ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 1997, p. 2352–2354 Vol. 41, No. 11
0066-4804/97/$04.00+0

Carbapenem Resistance in a Clinical Isolate of *Citrobacter freundii*

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Received 29 January 1997/Returned for modification 30 April 1997/Accepted 26 August 1997

Carbapenem resistance was studied in two sets of *Citrobacter freundii* strains: (i) strain CFr950, resistant to imipenem (MIC, 16 μg/ml) and isolated in vivo during imipenem therapy, and strain CFr950-Rev, the spontaneous, imipenem-susceptible revertant of CFr950 selected in vitro, and (ii) strains CFr801 and CFr802, two imipenem-resistant mutants selected in vitro from the susceptible clinical isolate CFr800. In all strains, whether they were imipenem-susceptible or -resistant strains, production of the cephalosporinase was derepressed and their K_m values for cephaloridine were in the range of 128 to 199 μM. No carbapenemase activity was detected in vitro. The role of cephalosporinase overproduction in the resistance was demonstrated after introduction of the *ampD* gene which decreased the level of production of cephalosporinase at least 250-fold and resulted in an 8- to 64-fold decrease in the MICs of the carbapenems. The role of reduced permeability in the resistance was suggested by the absence, in CFr950 and CFr802, of two outer membrane proteins (the 42- and 40-kDa putative porins whose levels were considerably decreased in CFr801) and the reappearance of the 42-kDa protein in imipenem-susceptible strain CFr950-Rev. This role was confirmed after introduction of the *ompF* gene of *Escherichia coli* into the CFr strains, which resulted in 8- to 16-fold decreases in the MICs of carbapenems for CFr802 and CFr950. We infer from these results that the association of reduced, porin-mediated permeability with high-level cephalosporinase production, observed previously in other gram-negative bacteria, may also confer carbapenem resistance on *C. freundii*.

Carbapenem resistance has been rarely described in members of the family *Enterobacteriaceae*. One mechanism of resistance is the production of plasmid- or chromosome-encoded carbapenem-hydrolyzing β-lactamases (16, 20, 21, 24, 27). Another mechanism of resistance, the association of reduced outer membrane permeability with high-level cephalosporinase production, has been reported in *Enterobacter aerogenes* (5, 6, 7, 10, 25, 26), *Enterobacter cloacae* (4, 11, 22), *Providencia* (*Proteus*) *rettgerii* [22], *Proteus mirabilis* (15), and more recently, *Klebsiella pneumoniae* strains producing a plasmid-mediated AmpC β-lactamase (3). Finally, low-level resistance to imipenem has been found in clinical isolates of *P. mirabilis* with alterations in penicillin-binding proteins (19).

In this study, we explored the mechanism responsible for an unusually high level of carbapenem resistance in a clinical isolate of *Citrobacter freundii*, a species in which none of the three former mechanisms has previously been described.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. The carbapenem-resistant strain *C. freundii* CFr950 was isolated after imipenem therapy (2 g/day for 9 days) for an intra-abdominal sepsis. CFr950-Rev was a spontaneous, in vitro-selected, susceptible revertant of CFr950. Two imipenem-resistant mutants of the imipenem-susceptible strain CFr800 were selected by two successive steps: strain CFr801 was selected on moxalactam (32 μg/ml), which yielded CFr802 after selection on imipenem (2 μg/ml). The strains were grown with aeration at 37°C in medium 3 (Difco Laboratories, Detroit, Mich), but the MIC determinations were carried out on Mueller-Hinton agar by using a multiple-inoculum replicator and ca. 10^4 CFU per spot. Transformation was carried out as described previously (13).

Antibiotics. The following antibiotics were kindly provided by the indicated companies: imipenem, Merck Sharp & Dohme-Chibret (Paris, France); moxalactam, Eli Lilly & Co. (Indianapolis, Ind.); and meropenem, Zeneca Pharmaceuticals (Cergy-Pontoise, France).

Characterization of outer membrane proteins. Outer membranes were prepared with 0.1% (wt/vol) *N*-laurylsarcosine (Sigma Chemical Co., St. Louis, Mo.) as described previously (8) and were separated on sodium dodecyl sulfate-containing polyacrylamide (12%) gels containing 20% urea.

β-Lactamase assays. Crude extracts of β-lactamase were obtained from the CFr strains as described previously (11). Analytical isoelectric focusing was performed by the method of Matthew et al. (14). One unit of β-lactamase was defined as the amount of enzyme that hydrolyzed 1 μmol of cephaloridine per min per mg of protein at 30°C. K_m values were calculated by computerized linear regression analysis of Woolf-Augustinsson-Hofstee plots (v versus v/s) (2).

Permeability assays. Outer membrane permeability coefficients of imipenem for the carbapenem-resistant strain CFr950 and the carbapenem-susceptible strain CFr950-Rev were determined as described previously (11) after introduction of the plasmid pPTN107 harboring the *smeA* gene which codes for a carbapenem-hydrolyzing β-lactamase (17). Briefly, the coefficients were calculated after measuring the rate of hydrolysis of imipenem by intact cells in a cuvette with an optical path length of 2 mm as well as by sonicated cells from the same culture and by using the K_m and V_{max} values of the enzyme for imipenem. The rates of hydrolysis measured with the intact cells were corrected for β-lactamase leakage by subtraction of the rates of hydrolysis measured with the supernatants of the sedimented cells, which were less than 25% of the total.

RESULTS AND DISCUSSION

Antibiotic susceptibility and β-lactamase content of the *C. freundii* strains. Compared with the MICs for the spontaneous, in vitro-selected revertant CFr950-Rev and the imipenem-susceptible strain CFr800, the MICs of imipenem and meropenem for the imipenem-resistant strain CFr950 were increased 32- to 128-fold and the MICs of moxalactam were increased 8-fold (Table 2). To determine whether the carbapenem resistance was typical of CFr950 or whether it could also be found in other strains of this species, we selected two imipenem-resistant mutants from the imipenem-susceptible strain CFr800 in

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant phenotype or genotype[a]	Reference
C. freundii		
CFr950	Imi^r; clinical strain isolated after imipenem therapy (2 g/day for 9 days) for an intra-abdominal sepsis	This study
CFr950-Rev	Imi^s; spontaneous in vitro revertant from strain CFr950	This study
CFr800	Imi^s; clinical isolate susceptible to moxalactam	18
CFr801	Imi^r; in vitro mutant of strain CFr800 selected on moxalactam	This study
CFr802	Imi^r; in vitro mutant of strain CFr801 selected on imipenem	This study
Plasmids		
pNH5	*Hpa*I fragment containing *ampD* from *E. coli* in pBS18	9
pPTN107	*Nsi*I-*Hin*dIII fragment containing *smeA* from *Serratia marcescens* in pK19	17
pMY2222	*Eco*RI-*Hin*dIII fragment containing *ompF* from *E. coli* in pBR322	23

[a] Imi^r, imipenem resistant; Imi^s, imipenem susceptible.

two successive steps. Strain CFr801 was selected on moxalactam, and the MICs of imipenem and meropenem were increased 8- and 32-fold, respectively (Table 2), while for CFr802, derived from CFr801 after selection on imipenem, there was a further 8- to 16-fold increase in the MICs.

Role of the β-lactamase. Examination of the β-lactamase contents of CFr950, CFr800, and their derivatives revealed only one β-lactamase with a pI of ca 9.2, in agreement with the pI of the cephalosporinase normally produced by this species (28). All the strains produced similarly high amounts of cephalosporinase, with K_m values similar to those for cephaloridine (Table 2). No significant carbapenemase activity against imipenem or meropenem could be detected. When other susceptible *C. freundii* strains were assayed for the selection of high-level imipenem-resistant mutants, these were only obtained if the carbapenem-susceptible strain initially produced high amounts of cephalosporinase (data not shown).

The possible contribution of the high amounts of cephalosporinase to the carbapenem resistance of the CFr strains was assessed after introduction of the plasmid-borne *ampD* gene, the product of which decreases *ampC* expression (9). In the presence of *ampD*, cephalosporinase production was decreased by more than 250-fold in all strains (data not shown). As indicated in Table 2, this was associated in the carbapenem-resistant strains with a significant decrease (8- to 64-fold) in the MICs of imipenem and meropenem almost to the levels observed for the susceptible strains CFr950-Rev and CFr800. Under these conditions, the MICs of moxalactam were reduced to the same level (4 μg/ml) for all strains.

Characterization of the outer membrane proteins and role of the permeability. Although it is obvious from the results presented above that the overproduction of the cephalosporinase plays a role in the carbapenem resistance of strains CFr950, CFr802, and CFr801, it was not sufficient to explain this resistance, since the carbapenem-susceptible strains CFr950-Rev and CFr800 produced similarly high amounts of cephalosporinase. Therefore, we investigated whether a change in the outer membrane structure was a factor contributing to the carbapenem resistance mechanism. The outer membrane proteins of the imipenem-susceptible strain CFr800 had three major proteins which appeared to be similar to those in *C. freundii* described previously (1). Two of these, of ca. 40 and 42 kDa and considered to be porins (1), were apparently absent from CFr950 and CFr802, and their levels were significantly decreased in CFr801 (Fig. 1). In CFr950-Rev, only the 42-kDa porin reappeared, which suggested that the presence of this protein alone is sufficient to decrease the MICs of the carbapenems. It is also likely that the low-level carbapenem resistance of CFr801 is due to the residual amounts of the 40- and 42-kDa porins (Fig. 1).

Further evidence of the influence of the putative porin-dependent permeability defect on the MICs of carbapenems was obtained after expression, in the different CFr strains, of the plasmid-borne *ompF* gene of *Escherichia coli* (23). As indicated in Table 2, 8- to 16-fold decreases in the MICs of carbapenems were observed for CFr950 and CFr802, while only 2- to 4-fold decreases in the MICs of moxalactam were found. Under these conditions, the MICs of the β-lactams for the carbapenem-resistant strains did not reach those observed for the susceptible strains, probably due to only modest expression of the exogenous *ompF*. In fact, examination of the outer membrane composition of CFr802 and CFr950 showed that exogenous OmpF was present, but only in small amounts (data not shown). These results and those of the MICs of moxalactam for the different mutants indicate that as expected for porins of members of the family *Enterobacteriaceae*, the 40- and 42-kDa porins are not specific for carbapenems. To evaluate the reduced permeability directly as part of the resistance

TABLE 2. MICs of β-lactam antibiotics, β-lactamase activities, and K_m values for different strains of *C. freundii* before and after introduction of the plasmid-borne *ampD* and *ompF* genes[a]

Strain	MIC (μg/ml)			β-Lactamase (mU/mg)[b]	K_m (μM)[b]	MIC (μg/ml) in the presence of *ampD*			MIC (μg/ml) in the presence of *ompF*		
	Mox	Imi	Mer			Mox	Imi	Mer	Mox	Imi	Mer
CFr950	256	16	8	2,940	129	4	1	0.12	64	2	1
CFr950-Rev	32	0.5	0.06	5,330	134	4	0.12	0.03	32	0.25	0.03
CFr800	16	0.25	0.03	4,480	193	4	0.12	0.03	8	0.12	0.03
CFr801	256	2	1	3,680	185	2	0.25	0.06	128	1	1
CFr802	256	32	8	6,580	199	4	0.5	0.12	128	2	1

[a] Mox, moxalactam; Imi, imipenem; Mer, meropenem.
[b] Cephaloridine was used as the substrate.

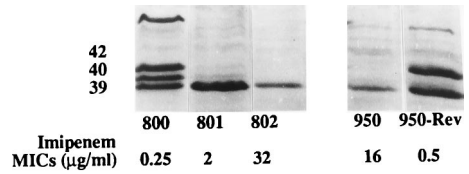

FIG. 1. Urea-sodium dodecyl sulfate-polyacrylamide gel electrophoresis of outer membrane proteins prepared from the following *C. freundii* strains: CFr800, clinical isolate susceptible to imipenem; CFr801, in vitro mutant of CFr800 selected on moxalactam and resistant to imipenem; CFr802, in vitro mutant of CFr801 selected on imipenem and resistant to imipenem; CFr950, imipenem-resistant clinical strain; CFr950-Rev, spontaneous in vitro revertant from CFr950, susceptible to imipenem. The numbers on the left are in kilodaltons.

mechanism, we determined the permeability coefficients of imipenem for CFr950 and its imipenem-susceptible variant CFr950-Rev after introduction of the plasmid-borne carbapenemase gene *smeA* (16) into both strains. Compared with CFr950-Rev, the permeability coefficient for CFr950 was about threefold lower, i.e., 50 instead of 160 nm/s. Finally, it should be emphasized that even though the 40- and 42-kDa proteins were absent from CFr950 and CFr802, no carbapenem resistance was observed when cephalosporinase overproduction was repressed in the presence of *ampD* (Table 2).

As previously described for *E. cloacae* (11) and *Pseudomonas aeruginosa* (12, 29), high-level cephalosporinase production associated with decreased permeability for carbapenems was responsible for the carbapenem resistance observed in the *C. freundii* strains studied here. These results underline the critical influence of the outer membrane permeability on the MICs of carbapenems for cephalosporinase-overproducing *C. freundii* strains as well and suggest that the selection of cephalosporinase-overproducing strains by broad-spectrum cephalosporins might subsequently facilitate the selection of carbapenem-resistant strains.

ACKNOWLEDGMENTS

This work was funded by a grant (CRI 95 0601) from the Institut National de la Santé et de la Recherche Médicale, Paris, France.

We thank P. Nordmann for providing plasmid pPTN107 and C. Harcour for secretarial assistance.

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