ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Oct. 1997, p. 2302–2304 Vol. 41, No. 10
0066-4804/97/$04.00+0

Involvement of a 43-Kilodalton Outer Membrane Protein in β-Lactam Resistance of *Shigella dysenteriae*

ALAK KANTI KAR, ANINDYA SUNDAR GHOSH, KAMLESH CHAUHAN, JASIMUDDIN AHAMED, JOYOTI BASU, PARUL CHAKRABARTI, AND MANIKUNTALA KUNDU*

Department of Chemistry, Bose Institute, Calcutta 700 009, India

Received 24 February 1997/Returned for modification 19 May 1997/Accepted 7 August 1997

A beta-lactam-sensitive strain (C152) of *Shigella dysenteriae* showed two major outer membrane proteins (OMPs) with M_rs of 43,000 and 38,000, while the clinical isolate M2 lacked the 43,000-M_r OMP, which acted as a channel for beta-lactam antibiotics. Permeability of beta-lactams across the outer membrane (OM) of M2 was lower than that across the OM of C152. Mutants deficient in the 43-kDa OMP could be selected in vitro from strain C152 in the presence of cefoxitin. All beta-lactam-resistant strains were sensitive to imipenem.

Shigella species represent a major cause of diarrheal diseases in developing countries (2). The frequency of strains multiply resistant to ampicillin, trimethoprim-sulfamethoxazole, streptomycin, chloramphenicol, and tetracycline is a cause of growing concern (2, 4). Resistance to antibiotics may arise through alteration of the drug targets (26), degradation of the drugs by enzymes such as the beta-lactamases (10), decrease in permeability of the cell envelope (8, 9, 14, 16, 23, 25), and extrusion of the drugs by efflux pumps of broad specificity (17). The permeability factor controlling the access of the antibiotic to its target is often critical in determining the efficacy of a beta-lactam against a gram-negative bacterium (18). We demonstrate here the association of beta-lactam resistance with the absence of a 43-kDa porin in *Shigella dysenteriae*.

Clinical isolates of sensitive (C152) and resistant (M2) strains of *S. dysenteriae* type I were obtained from the School of Tropical Medicine, Calcutta, India. The organisms were grown routinely in tryptic soy broth (Difco) at 37°C. The antimicrobial agents tested were cephaloridine, moxalactam, cefoxitin, and norfloxacin (Sigma, St. Louis, Mo.); tetracycline and chloramphenicol (Boehringer Mannheim); imipenem (gift from Jean-Marie Frere, University of Liege, Liege, Belgium); and aztreonam (gift from Bristol-Myers Squibb, Syracuse, N.Y.). MICs were determined by growing organisms in twofold serial dilutions of the antibiotics on Mueller-Hinton agar plates. A total of 10^6 CFU was spotted onto agar plates supplemented with antibiotics, and MICs were read after 18 h of incubation at 37°C. The concentration at which no visible growth was observed was recorded as the MIC. Cefoxitin-resistant mutants were isolated by adapting strain C152 to serially increasing concentrations of cefoxitin on Luria agar plates. Colonies growing at a particular concentration of cefoxitin were plated at the same concentration of antibiotic three times in succession before adaptation in the presence of the next twofold-higher concentration of cefoxitin.

For preparing outer membranes (OMs), cells were harvested, washed with 50 mM Tris-HCl–5 mM EDTA (pH 8) containing 1-μg/ml DNase, and disrupted by sonication (Labsonic 2000; Braun, Melsungen, Germany) with four 1-min pulses at 100 W. Undisrupted cells and cell debris were removed by centrifugation. Membranes were then pelleted by centrifugation at 16,000 × *g* for 30 min as described previously (12), treated with 2% Sarkosyl for 60 min at 30°C, and centrifuged at 100,000 × *g* for 30 min. The pellet which contained the outer membrane proteins (OMPs) was washed twice with 0.5% Sarkosyl and extracted successively with 1% (wt/vol) CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate} in the presence of 0.5 M NaCl overnight at 4°C and with 2% (wt/vol) Nonidet P-40 for 1 h at 30°C, followed by centrifugation at 100,000 × *g* for 40 min. The pellet was then treated with 2% (wt/vol) *N*-dodecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate (Zwittergent 3-12) overnight at 4°C and centrifuged as before. The supernatant was dialyzed against 20 mM Tris-HCl–5 mM EDTA containing 0.1% Zwittergent 3-12 (pH 8) (buffer A), and 0.7 mg of protein was loaded on a Mono Q HR5/5 (1-ml) column connected to a fast protein liquid chromatography system and equilibrated in buffer A. The protein was eluted with a gradient of 0 to 0.7 M NaCl at a flow rate of 1 ml/min. Fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (7). For better resolution of the OMPs, 3 M urea was included in the gels (11). Fractions containing mainly the 43-kDa OMP were pooled and stored for further use.

Liposome swelling assays were carried out as described by Nikaido and Rosenberg (20). A total of 2.5 μmol of egg phosphatidylcholine and 0.1 μmol of dicetyl phosphate were dried as a thin film. The film was suspended in 0.2 ml of buffer to which crude OM (100 μg of protein) or purified 43-kDa protein (25 μg) was added. The mixture was sonicated and dried under vacuum. Finally, the film was reconstituted with 0.4 ml of a solution containing 12 mM stachyose, 4 mM sodium NAD, and 1 mM imidazole-NAD buffer (pH 6) (9, 19) and diluted in isotonic solutions of beta-lactams. Liposome swelling was measured as the initial rate of decrease in absorbance at 400 nm. The concentration of stachyose used was isotonic for the above liposomes. The isotonic concentration of stachyose as well as that of the beta-lactam antibiotic used was determined as described by Yoshimura and Nikaido (28).

Beta-lactamase activity was assayed spectrophotometrically at 482 nm (21) with the chromogenic cephalosporin nitrocefin (Becton Dickinson Microbiology Systems, Cockeysville, Md.), with sonic extracts of exponentially growing cells. For penicillin binding assays, 100 μg of membrane proteins was incubated with 10^{-3} M benzyl[^{14}C]penicillin (0.5 mCi/mmol) (Amersham International, Buckinghamshire, United Kingdom) at 30°C for 10 min (12). The labeling was terminated with a 10-fold excess

* Corresponding author. Mailing address: Department of Chemistry, Bose Institute, 93/1 APC Road, Calcutta 700 009, India. Fax: 91-33-3506790.

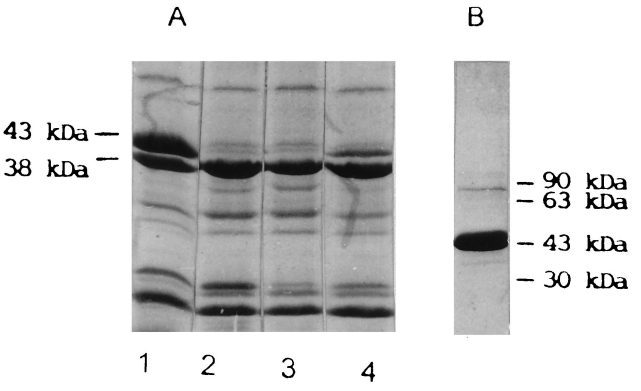

FIG. 1. SDS-PAGE of OMPs of *S. dysenteriae* C152 (lane 1), M2 (lane 2), M3 (lane 3), and M7 (lane 4) (A) and purified 43-kDa porin (B).

of nonradioactive penicillin. Membranes were analyzed by SDS-PAGE, followed by fluorography. In competition studies, membranes were first incubated for 10 min at 30°C with the nonradioactive beta-lactam, followed by incubation for 10 min with a saturating concentration (10^{-4} M) of benzyl[^{14}C]penicillin and termination of the reaction after 10 min as usual. For determination of the 90% inhibitory doses (ID_{90}), the intensities of the bands were analyzed by densitometric scanning of the fluorograms.

Automated N-terminal peptide sequencing with purified protein was performed on a Shimadzu PSQ-1 protein sequencer.

OM profiles and susceptibilities of the different strains of *S. dysenteriae* to beta-lactam and other antibiotics. On SDS-urea gels, *S. dysenteriae* C152 was found to contain two major prospective pore-forming proteins of 43,000 and 38,000 in M_r (Fig. 1). The clinical isolate M2 showed an almost complete absence of the 43-kDa OMP. Adaptation of C152 in the presence of cefoxitin in vitro gave rise to two porin-deficient mutants, M3 (with an OM profile similar to that of M2) and M7 (which showed a partial lack of the 43-kDa OMP in comparison to C152) (Fig. 1). With reference to C152, the 43-kDa-OMP-deficient strains M2, M3, and M7 were all resistant to a range of beta-lactams (Table 1). Interestingly, however, all four strains were sensitive to the carbapenem, imipenem. The sensitivity to the structurally unrelated antibiotics tetracycline, chloramphenicol, and norfloxacin was the same among all four strains.

Susceptibilities of the PBPs towards beta-lactam antibiotics and beta-lactamase activities. The penicillin-binding protein (PBP) profiles of all the strains were identical and similar to that reported earlier for *S. dysenteriae* (reference 12 and data not shown). There were no observable changes in the affinities of the PBPs of M2 or M7 compared to those of C152 in the case of antibiotics to which these strains were resistant (Table 2) with the exception of moxalactam, which appeared to have a higher affinity for PBP3 of C152 and M7 compared to that of M2. The beta-lactamase levels in all the strains were very low (≤2 nmol of nitrocefin hydrolyzed/min/mg of protein), and no beta-lactamase could be induced in the presence of the beta-lactams tested.

The 43-kDa porin as a channel for beta-lactam antibiotics. The 43-kDa porin was purified from the OM of *S. dysenteriae* C152 and was greater than 98% pure (Fig. 1). It had the N-terminal sequence AEVYNKD-N, which was typical of other enterobacterial porins (1). Permeabilities of beta-lactam antibiotics were measured with proteoliposomes containing crude OMs or purified porin. M2 showed a lower permeability in the case of piperacillin, cephaloridine, moxalactam, cefoxitin, and aztreonam compared to C152 (Table 3). Imipenem permeabilities of the two strains were comparable. M3, which

TABLE 1. Susceptibilities of different strains of *S. dysenteriae* towards beta-lactam and other antibiotics

Antibiotic	MIC (μg/ml) for strain:			
	C152	M2	M3	M7
Cephaloridine	4	80	80	100
Cefoxitin	5	1,000	1,000	1,000
Moxalactam	8	500	125	125
Piperacillin	8	1,000	1,000	1,000
Aztreonam	0.5	500	500	125
Imipenem	2	2	2	2
Tetracycline	200	200	200	200
Chloramphenicol	250	250	250	250
Norfloxacin	0.3	0.3	0.3	0.3

TABLE 2. Binding of different beta-lactam antibiotics to the PBPs of *S. dysenteriae*, represented as ID_{90} (μM)[a]

Antibiotic	Strain	PBP1	PBP2	PBP3	PBP5
Cephaloridine	C152	100	>100	>100	>100
	M2	100	>100	>100	>100
	M7	100	>100	>100	>100
Cefoxitin	C152	100	>100	>100	1
	M2	100	>100	>100	1
	M7	100	>100	>100	1
Aztreonam	C152	>100	>100	5	>100
	M2	>100	>100	5	>100
	M7	>100	>100	5	>100
Piperacillin	C152	>100	>100	5	>100
	M2	>100	>100	5	>100
	M7	>100	>100	5	>100
Imipenem	C152	>100	5	>100	>100
	M2	>100	5	>100	>100
	M7	>100	5	>100	>100
Moxalactam	C152	>100	>100	1	>100
	M2	>100	>100	10	>100
	M7	>100	>100	1	>100

[a] ID_{90} represents the concentration of beta-lactam antibiotic that reduces the binding of benzyl[^{14}C]penicillin by 90%. Results are the means of three determinations (standard deviation, ≤10%).

TABLE 3. Swelling rates of proteoliposomes reconstituted with OMPs from M2

Antibiotic	Relative swelling rate[a]
Cephaloridine	50 (40–59)
Cefoxitin	50 (42–59)
Moxalactam	59 (54–65)
Piperacillin	50 (43–57)
Aztreonam	60 (52–69)
Imipenem	100 (95–108)

[a] For each antibiotic, results are expressed as percentages of the swelling rate of liposomes reconstituted with OMs of C152. Data represent the averages of four sets of experiments performed with four different OM preparations. The ranges of values obtained are listed in parentheses.

has an antibiotic susceptibility profile similar to that of M2, showed, like this strain, almost no 43-kDa porin in SDS-urea gels. M7, which showed a partial lack of the 43-kDa porin, was also less susceptible than C152 to the antibiotics tested. A partial lack of the 43-kDa porin therefore appeared sufficient to confer high-level beta-lactam resistance to poorly permeable beta-lactams. With proteoliposomes containing the purified 43-kDa porin, this protein was confirmed to act as a channel for beta-lactams. Relative swelling rates, calculated as percentages of the rate of swelling of cephaloridine, were 50, 30, 15, and 5 for cefoxitin, moxalactam, aztreonam, and piperacillin, respectively. Permeability was, therefore, low in the case of the dianionic compounds aztreonam and moxalactam and more so in the case of piperacillin, which has a bulky side chain.

Antibiotic insensitivity is often associated with intrinsic low permeability of hydrophilic antibiotics through the porin channels, as in the case of *Pseudomonas aeruginosa* (15), or by the lack of expression of nonspecific porin channels (22, 27). In *S. dysenteriae*, the absence of the 43-kDa porin affected largely the susceptibility to slowly penetrating beta-lactams such as the dianionic moxalactam and aztreonam, as well as piperacillin, which has an extremely bulky side chain. This was not surprising since earlier reports have shown that *Escherichia coli* K-12 mutants that were highly resistant to moxalactam or dibasic beta-lactams were only slightly more resistant to more permeable antibiotics such as cephaloridine, cephacetrile, and ampicillin (5, 6). The OM penetration step is likely to comprise the rate-limiting step in the action of slowly penetrating antibiotics in *S. dysenteriae*, accounting for the high-level resistance of M2 to these antibiotics, compared to that of C152. The absence of cross-resistance to other classes of antibiotics suggests that efflux pumps (which confer multiple antibiotic resistance) are unlikely to be involved in the resistance mechanism of these mutants.

For *Enterobacter cloacae*, imipenem- and meropenem-resistant mutants have been reported to lack nonspecific porins (24). On the other hand, the absence of nonspecific porins does not directly correlate with the imipenem susceptibility of *Enterobacter aerogenes* (3). In *S. dysenteriae*, the absence of the 43-kDa porin was not sufficient to confer imipenem resistance, similar to the case of *Klebsiella pneumoniae*, for which mutants selected in vivo were resistant to cefoxitin, whereas imipenem maintained activity in these porin-deficient mutants (13). The speculation that there is an alternative pathway for uptake of carbapenems in *S. dysenteriae* is supported by the fact that imipenem permeabilities were comparable in C152 and M2. However, the imipenem channel, if any, remains to be identified.

This study was supported in part by grants from the Department of Science and Technology and the Council of Scientific and Industrial Research (CSIR), Government of India, to M.K. A.K.K. was supported by a fellowship from the CSIR. K.C. is a postdoctoral fellow of the Department of Biotechnology, Government of India.

We are grateful to H. S. Savithri, Indian Institute of Science, Bangalore, for help in protein sequencing.

REFERENCES

1. **Alberti, S., F. Rodriguez-Quinones, T. Schirmer, G. Rummel, J. M. Tomas, J. P. Rosenbusch, and V. J. Benedi.** 1995. A porin from *Klebsiella pneumoniae*: sequence homology, three-dimensional model, and complement binding. Infect. Immun. **63:**903–910.
2. **Bennish, M. L., and M. A. Salam.** 1992. Rethinking options for the treatment of shigellosis. J. Antimicrob. Chemother. **30:**243–247.
3. **Charrel, R. N., J.-M. Pages, P. De Micco, and M. Mallea.** 1996. Prevalence of outer membrane porin alteration in β-lactam antibiotic-resistant *Enterobacter aerogenes*. Antimicrob. Agents Chemother. **40:**2854–2856.
4. **Cohen, M. C.** 1994. Antimicrobial resistance: prognosis for public health. Trends Microbiol. **2:**422–425.
5. **Harder, K. J., H. Nikaido, and M. Matsuhashi.** 1981. Mutants of *Escherichia coli* that are resistant to certain beta-lactam compounds lack the *ompF* porin. Antimicrob. Agents Chemother. **20:**549–552.
6. **Komatsu, Y., K. Murakami, and T. Nishikawa.** 1981. Penetration of moxalactam into its target proteins in *Escherichia coli* K-12: comparison of a highly moxalactam-resistant mutant with its parent strain. Antimicrob. Agents Chemother. **20:**613–619.
7. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
8. **Lee, E. H., M. H. Nicolas, M. D. Kitzis, G. Pialoux, E. Collatz, and L. Gutmann.** 1991. Association of two resistance mechanisms in a clinical isolate of *Enterobacter cloacae* with high-level resistance to imipenem. Antimicrob. Agents Chemother. **35:**1093–1098.
9. **Lee, E.-H., E. Collatz, J. Trias, and L. Gutmann.** 1992. Diffusion of beta-lactam antibiotics into proteoliposomes reconstituted with outer membranes of isogenic imipenem-susceptible and resistant strains of *Enterobacter cloacae*. J. Gen. Microbiol. **138:**2347–2351.
10. **Livermore, D. M.** 1995. β-Lactamases in laboratory and clinical resistance. Clin. Microbiol. Rev. **8:**557–584.
11. **Lugtenberg, B., J. Meijers, R. Peters, P. van der Hoek, and L. van Alphen.** 1975. Electrophoretic resolution of the "major outer membrane protein" of *Escherichia coli* K12 into four bands. FEBS Lett. **58:**254–258.
12. **Mahapatra, S., J. Basu, J. van Beeumen, and M. Kundu.** 1994. Characterisation of a 38 kDa penicillin-binding protein and its possible involvement in maintaining stationary-phase cells of *Shigella dysenteriae*. Microbiology **140:** 3177–3182.
13. **Martinez-Martinez, L., S. Hernandez-Alles, S. Alberti, J. M. Tomas, V. J. Benedi, and G. A. Jacoby.** 1996. In vivo selection of porin-deficient mutants of *Klebsiella pneumoniae* with increased resistance to cefoxitin and expanded-spectrum cephalosporins. Antimicrob. Agents Chemother. **40:**342–348.
14. **Masuda, N., E. Sakagawa, and S. Ohya.** 1995. Outer membrane proteins responsible for multiple drug resistance in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **39:**645–649.
15. **Nakae, T.** 1995. Role of membrane permeability in determining antibiotic resistance in *Pseudomonas aeruginosa*. Microbiol. Immun. **39:**221–229.
16. **Nikaido, H.** 1994. Prevention of drug access to bacterial targets: permeability barriers and active efflux. Science **264:**382–388.
17. **Nikaido, H.** 1996. Multidrug efflux pumps of gram-negative bacteria. J. Bacteriol. **178:**5853–5859.
18. **Nikaido, H., and S. Normark.** 1987. Sensitivity of *Escherichia coli* to various beta-lactams is determined by the interplay of outer membrane permeability and degradation by periplasmic beta-lactamases: a quantitative predictive treatment. Mol. Microbiol. **1:**29–36.
19. **Nikaido, H., E. Y. Rosenberg, and J. Foulds.** 1983. Porin channels in *Escherichia coli*: studies with beta-lactam antibiotics in intact cells. J. Bacteriol. **153:**232–240.
20. **Nikaido, H., and E. Y. Rosenberg.** 1983. Porin channels in *Escherichia coli*: studies with liposomes reconstituted from purified proteins. J. Bacteriol. **153:**241–252.
21. **O'Callaghan, C. H., A. Morris, S. M. Kirby, and A. H. Shingler.** 1982. Novel method for the detection of beta-lactamases by using a chromogenic cephalosporin substrate. Antimicrob. Agents Chemother. **1:**283–288.
22. **Pangon, B., C. Bizet, A. Buri, F. Pichon, A. Philippon, B. Regnier, and L. Gutmann.** 1989. In vivo selection of a cephamycin-resistant, porin deficient mutant of *Klebsiella pneumoniae* producing TEM-3 beta-lactamase. J. Infect. Dis. **159:**1005–1006.
23. **Parr, T. R., R. A. Moore, L. V. Moore, and R. E. W. Hancock.** 1987. Role of porins in intrinsic antibiotic resistance of *Pseudomonas cepacia*. Antimicrob. Agents Chemother. **31:**121–123.
24. **Raimondi, A., A. Traverso, and H. Nikaido.** 1991. Imipenem- and meropenem-resistant mutants of *Enterobacter cloacae* and *Proteus rettgeri* lack porins. Antimicrob. Agents Chemother. **35:**1175–1180.
25. **Rice, L. B., L. L. Carias, L. Etter, and D. M. Shlaes.** 1993. Resistance to cefoperazone-sulbactam in *Klebsiella pneumoniae*: evidence for enhanced resistance resulting from the coexistence of two different resistance mechanisms. Antimicrob. Agents Chemother. **37:**1061–1064.
26. **Spratt, B. G.** 1994. Resistance to antibiotics mediated by target alterations. Science **264:**368–393.
27. **van de Klundert, J. A. M., M. H. van Gestel, G. Meerdink, and S. de Marie.** 1988. Emergence of bacterial resistance to cefamandole in vivo due to outer membrane protein deficiency. Eur. J. Clin. Microbiol. Infect. Dis. **7:**776–777.
28. **Yoshimura, F., and H. Nikaido.** 1985. Diffusion of beta-lactam antibiotics through the porin channels of *Escherichia coli* K-12. Antimicrob. Agents Chemother. **27:**84–92.