Application of a novel multi-screening signature-tagged mutagenesis assay for identification of \textit{Klebsiella pneumoniae} genes essential in colonization and infection

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\textit{Klebsiella pneumoniae} is a common cause of urinary tract infections (UTIs) and pneumonia, especially in immunocompromised individuals. Epidemiological studies have revealed that \textit{K. pneumoniae} infections are frequently preceded by gastrointestinal colonization and the gastrointestinal tract is believed to be the most important reservoir for transmission of the bacteria. To identify genes involved in the ability of \textit{K. pneumoniae} to colonize the intestine and infect the urinary tract, a novel multi-screening signature-tagged mutagenesis (MS-STM) assay was implemented. In the MS-STM assay, PCR-amplified tags present in the inoculum as well as recovered pools from each infection model are simultaneously subjected to hybridization using each specific tag as a probe. Therefore, screenings of a mutant library in more than one infection model is significantly eased compared to the traditional signature-tagged mutagenesis methodology. From a total of 1440 \textit{K. pneumoniae} transposon mutants screened, 13 mutants were identified as attenuated in intestinal colonization as well as the UTI model. In addition, six mutants attenuated only in the UTI model were identified. Transposon insertion sites in attenuated mutants were, among others, in genes encoding well-known \textit{K. pneumoniae} virulence factors such as lipopolysaccharide and capsule, as well as in genes of unknown function.

INTRODUCTION

\textit{Klebsiella pneumoniae}, a Gram-negative bacterium belonging to the \textit{Enterobacteriaceae}, is an important opportunistic pathogen and a frequent cause of urinary tract infections (UTIs) and pneumonia in immunocompromised individuals. Translocation of \textit{K. pneumoniae} from the initial focus of infection to the bloodstream is frequently observed. Thus, after \textit{E. coli}, it is the most common cause of Gram-negative septicaemia (Hansen \textit{et al}., 1998). Of particular concern is the worldwide occurrence of multi-resistant strains of \textit{K. pneumoniae}. Several outbreaks of infections associated with \textit{K. pneumoniae} strains expressing extended-spectrum \(\beta\)-lactamases conferring resistance to third-generation cephalosporins have been described (Shannon \textit{et al}., 1998; Verma \textit{et al}., 2001). Infections with such strains are difficult to eradicate, as the bacteria are frequently also resistant to other antimicrobial agents, including aminoglycosides. Epidemiological studies have revealed that \textit{K. pneumoniae} infections are often preceded by colonization of the hosts’ gastrointestinal (GI) tract and the GI tracts of patients are believed to be the most important reservoir of transmission of the bacteria (Montgomerie, 1979). A number of virulence factors have been suggested in \textit{K. pneumoniae}, including the prominent polysaccharide capsule expressed by the vast majority of clinical isolates as well as different adhesins, lipopolysaccharide (LPS) and iron-scavenging proteins (Podschun & Ullmann, 1998). Knowledge of the exact role of individual \textit{K. pneumoniae} virulence factors in different types of infection is, however, sparse and even less is known of the factors involved in the ability of the bacteria to colonize and reside in the GI tract of the host. Such knowledge is important in the search for new strategies to treat and prevent \textit{K. pneumoniae} infections.

Signature-tagged mutagenesis (STM) is an effective technique for genetic analysis of microbial factors involved in the infection or colonization of a host (Hensel \textit{et al}., 1995). STM is based on random transposon insertional mutagenesis but allows simultaneous screening of several mutants. This is accomplished by tagging each mutant with a unique short DNA sequence so that it can subsequently be identified.
within a pool of mutants by DNA–DNA hybridization analysis. The STM methodology has been adapted and used to identify virulence genes in several microbial pathogens (Shea et al., 2000). Modifications of the method include the use of pre-selected tagged transposons to construct the mutant library, which simplifies the screening significantly (Mei et al., 1997). STM screenings of a mutant library in more than one infection model have been performed (Coulter et al., 1998; Tsolis et al., 1999; Bispham et al., 2001; Lau et al., 2001). Such screenings provide valuable knowledge of whether the same virulence factors are involved in different types of infections. By use of the traditional STM methodology, screenings in more than one infection model are, however, very laborious. Here, we describe the development and implementation of a multi-screening STM (MS-STM) assay to identify K. pneumoniae genes involved in GI colonization and UTIs. The MS-STM assay is an effective alternative to the traditional STM methodology and reduces the workload significantly when a mutant library is screened in more than one infection model.

**METHODS**

**Bacterial strains, plasmids and mating.** Bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were routinely cultured at 37 °C in Luria–Bertani (LB) broth or on LB agar plates containing the appropriate antibiotics at the following concentrations: rifampicin, 50 μg ml⁻¹; kanamycin, 50 μg ml⁻¹; ampicillin, 100 μg ml⁻¹; streptomycin, 100 μg ml⁻¹.

Matings were performed by mixing equal volumes (10 μl) of overnight broth cultures of the donor E. coli S17-1 λpir and the recipient K. pneumoniae C3091Sm⁰Rif⁰ on LB agar without antibiotics. After overnight incubation at 37 °C, bacteria were harvested from the plates, suspended in 2 ml of 0.9% NaCl and plated onto selective medium containing rifampicin and kanamycin for selection of transconjugants. Individual transconjugants were picked and stored at -80 °C in the wells of microtiter plates containing LB broth supplemented with 10% (v/v) glycerol.

**Molecular techniques.** Chromosomal DNA was purified by use of the Easy-DNA Kit (Invitrogen) and plasmid DNA was prepared by use of the QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturers’ instructions. Restriction enzymes and T4 ligase were obtained from New England Biolabs; all restriction digests and ligations were performed as recommended by the manufacturer.

The tag region was amplified by PCR using the primers P2 (5'-TACCTACACCTCAAGCT-3') and P4 (5'-TACCCATTTCACTACAA- GC-3') (Hensel et al., 1995). PCR conditions were as follows: an initial denaturation at 95 °C for 4 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 1 min and elongation at 72 °C for 10 s. Synthesis of digoxigenin (DIG)-labelled tags was performed by use of the PCR DIG Synthesis Kit (Roche) according to the manufacturer’s instructions.

For dot-blot hybridizations, 50 μl of a 25× dilution of the PCR products were transferred onto a positively charged nylon membrane (Hybond-N⁺; Amersham) using a Bio-Dot microfiltration apparatus (Bio-Rad). DNA on the membranes was denatured by alkali treatment and fixed by UV cross-linking according to the manufacturer’s instructions. Membranes were pre-hybridized at 42 °C for 1 h in hybridization buffer [5× SSC, 0.1% N-laurylsarcosine, 0.02% SDS, 0.5% Blocking reagent (Roche)] followed by hybridization with the DIG-labelled probe for 1 h at 42 °C. The membranes were then washed twice for 5 min with low-stringency washing buffer (2× SSC, 0.1% SDS) at room temperature followed by two 30 min washes with high-stringency washing buffer (0.1× SSC, 0.1% SDS) at 50 °C.

Hybridized DIG-labelled probes were detected by an enzyme-linked immunoassay and a subsequent enzyme-catalysed colour reaction by use of the DIG Nucleic Acid Detection Kit (Roche) as described by the manufacturer, except that the incubation period with the antibody was extended to overnight.

Southern hybridization analysis was performed by standard methods on Xhol-digested genomic DNA using the kanamycin-resistance cassette from mini-Tn5Km2 as a probe (Auszubel et al., 1995).

**Animal models.** Six- to eight-week-old outbred albino female mice Ssc : CF1 [Statens Serum Institut (SSI)] were used for GI colonization experiments as described previously (Licht et al., 1996). Briefly, mice were individually caged and given sterile water containing 5 g streptomycin sulphate l⁻¹ and fed continuously. After 24 h, 100 μl bacterial suspensions containing approximately 10⁸ c.f.u. were given orally to the mice by pipette. During the colonization experiment, cages were changed daily and the mice continuously received water containing

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**Table 1. Strains and plasmids used in this study**

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Description</th>
<th>Source*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strain</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K. pneumoniae 3091</td>
<td>UTI isolate, capsule serotype K16</td>
<td>Oelschlaeger &amp; Tall (1997)</td>
</tr>
<tr>
<td>K. pneumoniae C3091Sm⁰Rif⁰</td>
<td>Spontaneous streptomycin- and rifampicin-resistant mutant of 3091</td>
<td>This study</td>
</tr>
<tr>
<td>E. coli C118 λpir</td>
<td>Δ(are-leu) araD ΔluxX74 galE galK phoA20 thi-1 rpsE argE recA1</td>
<td>Miller &amp; Mekalanos (1988)</td>
</tr>
<tr>
<td>E. coli S17-1 λpir</td>
<td>Tp⁰, Sm⁰; recA thi pro hsdR M⁰ RP4::2-Tc::Mu::Km</td>
<td>Herrero et al. (1990)</td>
</tr>
<tr>
<td>E. coli DH5</td>
<td>F⁻ supE44 hsdR17 recA1 gyrA96 endA1 thi-1 relA1 deoR λ⁻</td>
<td>ATCC 53868</td>
</tr>
<tr>
<td><strong>Plasmid</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pTag-mini-Tn5Km2</td>
<td>Ap⁰, Km⁰; pool of sequence-tagged mini-Tn5 transposons in pUT delivery plasmid</td>
<td>Hensel et al. (1995)</td>
</tr>
<tr>
<td>pUC18</td>
<td>Ap⁰</td>
<td>New England Biolabs</td>
</tr>
</tbody>
</table>

*ATCC, American Type Culture Collection, Manassas, VA 20108, USA.*
streptomycin. At days 1, 3 and 7 after inoculation, 0·5 g of faeces was collected and homogenized in 5 ml of 0·9% NaCl; dilutions were plated onto selective medium.

For UTI experiments, six- to eight-week-old outbred albino female mice Ssc: CF1 were used. A modification of the UTI model previously described was applied (Hvidberg et al., 2000). From 3 days before inoculation and throughout the experiment, the mice were given a 5% glucose solution as the sole source of drinking water, as this has been shown to promote UTIs in mice (Keane & Freedman, 1967). The mice were anaesthetized by intraperitoneal administration of 0·08 ml of a mixture of Hypnorm (0·315 mg fentanyl citrate ml⁻¹ and 10 mg fluanison ml⁻¹; Janssen Animal Health) and diazepam (5 mg ml⁻¹) in a ratio of 1:3. Anaesthetized mice were inoculated transurethrally with a 50 µl bacterial suspension containing approximately 5×10⁹ c.f.u. by use of plastic catheters. The catheter was carefully pushed horizontally through the urethral orifice until it reached the top of the bladder, and the bacterial suspension was slowly injected into the bladder. The catheter was immediately removed after inoculation and the mice were not subjected to further manipulations until they were killed. The mice were killed on day 3 after inoculation. For recovery of bacteria, the bladders were collected in 500 µl of PBS (2·7 mM KCl, 8 mM Na₂HPO₄, 1·5 mM KH₂PO₄, 137 mM NaCl, pH 7·4) and homogenized; dilutions were plated onto selective medium.

Screening of the STM transposon mutant library. Each pool of 48 mutants was grown in LB broth supplemented with streptomycin and kanamycin in a 96-well microtitre plate at 37 °C for 18 h. The bacteria were pooled, centrifuged at 6500 g for 10 min and resuspended at a concentration of approximately 10⁸ c.f.u. ml⁻¹ to form the inoculum used to infect pairs of mice as described above.

Chromosomal DNA was isolated from plate cultures of the inoculum and from the homogenized faeces and bladders of infected mice and was used as template for PCR with primers P2 and P4 (Hensel et al., 1995). The amplified tags from the inoculum and recovered pools were spotted onto 48 membranes and subjected to separate hybridization above.

Competition assays. For mixed infections, mice were inoculated with a bacterial suspension consisting of equal numbers of the mutant and wild-type strain. Dilutions of inoculum and of homogenized faeces and bladders of infected mice were plated onto media with and without kanamycin, to estimate the ratio of mutant to wild-type bacteria. The competitive index was calculated as the proportion of mutant to wild-type bacteria recovered from each animal divided by the proportion of mutant to wild-type bacteria in the inoculum.

For in vitro competition experiments, 10 ml of LB broth were inoculated with approximately 1×10⁹ c.f.u. of bacteria consisting of equal numbers of mutant and wild-type cells. The cultures were incubated at 37 °C for 18 h and serial dilutions were plated onto media with or without kanamycin. Experiments were performed in triplicate, and competitive indexes were calculated as described above.

Cloning and sequence analysis of attenuated mutants. Chromosomal DNA was purified from attenuated mutants and digested with EcoRI, PstI or Sall. The DNA fragments were ligated into linear pUC18 and the ligation reaction was used to transform E. coli DH5α to kanamycin resistance. Plasmids from kanamycin-resistant subclones were checked by restriction enzyme digestion and sequenced commercially by use of primer P6 (5’-CCTAGG-CGCCAGATCTGAT-3’) or P7 (5’-GCACCTGTGATAAGAGT-CAGT-3’) (Hensel et al., 1995).

Analysis of LPS and capsule expression. LPS was obtained by proteinase K digestion of whole cells as described by Hitchcock & Brown (1983). LPS samples were separated by SDS-PAGE and silver stained as described by Tsai & Frasch (1982).

Detection of capsule expression was performed by slide agglutination using K. pneumoniae K16 antiserum [Escherichia and Klebsiella Reference Centre (WHO), SSI, Copenhagen, Denmark] as described by Orskov & Orskov (1984).

RESULTS

Construction of signature-tagged K. pneumoniae mutant library

From the original pool of signature-tagged transposons, 48 uniquely tagged transposons were selected and used to construct the mutant library. The criteria of selection were efficient amplification and labelling and lack of cross-hybridization to other tags. The library was constructed by independent mating using each of the 48 pre-selected transposons and the mutants were arrayed in microtitre plates based on the tags they carried. A total of 1440 mutants were arrayed in 30 pools each containing 48 mutants. To confirm random insertion of mini-Tn5Km2 into K. pneumoniae C3091SmR RifR, Southern hybridization analysis of 30 mutants using the kanamycin-resistance cassette in mini-Tn5Km2 as the probe was used. Each mutant was found to have a single transposon insertion in a distinct site in the chromosome (data not shown).

Description of the MS-STM assay

To simplify screening of the mutant library in more than one infection model, an MS-STM assay was developed. In the traditional STM methodology, the PCR-amplified tags present in the inoculum and recovered pools are labelled by a second PCR and used to hybridize membranes bearing the tag sequences. In the MS-STM assay, the PCR-amplified tags present in the inoculum and recovered pools are spotted onto membranes and separate hybridizations are performed with each specific tag as a probe. Therefore, the number of hybridizations necessary to screen a mutant library in more than one infection model is significantly reduced, since pools of tags recovered from more than one infection model can be analysed simultaneously (Fig. 1). Also, fewer numbers of PCR amplifications are needed when the MS-STM assay is applied, as two rounds of PCR are no longer needed to obtain labelled tags. To avoid cross-hybridization, the invariant arms are removed from the tag region by restriction enzyme digestion in the traditional STM methodology. In the MS-STM assay, the presence of the invariant arms did not result in higher background levels of hybridization in negative controls compared to when the invariant arms were removed from the tag region before spotting the amplified tags onto the membranes.
(data not shown). Thus, no restriction enzyme digestions are necessary when the MS-STM assay is applied. A comparison of the total numbers of hybridizations, PCR amplifications and restriction enzyme digestions necessary to screen a mutant library in one or two infection models by use of the traditional STM methodology and the MS-STM assay is shown in Table 2.

**Screening for attenuated mutants in models of GI colonization and UTI**

The library of signature-tagged mutants was screened for loss of ability to colonize the GI tract and cause UTI by use of animal models. Day 3 after oral inoculation, *K. pneumoniae* C3091Sm\(^R\)Rif\(^R\) was found to colonize the GI tract of inoculated mice at levels of \(10^7\) to \(10^9\) c.f.u. (g faeces\(^{-1}\)). Hybridization analysis by use of the MS-STM assay revealed 22 putative colonization mutants that gave positive hybridization signals in the inoculum but which were not recovered from the faecal samples.

By use of a model of ascending UTI, mice were successfully infected with *K. pneumoniae* C3091Sm\(^R\)Rif\(^R\), as bladder cultures of all inoculated mice were positive at day 3 after inoculation. The bacterial counts in the vast majority of infected bladders were in the range of \(10^4\) to \(10^6\) c.f.u. In the UTI model, the initial screening identified 116 potential virulence mutants. These mutants were reassembled into 10 pools of 24 mutants, with each pool including mutants not identified as attenuated in the initial screening, and reassessed in the UTI model. Of the 116 mutants, 29 gave consistently negative hybridization signals in both rounds of screening.

All putative attenuated mutants identified by either animal model were tested in direct competition with the wild-type strain. In the GI colonization model, faeces from mice inoculated with equal numbers of mutant and wild-type bacteria were sampled and plated on days 1, 3 and 7 after inoculation to compare the colonization abilities of the mutant and wild-type strains. Attenuated colonization abilities could be confirmed for 13 of the 22 putative colonization mutants (Table 3). In the UTI model, attenuated infection abilities could be confirmed for 19 of the 29 putative attenuated mutants by bladder cultures day 3 after inoculation with equal numbers of mutant and wild-type bacteria (Table 3). To investigate whether *in vivo* attenuation was due to a general growth effect, the *in vitro* growth rate of the mutants was compared with that of the wild-type strain. The *in vitro* growth rate was significantly reduced only in mutant 14B11 (Table 3). Interestingly, all mutants attenuated in their ability to colonize the GI tract were also attenuated in the UTI model. Additionally, six mutants attenuated in the UTI model were identified. However, all six of them were just as effective at colonizing the GI tract as the wild-type strain (Table 3).

**Characterization of attenuated mutants**

The transposon inserts of 16 of the 19 attenuated mutants were cloned and the DNA sequences flanking the insertion points were determined. To identify the genes disrupted, DNA and protein database searches were undertaken. The results of these are shown in Table 3.

**Mutants attenuated in the GI colonization and UTI animal models.** Among the 13 mutant strains attenuated...
**Klebsiella pneumoniae virulence genes**

**Table 2.** Comparison of workload when screening a mutant library (30 pools of 48 mutants) in one or two animal models by use of the traditional STM methodology or the MS-STM assay

<table>
<thead>
<tr>
<th>Procedure</th>
<th>One animal model</th>
<th>Two animal models</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Traditional STM</td>
<td>MS-STM</td>
</tr>
<tr>
<td>PCR amplifications</td>
<td>120</td>
<td>108*</td>
</tr>
<tr>
<td>Enzyme digestions</td>
<td>60</td>
<td>0</td>
</tr>
<tr>
<td>Hybridizations</td>
<td>60</td>
<td>48</td>
</tr>
</tbody>
</table>

*Including synthesis of 48 DIG-labelled tags.

in their ability to colonize the intestine and infect the bladder, three were mutated in genes involved in LPS synthesis. In two of the mutants, the insertions were located in genes inside the waa gene cluster, which encodes the genes involved in core LPS biosynthesis. The waaL gene encodes an O-antigen polymer ligase that is responsible for ligation of the O-antigen to the core region, while waaE presumably encodes a transferase responsible for the addition of a *K. pneumoniae*-specific disaccharide to the core structure (Regue et al., 2001). In the third LPS-related mutant, the insertion was inside the *wbbO* gene, which encodes an enzyme essential in the biosynthesis of the *K. pneumoniae* O-antigen (Clarke et al., 1995). LPS was extracted from all three mutant strains and compared to the LPS of *K. pneumoniae* C3091Sm*Rif* by SDS-PAGE analysis. As expected, the *waaL* and *wbbO* mutants both failed to express the O-antigen, whereas the core portion of LPS was found to be altered in the *waaE* mutant (data not shown).

In three additional mutants, insertions were found in genes related to cell-membrane and cell-surface structures, namely *plsX, ompA* and *surA*. The *plsX* gene is involved in phospholipid and fatty acid biosynthesis, but the exact role of this gene is poorly understood (Zhang & Cronan, 1998). The *ompA* gene encodes the multifunctional outer-membrane protein A (OmpA), one of the major proteins in the outer membrane of many members of the *Enterobacteriaceae* (Nikado, 1996). The *surA* gene encodes a periplasmic protein required for the proper assembly and folding of several outer-membrane proteins (Lazar & Kolter, 1996). Transposon insertion sites were also located in genes homologous to *tufA*, one of two genes encoding the essential peptide chain elongation factor Tu in *E. coli* (Zuurmond et al., 1999), and *hupA*, which encodes the HU-2 protein, one of the two subunits of the histone-like protein HU (Wada et al., 1988). Mutant 26C5 was characterized by a significantly higher degree of attenuation in the gut colonization model than in the UTI model. The disrupted gene in mutant 26C5 was identified as a homologue of the *E. coli* arcB gene involved in the transcriptional regulation of genes implicated in metabolism in response to oxygen availability (Gunsalus & Park, 1994). In two mutants, the transposon insertion sites were in genes homologous to ORFs in the *E. coli K-12* genome.

**Mutants attenuated in the UTI model only.** In two of the six mutants attenuated in their ability to infect the bladder but not in their ability to colonize the GI tract, the disrupted genes were identified as *gmd* and *fcl*, which are involved in the biosynthesis of GDP-L-fucose (Andrianopoulos et al., 1998). Fucose is a residue in certain bacterial polysaccharides, including specific *E. coli* O- and K-antigens. No *K. pneumoniae* O-antigens contain fucose; however, it is a component of certain K-antigens including K16, the K-antigen of strain 3091 (Chakraborty et al., 1977; Ørskov & Ørskov, 1984). When grown on solid medium, both mutant strains exhibited non-mucoid colony morphologies that were distinctively different from the mucoid morphology of the wild-type strain. To investigate the expression of capsule in the two mutant strains, slide agglutination tests with K16 antiserum were performed. Neither of the mutant strains agglutinated in the presence of antiserum, whereas distinctive agglutination was observed for the wild-type strain. These results indicate that both of the mutant strains were impaired in expression of the K16 capsule polysaccharide as a result of the inability to synthesize fucose. In mutant strain 27G5, the obtained nucleotide sequence showed 60% similarity to the *fimB* gene of *E. coli*, which is involved in the regulation of type 1 fimbriae expression. However, the actual transposon insertion site in the mutant was located approximately 150 bp downstream of the *fimB* gene homologue, leaving the gene itself intact in the mutant. Finally, in one mutant, the transposon insertion sites were in genes homologous to ORFs in the *E. coli* K-12 genome, whereas the sequence flanking the transposon insertion site in mutant strain 4C9 showed no homology to sequences in any of the public databases.

**DISCUSSION**

This study describes the development and use of a novel MS-STM assay to identify *K. pneumoniae* genes implicated in the ability of the bacterium to colonize the intestine and to infect the urinary tract. It has been shown that *K. pneumoniae* infections in general are preceded by GI colonization of the patient (Montgomerie, 1979); therefore, such screenings are highly relevant. Knowledge of the bacterial factors implicated in GI colonization is valuable in the development of new strategies to prevent colonization.
### Table 3. Competitive indexes (CIs) and transposon insertion sites in attenuated mutants

<table>
<thead>
<tr>
<th>Attenuated mutants</th>
<th>GI colonization CI*</th>
<th>Bladder CI*</th>
<th>In vitro CI*</th>
<th>Disrupted gene†</th>
<th>Identity (%)‡, organism</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 3</td>
<td>Day 7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>GI and UTI models</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4H11</td>
<td>0.22</td>
<td>0.17</td>
<td>0.07</td>
<td>0.14</td>
<td>1.6</td>
<td>waaL</td>
</tr>
<tr>
<td>14B11</td>
<td>0.02</td>
<td>0.003</td>
<td>&lt;0.00008</td>
<td>&lt;0.02</td>
<td>0.09</td>
<td>waaE</td>
</tr>
<tr>
<td>22D1</td>
<td>0.36</td>
<td>0.14</td>
<td>0.01</td>
<td>0.06</td>
<td>1.8</td>
<td>wbbO</td>
</tr>
<tr>
<td>10A1</td>
<td>0.30</td>
<td>0.43</td>
<td>0.07</td>
<td>&lt;0.002</td>
<td>0.82</td>
<td>plsX</td>
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<tr>
<td>24B9</td>
<td>0.07</td>
<td>&lt;0.0007</td>
<td>&lt;0.3</td>
<td>&lt;0.00001</td>
<td>1.3</td>
<td>ompA</td>
</tr>
<tr>
<td>21E1</td>
<td>0.05</td>
<td>0.0005</td>
<td>0.002</td>
<td>&lt;0.000008</td>
<td>0.70</td>
<td>surA</td>
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<tr>
<td>5D9</td>
<td>0.03</td>
<td>0.03</td>
<td>&lt;0.002</td>
<td>0.002</td>
<td>0.62</td>
<td>tufA</td>
</tr>
<tr>
<td>14A1</td>
<td>0.03</td>
<td>0.03</td>
<td>&lt;0.003</td>
<td>0.0008</td>
<td>1.6</td>
<td>hupA</td>
</tr>
<tr>
<td>26C5</td>
<td>0.004</td>
<td>&lt;0.000001</td>
<td>&lt;0.000001</td>
<td>0.22</td>
<td>0.66</td>
<td>arcB</td>
</tr>
<tr>
<td>14B1</td>
<td>0.05</td>
<td>0.01</td>
<td>0.005</td>
<td>0.0002</td>
<td>1.0</td>
<td>ORF (b2512)</td>
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<tr>
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<td>0.09</td>
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<td>ORF (b1631)</td>
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<td>12G5$</td>
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<td>0.35</td>
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<tr>
<td>22B1$</td>
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<td>0.22</td>
<td>0.45</td>
<td>0.03</td>
<td>2.0</td>
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<tr>
<td>12H9</td>
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<td>1.5</td>
<td>1.3</td>
<td>0.0009</td>
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<td>gmd</td>
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<td>5B9</td>
<td>1.6</td>
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<td>1.6</td>
<td>&lt;0.001</td>
<td>0.60</td>
<td>fcl</td>
</tr>
<tr>
<td>27G5</td>
<td>0.91</td>
<td>0.82</td>
<td>0.70</td>
<td>0.005</td>
<td>1.2</td>
<td>fimB (D)</td>
</tr>
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<td>2.1</td>
<td>0.94</td>
<td>0.004</td>
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<td>ORF (ytfn)</td>
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<td>2.7</td>
<td>0.08</td>
<td>0.77</td>
<td>Unknown</td>
</tr>
<tr>
<td>20B1$</td>
<td>1.2</td>
<td>2.0</td>
<td>0.57</td>
<td>0.08</td>
<td>0.87</td>
<td></td>
</tr>
</tbody>
</table>

*CI, Output ratio of mutant to wild-type cells divided by the input ratio of mutant to wild-type cells; <, no mutants recovered from one or more mice.
†ORF code refers to the name given to the corresponding ORF in E. coli MG1655; (D), the transposon was located downstream of the specific gene.
‡Amino-acid identity of predicted protein over the region sequenced.
§Transposon insertion site not cloned.
and subsequent infection of susceptible patients with *K. pneumoniae*. Furthermore, by screening a mutant library in a colonization as well as an infection model, it is possible to differentiate between bacterial factors generally important for *in vivo* growth and survival of the bacteria, as during GI colonization, and factors specifically important when the bacteria act as pathogens, as during UTI. To ease simultaneous screenings of a mutant library in more than one infection model, a novel MS-STM assay was applied. When screenings in more than one infection model are performed by the traditional STM methodology a high number of hybridizations are needed as the inoculum and the recovered pools from each model are analysed separately. In the MS-STM assay, PCR-amplified tags present in the inoculum and the recovered pools from each infection model are simultaneously subjected to hybridization using each specific tag as a probe. Therefore, the numbers of hybridizations needed are significantly reduced when screening a mutant library in more than one model. Furthermore, previously reported high background levels occurring during hybridization when using DIG rather than radiolabelled tags (Fuller et al., 2000) were eliminated by using the MS-STM assay. Thus, the cumbersome step of removal of the invariant arms from the tag region by restriction enzyme digestion before each hybridization analysis is not necessary in the MS-STM assay. The MS-STM assay can be easily adapted for use with other microbial pathogens and represents an effective alternative to the traditional STM methodology, especially when screenings are performed in more than one infection model.

All of the *K. pneumoniae* mutants attenuated in their ability to colonize the GI tract were also attenuated in the UTI model. This suggests that mutations affecting the ability of *K. pneumoniae* to colonize the intestine also affect its ability to cause UTIs. As the GI tract is considered a natural habitat for *K. pneumoniae*, it is not surprising that mutants with reduced ability to grow and persist in the intestine are also impaired in other host environments. In the urinary tract, the natural host defences such as urine flow and the immunoresponse of the host demand the presence of specialized microbial factors for bacteria to be able to survive, grow and infect the host. Some of these factors might only be essential when the bacteria act as pathogens and not when the bacteria are present as commensals in their natural habitat. This was confirmed in the present study, as six of the *K. pneumoniae* mutants were significantly attenuated in their ability to infect the bladder but were not affected in their ability to colonize the GI tract. In contrast, none of the mutants attenuated in GI colonization was virulent in the UTI model.

Disrupted genes in attenuated mutants included genes encoding well-known *K. pneumoniae* virulence factors and cell-surface structures, as well as 'housekeeping genes'.

Our results confirmed that LPS is an important colonization and UTI virulence determinant in *K. pneumoniae*, since the transposon insertion sites in three attenuated mutants were in genes related to LPS biosynthesis. In *K. pneumoniae*, LPS has been shown to protect against serum bactericidal activity and to mediate adhesion to uroepithelial cells *in vitro* (Benedit et al., 1998; Merino et al., 1997). Furthermore, *K. pneumoniae* mutants deficient in O-antigen expression have been shown to be attenuated in their ability to colonize the intestine of germ-free chickens (Camprubi et al., 1993b) and to infect the urinary tract of rats (Camprubi et al., 1993a; Merino et al., 2000). In contrast, an O-antigen-deficient mutant was recently shown to be as virulent in a mouse model of pneumonia as the wild-type strain (Cortés et al., 2002). It is obvious to speculate whether a central function of LPS *in vivo* is protecting the bacterial cell against harmful substances in the host environment, e.g. bile salts in the intestine and antibacterial substances in urine.

The two mutants with insertions in the *ompA* and *surA* genes, respectively, were extensively attenuated in the GI colonization and UTI models. OmpA has previously been shown to contribute to the serum resistance and virulence of *E. coli* K1 as well as to the ability of the bacterium to invade cultured cells *in vitro* (Weiser & Gotschlich, 1991; Prasad Rao et al., 1996). The *surA* gene was first identified as a gene essential for late-stationary phase survival of *E. coli* (Tormo et al., 1990) but has subsequently been recognized as a periplasmic protein required for the proper assembly and folding of several outer-membrane proteins (Lazar & Kolter, 1996; Behrens et al., 2001). In *Salmonella typhimurium*, *surA* has been shown to be essential for adhesion to and invasion of eukaryotic cells *in vitro* as well as virulence when administered orally or intravenously to mice (Sydenham et al., 2000). The present study supports a general role for *SurA* in bacterial virulence. *SurA* may be essential for the correct assembly of proteins directly involved in host–bacteria interactions. However, the attenuation in virulence may also be related to non-optimal functioning of several outer-membrane proteins in general.

Only one mutant strain, 26C5, disrupted in *arcB*, exhibited a higher degree of attenuation in GI colonization than in UTI virulence. The *ArcA/ArcB* regulon is one of two systems for transcriptional regulation of the genes involved in metabolism in response to oxygen availability. *ArcA* acts primarily as a repressor of genes whose products are involved in aerobic metabolism; however, it also activates the transcription of some genes involved in anaerobic metabolism (Gansalus & Park, 1994). *ArcB* regulates the activity of *ArcA* in response to the respiratory state of the cell, but the exact nature of the signal to which *ArcB* responds is unknown. Given the low oxygen tension in the large intestine, it is not surprising that a mutation affecting adaptation to anaerobic metabolism has a crucial impact on the ability of the strain to colonize the GI tract. Also, the presence of the normal flora in the GI tract, competing for available nutrients, further stresses the importance of optimal metabolism in this host environment.

The vast majority of clinical *K. pneumoniae* isolates express a prominent polysaccharide capsule that covers the bacterial
cells. The capsule is generally considered to be a major virulence factor in *K. pneumoniae*, especially in systemic infections, whereas its role in serum-poor host environments is less clear (Podschun & Ullmann, 1998). In the present study, two mutant strains defective in the synthesis of fucose, a major component of the K16 capsule serotype expressed by *K. pneumoniae* C3091Sm<sup>R</sup>Rif<sup>R</sup>, were significantly attenuated in their virulence in the UTI model whereas both were as effective at colonizing the intestine as the wild-type strain. Our results thereby confirm the role of the capsule as an important virulence factor in *K. pneumoniae* and reveal that the capsule is not essential for *in vivo* survival of *K. pneumoniae* C3091Sm<sup>R</sup>Rif<sup>R</sup> in general. These findings are in accordance with those of a previous study, in which non-capsulated variants of *K. pneumoniae* strains were as effective at colonizing the intestine as their respective wild-type strains (Camprubi *et al*., 1993b), and add an important notion to the development of strategies for the prevention of GI colonization by *K. pneumoniae*. However, it has been shown that a capsule-defective mutant of *K. pneumoniae* LM21 was significantly attenuated in GI colonization, suggesting that the role of capsule in intestinal colonization may be strain-dependent (Favre-Bonte *et al*., 1999).

Type 1 fimbriae are expressed by many members of the *Enterobacteriaceae*, including *K. pneumoniae*, and are characterized by mediating adhesion to mannosylated structures. In *E. coli*, type 1 fimbriae have been identified as an important virulence factor in uropathogenic strains by several studies (Connell *et al*., 1996; Langermann *et al*., 1997; Sokurenko *et al*., 1998; Struve & Krogfelt, 1999). Our results suggest that type 1 fimbriae also have an important role in *K. pneumoniae*-associated UTIs, whereas expression of the fimbriae seems unimportant in GI colonization. However, as the transposon insertion site in the attenuated mutant was located downstream of the *fimB* homologue, the exact nature of the mutant strain is uncertain.

As in other STM studies, a relatively high percentage of the attenuated mutants were disrupted in genes of unknown function. In a recent study applying *in vivo* expression technology to identify *K. pneumoniae* genes specifically expressed during systemic infection of mice, 50% of the gene sequences identified were of unknown function (Lai *et al*., 2001). These results indicate the existence of many previously unrecognized factors in *K. pneumoniae* that influence the virulence and *in vivo* survival of the bacterium.

In a recent study, *K. pneumoniae* genes involved in the intestinal colonization of ampicillin-treated mice and adhesion to cultured epithelial cells were investigated by using STM (Maroncle *et al*., 2002). Most identified genes were involved in metabolic pathways, membrane transport, DNA metabolism and transcriptional regulation or were genes of unknown functions. It is striking that none of the genes was identical to genes identified in the present study and no genes encoding well-known *K. pneumoniae* virulence factors were identified. This suggests that the factors required during colonization of ampicillin-treated mice are fundamentally different from the factors required during colonization of streptomycin-treated mice. This difference is likely to reflect the different composition of the competing intestinal residual flora in the two models.

STM studies on GI colonization have also been performed in *E. coli* (Martindale *et al*., 2000) and *Vibrio cholerae* (Merrell *et al*., 2002). In accordance with the present study, several attenuated mutants were disrupted in genes involved in LPS biosynthesis and genes affecting anaerobic metabolism. Genes involved in acid tolerance, expected to be essential for bacterial survival during transit through the stomach, were also identified in these studies. The failure to isolate such genes in the present study might be related to the fact the mutagenesis was not saturated as only 1440 mutants were screened. To our knowledge, STM screening in a UTI model has previously only been performed in one study on *Proteus mirabilis* (Zhao *et al*., 1999). In this study only two attenuated mutants were identified, both of which were disrupted in genes of uncertain function.

In summary, we have developed and used a novel MS-STM assay to identify *K. pneumoniae* genes important for colonization of the intestine and infection of the urinary tract. By use of the MS-STM assay, screening of a mutant library in more than one infection model is significantly less cumbersome compared to the traditional STM methodology. Genes previously shown to be important for bacterial survival in a host were re-identified, thus validating the methodology of the MS-STM assay. Also, previously unknown genes important for *in vivo* survival of *K. pneumoniae* have been detected. Future studies will identify and characterize the function of these genes, which might play an important role in the virulence of *K. pneumoniae*.

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**REFERENCES**


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