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Penicillin-Binding Proteins of *Rhodococcus equi*: Potential Role in Resistance to Imipenem

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Rhodococcus equi **is a gram-positive coccobacillus which, like other members of the order** ***Actinomycetales*****, is increasingly reported as an opportunistic pathogen in patients with AIDS. The use of combinations of antibiotics that include imipenem (IMP) has been suggested for the treatment of patients infected with** ***R. equi*****. An antagonism between IMP, meropenem, cefoxitin, ceftriaxone, moxalactam, and oxacillin and other β-lactams, such as penicillin, amoxicillin, cephalothin, and ticarcillin, was detected in vitro both on Mueller-Hinton agar and in broth for all 10 IMP-susceptible** ***R. equi*** **strains examined. To study the mechanism of the antagonism between β-lactams, a mutant with decreased susceptibility to IMP (isolate IpR) was selected in vitro from a susceptible clinical isolate of** ***R. equi*** **(isolate IpS). IpR exhibited decreased susceptibility to IMP, meropenem, cefoxitin, ceftriaxone, moxalactam, and oxacillin but not to penicillin, amoxicillin, cephalothin, or ticarcillin. No β-lactamase was found in IpS, IpS cultured with antagonistic β-lactams, or IpR strains. Labeling of penicillin-binding proteins (PBPs) revealed four PBPs with molecular masses of ca. 59, 56, 43, and 26 kDa in IpS. In IpR, PBP 3 disappeared and was replaced by PBP 3a of 40 kDa. The 50% saturation of PBP 3 and PBP 3a by the carbapenems correlated with the MICs of these antibiotics, respectively, for IpS and IpR strains. However, PBP 3a was not detected in IpS when IpS was cultured in the presence of β-lactams, with which antagonism was observed. The present work describes the PBPs of** ***R. equi*** **and reports that IMP resistance in** ***R. equi*** **is related to an altered PBP pattern.**

Rhodococcus equi is a gram-positive coccobacillus and is well-known as a pulmonary pathogen in foals (10). It is increasingly isolated as an opportunistic pathogen in immunocompromised patients, especially those with AIDS (7, 8, 12, 13, 15). The optimal antimicrobial regimen is not yet known, since frequent relapses occur after therapy (7, 8, 12). Although there is little available information about the susceptibilities of human isolates of *R. equi* to β-lactams, it seems that these strains are resistant or of intermediate susceptibility to all β-lactams except the carbapenems (3, 6, 9, 16). Thus, different combinations of antibiotics, including imipenem (IMP), have been proposed for use in the treatment of these infections (2, 10, 11). However, acquired antibiotic resistance to β-lactams, including IMP, has been described in treated patients (3).

During the course of a study on the susceptibility to antibiotics of different clinical isolates of *R. equi*, we noticed that an antagonism toward IMP was revealed in the presence of other β-lactams by the disk diffusion method. This led us to select a stably IMP-resistant strain of *R. equi* in vitro in which the mechanism of resistance was studied.

MATERIALS AND METHODS

Bacterial strains and selection of mutants. Five clinical isolates of *R. equi*, PN 1001, PN 1002, PN 1003 R, PN 1004 R, and PN 1005 R, were isolated from AIDS patients suffering from pneumonia. The *R. equi* reference strains CIP 5869 and ATCC 6939 were provided by the Institut Pasteur of Paris; strains ATCC 33701, ATCC 33702, and ATCC 33705 were obtained from the American Type Culture Collection (ATCC; Atlanta, Ga.). The identification of the clinical isolates (gram-positive coccobacilli, salmon pink-colored colonies, catalase positive, oxidase negative, urease positive; CAMP test positive with *Listeria monocytogenes* [gift from C. Nauciel]) was obtained with the API Corynebacteria test (Biomerieux S.A., Marcy l'Etoile, France) and was verified by the identification center of the Institut Pasteur. Two isogenic strains were further studied by penicillin-binding protein (PBP) analysis: an IMP-susceptible strain, PN 1002 (IpS), and its isogenic IMP-resistant (IpR) derivative. The IpR mutant was selected in vitro by culturing an inoculum of 10^7 CFU of the IpS strain on Mueller-Hinton (MH) agar containing 1.25 μg of IMP per ml. After incubation at 30°C for 48 h, resistant clones were tested for their susceptibilities to IMP.

Antimicrobial agents and MIC determinations. The antimicrobial agents used in the present study were obtained from standard laboratory powders and were used immediately after solubilization. The agents and their sources were as follows: amoxicillin, clavulanic acid, and ticarcillin (Smith-Kline French-Beecham, Paris, France); moxalactam (Eli Lilly & Co, Indianapolis, Ind.); cephalothin and ceftriaxone (Roche S.A., Courbevoie, France); cefoxitin and IMP (Merck Sharp & Dohme-Chibret, Paris, France); oxacillin and meropenem (ICI Pharmaceuticals, Alderley Park, United Kingdom); and penicillin G (Bristol-Myers Squibb, Paris, France). [^{3}H]benzylpenicillin (ethylpiperidinium salt; 25.5 μCi/mg) was a gift from Rhône-Poulenc Recherche (Vitry, France), and [^{14}C]meropenem (87.7 μCi/mg) was a gift from ICI Pharmaceuticals.

MICs were determined by broth macrodilution in glass tubes containing 5 ml of MH broth (Diagnostics Pasteur,

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Marnes-La-Coquette, France). Inocula were prepared by using appropriate dilutions of MH broth cultures that were shaken overnight. A standard inoculum of 10^5 CFU/ml was used in all cases. All tubes were incubated at 37 and 30°C for 18 h. MICs were defined as the lowest concentrations yielding a complete lack of growth.

Detection of antagonism. To detect an antagonism between IMP and the other β-lactams tested, different disks were put next to an IMP disk on MH agar (Diagnostics Pasteur). Antagonism to other β-lactams was detected similarly. Additionally, the MICs of IMP and cefoxitin were determined for each IMP susceptible strain in MH broth in the presence of subinhibitory concentrations of cephalothin (0.25- and 0.12-fold the cephalothin MIC). To study the time course of antagonism development toward IMP, an overnight culture of PN 1002 (IpS) was diluted (1:100) into 100 ml of prewarmed MH broth. Ten-milliliter cultures were then grown for 48 h in the presence of 1.25 μg of IMP per ml alone or in combination with 1, 5, or 10 μg of cephalothin per ml or with 0.5, 2, or 5 μg of penicillin per ml. Cultures were grown in shaking water baths at 37 and 30°C since 30°C is the optimal temperature for growth of *R. equi*. Growth was monitored with a Perkin-Elmer Junior III spectrophotometer at 650 nm.

β-Lactamase assays. β-Lactamase activity was detected by the nitrocefin assay and was detected spectrophotometrically at 30°C with a double-beam spectrophotometer (Rodel 550) by using penicillin (233 nm), cephaloridine (260 nm), and IMP (299 nm) as substrates at a concentration of 100 μM in the presence of 10 μM $ZnCl_2$ and in its absence. The following 18-h broth cultures were tested: IpS, IpR, and IpS in the presence of cephalothin (5 μg/ml), with or without IMP (1.25 μg/ml). Cultures were centrifuged at 5,000 × *g* for 20 min. The culture supernatants were concentrated by ammonium sulfate (80%) precipitation, and the precipitate was dialyzed twice overnight at 4°C against 10 mM phosphate buffer. Bacterial pellets were analyzed after they were broken with glass beads as described below. Colonies (24 h) of each strain grown on MH agar were also tested with nitrocefin.

Preparations of membrane fractions. *R. equi* CIP 5869, PN 1001, IpS, and IpR were grown to the late logarithmic phase in 1,000 ml of brain heart broth at 30 and 37°C. In experiments for the detection of antagonism, strain IpS was grown for 18 h in the presence of 5 μg of cephalothin per ml or 1 μg of radioactive penicillin per ml, which were used as antagonistic drugs, with or without IMP (0.25 μg/ml). Cells were harvested by centrifugation at 8,000 × *g* at 4°C for 20 min and were washed twice in 10 ml of 15 mM phosphate buffer (pH 7). The pellets were suspended in 5 ml of the same buffer, 5 g of glass beads (Sigma, St. Louis, Mo.) was added, and the cells were mechanically disrupted in a cell disintegrator (Mickle Laboratories Engineering Corp., Gomshaw, United Kingdom) for 6 h at 4°C. After removal of the unbroken cells by centrifugation at 8,000 × *g* for 15 min, the cytoplasmic membranes were collected by centrifugation at 100,000 × *g* (30 min at 4°C), washed, and suspended in 100 μl of 15 mM phosphate buffer (pH 7).

Analysis of PBPs. A total of 10 μl of the membrane preparation (ca. 80 μg of proteins) was incubated in the presence of various concentrations of [^{3}H]benzylpenicillin (0.25 to 25 μg/ml) or [^{14}C]meropenem (0.06 to 128 μg/ml) for 10 min at 37°C. The reaction was stopped by adding an excess of nonradioactive benzylpenicillin, and samples were boiled after the addition of one-half volume of sample buffer (1). For competition experiments, membranes were first incubated with various concentrations of nonradioactive antibiotics (0.03 to 128 μg/ml) for 10 min at 37°C. Then, [^{3}H]benzylpenicillin was added to a final concentration of 25 μg/ml. Samples were incubated for an additional 10 min and were then processed as described above. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (7.5% acrylamide, 0.067% bisacrylamide). Gels were stained in 0.25% Coomassie blue in 40% methanol–5% acetic acid and were soaked successively for 30 min in dimethyl sulfoxide (twice), 60 min in diphenyl-2,5-oxazole-dimethyl sulfoxide (20%; wt/vol), 2 h in distilled water, and overnight in 1% glycerol–1% acetic acid (14). The fluorograms were exposed at −80°C for 2 weeks when the PBPs were labeled with penicillin and for 3 months when they were labeled with meropenem. The concentration of each antibiotic required for 50% saturation of the PBPs was estimated by measuring the band density by using a Cliniscan apparatus (Helena Laboratories, Beaumont, Tex.).

TABLE 1. MICs of β-lactam antibiotics for strains IpS and IpR

Antibiotic	MIC (μg/ml) for:	
	IpS	IpR
Amoxicillin	8	8
Amoxicillin-clavulanic acid[a]	8	8
Cephalothin	64	64
Cefoxitin	8	64
Cefoxitin-cephalothin[a]	64	64
Ceftriaxone	2	16
Imipenem	0.25	2
Imipenem-cephalothin[a]	2	2
Meropenem	0.50	4
Moxalactam	8	64
Oxacillin	64	512
Penicillin	8	8
Ticarcillin	256	256

[a] Clavulanic acid and cephalothin were used at fixed concentrations of 5 μg/ml.

RESULTS

In vitro susceptibility and selection of resistant mutants. All *R. equi* strains showed the same pattern of β-lactam susceptibility as strain IpS (Table 1). No difference was found when MICs were determined at 30 or 37°C. Selection of IMP-resistant clones from IpS was obtained at a frequency of 2×10^{-6}. One of them, designated IpR, was chosen for further studies. IpR exhibited a stably decreased susceptibility not only to IMP but also to meropenem, cefoxitin, moxalactam, oxacillin, and ceftriaxone (Table 1). In contrast, the susceptibilities of strains IpR and IpS to amoxicillin, amoxicillin-clavulanic acid, ticarcillin, penicillin, and cephalothin were identical (Table 1).

Antagonism between IMP and other β-lactam antibiotics. An antagonism between IMP and the other β-lactam antibiotics tested was noted by the disk diffusion technique with all IMP-susceptible *R. equi* strains tested. The image of antagonism was noted inside the IMP susceptibility zone (Fig. 1). Two groups of β-lactams could be differentiated: those which were considered to be antagonistic drugs, i.e., amoxicillin, penicillin, cephalothin, and ticarcillin, and those whose activities were reduced by the antagonistic drugs, i.e., IMP, meropenem, moxalactam, cefoxitin, oxacillin, and ceftriaxone. No antagonism was detected between members of each group. The MICs of IMP and cefoxitin for IpS measured in the presence of subinhibitory concentrations of

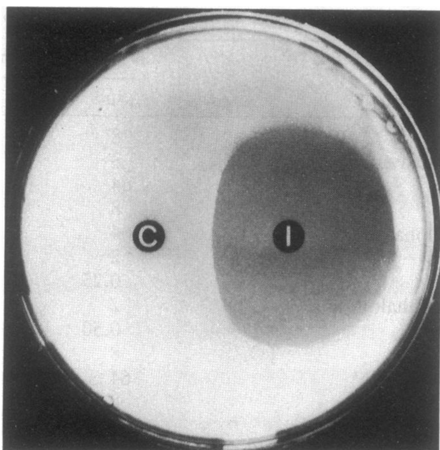

FIG. 1. Antagonism between IMP and cephalothin in *R. equi* visualized on an MH plate. A suspension of 10^5 CFU of *R. equi* IpS per ml was poured into the plate and then dried. Then, a disk of imipenem (disk I, 10 μg) was put 2 cm away from a disk of cephalothin (disk C, 30 μg). The plate was incubated for 24 h at 37°C.

cephalothin were identical to those found for IpR (Table 1). Interestingly, all the β-lactam antibiotics whose MICs were increased for IpR were those which were antagonized in IpS. In IpR, no antagonism toward IMP was detected with any of the β-lactam antibiotics tested.

As shown by the time course of growth (Fig. 2), subinhibitory concentrations of cephalothin antagonized the antibacterial effect of IMP in IpS at 37°C. The same antagonism was obtained at 30°C or when penicillin was used as an antagonistic drug (data not shown). After centrifugation and washing of the cells which had been exposed to the combination of IMP plus cephalothin as the antagonistic β-lactam, their antibiotic susceptibilities were tested. They showed the same IMP susceptibility pattern as that of the unexposed strain. Therefore, the decreased antibacterial effect of IMP

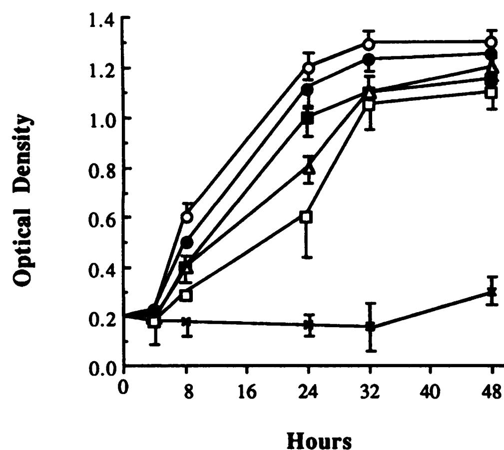

FIG. 2. Time course of the antagonism toward IMP by cephalothin in *R. equi* in MH broth. *R. equi* IpS was grown in MH broth in a shaking water bath at 37°C under the following conditions: without antibiotics (○), with cephalothin (CEF) at 10 μg/ml (●), with IMP at 1.25 μg/ml (×), and with IMP at 1.25 μg/ml in the presence of cephalothin at 1 μg/ml (△), 5 μg/ml (■), and 10 μg/ml (□). Growth was measured spectrophotometrically.

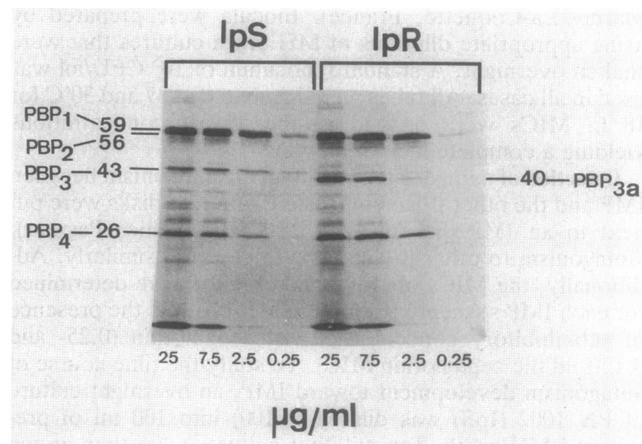

FIG. 3. PBPs of *R. equi* IpS and IpR labeled with [^{3}H]benzylpenicillin. Concentrations of [^{3}H]benzylpenicillin are shown at the bottom. The molecular masses (in kilodaltons) of the PBPs are given on the left and right margins.

observed in the presence of cephalothin was reversible when the antagonistic drug was removed from the culture medium.

β-Lactamase assays. IpS alone or in the presence of 5 μg of cephalothin per ml, with or without IMP (1.25 μg/ml), and IpR had no detectable β-lactamase activity.

PBPs of IMP-susceptible and -resistant *R. equi*. Since no β-lactamase was detected, PBP profiles and PBP affinities for different β-lactams of strains IpS and IpR were determined. As shown in Fig. 3, four major PBPs were present in the susceptible strain, with molecular masses ranging from 59 to 26 kDa. The PBP profile of the resistant strain IpR was very similar, but there was an almost total disappearance of PBP 3 and the appearance of a new PBP, PBP 3a, with a lower molecular mass (40 kDa).

Since the new PBP, PBP 3a, showed a 50% saturation with [^{3}H]benzylpenicillin (9 μg/ml) which was very similar to that observed for PBP 3 (4 μg/ml), it was expected that no significant change in the MIC of penicillin for the IpR strain would occur. This was indeed the case (Table 1). To assess whether a particular PBP was associated with the resistance to the carbapenems, competition experiments with IMP were performed. As shown in Table 2, while the 50% saturation of PBPs 1, 2, and 4 was obtained with less than

TABLE 2. Affinities of PBPs of strains IpS and IpR for β-lactam antibiotics

PBP	50% saturation (μg/ml)[a]					
	[^{3}H]penicillin G		Imipenem[b]		[^{14}C]meropenem	
	IpS	IpR	IpS	IpR	IpS	IpR
1/2	2	1	<0.12	<0.12	0.09	0.13
3	4	—[c]	ND[d]	—	0.12	—
3a	—	9	—	3	—	4
4	5	4	<0.12	<0.12	0.07	0.05

[a] The MICs of [^{3}H]penicillin G, imipenem, and [^{14}C]meropenem for the IpS and IpR strains were 8 and 8 μg/ml, 0.25 and 2 μg/ml, and 0.5 and 4 μg/ml, respectively.

[b] A competition experiment was performed.

[c] —, absent or barely visible.

[d] ND, not detectable, since this PBP always disappeared during the competition experiments.

0.12 μg of IMP per ml, PBP 3a showed a 50% saturation by IMP of 3 μg/ml, a value very similar to the MIC of imipenem for IpR. Unfortunately, it was never possible to visualize PBP 3 in strain IpS by the competition assay because of the lability of the nonsaturated fraction of PBP 3 at 37°C during the incubation with nonlabeled IMP. The temperature lability of PBP 3 was detected by incubating labeled and nonlabeled IpS membranes at 37°C for 15 to 30 min. The disappearance of PBP 3 from nonlabeled membranes was detected. Therefore, direct labeling was obtained with [^{14}C]meropenem. As shown in Table 2, the 50% saturation of the different PBPs, including PBP 3, was obtained with 0.12 μg/ml or less, while PBP 3a in IpR showed 50% saturation with only 4 μg/ml.

Finally, since PBP 3a was apparently the PBP target responsible for the stable resistance to IMP in IpR, we looked to see whether the antagonism to IMP observed in the presence of cephalothin and penicillin was correlated with the appearance of PBP 3a. Labeling of the IpS PBPs with 25 μg of [^{3}H]benzylpenicillin per ml was carried out with membranes extracted from cells grown for 18 h in the presence of either cephalothin (5 μg/ml), with or without IMP (0.25 μg/ml), or [^{3}H]benzylpenicillin (1 μg/ml). These analyses failed to detect PBP 3a.

DISCUSSION

In this report we described IMP resistance in *R. equi*. This resistance was of a low level. Antagonism to IMP was detected in the presence of β-lactams such as cephalothin or penicillin and to other β-lactams such as meropenem, cefoxitin, moxalactam, ceftriaxone, and oxacillin. This antagonism was found for all strains studied. Since no β-lactamase was detected and since *R. equi* is a gram-positive bacterium, a likely explanation is that an inducible modification of a PBP would occur. In order to test this possibility, a constitutive mutant, IpR, which was resistant to IMP, was selected in vitro and its PBPs were studied.

Interestingly, while one PBP, PBP 3, disappeared from strain IpR, a new PBP, PBP 3a, appeared. Moreover, PBP 3a was the only PBP to show a 50% saturation by the carbapenems which correlated with their MICs. Since PBP 3a was present in the constitutive strain IpR and since the MICs of IMP or cefoxitin in the presence of subinhibitory concentrations of antagonistic β-lactams were identical to those of the stable IMP-resistant strain IpR, it was tempting to demonstrate, as an explanation of the antagonism, that PBP 3a could be induced in strain IpS and that a switch of essential PBPs would therefore occur. Under the experimental conditions used in the present study, this was not possible. One explanation could be that the antagonistic drug itself partially saturated PBP 3a. However, one cannot rule out the possibility that another nonexplained mechanism of resistance could be responsible for the antagonism observed.

Finally, several interesting observations emerged from the present study. (i) This is the first report of PBPs in *Rhodococcus* spp. (ii) The description of antagonism between IMP and other β-lactams is, to the best of our knowledge, novel in gram-positive bacteria. (iii) This antagonism seems to be widespread among *R. equi* isolates. Its detection in the clinical laboratory may help to differentiate *R. equi* isolates (which should be considered opportunistic pathogens) from other coryneform bacteria in which a similar antagonism has not been described (particularly in *R. rhodochrous*, *R. erythropolis*, and *Gordona bronchialis*, in which it has not been found [data not shown]). (iv) Because mechanisms of inducible resistance to rifampin and fusidic acid have been reported in *R. erythropolis* (4, 5), it is likely that inducible mechanisms of antibiotic resistance are widespread in members of the order *Actinomycetales*. Such mechanisms of resistance may represent survival mechanisms against antibiotics which are produced by numerous members of the order *Actinomycetales*, for example, the production of thienamycin by *Streptomyces cattleya*.

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