Contribution of outer membrane protein K36 to antimicrobial resistance and virulence in Klebsiella pneumoniae

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Objectives: Loss of outer membrane protein (Omp) is commonly encountered in multidrug-resistant Klebsiella pneumoniae. However, little is known about the association between Omp loss and virulence. In the present study, this association was investigated in K. pneumoniae.

Methods: An OmpK36-deficient mutant (D OmpK36) was derived from a virulent clinical isolate by targeted gene insertion. Antimicrobial susceptibility was tested by microbroth dilution and disc diffusion. Virulence was assessed by serum resistance, phagocytosis, clearance of viable bacteria in the liver and lethality in mice following inoculation with bacteria.

Results: Susceptibility tests showed that D OmpK36 contributed to the resistance to cefazolin and cefoxitin but not to resistance to late-generation cephalosporins. In vitro assays demonstrated that loss of OmpK36 decreased the resistance to neutrophil phagocytosis and increased the resistance to serum killing during the first hour of the assay, but did not influence the growth rate when compared with the parental strain. Intraperitoneal injection of similar doses (~4 x 10^4 cfu) of the parental strain and D OmpK36 led to significantly fewer viable bacteria in the liver 24 h post-inoculation in D OmpK36-inoculated mice. In the mice LD_50 (the bacterial dose that caused 50% death) assay, the parental strain was ~100-fold more lethal (~10^5 cfu) than the D OmpK36 mutant (~10^5 cfu).

Conclusions: Loss of OmpK36 in K. pneumoniae resulted in increased antimicrobial resistance, increased susceptibility to neutrophil phagocytosis, increased resistance to serum killing and reduced virulence.

Keywords: K. pneumoniae, Ompk36, neutrophil phagocytosis

Introduction

The outer membrane protein (Omp) serves as a channel to regulate the exchange of extra- and intracellular substances, such as iron, nutrients and antibiotics, in Gram-negative bacteria. The loss of Omp can be found frequently in clinical isolates, especially in extended-spectrum β-lactamase (ESBL)-producing Enterobacteriaceae. In Klebsiella pneumoniae, the absence or reduced expression of the two major porins, OmpK35 and OmpK36, is often reported to be associated with a wide range of antimicrobial resistance. The consequence of dual loss of OmpK35 and OmpK36 in AmpC β-lactamase-producing K. pneumoniae has contributed to the extensive resistance to imipenem, an effective drug for ESBL producers.

Previous studies have shown that Omps in bacteria also play a role in the inflammatory response during bacterial infections. However, virulence associated with loss of Omps in K. pneumoniae is yet to be completely understood. In this study, we investigated the role of OmpK36 in virulence and antimicrobial resistance in K. pneumoniae.

Materials and methods

Identification and sequencing of the OmpK36 gene from a wild-type K. pneumoniae strain

To detect the ompK36 gene in the wild-type K. pneumoniae isolate used in this study, a gene-specific primer set, OmpK36-F (5′-GAAGGG TAATCAGTAAGCAGTGGC-3′) and OmpK36-R (5′-CCGTCGTAGAACTGTTGTA-3′) were used.

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AAAC-3), was used to determine the presence of the omp36 gene. The search for homologous sequences was performed at the GenBank database using the basic local alignment search tool (BLAST) through the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/).

**Construction and confirmation of the OmpK36-deficient mutant**

A clinical serotype K1 *K. pneumoniae* isolate NVT1002 that was susceptible to all antibiotics, except for an intrinsic resistance to ampicillin, was used for this study. An OmpK36-deficient mutant (ΔompK36-NVT1002) was generated from NVT1002 using a target insertional method as follows. A partial DNA fragment of omp36 was amplified using primers OmprK36F (5′-GGAATTCCATCGGTAGCGGCCTGAAA-3′) and OmprK36R (5′-ATACGATGCGCCGCCGTCGCTCTGTTGC-3′). PCR-amplified fragments were then transformed into *Escherichia coli* S17-1, followed by conjugation with the wild-type *K. pneumoniae* strain. The mutants with an insertion of the plasmid DNA fragment were confirmed by alignment-specific primer pairs, OmprK36F/pUUT (5′-CGGGATCAGCGG TAAAATGC-3′) and OmprK36R/pUTR (5′-AAGTTTACGATTGGGAC-3′). PCR products of OmprK36F/OmprK36R and pUTF/pUTR were also used for further confirmation that ΔompK36-NVT1002 was obtained.

To confirm ΔompK36-NVT1002, bacterial Omps were extracted with a 1% solution of sodium n-lauryl sarcosine (Sigma, USA) in PBS (pH 7.2) by sonication of bacteria grown in nutrient broth and analysed by SDS–PAGE. PFGE was also applied to confirm the genetic correlation between NVT1002 and ΔompK36-NVT1002; PFGE was performed as described previously.5

**Antimicrobial susceptibility testing**

Antimicrobial susceptibility was determined by the microbroth dilution test and disc diffusion according to the CLSI. The following antimicrobial agents were used: ampicillin; cefazolin; amoxicillin/clavulanic acid; cefoxitin; cefotaxime; ceftazidime; aztreonam; imipenem; amikacin; gentamicin; ciprofloxacin; and trimethoprim/sulfamethoxazole.7

**Virulence studies**

In vitro assays to assess virulence were performed by growth rate analysis, neutrophil phagocytosis and susceptibility to serum killing of NVT1002 and ΔompK36-NVT1002 strains. The growth curves were determined with 1 mL samples in an Eppendorf BioPhotometer, where optical density at 600 nm was measured every 30 min for 6 h. Neutrophil phagocytosis assays were performed as described previously.8 Serum bactericidal activity was measured using the method of Podschun et al.9 Results were expressed as a percentage of the inoculum, and responses in terms of viable counts were graded from 1 to 6. Grade 1 represented 100% of the inoculum after 1 h, but were <100% after 2 and 3 h. Grade 4 represented viable counts >100% of the inoculum after both 1 and 2 h, but <100% after 3 h. Grade 5 represented viable counts >100% of the inoculum after 1, 2 and 3 h, but which decreased during the third hour. Finally, grade 6 represented viable counts that exceeded those of the inoculum after 1, 2 and 3 h, and which increased throughout this time period. Each isolate was classified as highly susceptible (grade 1 or 2), immediately susceptible (grade 3 or 4) or resistant (grade 5 or 6).

An in vivo animal model to assess virulence was performed by assessing bacterial burden in the liver after intraperitoneal (ip) injection of NVT1002 and ΔompK36-NVT1002 and determination of the inoculum of *K. pneumoniae* causing a 50% death rate (LD50) in mice. Mice were sacrificed using CO2 after inoculation (~104 cfu) at 3–72 h. Livers were removed aseptically and homogenized with a tissue homogenizer in 5 mL of sterile saline. Bacterial colonies in liver were counted and counts of viable bacteria were calculated. Experiments were repeated three times to obtain the mean value of viable bacterial counts. All animal care procedures and protocols were approved by the Institutional Animal Care and Use Committee of the National Health Research Institute (NHRI-IACUC-096004-A).

For the determination of LD50 in mice, a 10-fold serial dilution of cfu of *K. pneumoniae* was made and the adult BALB/c mice were injected ip with 0.1 mL of each concentration. Ten mice were used as a sample population for each bacterial concentration. Symptoms and signs of infection were observed for 14 days. Survival of the inoculated livers was recorded and the LD50 was calculated using SigmaPlot version 7.0 from SPSS Inc. (Chicago, IL, USA).

**Statistical analysis**

The Student’s t-test was used for statistical analysis. Data are presented as means ± SD. P values of <0.05 were considered statistically significant.

**Results**

**Determination of OmpK36 expression in the ΔompK36-NVT1002 mutant**

Incorporation of pUT-km carrying a partial sequence of omp36 to target chromosomal omp36 by homologous recombination was confirmed using four PCR primer sets. Positive DNA fragments were observed using the pUT-F/OmpK36-F and pUT-R/OmpK36-R primer sets, respectively. No DNA amplification was observed, as predicted, with the OmpK36-F/OmpK36-R and pUT-F/pUT-R primer sets. The ΔompK36 mutant was further ascertained as deriving from parental *K. pneumoniae* by comparative PFGE. Loss of OmpK36 expression was also confirmed and determined by SDS–PAGE.

**Comparison of antimicrobial susceptibility and growth rate between NVT1002 and ΔompK36-NVT1002**

The parental strain, NVT1002, was susceptible to all tested antibiotics, except for the intrinsic resistance to ampicillin conferred by the blaTEM in *K. pneumoniae*. The ΔompK36-NVT1002 mutant showed additional resistance to cefazolin and cefoxitin when compared with the parental strain. Absence of OmpK36 expression did not result in a significant difference in the growth rate of ΔompK36 when compared with NVT1002 (Figure 1a).

**Contribution of OmpK36 to virulence in *K. pneumoniae***

Among the NVT1002, ΔompK36-NVT1002 and ATCC 13883 strains, NVT1002 exhibited the highest resistance to neutrophil phagocytosis, while ATCC 13883 exhibited the lowest resistance. Although ΔompK36-NVT1002 was relatively more resistant to phagocytosis than ATCC 13883, its phagocytic resistance was significantly (P<0.05) lower than that of NVT1002 (Figure 1b).

The NVT1002 and ΔompK36-NVT1002 strains were both susceptible to serum inhibition and were classified as grade 2. The viable counts of ΔompK36-NVT1002 were shown to decline less rapidly than those of NVT1002 within the first hour of the
and the inhibition of growth was comparable between the parental and ΔOmpK36-NVT1002 strains thereafter (Figure 1c).

**Bacterial burden of NVT1002 and ΔOmpK36-NVT1002 in the liver after ip injection**

The count of viable bacteria from the liver of mice injected with NVT1002 was elevated in a time-dependent manner, peaking at 2–3 days after injection. In mice injected with ΔOmpK36-NVT1002, the bacterial count was elevated slightly within 3 h and subsequently decreased throughout the course of the experiment ($P<0.05$) (Figure 2a).

**Determination of lethality in mice for NVT1002 and ΔOmpK36-NVT1002**

The LD$_{50}$ of NVT1002 was $5\times10^2$ cfu. The OmpK36-deficient strain, ΔOmpK36-NVT1002, was less virulent than NVT1002, as $10^5$–$10^6$ cfu more bacteria were required to achieve the LD$_{50}$ (Figure 2b).

**Discussion**

Loss of Omp is one of the important mechanisms that contributes to antimicrobial resistance in Gram-negative bacteria. Several studies have indicated that two major porins in K. pneumoniae, OmpK35 and OmpK36, are often missing in multidrug-resistant strains, especially in combination with ESBL producers. Concomitance of OmpK35 and OmpK36 deficiencies and AmpC β-lactamase production can confer resistance to imipenem, which is an effective drug for multidrug-resistant bacteria. Our results demonstrated a significant increase in the MICs of cefazolin and cefoxitin in the ΔOmpK36-NVT1002 strain, which exhibited significant resistance to these antibiotics. Although deletion of OmpK36 expression without ESBL production can lead to resistance to cefazolin and cefoxitin, this mutant was still susceptible to extended-spectrum cephalosporins. This suggests that intrinsic SHV-1 in combination with loss of OmpK36 is insufficient to cause resistance to extended-spectrum cephalosporins.

It has been reported that K. pneumoniae OmpK36 can activate the classical complement pathway via antibody-independent binding to C1q. Presumably, the OmpK36-deficient strain is more resistant to serum killing. Although knockout of ompK36 can modify the surface structure of K. pneumoniae and this modification may alter the receptor binding of phagocytes leading to increased susceptibility to phagocytosis, the exact mechanism is yet to

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**Figure 1.** Effect of OmpK36 on cell growth (a), phagocytosis (b) and susceptibility to serum killing (c). (a) The cell growth curves were determined by spectrophotometry, measuring optical density at 600 nm. No significant difference was observed between NVT1002 and ΔOmpK36-NVT1002 within 150 min. (b) The phagocytosis rate was calculated as the percentage of neutrophil-ingested fluorescein isothiocyanate (FITC)-labelled bacteria over time. Values are expressed as means±SD. *$P<0.05$, NVT1002 versus ΔOmpK36-NVT1002. (c) There was no significant difference in the number of viable bacteria between NVT1002 and ΔOmpK36-NVT1002 after exposure to serum over 3 h.
be determined. Since Omp is responsible for the exchange of substances, including nutrients and toxic metabolites, the reduced virulence may be due to the accumulation of internal toxic metabolites or blocking the entry of nutrients. However, since there was no observable effect on ΔompK36 growth (Figure 1a), it is possible that OmpK35 or another porin is sufficient for exchanging internal and external substances.

Comparing both strains, viable counts of bacteria in the liver for ΔompK36-NVT1002 were slightly increased after ip injection and peaked at 3 h after injection before being cleared rapidly (Figure 2a). In contrast, the viable wild-type parental strain persisted and progressively proliferated beyond day 3 after inoculation. Disruption of ompK36 reduced virulence, as indicated by the rapid clearance of the mutant bacterial burden from the liver. This reduction in virulence is further supported by our mice lethality study, as NVT1002 was ∼100-fold more lethal than ΔompK36-NVT1002 (∼10^5 cfu) in the LD50 assay (Figure 2b). These data suggest that OmpK36 plays a significant role in the virulence of K. pneumoniae.

In conclusion, we have demonstrated that OmpK36 contributes to both the antimicrobial resistance and virulence of K. pneumoniae. Omp deficiency in K. pneumoniae could result in resistance to antimicrobial agents, but diminished virulence.

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Transparency declarations
None to declare.

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