional mutagenesis in wild-type and *piuB*, *piaA*, and *piuB*/*piaA* mutant backgrounds. Strains containing insertions in *piuB*, *piaA*, and both *piuB* and *piaA* have been described previously (5). Mutations were confirmed by Southern hybridization and by PCR (data not shown).

TABLE 2. Proteins to which PitADBC have close identity and similarity

Pit protein	Protein name or accession no.	Function	Organism	% Identity/ similarity	Length of amino acids compared
PitA	AE006550	Putative iron uptake lipoprotein	*Streptococcus pyogenes*	52/73	323
	BitA	Putative iron uptake lipoprotein	*B. hyodysenteriae*	42/63	306
	BitB	Putative iron uptake lipoprotein	*B. hyodysenteriae*	42/60	306
	BitC	Putative iron uptake lipoprotein	*B. hyodysenteriae*	42/61	306
	AfuA	Putative iron uptake lipoprotein	*Actinobacillus pleuropneumoniae*	30/46	316
	AfuA	Putative iron uptake lipoprotein	*Haemophilus influenzae*	28/48	284
PitD	BitD	ATPase	*B. hyodysenteriae*	51/68	366
	MalK	ATPase	*Vibrio cholerae*	38/59	359
	PotA	ATPase	*Borrelia burgdorferi*	42/65	294
PitB	BitE	Transmembrane protein	*B. hyodysenteriae*	47/68	191
	HitB	Transmembrane protein	*Haemophilus influenzae*	26/44	166
	AfuB	Transmembrane protein	*Actinobacillus pleuropneumoniae*	25/42	181
PitC	BitF	Transmembrane protein	*B. hyodysenteriae*	48/74	189
	AfuB	Transmembrane protein	*Haemophilus influenzae*	31/51	188
	AfuB	Transmembrane protein	*Actinobacillus pleuropneumoniae*	31/51	188

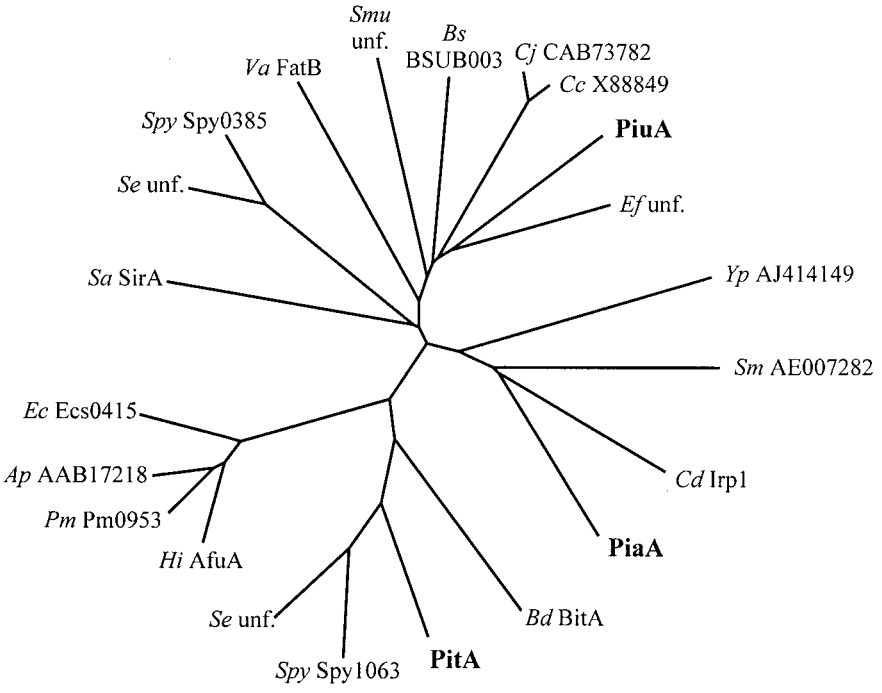

FIG. 2. Cladogram showing the relationship of PitA, PiuA, and PiaA to each other and their close homologs in the finished and unfinished bacterial genomes (marked by their name if published or accession or annotated genome number if not). unf., unfinished genome data; *Ap*, *Actinobacillus pleuropneumoniae*; *Bd*, *B. hyodysenteriae*; *Bs*, *Bacillus subtilis*; *Cc*, *Campylobacter coli*; *Cj*, *Campylobacter jejuni*; *Cd*, *C. diphtheriae*; *Ef*, *Enterococcus faecalis*; *Ec*, *E. coli*; *Hi*, *Haemophilus influenzae*; *Pm*, *Pasteurella multocida*; *Se*, *S. equi*; *Sa*, *S. aureus*; *Sm*, *Sinorhizobium meliloti*; *Smu*, *Streptococcus mutans*; *Spy*, *S. pyogenes*; *Va*, *Vibrio anguillarum*; *Yp*, *Y. pestis*.

Transcriptional analysis of *pitADBC*. The *pitD* ORF starts 15 bp after the stop codon of *pitA*, *pitB* overlaps *pitD* by 4 bp, and *pitC* overlaps *pitB* by 8 bp, suggesting that *pitADB* and *pitC* are transcribed as a single operon. In addition, there is a hairpin loop 216 bp 3′ to *pitC* (AAAAACAGCCGAAAGGA GTGCCCTCGGCTGTTTTT; ΔG, -17.0 kcal mol^{-1}) but none 3′ to the other *pit* genes. In order to confirm that *pitADBC* are transcribed as a single operon, the mRNA structure of the *pit* genes was analyzed by RT-PCR with combinations of primers designed to amplify across the junctions of the *pitADBC* ORFs. Control reactions containing heat-killed reverse transcriptase did not generate any products, demonstrating that there was no DNA contamination of the RNA samples. As expected, RT-PCRs across the *pitA*/*pitD*, *pitD*/*pitB*, and *pitB*/*pitC* junctions gave products identical in size to those of the positive control reactions with the same primers and genomic DNA as the template (Fig. 1B). For reasons which are unclear, RT-PCRs with three different pairs of primers designed to amplify the junction of *pitD* and *pitB* consistently resulted in only a low yield of product (results for one pair of primers presented in Fig. 1B). However, Southern hybridization with a probe made from a PCR product containing all four *pit* genes (amplified with primers IT3.10 and IT3.11 and genomic DNA as the template) demonstrated that the RT-PCR products, including those for the *pitD*/*pitB* junction, represented amplified portions of the *pit* transcript (Fig. 1B). RT-PCR with primers which bind 672 bp 5′ or 519 bp 3′ to the *pit* locus matched with a primer within *pitA* or *pitC*, respectively, resulted in no product (Fig. 1B), confirming that the *pit* genes are not transcriptionally linked to genes 5′ or 3′ to the *pit* locus. Hence, a phenotype exhibited by an insertional mutation of *pitA* should be due to disruption of the *pit* operon alone and should not affect transcription of genes 3′ to the *pit* locus.

Effect of *pitA* mutations on growth in iron-deficient medium. Growth of *pitA* mutant strains was compared to that of their parent strains (wild type and *piuB*/*piaA* mutant) in an undefined complete medium, THY (iron content, 0.78 ppm ± standard deviation [SD] of 0.01), and in three iron-deficient media: Chelex-THY (THY which has been treated with Chelex-100 to remove cations; iron content, 0.17 ppm ± SD of 0.01), THY containing EDDA (a cation-chelating agent with relative specificity for iron), and RPMIm (a defined medium based on RPMI containing no added iron; iron content, 0.30 ppm ± SD of 0.01). As previously described for the *piuB* and *piaA* single mutant strains, growth of the *pitA* mutant strain was not significantly different from that of the wild-type strain in THY, RPMIm, or Chelex-THY (Fig. 3A and D and data not shown). However, growth of the *pitA* mutant strain in THY containing 200 μM EDDA was delayed and reached a lower maximum OD than did the wild-type strain (maximum OD_{580} for the wild-type strain was 0.54 ± SD of 0.03 and for the *pitA* mutant strain was 0.37 ± SD of 0.05, $P = 0.02$) (Fig. 3B). The impaired growth of the *pitA* mutant strain relative to the wild-type strain in THY-EDDA medium was partially restored by supplementing the medium with 50 μM $FeCl_3$ (maximum OD_{580} for the wild-type strain was 0.77 ± SD of 0.04 and for the *pitA* mutant strain was 0.69 ± SD of 0.04, $P = 0.07$). The results of growth of the wild-type and *pitA* mutant strains in THY-EDDA me-

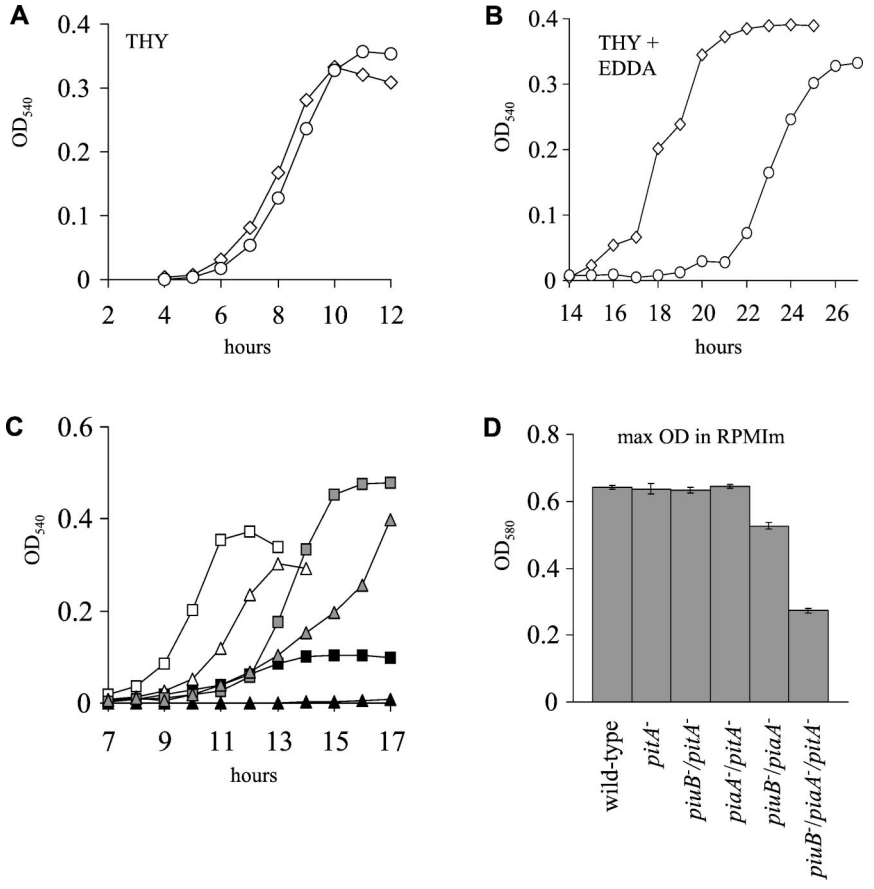

FIG. 3. (A and B) Growth curves as measured by OD of the *pitA* mutant (circles) and wild-type (diamonds) strains in THY (A) and in THY plus 200 μM EDDA (B). (C) Growth curves as measured by OD of the *piuB*/*piaA* (squares) and the *piuB*/*piaA*/*pitA* (triangles) mutant strains in THY (open symbols), Chelex-THY (black symbols), and Chelex-THY–50 μm $FeCl_3$ (grey symbols). Results for panels A to C are representative curves from experiments performed three times. (D) Maximum OD during growth in RPMIm broth for *pitA* mutant strains and strains containing double mutations in two of the three genes *piuB*, *piaA*, and *pitA* compared to the wild-type strain. Results are the means of three separate samples, and the bars represent SDs. For the difference in maximum OD between the *piuB*/*piaA* and the *piuB*/*piaA*/*pitA* mutant strains, P was <0.001.

dium when supplemented with $MnCl_2$ or $ZnCl_2$ were variable and inconsistent (data not shown).

To investigate further whether *pit* encodes an iron transporter, we examined the additive effects of mutation of *pitA* on the in vitro growth phenotype of a strain already containing mutations in both the *piu* and *pia* loci. Addition of a mutation in *pit* to the double mutant *piuB*/*piaA* strain increased the growth defect of this strain in the iron-deficient media Chelex-THY and RPMIm (Fig. 3C and D). Growth of the triple mutant strain in Chelex-THY was particularly poor but was partially restored by the addition of $FeCl_3$ to the medium (Fig. 3C). In addition, this strain consistently reached a lower maximum OD_{580} in the complete medium THY than did the wild-type and *piuB*/*piaA* double mutant strains (Fig 3C). The relative contribution of each ABC transporter system to the growth of *S. pneumoniae* in iron-deficient medium was investigated by comparison of the maximum ODs obtained in RPMIm for the three strains containing each double mutation combination. Of the three double mutation strains, only the growth of the *piuB*/*piaA* mutant strain had a lower maximum OD than did the wild-type strain in RPMIm (Fig. 3), indicating that the presence of either a functioning Piu or a functioning Pia is sufficient for growth under these conditions.

Effect of *pitA* mutations on streptonigrin sensitivity. The antibiotic streptonigrin requires intracellular iron for its bactericidal effect. Hence, the degree of susceptibility to streptonigrin is a sensitive measure of intracellular levels of iron and can be used to identify bacterial strains with a reduced ability to acquire environmental iron (4, 34). The sensitivity to streptonigrin of the *pitA* single mutant strain was compared to those of the wild-type and *piuB* and *piaA* mutant strains by measuring the zone of growth inhibition surrounding an antibiotic disk impregnated with 5 μg of streptonigrin on an RPMIm plate with and without supplementation with the iron chelator DIP. The *pitA* mutant strain had increased resistance to streptonigrin compared to the wild-type strain, indicating that this strain has a lower level of intracellular iron than does the wild-type strain (Fig. 4A). The *piuB* and *pitA* mutant strains had similar levels of resistance to streptonigrin, but the *piaA* mutant strain was markedly more resistant to streptonigrin when grown on RPMIm containing DIP than either the *piuB* or *pitA* mutant strains, suggesting that under these conditions Pia is the dominant iron transporter (Fig. 4A). The addition of a mutation in *pit* to a *piuB*/*piaA* mutant strain resulted in increased resistance to high-dose (20-μg) streptonigrin disks, providing further ev-

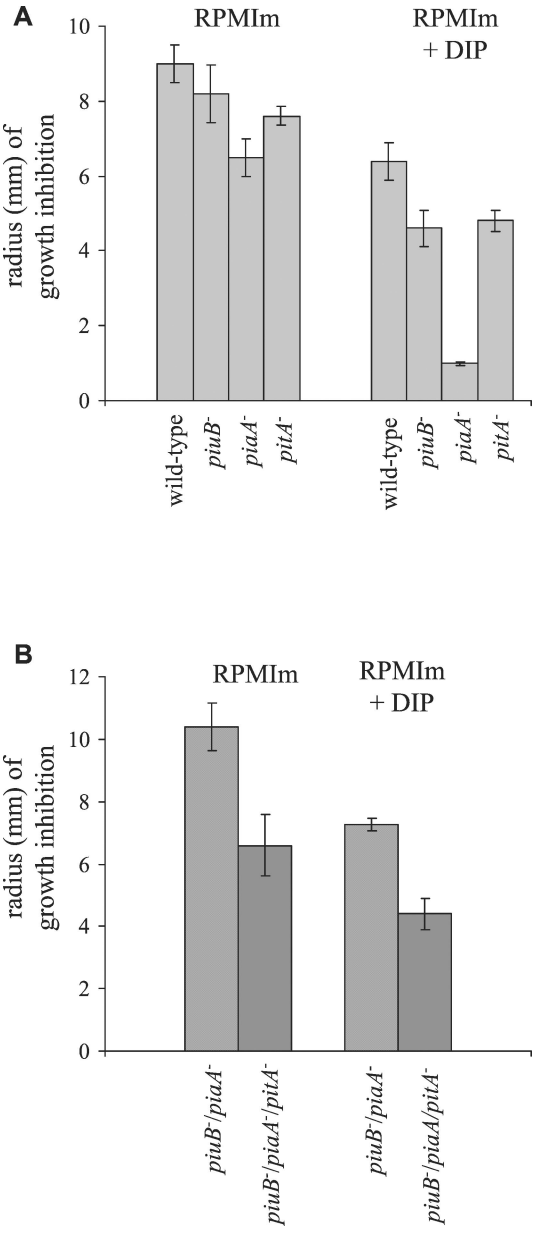

FIG. 4. Sensitivity to streptonigrin of the *pitA* mutant strains. Results are expressed as the radius of growth inhibition (millimeters) surrounding an antibiotic disk impregnated with streptonigrin. (A) Comparison of the results with 5-μg streptonigrin disks for the *piuB*, *piaA*, and *pitA* single mutant strains cultured on RPMIm plates and on RPMIm plates containing 400 μM DIP. For the *pitA* mutant strain versus the wild-type strain, *P* was <0.001 (on RPMIm) and 0.001 (on RPMIm plus DIP); for the *piaA* mutant strain versus the *piuB* or *pitA* mutant strain, *P* was <0.05 (on RPMIm) and <0.001 (on RPMIm plus DIP). (B) Comparison of the results with 20-μg streptonigrin disks for the *piuB*/*piaA*/*pitA* triple mutant strain and the *piuB*/*piaA* double mutant strain cultured on RPMIm plates and on RPMIm plates containing 400 μM DIP. For the *piuB*/*piaA* mutant strain versus the *piuB*/*piaA*/*pitA* mutant strain, *P* was 0.09 (on RPMIm) and <0.001 (on RPMIm plus DIP). Error bars represent the SDs.

idence that *pit* is required for acquisition of intracellular iron by *S. pneumoniae* (Fig. 4B).

$^{55}FeCl_3$ uptake by *pitA* mutant strains. The effects of disruption of the *pit* locus on iron acquisition were measured

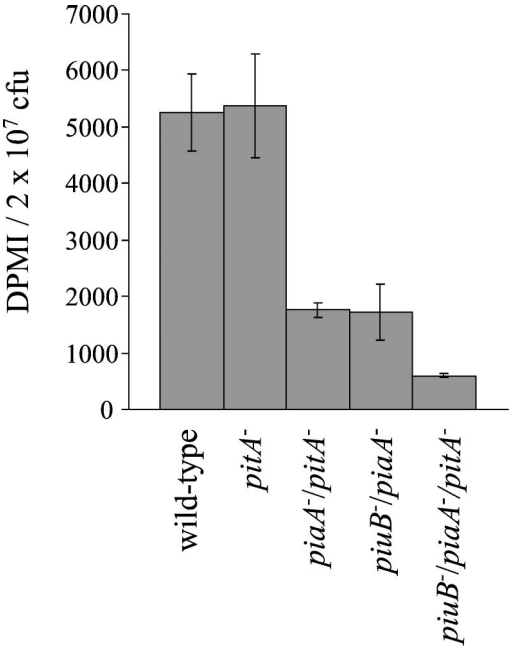

FIG. 5. Comparison of the $^{55}FeCl_3$ content after 30 min of incubation for the wild-type, *pitA* mutant, *piaA*/*pitA* and *piuB*/*piaA* double mutant, and *piuB*/*piaA*/*pitA* triple mutant strains. Mean data from three assays per strain are presented and normalized for bacterial CFU. For the differences between the wild-type strain and the *pitA* mutant strain, *P* was 0.9; for the differences between the wild-type strain and the *piaA*/*pitA*, *piuB*/*piaA*, and the *piuB*/*piaA*/*pitA* mutant strains, *P* was ≤0.001; for the differences between the *piuB*/*piaA* mutant strain and the *piuB*/*piaA*/*pitA* mutant strain, *P* was 0.02. Error bars represent the SDs.

directly with an $^{55}FeCl_3$ uptake assay. After 30 min of incubation in the presence of ^{55}Fe, the accumulation of ^{55}Fe by the *pitA* single mutant strain was no different from that of the wild-type strain, a result similar to that described for the *piuB* and *piaA* single mutant strains (5) (Fig. 5). The *piuB*/*piaA* mutant strain has previously been demonstrated to have a strongly reduced rate of ^{55}Fe accumulation compared to that of the wild-type strain (5), and these results were reconfirmed. In addition, the *piaA*/*pitA* mutant strain had a much reduced ^{55}Fe content after 30 min compared to the wild-type strain, similar to the ^{55}Fe content of the *piuB*/*piaA* mutant strain. The ^{55}Fe content of the triple mutant strain was much reduced compared to those of all the other strains: between 5 and 30 min the ^{55}Fe content of the triple mutant strain increased by only 56%, compared to 544% for the *piuB*/*piaA* mutant strain and 1,600% for the wild-type strain (Fig. 5). These results confirm that Pit is required for iron acquisition, at least in the absence of Piu and Pia.

Effects of the *pitA* mutation on virulence. In order to assess the importance during in vivo growth of Pit, we investigated the virulence of the *pitA* single mutant strain and the *piuB*/*pitA* and *piaA*/*pitA* mutant strains by using mixed infections and determining the CI (Table 3). The *pitA* mutant strain was no less virulent than the wild-type strain for pulmonary infection but was less virulent than the wild-type strain during systemic infection (CI of 0.42), indicating that during in vivo growth iron acquisition by Pit is important for systemic but not intrapulmonary growth (Table 3). This level of attenuation would

TABLE 3. Virulence in mice of *pitA* mutant strains when assessed by mixed infection against the wild-type strain

Mutant strain used against wild type	Inoculation route	CI (±SD)	*n*	*P* value
pitA	i.n.	1.0 (1.1)	4	0.52[a]
pitA	i.p.	0.42 (0.2)	5	0.001[b]
piuB	i.p.	1.13 (0.34)	9	—[c]
piaA	i.p.	0.28 (0.11)	12	—[c]
piuB/*pitA*	i.p.	0.39 (0.1)	9	0.001[b]
piaA/*pitA*	i.p.	0.45 (0.2)	5	0.003[b]
piuB/*piaA*	i.p.	< 0.001	4	—[c]

[a] Compared to 1.0, the expected CI for a strain with the same virulence as the wild-type strain.
[b] Compared to 1.2 (SD of 0.2), the CI for a strain containing a mutation in SP1429 (TIGR genome annotation) which is not attenuated after i.p. inoculation.
[c] —, previously reported data (5).

probably not be detectable by comparison of survival curves for mice given a pure inoculum of the *pitA* mutant strain or the wild-type strain (5). In contrast to the strain containing mutations in both *piuB* and *piaA*, which had CIs of <0.001 in models of systemic infection and pneumonia (5), combining mutations of *pitA* with those of either *piuB* or *piaA* had no additive effect on virulence attenuation during systemic infection (Table 3). The strong attenuation of virulence caused by loss of both *piuB* and *piaA* precluded investigation of any additional attenuation conferred by the *pitA* mutation in the triple mutant strain.

Relative abundance of *piu*, *pia*, and *pit* RNA transcripts. The varying importance of the Piu, Pia, and Pit iron uptake ABC transporters for in vitro and in vivo growth may be due to different levels of expression of their corresponding genes. We therefore assessed the level of expression of the *piuB*, *piaA*, and *pitA* genes (the first genes of each operon) by using RT-PCR and primers for the internal portions of each gene (Smt6.1-Smt6.2, IRP1-IRP2, and IT3.5-IT3.6, respectively) (5). To ensure that PCRs with these primers were of approximately equal efficiency, the quantity of specific PCR product for each primer pair was compared after 12, 16, 20, and 24 PCR cycles with equal quantities of DNA as the template. Each of the three primer pairs resulted in a PCR product of approximately equal intensity when assessed by ethidium bromide staining of an agarose gel (Fig. 6A). However, when cDNA made from RNA extracted from the wild-type strain grown in Chelex-THY to an OD of 0.2 (mid-log growth phase) was used as the target, a strong product was consistently obtained for *piaA* but only low quantities of products were obtained for *piuB* and *pitA* (Fig. 6B). Similar results were obtained with cDNA made from three different RNA preparations extracted from bacteria grown in Chelex-THY and from RNA preparations extracted from wild-type bacteria grown in THY to an OD of 0.5 as the template (data not shown). These results show that *pia* mRNA transcripts are present at a higher level than are *piu* and *pit* mRNA transcripts and provide a partial explanation for the dominance of the Pia iron transporter in the conditions that we have investigated.

DISCUSSION

We have previously described two *S. pneumoniae* four-gene operons, *piu* and *pia*, which encode iron uptake ABC transporters (5). In this paper we have characterized a third four-gene operon, *pit*, encoding proteins which are similar to components of iron uptake ABC transporters. Redundancy of iron transporters within a species has often hampered investigation of a particular iron transporter's function, with single mutations affecting an iron transporter frequently resulting in no discernible in vitro or in vivo phenotype (5, 16, 31). Hence, it is not surprising that the *pitA* single mutant strain had no growth defect in RPMIm and Chelex-THY and a similar rate of ^{55}Fe uptake as the wild-type parental strain. However, this mutant strain did have delayed growth in medium containing a cation chelator (possibly a more stringent test of iron transporter function than Chelex-THY medium, as the iron chelator can compete with bacterial iron uptake mechanisms for the available iron during bacterial growth) and increased resis-

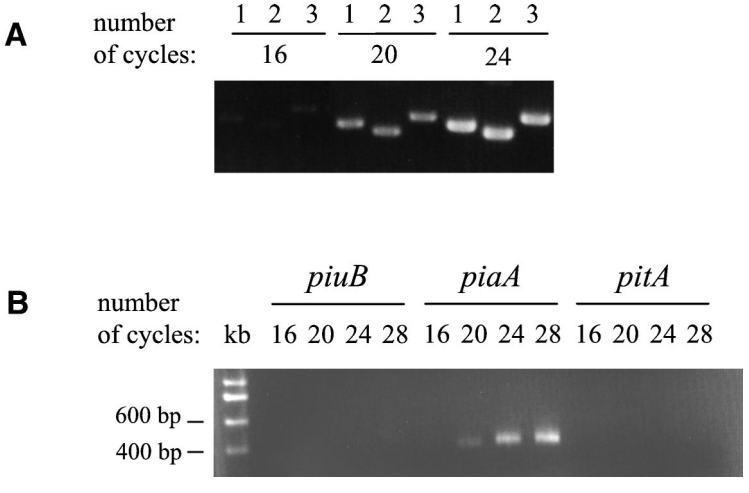

FIG. 6. Relative abundance of *piu*, *pia*, and *pit* mRNA transcripts. (A) Efficiency of PCRs for *piuB* (lanes 1), *piaA* (lanes 2), and *pitA* (lanes 3) with DNA as the template. Product quantity was assessed by running 8 μl of product after 16, 20, and 24 cycles on a 1.5% agarose gel and staining the product with ethidium bromide. (B) PCRs for *piuB*, *piaA*, and *pitA* with cDNA made from RNA obtained from wild-type bacteria grown in Chelex-THY. Product quantity was assessed by running 8 μl of product after 16, 20, 24, and 28 cycles on a 1.5% agarose gel and staining the product with ethidium bromide. The kilobase ladder is presented in the lane marked kb.

tance to the iron-dependent antibiotic streptonigrin, indicating that the *pit* operon also encodes an iron transporter. Furthermore, addition of a *pitA* mutation to a strain already carrying mutations in both *piu* and *pia* further impaired this strain's ability to grow in two varieties of iron-depleted media, increased its resistance to streptonigrin, and reduced its rate of $^{55}FeCl_3$ uptake. These results confirm that the *S. pneumoniae* *pit* locus is involved in iron acquisition and provide further evidence that the *pit* homologs in *B. hyodysenteriae* and *S. pyogenes* also encode iron transporters (13, 14). Iron uptake transporters, including *pia*, are frequently encoded on horizontally acquired regions of genomic DNA called either genomic islands or, if important for virulence, pathogenicity islands (5, 17, 18, 22, 45). However, the region of DNA including and surrounding the *pit* locus has none of the features of a genomic island, such as a G+C content different from the average for the whole genome, the presence of genetic mobility genes, or variable distribution among strains of the same species (18), and there is no evidence to suggest that *pit* was horizontally acquired by *S. pneumoniae*.

Multiple iron transporters with different substrates are well described for gram-negative bacteria. For example, the genome of *Y. pestis* contains seven separate iron uptake operons in addition to those that have been previously reported (1, 2, 16, 32), and *Neisseria* species have mechanisms for acquiring iron from transferrin and heme compounds as well as producing siderophores (37). The iron acquisition systems of gram-positive pathogens are less well described but include ABC transporters involved in Fe^{3+} uptake by *S. pyogenes* (23), siderophore uptake by *Staphylococcus aureus* (7, 20, 31), and hemin uptake by *Corynebacterium diphtheriae* (12), siderophores produced by mycobacteria (10, 11), and a transferrin receptor of *S. aureus* (30). In this and our previous study (5) we have now identified a total of three separate and seemingly unrelated *S. pneumoniae* iron uptake ABC transporters, *piu*, *pia*, and *pit*. In the published TIGR *S. pneumoniae* serotype 4 strain genome sequence these three ABC transporters are the only genetic loci with similarity to known iron uptake transporters (41). The reduced ability of the triple mutant strain to grow in a variety of cation-depleted media and its low rate of ^{55}Fe uptake provide additional evidence that these three ABC transporters account for the iron uptake mechanisms of *S. pneumoniae*. Loss of the majority of its iron uptake transporters might also explain why the *piaB*/*piuA*/*pitA* triple mutant strain has a growth defect even in THY, a medium which is relatively iron replete. However, there may be other *S. pneumoniae* genes whose products are involved in iron acquisition, either through the Piu, Pia, or Pit transporters (possibly acting as intermediates between the substrate and the ABC transporter lipoprotein) or by a separate mechanism, but which have no homology to known iron uptake proteins.

In general, the relative contribution to growth and for virulence of the various iron transporters present in gram-positive pathogens is unclear. By comparing the phenotypes of the *pitA*, *piuB*, and *piaA* mutant strains, we have been able to assess the relative importance of each *S. pneumoniae* iron transporter during in vitro and in vivo growth. Three lines of evidence suggest that Piu and especially Pia are the most important *S. pneumoniae* iron transporters for iron acquisition during both in vitro and in vivo growth: (i) the only double iron transporter mutant strain with impaired growth in RPMIm is the *piuB*/*piaA* mutant strain, (ii) in the presence of the chelator DIP the *piaA* mutant strain has a markedly lower sensitivity to streptonigrin than do the *piuB* and *pitA* mutant strains, and (iii) *piaA* mutant strains have the highest degree of attenuation in virulence in mouse models of nasal and systemic infection (5). Furthermore, the three combinations of double mutations in these genes have strikingly different effects on virulence. Combining a mutation in *pit* with a mutation in either *piu* or *pia* had no additive effect on virulence attenuation when assessed by mixed infection experiments, one of the most sensitive methods available for identifying subtle differences in virulence (3, 5). However, combining disruption of *piu* with disruption of *pia* causes a dramatic reduction in *S. pneumoniae* virulence in mouse models of pneumonia and systemic infection (5). Taken together, these results show that, if either the Piu or Pia iron transporter is present, then Pit has only a relatively small role during growth in vivo, a finding which is consistent with the in vitro growth data. The variable importance of different iron transporters for growth and virulence has previously been described for *Y. pestis* and is likely to occur in other pathogens containing multiple iron acquisition systems (2, 15, 16). Analysis of the relative abundance of *piu*, *pia*, and *pit* RNA transcripts demonstrated that in vitro there are abundant quantities of *pia*, but not *piu* or *pit*, RNA, hence providing one explanation of why Pia is the dominant iron transporter. Further investigation is required to explain why there are such marked differences in the roles of the *S. pneumoniae* iron uptake ABC transporters, including identification of their substrates and control of their expression in vivo.

Identification of *pit* and the demonstration that it encodes an iron transporter provide further evidence that iron acquisition is important for growth in vitro and in vivo of gram-positive as well as gram-negative pathogens. Defining the genes whose products allow *S. pneumoniae* and other pathogens to acquire micronutrients in vivo will improve our understanding of how microbial pathogens can grow within internal organs and cause disease and may identify potential candidates for new vaccine antigens (6) or novel targets for antimicrobial therapy. Detailed biochemical characterization of Pit, Piu, and Pia function is now required in order to identify their substrates and the exact mechanisms by which iron is transferred into the cell.

ACKNOWLEDGMENTS

We are grateful to Stephen Bowyer for performing the flame atomic absorption spectroscopy.

This work was supported by a Wellcome Trust Advanced Research Fellow grant awarded to J. S. Brown.

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Editor: E. I. Tuomanen

INFECTION AND IMMUNITY, Nov. 2004, p. 6754 Vol. 72, No. 11
0019-9567/04/$08.00+0 DOI: 10.1128/IAI.72.11.6754.2004

ERRATUM

Characterization of Pit, a *Streptococcus pneumoniae* Iron Uptake ABC Transporter

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Volume 70, no. 8, p.4389–4398, 2002. Page 4391, column 1, lines 4 and 5 from bottom: "Two of these operons, *pia* (SP1869–1872) and *piu* (SP1032–1035)" should read "Two of these operons, *piu* (SP1869–1872) and *pia* (SP1032–1035)."