J. Microbiol. Biotechnol. (2005), **15**(2), 421–424

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Expression of Newer Outer Membrane Proteins (OMPs) Induced by Cephalosporins and Quinolone Group of Antibiotics in *Klebsiella pneumoniae*

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Received: September 26, 2003
Accepted: May 10, 2004

Abstract Effect of antibiotics belonging to three different groups, including third generation cephalosporins, aminoglycosides, and quinolones, on the outer membrane protein (OMP) profile of *Klebsiella pneumoniae* was examined. It was found that a new OMP (porins) of 40 kDa molecular mass was expressed in *Klebsiella pneumoniae*, when grown in the presence of ceftazidime, whereas new proteins with 30 kDa and 22 kDa masses were detected in the presence of ofloxacin. The immunoblot analysis showed that the new proteins of 40 kDa and 30 kDa molecular masses were expressed on the outer envelope, when being exposed to antibiotics ceftazidime and ofloxacin, respectively. This finding is important, as the outer surface comes in contact with the immune system, and therefore may have a bearing on the outcome of the disease.

Key words: *Klebsiella pneumoniae*, outer membrane proteins (OMPs), antibiotics

Klebsiella pneumoniae is an important human pathogen which has become a leading cause of morbidity and mortality in hospital acquired infections [11]. Most studies have defined the role of LPS and capsule in the pathogenicity and other biological phenomenon, whereas the outer membrane proteins (OMPs) have attracted little attention [18]. Most of the studies conducted on OMPs have been carried out with *E. coli* as an organism [1], and its expression has been shown to be influenced by certain environmental conditions.

The indiscriminate use of antibiotics has led to the emergence of multiple drug resistant mutants among *Klebsiella pneumoniae*. Therefore, the focus has been made on the newer antibiotics, especially those belonging to the third generation cephalosporins, aminoglycosides, and quinolones. One of the mechanisms of co-resistance to β-lactam and the fluoroquinolone group of antibiotics in *K. pneumoniae* is decrease of permeability of the outer membrane to the antibacterial agents due to porin alterations [9]. It has also been demonstrated that exposure of bacteria to antibiotics can unmask/induce expression of newer protein (OMP) epitopes [7, 8, 15].

Thus, this study was undertaken to ascertain whether antibiotics belonging to three different groups can affect the OMP profile of a heavily encapsulated *K. pneumoniae* ATCC 43816, when grown in the presence of three different antibiotics with different modes of action.

Antibiotic Susceptibility Test

Susceptibility test of the bacterial strain (*Klebsiella pneumoniae* ATCC 43816, obtained from Dr. David P. Speert from the Department of Paediatrics, University of British Columbia, Vancouver, Canada) was carried out by following the method of the Bauer-Kirby disc diffusion test [2]. The antibiotics used and their concentration per disc were amikacin (10 μg), ceftazidime (30 μg), ceftrioxone (30 μg), ciprofloxacin (5 μg), gentamicin (10 μg), ofloxacin (2 μg), ceftrizoxime (30 μg), tobramicin (30 μg), and norfloxacin (10 μg). Readymade discs were obtained from Hi-Media, Mumbai, India. The minimum inhibitory concentration (MIC) of the antibiotics for *K. pneumoniae* was determined according to National Committee for Clinical Laboratory Standards Guidelines [4]. Antibiotic susceptibility test showed that *Klebsiella pneumoniae* ATCC 43816 was sensitive to all the third generation cephalosporins, except ceftazidime which showed minimum inhibitory concentration (MIC) of 40 μg/ml. Among the aminoglycosides and quinolones, amikacin and ofloxacin gave MICs of 10 μg/ml and 0.9 μg/ml, after repeated passages in respective antibiotic. The MICs of these test antibiotics were used in the rest of the experiments.

Extraction and Electrophoresis of Outer Membrane Proteins (OMPs)

The outer membrane proteins, particularly porins, have been implicated in the permeability to antimicrobial agents

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[10], and the loss of porin is an important cause of resistance to some of the antimicrobials, particularly β-lactam antibiotics [13, 10]. It has recently been reported that new porins may replace the function of the lost porins[13]. Therefore, in this study, we evaluated the effect of three antibiotics, such as ceftazidime, amikacin, and ofloxacin belonging to different groups, on the OMP (particularly porins) profiles of heavily encapsulated *K. pneumoniae* ATCC 43816. Outer membrane proteins were extracted from bacteria by the method of Sato *et al.* [16]. Briefly, cells were grown in the presence and absence of test antibiotics (MIC of ceftazidime, amikacin, and ofloxacin) overnight at 37°C in a shaking incubator (150 rpm). Bacteria were harvested, washed twice, suspended in Tris-buffer (0.1 M, pH 8.0), and sonicated. Unbroken cells and debris were eliminated by centrifugation at 3,000 ×*g* for 10 min, and cell envelopes in the supernatant were removed at 1,00,000 ×*g* for 2 h. After solubilization in 2% (w/v) N-lauroylsarcosine (Sigma Chemical Co., St. Louis, MO, U.S.A.) for 30 min at 37°C, OMPs were recovered as sodium lauroyl sarcosine-insoluble material by ultracentrifugation (1,00,000 ×*g* for 2 h) and stored at -20°C. Protein concentration of the OMPs was determined by the method of Lowry *et al.* [6]. Equal amounts of OMPs were loaded onto each well, and they were resolved on a 12% SDS-PAGE gel. In each case, OMPs of the parent bacteria were compared with the OMPs of bacteria exposed to the respective

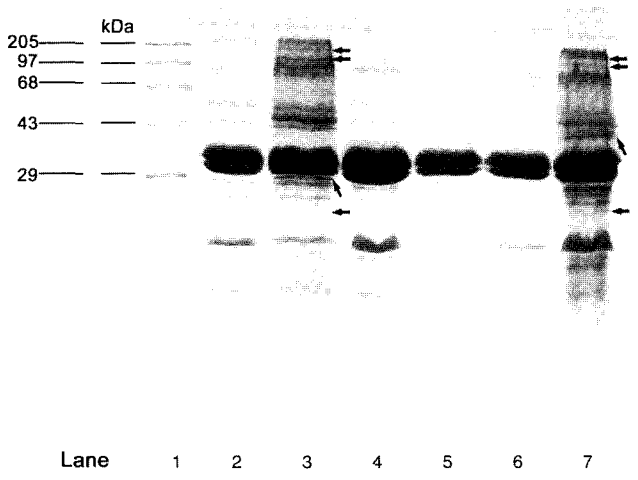

Fig. 1. Photographic representation of electrophoretic pattern of outer membrane proteins (OMPs) (lanes 2, 4, 6) expressed in the absence of antibiotics ofloxacin, amikacin, and ceftazidime, respectively; (lanes 3, 5, 7) expressed in the presence of antibiotics ofloxacin, amikacin, and ceftazidime, respectively, and standard molecular weight markers (lane 1) in 12% SDS-polyacrylamide gel stained with Coomassie blue.

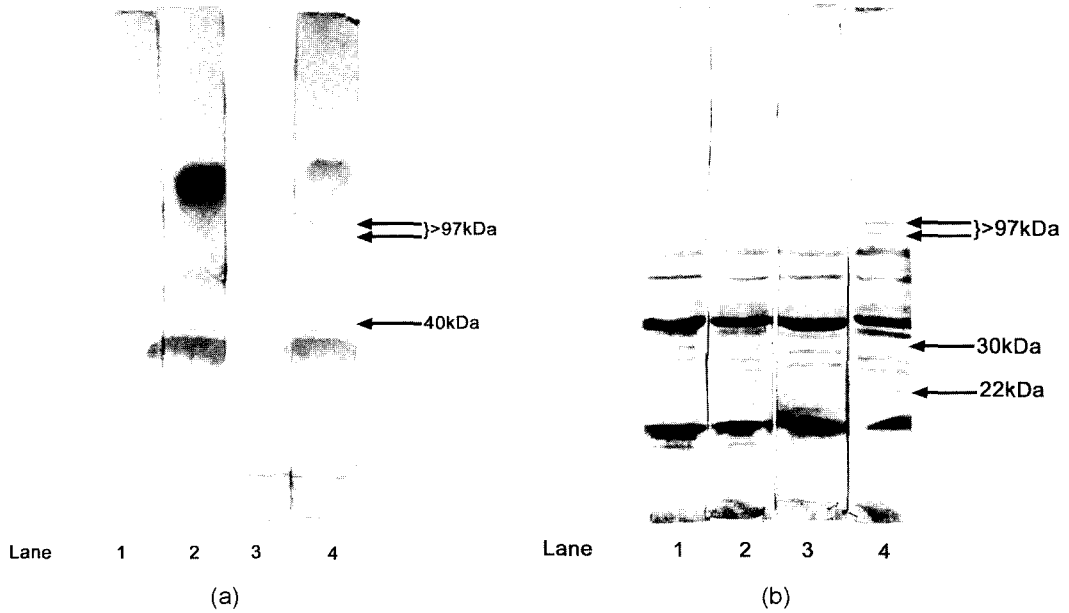

Fig. 2. (a) Immunoblot showing the presence of two proteins with molecular mass more than 97 kDa and a 40 kDa in the outer membrane proteins (OMPs) from the cells grown in the presence of antibiotic ceftazidime (test OMPs), but not in the OMPs from the cells grown in the absence of antibiotic (control OMPs). Lanes 1 and 3, OMPs from the cells grown in the absence of ceftazidime; lanes 2 and 4, OMPs from the cells grown in the presence of ceftazidime; lanes 1 and 2, Reacted with sera against control OMPs; lanes 3 and 4, Reacted with sera against test OMPs. (b) Immunoblot showing the presence of two proteins with molecular mass more than 97 kDa and two proteins of 30 kDa and 22 kDa in the OMPs from the cells grown in the presence of antibiotic ofloxacin (test OMPs), but not in the OMPs from the cells grown in the absence of antibiotic (control OMPs). Lanes 1 and 3, OMPs from the cells grown in the absence of ofloxacin; lanes 2 and 4, OMPs from the cells grown in the presence of ofloxacin; lanes 1 and 2, Reacted with sera against control OMPs; lanes 3 and 4, Reacted with sera against test OMPs.

antibiotics. The expression of newer proteins was noted, and their molecular size were calculated with the help of molecular weight markers after Coomassie blue staining of the gel. No difference in the OMP profile of the bacteria grown in presence and absence of amikacin was observed on Coomassie blue staining, whereas changes were observed in the OMP profile of the bacteria grown in the presence of ceftazidime and ofloxacin (Fig. 1).

Raising Immune Sera

The immune sera were raised in different groups of four mice each against the total OMP fractions or formalin-fixed whole cells which were grown in the presence or absence of the respective antibiotics. Formalin-fixed whole cells were prepared by treating cells (O.D. adjusted to 0.5 at 600 nm) with 0.5% formalin in PBS overnight at 37°C with shaking. Cells were then washed three times with PBS to remove the residual formalin. For raising immune sera, mice were immunized intramuscularly with two doses of 0.05 ml of formalin-fixed bacteria at 14-days interval. Mice were then bled on the 14th day after the second dose, and sera were separated, stored at -20°C, and used for the immunoblot experiment. Immune sera against the total OMP fractions in mice were raised in a similar way, as described for formalin killed cells.

Immunobloting

OMP preparations expressed in the presence or absence of respective antibiotics were resolved on gel electrophoresis as deseribed above, and they were then transferred onto nitrocellulose paper by the method of Towbin *et al.* [17]. Blotting was carried out at 14 V constant voltage overnight at 4°C in the electroblotting buffer. Transferred protein bands were visualized by amidoblack staining. Blots were blocked by immersion in PBS containing 1% BSA and treated at 4°C overnight with respective mouse antiserum. The blots were then detected by rabbit antimouse IgG-horseradish peroxidase conjugate (Biogene, Banglore, India). Strips were developed with diaminobenzidine (DAB). Immunoblot analysis with the test sera (raised against the OMPs extracted from the cells grown in the presence of respective antibiotics) revealed additional bands of molecular size of 40 kDa, and two bands larger than 97 kDa with ceftazidime test sera. Bands of molecular sizes 30 kDa and 22 kDa and two bands of molecular size above 97 kDa were observed with ofloxacin test sera. These bands were not detected with control sera (Figs. 2a and 2b). Immunoblot study with amikacin test sera revealed no additional band, thus confirming the previous result that no change was observed when OMP profiles of bacteria grown in the presence and absence of the amikacin were compared. The present results are in agreement with the result of Lun *et al.* [8], who demonstrated difference in recognition of protein epitopes by the sera which were raised against the bacteria preexposed and unexposed to the sub-MIC concentration of aztreonam. The reason for the observed difference in the OMP profile by antibiotic treatment may be due to rearrangement of bacterial proteins, revealing new antigenic determinants [7, 14, 12]. Also, it is likely that the antibiotic treatment causes reduced production of capsular polysaccharide, thus resulting in exposure of OM antigens which are otherwise occluded [5].

In order to further analyze whether the proteins expressed in the presence of antibiotics are present on the surface of bacteria, serum was raised against killed cells [originally grown in the presence of antibiotic ceftazidime (CD-Ts) and ofloxacin (OF-Ts), respectively] and was adsorbed to cells grown in the absence of the respective antibiotics. Immunoblot studies with CD-Ts and OF-Ts antisera revealed a single band of molecular mass of 40 kDa and 30 kDa in the test OMPs, respectively, but not in the control OMPs (Figs. 3a and 3b). This in fact confirms the surface location of these two proteins.

In conclusion, in this study, we have identified two new proteins (porins) with molecular masses of 40 kDa and 30 kDa, which are expressed on the surface of *K. pneumoniae*

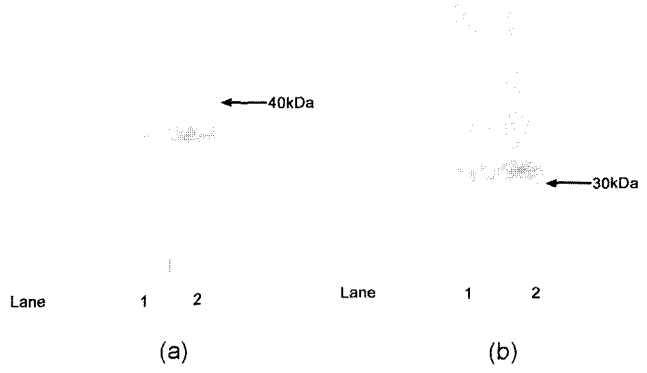

Fig. 3. (a) Immunoblot showing the presence of a single new protein of 40 kDa in the OMPs from the cells grown in the presence of antibiotic ceftazidime (test OMPs), but not in the OMPs from the cells grown in the absence of antibiotic (control OMPs). Lane 1, OMPs from the cells grown in the absence of ceftazidime; lane 2, OMPs from the cells grown in the presence of ceftazidime; the OMPs both lanes were reacted with antisera raised against killed cells grown in the presence of antibiotic ceftazidime and adsorbed to cells grown in the absence of antibiotic. (b) Immunoblot showing the presence of a single new protein of 30 kDa in the OMPs from the cells grown in the presence of antibiotic ofloxacin (test OMPs), but not in the OMPs from the cells grown in the absence of antibiotic (control OMPs). Lane 1, OMPs from the cells grown in the absence of ofloxacin; lane 2, OMPs from the cells grown in the presence of ofloxacin; the OMPs in both lanes were reacted with antisera raised against killed cells grown in the presence of antibiotic ofloxacin and adsorbed to cells grown in the absence of antibiotic.

in the presence of ceftazidime and ofloxacin, respectively. Further characterization of these new proteins will provide their possible role in antibiotic pressure.

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