Screening of *Streptococcus pneumoniae* ABC Transporter Mutants Demonstrates that LivJHMGF, a Branched-Chain Amino Acid ABC Transporter, Is Necessary for Disease Pathogenesis


Updated information and services can be found at: http://iai.asm.org/content/77/8/3412

**REFERENCES**

This article cites 43 articles, 25 of which can be accessed free at: http://iai.asm.org/content/77/8/3412#ref-list-1

**CONTENT ALERTS**

Receive: RSS Feeds, eTOCs, free email alerts (when new articles cite this article), more»

Information about commercial reprint orders: http://journals.asm.org/site/misc/reprints.xhtml
To subscribe to another ASM Journal go to: http://journals.asm.org/site/subscriptions/
Screening of *Streptococcus pneumoniae* ABC Transporter Mutants Demonstrates that LivJHMGF, a Branched-Chain Amino Acid ABC Transporter, Is Necessary for Disease Pathogenesis^7^

Shilpa Basavanna,1 Suneeta Khandavilli,1 Jose Yuste,1 Jonathan M. Cohen,1,2 Arthur H. F. Hosie,3 Alexander J. Webb,3 Gavin H. Thomas,4 and Jeremy S. Brown1^*

Centre for Respiratory Research, Department of Medicine, Royal Free and University College Medical School, Rayne Institute, London WC1E 6JJ, United Kingdom; Infectious Diseases and Microbiology Unit, Institute of Child Health, University College London, 30 Guilford Street, London WC1N 1EH, United Kingdom; Department of Microbiology, King’s College London Dental Institute, Guy’s Campus, London SE1 9RT, United Kingdom; and Department of Biology (Area 10), University of York, P.O. Box 373, YO10 5YW York, United Kingdom

Received 19 December 2008/Returned for modification 28 January 2009/Accepted 5 May 2009

Bacterial ABC transporters are an important class of transmembrane transporters that have a wide variety of substrates and are important for the virulence of several bacterial pathogens, including *Streptococcus pneumoniae*. However, many *S. pneumoniae* ABC transporters have yet to be investigated for their role in virulence. Using insertional duplication mutagenesis mutants, we investigated the effects on virulence and in vitro growth of disruption of 9 *S. pneumoniae* ABC transporters. Several were partially attenuated in virulence compared to the wild-type parental strain in mouse models of infection. For one ABC transporter, required for full virulence and termed LivJHMGF due to its similarity to branched-chain amino acid (BCAA) transporters, a deletion mutant (ΔlivHMGF) was constructed to investigate its phenotype in more detail. When tested by competitive infection, the ΔlivHMGF strain had reduced virulence in models of both pneumonia and septicemia but was fully virulent when tested using noncompetitive experiments. The ΔlivHMGF strain had no detectable growth defect in defined or complete laboratory media. Recombinant LivJ, the substrate binding component of the LivJHMGF, was shown by both radioactive binding experiments and tryptophan fluorescence spectroscopy to specifically bind to leucine, isoleucine, and valine, confirming that the LivJHMGF substrates are BCAAs. These data demonstrate a previously unsuspected role for BCAA transport during infection for *S. pneumoniae* and provide more evidence that functioning ABC transporters are required for the full virulence of bacterial pathogens.

Bacterial ABC transporters are an important class of transmembrane transporters that are involved in the import and export of a wide variety of substrates, including sugars, amino acids, peptides, polyamines, and cations (11, 12, 17, 39). A typical ABC transporter consists of four membrane-associated proteins consisting of two ATP-binding proteins (ATPases) and two membrane-spanning proteins (permeases) (11, 17). These may be fused in a variety of ways to form multidomain polypeptides, but typically permeases consist of six putative α-helical transmembrane segments that act as a channel through which substrates are transported across the membrane (11, 17). ABC transporters that import their substrate also contain a substrate-binding protein (SBP) that is present in the periplasm of gram-negative bacteria and most often as a lipid-protein bound to the outer surface of the membrane in gram-positive bacteria (17). These SBPs bind to the substrate before it is transferred across the cell membrane and therefore confer substrate specificity for the ABC transporter. Approximately 5% of the *Escherichia coli* and *Bacillus subtilis* genomes encode components of ABC transporters, highlighting the importance of ABC transporters for the physiology of both gram-positive and gram-negative bacteria (12, 30). ABC transporters are known to influence many cellular processes, including antibiotic resistance, nutrient acquisition, adhesion, protein secretion, environmental sensing, spore formation, conjugation, and growth under stress conditions (39). As a consequence, many ABC transporters have been shown by signature-tagged mutagenesis (STM) screens to be important for the virulence of a range of bacterial pathogens, including *Yersinia* spp., *Staphylococcus aureus*, and *Streptococcus pneumoniae* (9, 15, 22, 26), and these data have been supported by publications on the functions of individual ABC transporters (6, 7, 38, 45).

The annotated genome sequence of the TIGR4 strain of the common gram-positive pathogen *S. pneumoniae* contains 73 ABC transporters (4, 13). Several ABC transporters required for substrate uptake have been described in some detail previously, and some of these are known to be important for full virulence, including the cation transporters PsaN, PiuA, PiaA, and PitA (6, 7, 27, 34) and the polyamine transporter PotABCD.

* Corresponding author. Mailing address: Centre for Respiratory Research, Department of Medicine, University College Medical School, Rayne Institute, 5 University Street, London WC1E 6JJ, United Kingdom. Phone: 44 20 7679 6008. Fax: 44 20 7679 6973. E-mail: jeremy.brown@ucl.ac.uk.

^7^ Published ahead of print on 26 May 2009.
eral ABC transporters were identified as required for full virulence of S. pneumoniae. This hypothesis was supported by the demonstration that disruption of one of these ABC transporters, termed livHMGF, during in vivo, some of the S. pneumoniae ABC transporters that have not yet been investigated are also likely to influence the pathogenesis of S. pneumoniae infections. Using mouse models of infection, we have therefore assessed the potential role during virulence of nine S. pneumoniae ABC transporters that have not, as far as we are aware, previously been investigated. Several ABC transporters were identified as required for full S. pneumoniae virulence in models of septicaemia or pneumonia. The function of one of these ABC transporters, termed livHMGF, which BLAST searches suggest is a member of the hydrophobic amino acid transporter subfamily and is likely to be a branched-chain amino acid (BCAA) transporter (40), was investigated in more detail.

**MATERIALS AND METHODS**

**Bacterial strains, media, and growth conditions.** The E. coli strains DH5α, Novablu (Novagen), JM109 (Promega), and M15 (Qiagen) were used for cloning procedures. The capsular serotype 3 S. pneumoniae strain 0100993, originally isolated from a patient with pneumonia and obtained from SmithKline Beecham, Plc. (22), was used to construct S. pneumoniae mutant strains for the majority of the in vitro and in vivo phenotype analysis, with additional experiments performed using the capsular serotype 2 strain D39 and the serotype 4 strain TIGR4. E. coli was cultured at 37°C using Luria-Bertani broth or agar plates, and S. pneumoniae strains were cultured in the presence of 5% CO2 at 37°C on Columbia agar (Oxoid) supplemented with 5% horse blood. The conditions for the second PCR were as follows: a reaction volume of 20 μl containing 3.7 μl of nucleic acid, 1 μl of primer, 1 μl of 50 mM MgSO4, 0.2 μl of Taq polymerase (Bioline), and approximately 50 ng of each PCR product with no primers and an initial denaturing step at 94°C for 2 min, followed by 10 cycles of 94°C for 20 s, 55°C for 30 s, and 72°C for 1 min, with a final extension at 72°C for 10 min. Cleaned individual PCR products were fused by using a two-step PCR, with an initial PCR to fuse the 5′-flanking DNA fragment with er, by adding of the 3′-flanking DNA. The conditions for the first PCR were as follows: reaction volume of 20 μl containing 5 μl of DNA solution, 1 μl of primer, 1 μl of 50 mM MgSO4, 0.2 μl of Taq polymerase (Bioline), and approximately 50 ng of each PCR product with no primers and an initial denaturing step at 94°C for 2 min, followed by 10 cycles of 94°C for 20 s, 55°C for 30 s, and 72°C for 1 min. The conditions for the second PCR were as follows: reaction volume of 100 μl containing 68.2 μl of nuclease-free water, 10 μl of buffer, 10 μl of 2 mM concentrations of dNTPs, 4 μl of 50 μl MgSO4, 100 pmol of primers Sp0749F and Sp0754R, and 3 μl of purified PCR product from the first PCR, and 0.8 μl of Taq polymerase and an initial denaturing step at 94°C for 2 min, followed by 35 cycles of 94°C for 20 s, 50°C for 30 s, and 72°C for 2.5 min, with a final extension at 72°C for 3 min. The fusion PCR products were then analyzed by a 1% agarose gel, and the desired DNA band was excised and purified by using Qiagen QIAquick columns and transformed into the S. pneumoniae 0100993, D39, and TIGR4 strains (22). Plasmid and mutant strains were used for cloning procedures. The capsular serotype 3 S. pneumoniae strain 0100993, originally isolated from a patient with pneumonia and obtained from SmithKline Beecham, Plc. (22), was used to construct S. pneumoniae mutant strains for the majority of the in vitro and in vivo phenotype analysis, with additional experiments performed using the capsular serotype 2 strain D39 and the serotype 4 strain TIGR4. E. coli was cultured at 37°C using Luria-Bertani broth or agar plates, and S. pneumoniae strains were cultured in the presence of 5% CO2 at 37°C on Columbia agar (Oxoid) supplemented with 5% horse blood (TCS Biosciences) or in Todd-Hewitt broth supplemented with 0.5% yeast extract (Oxoid) or CDEM medium (42). Plasmids and mutant strains were selected for using appropriate antibiotics (10 μg of chloramphenicol, 100 μg of carbenicillin, and 25 μg of kanamycin ml−1 for E. coli and 10 μg of chloramphenicol and 0.2 μg of erythromycin ml−1 for S. pneumoniae). Stocks of S. pneumoniae were stored as single-use 0.5-ml aliquots of THY broth culture (optical density at 580 nm [OD580] of 0.3 to 0.4) at −70°C in 10% glycerol. The growth of S. pneumoniae strains in broth was monitored by measuring the OD580.

**Nucleic acid manipulations and RT-PCR.** S. pneumoniae genomic DNA was extracted from bacteria grown in THY by using a modified Wizard genomic DNA kit (Promega), and RNA was extracted by using an SV total RNA extraction kit (Promega) as previously described (6). Restriction digests, ligation of DNA fragments, fractionation of DNA fragments by electrophoresis, and transformation of E. coli (by heat shock) were performed according to established protocols (33). DNA fragments were purified from electrophoresis gels by using the BigDye terminator technique and gene-specific PCR primers). The expression of one of these ABC transporters, termed livHMGF, which BLAST searches suggest is a member of the hydrophobic amino acid transporter subfamily and is likely to be a branched-chain amino acid (BCAA) transporter (40), was investigated in more detail.

**Expression and characterization of His6-LivJ lipoprotein.** The lipoprotein LivJ (Sp0749) was expressed in E. coli and purified by using an N-terminal His-tagged QIAExpressionist system (Qiagen). Primers Sp0749Fwd and Sp0749Rv amplified a full-length hvJ (excluding the 5′ portion encoding the predicted N-terminal signal peptide), which was ligated into the pQE30 expression vector to make the plasmid pQChvJ and transformed into E. coli strain M15. Protein expression was induced with IPTG (isopropyl-β-D-thiogalactopyranoside) and native His6-LivJ purified using Ni-NTA affinity columns according to the QIAexpressionist manual. Purification products were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and shown to contain a ≥95% purity of protein of the expected size for His6-LivJ, and the identity of the recombinant protein was confirmed by peptide mass fingerprinting as reported previously (35).

**14C]leucine, [14C]isoleucine, and [14C]maltose uptake and binding assays.** Radioactive uptake assays were performed by the rapid filtration method as previously described (45) with minor modifications. S. pneumoniae strains were grown in THY medium until the OD600 reached 0.5. Uptake assays were performed using a capsular serotype 2 S. pneumoniae strain (D39) since the mucoid colonies of the capsular serotype 3 strain prevented effective pelleting of the bacteria for these assays. Bacteria were harvested at 13,000 × g for 20 min and resuspended in 50 mM potassium phosphate buffer (pH 7.2) with 1 mM...
MgCl₂ to an OD₆₀₀ between 0.8 and 1.1. The uptakes of leucine, isoleucine, and maltose were determined in 1-ml assays containing 0.85 ml of bacterial cells and a final concentration of 25 μM maltose, isoleucine, or leucine containing 0.125 μCi of ¹⁴C-labeled substrate ([¹⁴C]isoleucine, [¹⁴C]leucine, and [¹⁴C]maltose; GE Healthcare, United Kingdom) determined that an OD₆₀₀ of 1 was equivalent to 15,000 cpm. The uptake was incubated with a 5 μM substrate containing 0.1 μCi of ¹³C-labeled ligand (GE Healthcare) and further incubated on ice for 10 min. To this mixture, 1 ml of saturated ammonium sulfate was added, followed by incubation on ice for 20 min, and then filtered onto glass fiber filter papers (GF/F, Whatman). The filters were then washed with 4 ml of saturated ammonium sulfate and dried for 5 min. The filters were allowed to equilibrate in Ready-Safe scintillation cocktail for 20 min, and the radioactivity was determined by using a Wallac 1214 RackBeta liquid scintillation counter. A bicinchoninic acid protein assay (Sigma (Beckman Coulter), and the radioactivity was determined by using a Wallac 1214 RackBeta liquid scintillation counter. A bicinchoninic acid protein assay (Sigma Aldrich, United Kingdom) determined that an OD₆₀₀ of 1 was equivalent to 0.258 μg of protein, and this figure was used to convert the radioactivity counts to nmol of solute per μg of protein.

For radioactive substrate binding assays, 100 μl of purified His₆-LivJ (27.5 μg) was incubated with a 5 μM substrate containing 0.1 μCi of ¹³C-labeled ligand (GE Healthcare) and further incubated on ice for 10 min. To this mixture, 1 ml of saturated ammonium sulfate was added, followed by incubation on ice for 20 min, and then filtered onto glass fiber filter papers (GF/F, Whatman). The filters were then washed with 4 ml of saturated ammonium sulfate and dried for 5 min. The filters were allowed to equilibrate in Ready-Safe scintillation cocktail for 20 min, and the radioactivity was determined by using a Wallac 1214 RackBeta liquid scintillation counter (31).

Tryptophan fluorescence spectroscopy. Purified His₆-LivJ was used for tryptophan fluorescence spectroscopy using a Hitachi F-2500 spectrofluorimeter at an excitation wavelength of 280 nm (slit width, 3 nm) and an emission wavelength of 309 nm (slit width, 3 nm) (41). The assay was performed by adding test amino acids (Sigma) dissolved in 10 mM NaH₂PO₄ to 0.5 μM His₆-LivJ in 1.5 ml of 50 mM Tris-HCl (pH 8) in a sample cuvette maintained at 25°C in the spectrofluorimeter with continuous stirring. The slit width of the spectrofluorimeter was adjusted to reduce photobleaching of the protein (41).

Animal models of infection. Infection experiments were performed in age- and sex-matched groups of outbred CD1 mice (Charles River Breeder). Between 4 to 8 weeks old. For mixed infections, equivalent numbers of bacteria from stocks of wild-type and mutant S. pneumoniae strains were mixed and diluted to the appropriate concentration. For the nasopharyngeal colonization model, 10⁶ CFU of bacteria in 10 μl were administered by intranasal (i.n.) inoculation under halothane general anesthesia, and nasal washes were obtained after 2 days. For the systemic model of infection, 10⁷ CFU of bacteria in 100 μl were inoculated by intraperitoneal (i.p.) injection, and spleen homogenates were obtained at 24 h (6, 21). For the pneumonia model, 5 x 10⁴ CFU of bacteria in 40 μl were given by i.n. inoculation under halothane general anesthesia, and lung and spleen homogenates were obtained at 48 h (8, 21). Aliquots from samples recovered from mice were plated on plain and Cm containing plates to allow calculation of a competitive index (CI). The CI was calculated as follows: the ratio of mutant to wild-type strain recovered from mice divided by the ratio of mutant to wild-type strain in the inoculum (5). A CI of <1 indicates that the mutant strain is attenuated in virulence compared to the wild-type strain, and the lower the CI the more attenuated the mutant strain. Similar experiments were performed with a pure inocula of wild-type or livHMGF strain bacteria to calculate the bacterial CFU for each strain at specific time points or to monitor the progress of infection identifying mice likely to progress to fatal disease according to previously established criteria (6).

Statistical analysis. All of the in vitro growth curves were performed in triplicates and are represented as means and standard deviations. The results of growth curves, radioactive uptake, and binding assays were analyzed by using two-tailed Student t tests. The results of survival experiments were compared by using the log-rank method, and target organ CFU levels were analyzed by using the Mann-Whitney U test. The statistical validity of the results for Cls is represented by 95% confidence intervals.

RESULTS

Selection of ABC transporters for investigation. The ABC transporters chosen for these studies were identified from the annotated genome of the capsular serotype 4 strain of S. pneumoniae (TIGR4) (40). This genome contains 24 loci consisting of three or more adjacent genes that encode putative components of ABC transporters and are likely to be transcribed as an operon, 10 of which have been previously investigated. Eleven of the previously undescribed ABC transporters

---

**TABLE 1. Strains and plasmids constructed and/or used in this study**

<table>
<thead>
<tr>
<th>Plasmids or strain</th>
<th>Description (source or reference)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pID701</td>
<td>Shuttle vector for IDM transformation of S. pneumoniae: Cm⁺ (22)</td>
</tr>
<tr>
<td>pPC110</td>
<td>pID701 containing an internal portion of Sp0090 in the XbaI site: Cm⁺ (this study)</td>
</tr>
<tr>
<td>pPC111</td>
<td>pID701 containing an internal portion of Sp0149 in the XbaI site: Cm⁺ (this study)</td>
</tr>
<tr>
<td>pPC112</td>
<td>pID701 containing an internal portion of Sp0610 in the XbaI site: Cm⁺ (this study)</td>
</tr>
<tr>
<td>pPC113</td>
<td>pID701 containing an internal portion of Sp0710 in the XbaI site: Cm⁺ (this study)</td>
</tr>
<tr>
<td>pPC114</td>
<td>pID701 containing an internal portion of Sp1796 in the XbaI site: Cm⁺ (this study)</td>
</tr>
<tr>
<td>pPC115</td>
<td>pID701 containing an internal portion of Sp1824 in the XbaI site: Cm⁺ (this study)</td>
</tr>
<tr>
<td>pPC116</td>
<td>pID701 containing an internal portion of Sp0750 in the XbaI site: Cm⁺ (this study)</td>
</tr>
<tr>
<td>pPC117</td>
<td>pID701 containing an internal portion of Sp1690 in the XbaI site: Cm⁺ (this study)</td>
</tr>
<tr>
<td>pPC118</td>
<td>pID701 containing an internal portion of Sp0846 in the XbaI site: Cm⁺ (this study)</td>
</tr>
<tr>
<td>pPC119</td>
<td>pID701 containing an internal portion of Sp2084 in the XbaI site: Cm⁺ (this study)</td>
</tr>
<tr>
<td>pPC120</td>
<td>pID701 containing an internal portion of Sp2108 in the XbaI site: Cm⁺ (this study)</td>
</tr>
<tr>
<td>pPC139</td>
<td>pQE30 carrying full length livJ: Km⁺ Amp⁺ (this study)</td>
</tr>
</tbody>
</table>

**Strains**

<table>
<thead>
<tr>
<th>Strains</th>
<th>Description (source or reference)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0100993</td>
<td>S. pneumoniae capsular serotype 3 clinical isolate (22)</td>
</tr>
<tr>
<td>ΔSp0090</td>
<td>0100993 containing an insertion made with plasmid pPC110: Cm⁺ (this study)</td>
</tr>
<tr>
<td>ΔSp0149</td>
<td>0100993 containing an insertion made with plasmid pPC111: Cm⁺ (this study)</td>
</tr>
<tr>
<td>ΔSp0610</td>
<td>0100993 containing an insertion made with plasmid pPC112: Cm⁺ (this study)</td>
</tr>
<tr>
<td>ΔSp0750</td>
<td>0100993 containing an insertion made with plasmid pPC116: Cm⁺ (this study)</td>
</tr>
<tr>
<td>ΔSp0846</td>
<td>0100993 containing an insertion made with plasmid pPC118: Cm⁺ (this study)</td>
</tr>
<tr>
<td>ΔSp1690</td>
<td>0100993 containing an insertion made with plasmid pPC117: Cm⁺ (this study)</td>
</tr>
<tr>
<td>ΔSp1796</td>
<td>0100993 containing an insertion made with plasmid pPC119: Cm⁺ (this study)</td>
</tr>
<tr>
<td>ΔSp1824</td>
<td>0100993 containing an insertion made with plasmid pPC20: Cm⁺ (this study)</td>
</tr>
<tr>
<td>ΔlivHMGF</td>
<td>0100993 containing the livHMGF deletion construct: Erm⁺ (this study)</td>
</tr>
<tr>
<td>T4ΔlivHMGF</td>
<td>TIGR4 containing the livHMGF deletion construct: Erm⁺ (this study)</td>
</tr>
</tbody>
</table>

* Cm⁺, chloramphenicol resistance; Km⁺, kanamycin resistance; Erm⁺, erythromycin resistance.
**TABLE 2. Primers used in this study**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sp0090.1</td>
<td>GCTCTAGACATTCGAGGAGCAACTGG</td>
</tr>
<tr>
<td>Sp0090.2</td>
<td>CGCTGACATGGAAGAGATAAGGAGGAC</td>
</tr>
<tr>
<td>Sp0149.1</td>
<td>GCTCTAGAGCTGCTTCATGTCAGG</td>
</tr>
<tr>
<td>Sp0149.2</td>
<td>CGCTGACATGCACTGCTGCAAGTACAAGGCA</td>
</tr>
<tr>
<td>Sp0610.1</td>
<td>GCTCTAGATTCGTCAGTGACAGT</td>
</tr>
<tr>
<td>Sp0610.2</td>
<td>GCTCTAGATTCGTCAGTGACAGT</td>
</tr>
<tr>
<td>Sp0710.1</td>
<td>GCTCTAGATTCGTCAGTGACAGT</td>
</tr>
<tr>
<td>Sp0710.2</td>
<td>GCTCTAGATTCGTCAGTGACAGT</td>
</tr>
<tr>
<td>Sp1791.1</td>
<td>GCTCTAGATTCGTCAGTGACAGT</td>
</tr>
<tr>
<td>Sp1791.2</td>
<td>GCTCTAGATTCGTCAGTGACAGT</td>
</tr>
<tr>
<td>Sp1826.1</td>
<td>GCTCTAGATTCGTCAGTGACAGT</td>
</tr>
<tr>
<td>Sp1826.2</td>
<td>GCTCTAGATTCGTCAGTGACAGT</td>
</tr>
<tr>
<td>Sp1796.1</td>
<td>GCTCTAGATTCGTCAGTGACAGT</td>
</tr>
<tr>
<td>Sp1796.2</td>
<td>GCTCTAGATTCGTCAGTGACAGT</td>
</tr>
<tr>
<td>Sp0750.1</td>
<td>GCTCTAGATTCGTCAGTGACAGT</td>
</tr>
<tr>
<td>Sp0750.2</td>
<td>GCTCTAGATTCGTCAGTGACAGT</td>
</tr>
<tr>
<td>Sp0749.3</td>
<td>GCTCTAGATTCGTCAGTGACAGT</td>
</tr>
<tr>
<td>Sp0749.4</td>
<td>GCTCTAGATTCGTCAGTGACAGT</td>
</tr>
</tbody>
</table>

* Restriction enzyme sites in 5' linkers are underlined.

**Phenotypes of mutant strains**

Construction of ABC transporter mutant strains. To investigate the role of the selected ABC transporters during in vivo growth and virulence, mutant strains of the 0100993 strain of *S. pneumoniae* were selected for further investigation (Sp0090-3, Sp0148-52, Sp0607-10, Sp0708-11, Sp0749-53, Sp0846-8, Sp1688-90, Sp1796-8, Sp1824-6, Sp2084-7, and Sp2108-10). The results of BLAST searches using the derived amino acid sequence for each gene within the putative operons are shown in Table 3. Most of these ABC transporter proteins have >90% identity and similarity at the amino acid level to proteins encoded by genes in the *S. pneumoniae* R6 strain, an avirulent laboratory strain of *S. pneumoniae* derived from a capsular serotype 2 strain. However, there are no homologues of the Sp1796-8 operon in the R6 genome, and BLAST searches versus the 18 other available *S. pneumoniae* genomes demonstrated that the Sp1796-8 operon was also absent from the genomes of a further five strains (CDC1087-00, D39, G54, Hungary 19A-6, and Sp23-BS72). In contrast, the SBPs for almost all of the remaining ABC transporters were highly conserved between *S. pneumoniae* strains, with 97% or greater levels of identity between the deduced amino acid sequence of the TIGR4 SBP with the sequence of the equivalent SBP for all 19 *S. pneumoniae* strains with available genome data. The exception was Sp1826, which had between 93 and 95% identity to proteins encoded by genes present in 18 strains and 99% identity to the remaining strain (CDC0288-44). As expected, the majority of the predicted proteins have at least 50% identity and 60% similarity to proteins encoded by other streptococci (Table 3). In contrast, the genes in the putative Sp1688-90, Sp1824-6, and Sp2084-87 operons have no close homologues in streptococci. Sp1688-90 has >70% identity and >85% similarity to the amino acid sequence of predicted proteins encoded by Pm1760-62 of the gram-negative bacteria *Pasteurella multocida*, and Sp1824-6 and Sp2084-87 both have lower levels of identity and similarity to predicted proteins from a variety of unrelated bacteria. The mean G+C contents of Sp1688-90 and Sp1824-6 are 37 and 33.5%, respectively, which is significantly lower than the G+C content of the complete genome of TIGR4 (39.7%) (6, 40). The mean G+C content of Sp2084-7 was 39.0%, which is not significantly different from that of the complete genome of TIGR4. These data suggest that Sp1688-90, Sp1824-6, and possibly Sp2084-7 are contained within regions of the *S. pneumoniae* genome that could have been acquired by horizontal transfer from an unrelated species.

Phenotypes of mutant strains containing disruptions in genes encoding ABC transporters. The phenotypes of the mutant strains were investigated using mixed inocula and CIs to identify subtle defects affecting in vitro growth and to assess their virulence compared to the wild-type strain in animal models of infection. CIs were obtained using bacteria recovered from the lungs and spleen, respectively, to calculate the CI (Table 4). We were unable to establish a nosopharyngeal colonization model for the serotype 3 strain in which the mutants were constructed, preventing assessment of the role of these ABC transporters during col-
Most of the mutants had CIs close to 1.0 in THY, suggesting that disruption of the target ABC transporters had no effect on growth in complete medium. The exception was ΔSp0750 which had mildly impaired growth in THY. In contrast, in normal physiological fluid such as human blood, the mutants ΔSp0090, ΔSp0149, ΔSp0750, ΔSp1824, and (to a lesser extent) ΔSp0610 had reduced CIs, suggesting that these mutants have a particular problem growing in physiological fluid compared to THY. The in vivo CIs for most mutant strains mirrored the CIs for growth in blood, with these strains showing no impairment in CI in blood also being fully virulent (ΔSp0846, ΔSp1690, and ΔSp1796), whereas strains with impaired CIs in blood also had a reduced CI during infection. These data indicate that for these mutant strains, impaired growth under physiological conditions is associated with a reduced ability to cause invasive infection. In addition, ΔSp2108 had some impairment in virulence after i.p. inoculation without any impairment in CI in growth in blood. For both systemic and pulmonary infections, the ΔSp0750 and ΔSp0149 strains were markedly more attenuated in virulence than the other mutant strains with a CI reduced out of proportion to the reduced CI in blood.

**Construction of a deletion mutant of Sp0750-53.** The data obtained with the disruption mutant strains suggested that the ABC transporters encoded by Sp0149-52 and Sp0749-53 have the most crucial roles during *S. pneumoniae* infection (Table 4). BLAST alignments of the predicted proteins encoded by Sp0149-52 suggested these genes encode a methionine ABC transporter protein, but the function of this ABC transporter was not investigated further at this stage (37). BLAST alignments of the most crucial roles during *S. pneumoniae* infection (Table 4). BLAST alignments of the predicted proteins encoded by Sp0149-52 suggested these genes encode a methionine ABC transporter protein, but the function of this ABC transporter was not investigated further at this stage (37). BLAST alignments of the predicted proteins encoded by Sp0149-52 suggested these genes encode a methionine ABC transporter protein, but the function of this ABC transporter was not investigated further at this stage (37). BLAST alignments of the predicted proteins encoded by Sp0149-52 suggested these genes encode a methionine ABC transporter protein, but the function of this ABC transporter was not investigated further at this stage (37). BLAST alignments of the predicted proteins encoded by Sp0149-52 suggested these genes encode a methionine ABC transporter protein, but the function of this ABC transporter was not investigated further at this stage (37). BLAST alignments of the predicted proteins encoded by Sp0149-52 suggested these genes encode a methionine ABC transporter protein, but the function of this ABC transporter was not investigated further at this stage (37). BLAST alignments of the predicted proteins encoded by Sp0149-52 suggested these genes encode a methionine ABC transporter protein, but the function of this ABC transporter was not investigated further at this stage (37).
pseudomonallei (3, 24) but, as far as we are aware, no role has yet been described for BCAA transporters in bacterial virulence. We therefore elected to investigate the Sp0749-53 transporter in more detail and have named the genes livJHMGF to correspond to their E. coli orthologues (1).

To confirm the genetic organization of Sp0749-0753, RT-PCR was performed using RNA from strain 0100993 as the template and primers designed to amplify a product that spans the junctions of the genes present in the putative operon (Table 2). The lack of DNA contamination was confirmed by the absence of products for reactions with no added RT enzyme (data not shown), and RT-PCR products were sequenced to confirm their identities. The livJHMGF/Sp0749-53 region is 4,831 bp in length and consists of five genes, livJ/Sp0749 (encoding an SBP lipoprotein), livH/Sp0750 (encoding a membrane protein permease), livM/Sp0751 (encoding a membrane protein permease), livG/Sp0752 (encoding an ATPase) and livF/Sp0753 (encoding an ATPase) (Table 3 and Fig. 2A). The RT-PCR results matched with the operon structure deduced from the TIGR4 genome sequence, suggesting that livJ is not cotranscribed with livH (corresponding to the relatively large intergenic region between these two genes of 268 bp) and that livH, livM, livG, and livF are all cotranscribed (Fig. 2A and B).

Although the RT-PCR product obtained using primers spanning the junction of livM and livG was smaller than the expected size, sequencing confirmed it represented a product of the 5′/H11032 region of livM and 3′/H11032 region of livG and conformed exactly in length and almost exactly in nucleotide sequence to that predicted from the genome data for the TIGR4 strain. RT-PCR between livF and Sp0754 did generate a cDNA fragment, but sequencing demonstrated this was a nonspecific product. For further investigation of the function of livJHMGF, a mutant strain in the S. pneumoniae 0100993 background was constructed in which livHMGF were deleted using a construct made by overlap extension PCR (Fig. 2C and D). Correct deletion of these genes in the ΔlivHMGF mutant strain was confirmed by PCR and sequencing.

In vivo phenotypes of the ΔlivHMGF mutant strain. To investigate the effect of deletion of livHMGF on the virulence of S. pneumoniae, CIs were determined in the pulmonary and systemic mouse models of infection. The CIs for the ΔlivHMGF deletion mutant were reduced in models of pneumonia and septicemia showing a significant attenuation in virulence in these models of infection (Table 5), although the results were
higher than those obtained with the ΔSp0750 IDM mutant strain (Table 4). CIs were also obtained for a ΔlivHMGF strain in the TIGR4 background in models of nasopharyngeal colonization, systemic infection, and pneumonia (Table 5). The TIGR4 ΔlivHMGF strain was significantly attenuated in virulence in the pneumonia model but not in the nasopharyngeal colonization model and, in contrast to the serotype 3 0100993 ΔlivHMGF strain, had only a statistically not significant (the confidence intervals overlapped with 1.0) small decrease in the CI for the systemic model of infection. To assess the ability of the ΔlivHMGF S. pneumoniae strain to cause fatal disease, groups of 10 CD1 mice strain were inoculated i.n. with 10⁷ CFU of the wild-type S. pneumoniae 0100993 strain or the ΔlivHMGF deletion strain, and the progress of infection was monitored (6) (Fig. 3A). There was no difference in the survival of mice inoculated with the wild-type and ΔlivHMGF strains, demonstrating that despite the impaired virulence of the ΔlivHMGF strain when in competition with the wild-type strain this strain is still able to cause fatal disease. In addition, bacterial CFU in mouse lungs culled 48 h after inoculation of either the wild-type or ΔlivHMGF strains were not significantly different (Fig. 3B). These data show that the loss of LivJHMGF only impairs virulence significantly in a competitive model of infection.

In vitro phenotypes of the ΔlivHMGF strain. When compared using the OD₅₈₀, the growth of the ΔlivHMGF mutant strain was similar to the growth of the wild-type strain in THY and in a defined chemical medium CDEM with or without supplementation with the BCAAs valine, isoleucine, or leucine (data not shown). As shown for the ΔSp0750 IDM mutant strain (Table 4), the CI of the ΔlivHMGF mutant strain compared to the wild-type strains in vitro after growth in blood was impaired (CI = 0.56, confidence intervals of 0.34 to 0.78), but this was not corrected by the addition of 10 mg of valine, isoleucine, or leucine/ml impaired (CI = 0.44, confidence intervals of 0.15 to 0.74). Uptake of azaleucine by BCAA ABC transporters causes toxicity and impaired growth of gram-negative bacteria (14, 18), which is reduced if BCAA uptake is inactivated. However, azaleucine in concentrations of up to 100 μg ml⁻¹ was not toxic to S. pneumoniae (data not shown), and azaleucine toxicity could not therefore be used to assess

## Table 5. In vivo phenotype analysis of the ΔlivHMGF S. pneumoniae strains compared to the wild-type strain using CI values for three different serotype backgrounds

<table>
<thead>
<tr>
<th>Capsular serotype</th>
<th>CI (95% confidence interval) obtained by various routes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n.p. (nasal wash)</td>
</tr>
<tr>
<td>3</td>
<td>NDᵇ</td>
</tr>
<tr>
<td>4</td>
<td>1.05 (0.68–1.41)</td>
</tr>
</tbody>
</table>

ᵃ For these experiments, n = 5 to 10. n.p., nasopharyngeal.
ᵇ ND, not done (there was no nasopharyngeal model for this strain).
dimer of His6-LivJ. The ligand-binding properties of His6-LivJ wild-type or the /H9004 demonstrated that most of the purified protein was found in a single dodecyl sulfate-polyacrylamide gel electrophoresis demonstrated that the wild-type strain, precluding the use of uptake assays to assess the function of LivJHMGF. Since the SBP component provides substrate specificity for ABC transporters, to identify the substrate(s) of the LivJHMGF ABC transporter the binding of labeled amino acids to the LivJ (Sp0749) SBP was assessed. An N-terminally histidine-tagged LivJ (His6-LivJ) was expressed in E. coli and purified to ca. 95% purity. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis demonstrated that most of the purified protein was found in a single band of ~40 kDa, which is compatible with the expected mass for His6-LivJ. There was an additional band with a mass of ~80 kDa. Peptide mass fingerprinting confirmed that the 40-kDa band was His6-LivJ and that the 80-kDa band consisted of a dimer of His6-LivJ. The ligand-binding properties of His6-LivJ were investigated by measuring changes in intrinsic protein fluorescence, using tyrosine fluorescence (excitation at 280 nm) since the protein lacks any tryptophan residues. We were able to observe small but reproducible protein-dependent fluorescence changes in His6-LivJ upon the addition of 0.32 μM isoleucine, 3.2 μM leucine, and 16 μM valine (Fig. 4). There was no further fluorescence change with the addition of excess ligand, suggesting binding was saturated (Fig. 4). There were also no changes in fluorescence with the addition of 50 μM concentrations of the non-BCAAs proline, glycine, and alanine (data not shown). To estimate a relative affinity of the protein toward these three BCAAs, the lowest concentration of ligand for which we could detect a fluorescence change over the slow decrease in signal due to photobleaching was determined. Using 1.6 μM concentrations of ligand we could only detect isoleucine and leucine binding, and using 0.32 μM concentrations of ligand we could only detect isoleucine binding (Fig. 4 and data not shown). These data suggest that LivJ (Sp0749) is able to bind BCAA with a preference for isoleucine over valine and that the protein is likely to bind isoleucine in the submicromolar range that is typical for the physiological substrate of other ABC transporters.

The ligand-binding properties of His6-LivJ were also investigated by using a radioactive binding assay. The His6-LivJ lipoprotein bound to [14C]leucine and to a lesser extent [14C]leucine but not to the negative control ligand [α-[14C]aminoisobutyric acid (AIB) (Fig. 5A). Competitive binding experiments using putative ligands to inhibit [14C]leucine binding to His6-LivJ protein demonstrated that leucine and, to a lesser extent, valine and threonine inhibited [14C]isoleucine binding (Fig. 5B). Overall, the results of the fluorescence and radioactive binding studies show that the substrates of the S. pneumoniae LivJHMGF ABC transporter are BCAAs, with high-affinity binding of LivJ to isoleucine, moderate affinity binding to leucine and the least affinity toward valine.

**DISCUSSION**

Genes encoding components of ABC transporters make up a significant portion of many bacterial genomes, including S. pneumoniae (12, 40). Through controlling uptake or export of a wide range of substrates, ABC transporters have a central role in modulating the bacterial interactions with the environment, including the host for pathogens. Hence, it is perhaps not surprising that STM screens for virulence determinants frequently have identified ABC transporters (9, 10, 15, 22, 26) and that detailed characterization of individual S. pneumoniae ABC transporters have shown that several affect the development of infection through a variety of mechanisms (6, 17, 27, 34, 44). We have screened 11 S. pneumoniae ABC transporters that have not, as far as we are aware, previously been investigated for their roles in virulence. Mixed infections and CIs are

<table>
<thead>
<tr>
<th>Strain</th>
<th>[14C]leucine Uptake (mmol/mg of protein) at:</th>
<th>[14C]maltose Uptake (mmol/mg of protein) at:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>60 s</td>
<td>120 s</td>
</tr>
<tr>
<td>Wild type</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.44</td>
<td>0.47</td>
</tr>
<tr>
<td>ΔlivHMGF</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.41</td>
<td>0.62</td>
</tr>
</tbody>
</table>

**TABLE 6.** [14C]leucine and [14C]maltose uptake by wild-type and ΔlivHMGF strains
highly sensitive at identifying virulence defects and only require relatively small numbers of animals and so were used to assess the relative virulence of mutant strains containing disrupted ABC transporter genes to the wild-type strains (5, 6). We were unable to obtain any mutants affecting the putative amino acid transporter encoded by Sp0707-0711, perhaps since this transporter is important for *S. pneumoniae* viability, and the insertional duplication mutant affecting the putative phosphate transporter encoded by Sp2084-2087 was too unstable for further investigation. Of the nine ABC transporter mutant strains successfully constructed, six were impaired in full virulence in mouse models of sepsis and/or pneumonia (ΔSp0090, ΔSp0149, ΔSp0610, ΔSp0750, ΔSp1824, and ΔSp2108). Their putative roles included sugar, cation, BCAA, and other amino acid transport, and for the majority the CIs in blood were similar to the CIs in the infection model, suggesting that their effects on virulence are likely to be due to their role during growth under physiological conditions. These results indicate that ABC transporters frequently influence *S. pneumoniae* disease pathogenesis, demonstrating why mutations affecting lipoprotein and therefore SBP processing by *S. pneumoniae* affect virulence (21, 29). The majority of the loci encoding ABC transporters investigated in the present study have equivalents in other streptococci, and it is likely that some of these will also be required for full virulence.

For four of the ABC transporter mutant strains the impairment in virulence was relatively small, comparable to the effect of the loss of a single iron transporter (17), and this may be because their functions are partially redundant. For example, BLAST alignments suggest that several of the ABC transporters we have investigated encode sugar transporters, and disrupting the function of one could be compensated for by the others or even by non-ABC transporter uptake mechanisms such as phosphoenolpyruvate-dependent sugar transporters (40). In addition, in the host there are a variety of sugar substrates available, and the inability of *S. pneumoniae* to use a single sugar due to impaired uptake may not be critical due to the availability of other sugars. Dual mutations in genes encoding components of ABC transporters with related functions may have a much more marked effect on virulence as has been shown for the Piu and Pia iron transporters (6). However, too few functional data on the ABC transporters were investigated here to be able to determine which ABC transporters should be selected for dual mutation. The effects of disruption of these ABC transporters on virulence indicates bacterial functions that are likely to be important during infection, which can be suggested includes sugar, amino acid, and cation uptake. Although cation uptake is a well-recognized requirement for the virulence of *S. pneumoniae* and other bacteria (6, 17, 20, 25), sugar and amino acid transport is less well recognized. Identification of the specific substrate for each transporter will help define the precise physiological requirements for *S. pneumoniae* virulence but will require painstaking screening of a range of potential substrates using in vitro phenotypes and binding and uptake assays. In addition to ABC transporters encoded for by groups of genes investigated in this and previous studies, there are many additional ABC transporter components encoded by isolated single genes or gene pairs within the TIGR4 genome, some of which STM screens suggest may affect virulence (15, 22). These ABC transporter components also warrant further investigation, although their specific putative functions will in general be even less apparent than those encoded by several adjacent genes in putative operons.

Two mutant strains had a marked effect on virulence when analyzed using CIs. One contained a disrupted copy of Sp0149, part of a potential methionine transporter which is the subject of continued investigation in our laboratory. The second contained a disrupted copy of Sp0750, part of an operon whose

---

**FIG. 4.** Tryptophan fluorescence spectroscopy of purified His<sub>6</sub>-LivJ after addition of 0.32 μM isoleucine (A), 3.2 μM leucine (B), or 16 μM valine (C) (marked by the first arrow). The addition of excess ligand (marked by the second arrow) had no further effect on fluorescence, nor did the addition of 50 μM proline, glycine, or alanine (data not shown). Fluorescence was measured by using arbitrary units and an excitation wavelength of 280 nm with an emission wavelength of 309 nm.
function is strongly indicated by BLAST searches to be BCAA uptake. The genes at the Sp0749-53 loci are annotated as LivJHMGF in the TIGR4 genome (40) and are organized in a fashion similar to that of the equivalent BCAA of E. coli, with the SBP component encoded by livJ that is transcribed as a separate transcript to the remaining genes within the operon livHMGF (19). Tryptophan fluorescence spectroscopy and radioactive binding assays using [14C]leucine, [14C]isoleucine, and [14C]valine demonstrated that LivJ binds specifically to BCAA, strongly supporting that livJHMGF encodes a BCAA ABC transporter. Both the tryptophan fluorescence spectroscopy and the radioactive binding assays suggest that LivJ has the highest affinity for isoleucine and then leucine, with the least affinity for valine.

Growth of the ΔlivHMGF strain was not affected in media depleted in BCAA, the toxic BCAA analogue azaleucine did not impair S. pneumoniae growth, and we were unable to identify significant uptake of the BCAA leucine by S. pneumoniae. Hence, in the conditions used for these experiments (i.e., the complete medium THY and the defined medium CDEM) there seems to be very little BCAA uptake by S. pneumoniae. In contrast, in mouse models of infection the sbp component encoded by livJ is transcribed as a separate transcript to the remaining genes within the operon livHMGF does not have a powerful effect on S. pneumoniae disease pathogenesis, perhaps because the S. pneumoniae genome contains genes encoding enzymes required for BCAA synthesis (Sp0445-50) (24, 40). These may partially compensate for impaired BCAA uptake in vivo, perhaps to a variable degree between strains, so the effects of the ΔlivHMGF mutation on in vivo growth are only detectable if competing against a wild-type strain and depend on strain background. Exactly why the loss of BCAA affects virulence is not clear. The most obvious explanation is a nutritional requirement for BCAA in vivo, and the differences in CI between sites could be explained by variations in bacterial demand for BCAA, perhaps related to differences in bacterial replication rate in combination with host physiology. However, there was a marked difference in CI between growth in blood and during infection for the ΔSp0750 mutant strain, whereas other mutant strains with reduced CIs in blood only had weakly reduced CIs during infection. These data perhaps indicate that the loss of virulence associated with mutation of LivJHMGF may be more complex than simple impaired growth under physiological conditions. The impaired CI of the ΔlivHMGF strain compared to the wild-type strain when cultured in blood was not improved by the addition of exogenous BCAAs. This would be the expected result if LivJHMGF is a BCAA ABC transporter and there are no alternative S. pneumoniae low-affinity BCAA transporter systems that could compensate for a BCAA transport defect when BCAAs are present in high concentrations in the environment. Genes from both the livJHMGF and the Sp0445-50 operons have increased expression in cerebrospinal fluid in a rabbit model of meningitis (28) and are under the negative control of the transcriptional regulator CodY (16), but the relationship
between CodY repression, in vitro and in vivo expression of livHMGF, and BCAA transport requires further investigation.

To conclude, we have investigated whether a range of *S. pneumoniae* ABC transporters affect virulence and identified that six of the nine investigated had some effect on the pathogenesis of infection compared to the wild-type strain using microarray analysis. The functions of the majority of the ABC transporters affecting virulence were not clear, but we have characterized one encoded by livHMGF in more detail and confirmed that it encodes a BCAA ABC transporter. Further investigation is required to identify the substrates of the remaining ABC transporters and to define why BCAA uptake is important for *S. pneumoniae* virulence.

ACKNOWLEDGMENTS

This study was supported by the British Lung Foundation (grant P02/3) and the Wellcome Trust (grant 076442).

REFERENCES

29. Sutcliffe, I. C., and R. R. Russell. 1995. Lipoproteins of gram-positive bac-

Tests downloaded from http://iai.asm.org/ on June 3, 2012 by guest


Editor: J. N. Weiser