JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 1994, p. 377–383
0095-1137/94/$04.00+0

Vol. 32, No. 2

Correlation between Antibiotic Resistance, Phage-Like Particle Presence, and Virulence in *Rhodococcus equi* Human Isolates

PATRICE NORDMANN,[1]* MARCEL KELLER,[2] FLORENCE ESPINASSE,[1] AND ESTHEL RONCO[1]

Service de Microbiologie, Hôpital Raymond Poincaré, Faculté de Médecine Paris-Ouest, Université Paris 5, 92380 Garches, France,[1] and Abteilung Mikrobiologie, Biozentrum der Universität Basel, 4056 Basel, Switzerland[2]

Received 30 August 1993/Returned for modification 22 October 1993/Accepted 18 November 1993

***Rhodococcus equi* is a gram-positive coccobacillus that appears to be emerging as a pulmonary pathogen in AIDS patients. In four human clinical isolates, two antibiotic resistance phenotypes were found to coexist: one β-lactam resistant and the other β-lactam susceptible. In vitro, β-lactam-resistant mutants were obtained at a frequency of 1×10^{-5} to 5×10^{-5} from β-lactam-susceptible strains on cephalothin-containing plates. Neither β-lactamase nor plasmid DNA was detected in β-lactam-resistant or -susceptible strains. The penicillin-binding protein patterns for the two types of strains were identical. Electron microscopy revealed that the β-lactam-resistant strains possessed cell-surface-associated appendages and produced phage-like particles. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of total cell protein showed at least three additional bands of 42, 39, and 30 kDa found only in the β-lactam-resistant strains. Testing for virulence in Swiss mice revealed that (i) phage-like-particle-producing strains had lower 50% lethal doses when injected intravenously in euthymic and nude mice than the non-phage-like-particle-producing strains did and (ii) intravenous inoculation of a sublethal dose (5×10^6 CFU) in nude mice led to chronic infection by the phage-like-particle-producing bacteria only. Finally, in vitro growth curves indicated that the phage-like-particle-producing strains possessed an ecological selection advantage. These results suggest that, among *R. equi* human isolates, the antibiotic resistance phenotype is associated with virulence and may be phage mediated.**

Rhodococcus equi is a gram-positive coccobacillus classified in the order *Actinomycetales* (8). Since the start of the AIDS epidemic, there has been an increase in the number of cases of pneumonia, pulmonary abscess, and systemic infection caused by this opportunistic pathogen (7, 10, 26, 35). *R. equi* has been well-known to veterinary microbiologists since its initial isolation by Magnusson in 1923 (16). *R. equi* frequently appears as a frank pathogen in foals, principally causing subacute and chronic suppurative bronchopneumonia, but is also a significant opportunist in adult animals (2, 25). *R. equi* is a facultative intracellular pathogen infecting both macrophages and polymorphonuclear leukocytes. Its persistence in macrophages contributes to its ability to escape normal pulmonary defense mechanisms (12, 25, 31). Cellular mediators of immunity, such as $CD4^+$ and $CD8^+$ lymphocytes, play a major role in defense against this pathogen (13, 23, 25). In *R. equi* animal isolates, the presence of 15- to 17-kDa antigens, bound to the cell surface is associated with the presence of an 85-kb plasmid and virulence in mice (29, 30, 32, 33). The virulence of human *R. equi* isolates has not yet been studied. Most human strains are resistant to the β-lactams, except for the carbapenems (imipenem and meropenem) (22, 25). The primary aim of this work was to analyze the β-lactam resistance mechanism observed in four human clinical isolates and to determine its influence on virulence.

* Corresponding author. Mailing address: Service de Microbiologie, Hôpital Raymond Poincaré, 104 Blvd. Raymond Poincaré, 92380 Garches, France. Phone: 33 1 47 10 79 00, ext. 5461. Fax: 33 1 47 10 78 23.

MATERIALS AND METHODS

Mice. Six- to 8-week-old female euthymic and congenitally athymic nude (*nu/nu*) Swiss mice were purchased from Iffa Credo (L'Arbresle, France) and maintained in a pathogen-free environment. Food and water were provided ad libitum.

Bacterial strains and selection of mutants. *R. equi* reference strains ATCC 33701, ATCC 33702, ATCC 33703, and ATCC 33705 (all of animal origin) were obtained from the American Type Culture Collection (Rockville, Md.) (Table 1). The *R. equi* clinical strains were isolated from blood samples from AIDS patients with pulmonary abscesses (patients from different areas of France) (Table 1). Clinical isolates (gram-positive coccobacillus, salmon-pink mucoid colonies, oxidase negative, and CAMP test positive with *Listeria monocytogenes* [a gift from C. Nauciel]) were also identified by the API Corynebacteria test results (nitrate reduction positive, urease positive, alkaline phosphatase positive, alpha-glucosidase positive, catalase positive, and sugar fermentation negative) (Biomérieux, Marcy l'Etoile, France) (25).

From each β-lactam-susceptible isolate, in vitro selection of β-lactam-resistant mutants was performed by plating liquid cultures grown for 48 h (30°C, Luria-Bertani broth [LB]) on cephalothin (10 μg/ml)-containing Luria agar (LA). β-Lactam-resistant mutants were retained for further analysis (*R. equi* PN1003 M, PN1004 M, PN1005 M, and PN1006 M).

Antimicrobial agents and determination of MICs. The antimicrobial agents used in this study were obtained as powders, dissolved, and used immediately. The antimicrobial agents and their sources were as follows: oxacillin and penicillin G (Bristol); amoxicillin, clavulanic acid, and ticarcillin (Smith-Kline French Beecham); moxalactam and vancomycin (Eli Lilly); cephalothin (Glaxo); ceftriaxone

TABLE 1. Comparison of properties of human and animal *R. equi* isolates

Strain	Source	β-Lactam susceptibility or resistance[a]	Cell-surface-associated appendages	PLP	50% Lethal dose[b] (CFU)	Chronic infection[c]
Animal						
ATCC 33701	Horse lung	R	+	+	2×10^7	+
ATCC 33702	Dog skin	R	−	−	$>5 \times 10^9$	−
ATCC 33703	Pig lymph nodes	S	−	−	$>5 \times 10^9$	−
ATCC 33705	Pig lymph nodes	R	+	+	5×10^7	+
Human						
PN1003 R	Blood	R	+	+	1.5×10^8	+
PN1003 S	Blood	S	−	−	5×10^9	−
PN1004 R	Blood	R	+	+	1×10^8	+
PN1004 S	Blood	S	−	−	$>5 \times 10^9$	−
PN1005 R	Blood	R	+	+	2×10^7	+
PN1005 S	Blood	S	−	−	$>5 \times 10^9$	−
PN1006 R	Blood	R	+	+	5×10^8	+
PN1006 S	Blood	S	−	−	$>5 \times 10^9$	−

[a] R, resistant; S, susceptible.
[b] Expressed in *R. equi* CFU after i.v. inoculation of euthymic or nude mice.
[c] Chronic infection was determined in nude mice after i.v. inoculation of a sublethal dose of 5×10^6 *R. equi* CFU.

(Roche); erythromycin (Abbott); imipenem (Merck Sharp & Dohme); gentamicin (Schering); cefotaxime and chloramphenicol (Roussel); pefloxacin (Roger & Bellon); rifampin and kanamycin (Sigma Chemical Co., St. Louis, Mo.); tetracycline base (Pfizer); and meropenem (ICI Pharma). MICs were determined by the broth macrodilution technique in glass tubes containing 5-ml portions of Mueller-Hinton broth (Diagnostics Pasteur, Marnes-La-Coquette, France) (22).

β-Lactamase assays. β-Lactamase activity was detected by the nitrocefin assay (Oxoid) and by spectrophotometry at 30°C with a double-beam spectrophotometer (Rodel 550), using cephalothin (260 nm) as the substrate (27) (100 μM) in the presence or absence of 10 μM $ZnCl_2$ (in case a metalloenzyme was present). *R. equi* reference strains and the β-lactam-susceptible and -resistant clinical strains were cultured for 48 h in 500-ml portions of LB and then centrifuged at 5,000 × *g* for 20 min. The culture supernatants were concentrated by ammonium sulfate (80%) precipitation and dialysis overnight at 4°C against 10 mM phosphate buffer, and the culture pellets were tested for β-lactamase activity. Culture pellets were broken up with glass beads (Sigma) and analyzed (21). Similarly, cultures grown in LA for 48 h were tested for β-lactamase activity by examining whether a chromophore was released when the culture was incubated with nitrocefin.

Analysis of PBPs. *R. equi* PN1003 S and PN1003 R were grown for 18 h in 500 ml of LB at 30 and 37°C. Cells were then collected and analyzed for penicillin-binding protein (PBP) content (21). Briefly, pellets were broken up with glass beads, and membranes were collected by ultracentrifugation (100,000 × *g* for 30 min at 4°C). Cytoplasmic membranes were incubated with various concentrations of [^{3}H]benzylpenicillin (0.25 to 25 μg/ml). PBPs were revealed after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorogram exposure. The concentration of benzylpenicillin required for 50% saturation of the PBPs was estimated by measuring the band density on a Cliniscan apparatus (Helena Laboratories, Beaumont, Tex.).

Plasmid analysis. All *R. equi* strains were grown in 200-ml portions of LB for 48 h at 30 and 37°C. Plasmid isolation was performed as described by Takai et al. (29). Briefly, the bacteria were centrifuged and incubated at 37°C for 2 h in a buffer containing 0.05 M Tris hydrochloride, 0.01 M EDTA, 0.05 M NaCl, and 20% (wt/vol) sucrose (pH 8.0) plus 5 mg of lysozyme. Cells were then lysed in 3.0% (wt/vol) SDS in 0.05 M Tris hydrochloride buffer (pH 12.6) at 55°C for 2 h. Chromosomal DNA was precipitated with 5 M potassium acetate–acetate buffer (pH 4.8) and centrifuged at 10,000 × *g* for 15 min. The DNA was submitted to electrophoretic analysis for detection of plasmids. *R. equi* ATCC 33701, known to harbor an 85-kb plasmid, was used as the positive control.

In vitro growth rates. Cultures grown overnight were diluted (1:50) in 25-ml portions of prewarmed LB. Cultures were grown in a shaking water bath at 37 and 30°C for 72 h. Samples were collected at 12, 28, 24, 36, 44, 48, 60, 66, and 72 h. Growth was monitored in a Perkin-Elmer Junior III spectrophotometer at 650 nm. CFU counts at the stationary phase were determined by preparing serial dilutions in saline and plating the cells onto LA.

Electron microscopy. *R. equi* strains were grown in 5-ml portions of LB for 72 h at 30 and 37°C in a shaking water bath. Samples were collected at 12, 24, 48, and 72 h. Strains were also cultured in LA for 72 h at 30 and 37°C. Each plate was scraped, and the scraped cells were resuspended in 0.2 ml of sterile water. The electron microscopy procedure was modified from a previously reported method (36). Samples (5 μl) of bacterial suspension were adsorbed to carbon-coated collodion copper grids by floating the grids on drops of cell suspensions for 1 min. The grids were washed twice for 30 s with a solution of 10 mM Tris hydrochloride (pH 7.0), 10 mM NaCl, and 1 mM Mg_2SO_4. The grids were negatively stained with 0.5% sodium phosphotungstate (pH 7.0) twice for 30 s and examined with a Zeiss 109 transmission electron microscope.

Protein electrophoresis. *R. equi* strains were grown at 30 and 37°C in LB and LA for 72 h. In the case of LA cultures, cells were scraped off plates and resuspended in 0.2-ml portions of sterile water. Cells were centrifuged at 8,000 × *g* for 10 min at 4°C. The supernatant and pellet (50 μl) were solubilized in SDS reducing buffer (0.06 M Tris hydrochloride [pH 6.8], 10% [vol/vol] glycerol, 2% SDS, 5% 2-mercaptoethanol, 0.02% bromophenol blue) and boiled for 4 min. Protein analysis was carried out as previously described (14) with a 12.5% polyacrylamide vertical slab gel

and a 3% polyacrylamide stacking gel. Each sample was loaded onto a lane and electrophoresed at 50 V for 4 h. Gels were stained in 0.25% Coomassie blue in 40% methanol–5% acetic acid and destained overnight in 1% glycerol–1% acetic acid–5% methanol.

Mouse pathogenicity. The 50% lethal dose of the *R. equi* isolates after intravenous (i.v.) inoculation to euthymic and athymic Swiss mice was determined as previously described (30). Athymic nude mice were inoculated i.v. with a sublethal dose (5×10^6 CFU) of each strain shown in Table 1 or 1 of 10 β-lactam-resistant mutants of the PN1003 series (PN1003 M1 to M10). Groups of three animals were killed at various intervals. Spleens, livers, and lungs were homogenized in 2 ml of distilled water. Samples of the homogenates and 10-fold serial dilutions in saline were plated onto LA. Colonies were counted after 48-h incubation at 30°C (the temperature for optimal *R. equi* growth [25]).

Statistical analysis. Standard randomization procedures were used to select which animals were to be killed at a given interval. CFU counts of each infected group were compared by analysis of variance. Analysis of variance was performed on the basis of the results of the parametric Tukey's studentized range test (SAS/stat [26a]) for a fixed value of $P = 0.05$. Growth curve comparisons were made with the same statistical test.

RESULTS

Selection of mutants and analysis of antibiotic susceptibility of the *R. equi* strains. When clinical strains were analyzed by the agar disk diffusion method, two antibiotic susceptibility patterns were found to coexist. β-Lactam susceptibilities for susceptible and resistant strains cloned from each isolate (PN1003 S and PN1003 R, PN1004 S and PN1004 R, PN1005 S and PN1005 R, and PN1006 S and PN1006 R, respectively) were confirmed by MIC testing according to the guidelines (19) of the National Committee for Clinical Laboratory Standards (Table 2). The pattern of β-lactam resistance or susceptibility was very similar from one clinical strain to the other, facilitating classification of each strain as susceptible or resistant. Replication of the original blood isolates on plates containing antibiotic (cephalothin [10 μg/ml]) revealed that 6 to 8% of the colonies were β-lactam susceptible and 94 to 92% were resistant. Colonial pigmentation, texture, and morphology (salmon pink, mucoid) were unaffected by changes in β-lactam susceptibility. All strains, regardless of the response to β-lactams, remained susceptible to carbapenems. A survey of the *R. equi* animal type cultures revealed that ATCC 33701, ATCC 33702, and ATCC 33705 were β-lactam resistant and ATCC 33703 was β-lactam susceptible.

From *R. equi* β-lactam-susceptible strains (PN1003 S, PN1004 S, PN1005 S, and PN1006 S), β-lactam-resistant mutants were obtained in vitro at a frequency of 1×10^{-5} to 5×10^{-5} on cephalotin-containing LA plates. MICs from β-lactam-resistant mutants PN1003 M1 to M10, PN1004 M, PN1005 M, and PN1006 M were compared with those of the parent type cultures and clinical isolates and were found to have the same β-lactam resistance pattern as PN1003 R, PN1004 R, PN1005 R, and PN1006 R (Table 2, resistance pattern). All β-lactam-resistant strains showed cross-resistance toward tetracycline and pefloxacin (except for strains PN1004 S and PN1004 R, which were already fluoroquinolone resistant at high levels [pefloxacin MIC of 64 μg/ml] as a result of in vivo selection after fluoroquinolone therapy [24]).

TABLE 2. Two antibiotic susceptibility patterns in *R. equi* shown by MIC testing according to the National Committee for Clinical Laboratory Standards guidelines (19)

Antibiotic	MIC range (μg/ml) for strain	
	Susceptible	Resistant
β-Lactams		
Amoxicillin	0.06–0.12	4–8
Amoxicillin-clavulanic acid[a]	0.06–0.12	4–8
Cefoxitin	0.25–1	16–32
Ceftriaxone	0.03–0.24	1–4
Cephalothin	0.12–0.24	64–128
Imipenem	0.01–0.03	0.12–0.25[b]
Meropenem	0.03–0.06	0.25–0.5[b]
Moxalactam	0.5–1	16–32
Oxacillin	16–32[c]	64–128
Penicillin G	0.12–0.25[c]	2–16
Ticarcillin	8–16	256–512
Other		
Chloramphenicol	8–32	8–32[b]
Erythromycin	0.06–0.25	0.06–0.25[b]
Gentamicin	0.50–1	0.50–1[b]
Kanamycin	1–2	1–2[b]
Pefloxacin	2–4[c]	8–64
Tetracycline	0.12–0.25	1–4[b]
Vancomycin	0.12–0.25	0.12–0.25[b]

[a] Clavulanic acid at a fixed concentration of 5 μg/ml.
[b] *R. equi* strains exhibiting the antibiotic resistance pattern were susceptible to this antibiotic.
[c] *R. equi* strains exhibiting the antibiotic susceptibility pattern were resistant to this antibiotic.

β-Lactam resistance. Human strains, reference animal isolates, and in vitro β-lactam-resistant mutants did not show β-lactamase activity. *R. equi* PN1003 S and PN1003 R PBP patterns were identical (Fig. 1). Benzylpenicillin labeling showed that the 50% saturation values for PBP 1, 2, 3, and 4 were 2, 2, 4, and 5 μg/ml, respectively, in both strains.

Plasmid analysis. Despite repeated testing after culture in LB at 30 or 37°C, plasmids were not detected in any of the *R. equi* clinical strains. As previously reported (32, 33), we found that *R. equi* ATCC 33701 harbored an 85-kb plasmid, ATCC 33702 harbored a 5-kb plasmid, ATCC 33703 harbored an 100-kb plasmid, and ATCC 33705 harbored an

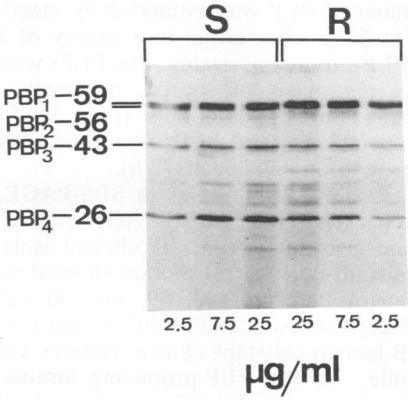

FIG. 1. PBP of *R. equi* β-lactam-susceptible PN1003 S (S lanes) and β-lactam-resistant PN1003 R (R lanes) labeled with [^{3}H]benzylpenicillin. Concentrations of [^{3}H]benzylpenicillin (in micrograms per milliliter) are shown at the bottom. The PBP molecular masses (in kilodaltons) are given in the left margin.

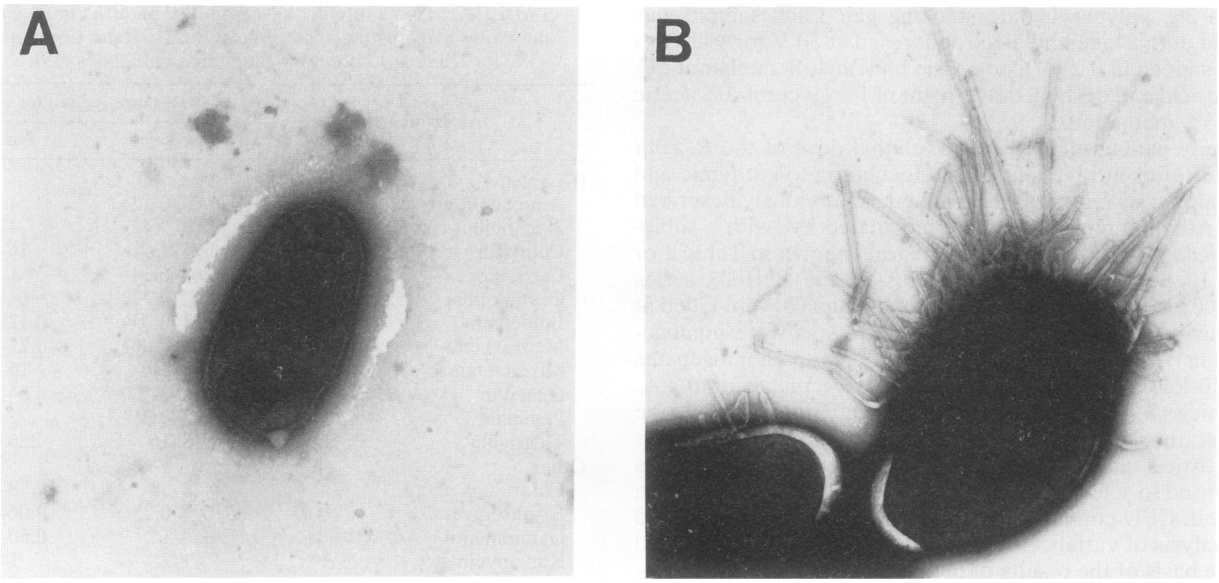

FIG. 2. Electron microscopy of 48 h LA cultures of β-lactam-susceptible *R. equi* PN1003 S (A) and β-lactam-resistant PN1003 R (B) coexisting within the same blood isolate. Note the presence of cell-surface-associated appendages in *R. equi* PN1003 R only. Similarly, cell-surface-associated appendages were detected in the other β-lactam-resistant human clinical strains, in *R. equi* ATCC 33701 and ATCC 33705 reference strains, and in in vitro-obtained β-lactam-resistant PN1003 M1 and PN1003 M2 mutants. Magnification, ×3,000.

80-kb plasmid (data not shown). Indirect procedures for plasmid detection such as ethidium bromide and acridine orange curing assays or electron microscopic visualization were not performed.

Electron microscopy. The most notable feature revealed by electron microscopy was cell-surface-associated appendages (Fig. 2). These structures were irregularly distributed over the entire bacterial surface: their length was approximately 1 μm, and both the length and width were variable. Among the 10 β-lactam-resistant mutants from the PN1003 series (PN1003 M1 to M10), 2 resistant mutants (PN1003 M1 and M2) harbored cell-surface-associated appendages, as did all clinical isolates and ATCC 33701 and ATCC 33705. Cell surface appendages were not produced by β-lactam-susceptible clinical strains or by *R. equi* β-lactam-resistant ATCC 33702 and β-lactam-susceptible ATCC 33703 strains (Fig. 2A). Bacteriophage-like particles (PLP) were detected in all cultures which had cell-surface-associated appendages. The average number of PLP was estimated by electron microscopy at 30 to 50 per bacterium in a survey of 20 different cultures of PLP-producing strains. The PLPs were similar in morphology, regardless of source, possessing hexagonal isometric heads and flexible tails (Fig. 3). Cell-surface-associated appendages and PLPs were best detected after prolonged growth on solid agar (72 h).

Presence of additional proteins in SDS-PAGE. Comparisons of proteins separated by SDS-PAGE showed that the *R. equi* β-lactam-resistant PN1003 R clinical isolate and the β-lactam-resistant PN1003 M1 mutant showed at least three additional major bands of 42, 39, and 30 kDa that the β-lactam-susceptible isolate (PN1003 S) did not have (Fig. 4). All the β-lactam-resistant clinical isolates exhibited this protein profile. All the PLP-producing strains possessed these additional protein bands after prolonged culture in LA (72 h).

Mouse virulence. β-Lactam-resistant and -susceptible human and reference animal strains were analyzed for their virulence in euthymic and nude mice. The PLP-producing strains had 50% lethal doses that were higher than those of the non-PLP-producing strains in euthymic and nude mice (Table 1). For a given strain, 50% lethal doses in euthymic and nude mice were identical. Nude mice were then inoculated with a sublethal dose (5×10^6 CFU) of the strains. Only PLP-producing bacteria such as *R. equi* PN1003 R gave chronic infections in nude mice; non-PLP-producing strains such as *R. equi* PN1003 S were eliminated from the examined organs within 72 h (Fig. 5). Differences in CFU counts were statistically significant at all times in all organs examined, except for the lungs at day 1 ($P = 0.05$). Of the 10 in vitro β-lactam-resistant mutants derived from the PN1003 series, only 2 mutants (*R. equi* PN1003 M1 and PN1003 M2) induced chronic infections and possessed 50% lethal doses similar to that of *R. equi* β-lactam-resistant PN1003 R (Fig. 5).

Growth curves. Similar growth curves were obtained at 30 and 37°C. The PLP-producing strains reached stationary phase earlier than the non-PLP-producing strains did and had a higher density, as shown for *R. equi* PN003 set (M1 and R compared with S [Fig. 6]). The stationary phase corresponded to higher CFU values in PLP-producing strains [$(2 \pm 1) \times 10^9$ CFU/ml] than in non-PLP-producing strains [$(3 \pm 1.2) \times 10^8$ CFU/ml] after 72 h of culture [initial inoculum of $(1.2 \pm 0.2) \times 10^5$ CFU/ml].

DISCUSSION

While previous studies on *R. equi* virulence have focused on animal isolates, we explored the clinical and physical characteristics of *R. equi* human isolates. The most interesting aspect of these human isolates was the fact that they exhibited two antibiotic resistance patterns, sometimes coexisting within the same blood isolate. The β-lactam-resistant strains predominated over the β-lactam-susceptible strains. Once coexisting strains were separated, both phenotypes were stable after subculture at 37 or 30°C for 20 passages (data not shown). In vitro, β-lactam-resistant mu-

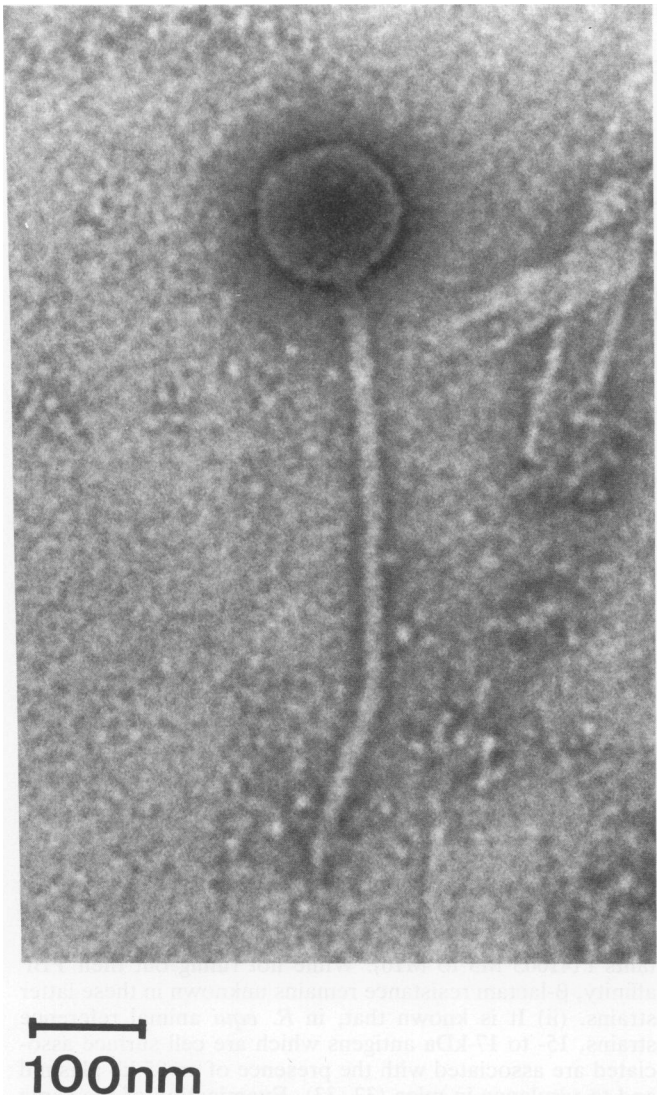

FIG. 3. PLP obtained from *R. equi* β-lactam-resistant PN1003 R cultured on LA for 72 h. Similar PLP were detected in the other β-lactam-resistant human clinical strains, in ATCC 33701 and ATCC 33705 reference strains, and in vitro in β-lactam-resistant PN1003 M1 and PN1003 M2 mutants.

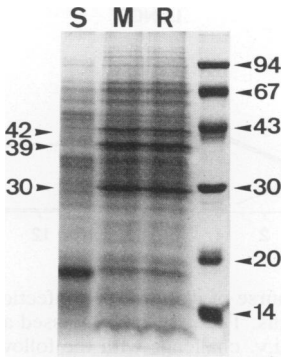

FIG. 4. Coomassie blue-stained SDS-PAGE pattern of whole-cell proteins from *R. equi* clinical strains of the PN1003 series grown at 37°C in LA. Lane S, β-lactam-susceptible PN1003 S clinical isolate; lane M, in vitro-obtained β-lactam-resistant PN1003 M1 mutant; lane R, β-lactam-resistant PN1003 R clinical isolate. The rightmost lane shows molecular mass markers (in kilodaltons). The arrows on the left indicate three additional major bands present in *R. equi* PN1003 R and PN1003 M1 not in the PN1003 S protein profile. Both β-lactam-resistant strains harbored cell-surface-associated appendages and produced PLP.

tants were obtained from β-lactam-susceptible clinical isolates at a relatively high frequency on cephalothin-containing plates. These β-lactam-resistant mutants showed the same pattern of β-lactam resistance, combined with cross-resistance to tetracycline and fluoroquinolones, as their resistant clinical counterparts did.

The mechanism of β-lactam resistance in *R. equi* remains unclear. The two main mechanisms known to be involved in β-lactam resistance in gram-positive bacteria, β-lactamase presence and differences in PBP affinities, were ruled out. Differences in β-lactam uptake and/or active β-lactam expulsion or modification of peptidoglycan precursors might explain the observed resistance. In this respect, it is interesting that (i) cross-resistance with antibiotic classes other than β-lactams has been observed (18), (ii) porins have recently been reported in a related bacterium, *Mycobacterium chelonei* (34), and (iii) active expulsion of chloramphenicol is known to occur in another *Rhodococcus* species, *R. fascians*, a plant pathogen (4).

Electron microscopy revealed the presence of cell-surface-associated appendages only in the β-lactam-resistant strains. These structures were distributed all over the bacterial surface and were bigger than the pili described in *R. equi* ATCC 6939 reference strain (37). The presence of pili did not correlate with shredding of the bacterial surface (13a). These cell-surface-associated appendages resembled deficient proteins of the structural components of either the head or tail of bacteriophage lambda (36). They were also associated with the presence of PLP within the bacteria which share the morphological structure of *Styloviridae* viruses (17). These PLP resembled bacteriophage lambda and the previously described phages in an *R. equi* isolate (11). Current work is directed to the isolation of PLP. Unfortunately, attempts to detect PFU when cultures of *R. equi* PLP-producing strains were plated onto non-PLP-producing strains have failed. Indicator strains used so far were non-PLP-producing *R. equi* human isolates, non-phage-producing *Rhodococcus erythropolis* (3), and *Gordona bronchialis*, *L. monocytogenes*, and *Staphylococcus aureus* reference strains (data not shown).

In *Escherichia coli*, phage-associated ampicillin resistance is known to exist but the resistance mechanism is not clear (28). Moreover, *E. coli* phages may produce porin-like outer membrane proteins during lysogeny (1). In the β-lactam-resistant *R. equi* human isolates, structural modifications of the cell wall components, tied to the PLP presence in situ, may explain the observed antibiotic resistance. However, attempts to transfer the β-lactam resistance marker to β-lactam-susceptible *R. equi* strains by filtrates from β-lactam-resistant strain supernatants have so far failed, as have mitomycin and UV light PLP induction experiments in β-lactam-susceptible strains (data not shown).

The β-lactam-resistant human isolates possessed a similar whole-cell protein pattern, with at least three more proteins than the β-lactam-susceptible clinical strains had. Attempts to purify these additional proteins are currently being made in order to raise antibodies and determine whether one or all

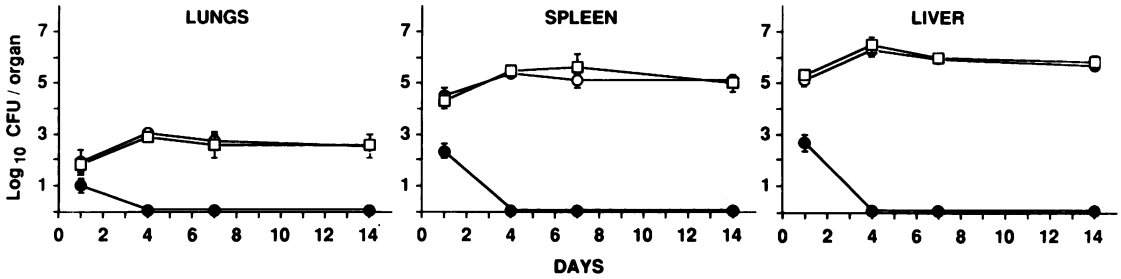

FIG. 5. Time course of disseminated infections in nude mice following i.v. inoculation with a sublethal dose of 5×10^6 CFU of *R. equi* PN1003 series strains. The data are expressed as geometric means of CFU (± standard deviations, three mice per point) in livers, spleens, and lungs after an i.v. challenge with the following *R. equi* strains: β-lactam-resistant PN1003 R clinical isolate (○), β-lactam-susceptible PN1003 S isolate (●), and in vitro β-lactam-resistant PN1003 M1 mutant (□). Both β-lactam-resistant strains harbored cell-surface-associated appendages and produced PLP.

of them react immunologically with the cell-surface-associated appendages and/or components of the PLP.

By using the 50% i.v. lethal dose, the PLP-producing *R. equi* human isolates (β-lactam resistant) were clearly more virulent in euthymic and nude mice than the non-PLP-producing isolates (susceptible) and were the only bacteria giving rise to chronic infection in all organs examined. However, only two of the β-lactam-resistant mutants analyzed, PN1003 M1 and PN1003 M2, led to chronic infection in nude mice and these were also the only mutants which produced PLP. Thus, it seems that PLP presence may therefore be associated with virulence in *R. equi* human isolates. It would also be interesting to study the mycolic acid content of the *R. equi* β-lactam-resistant and -susceptible strains, since virulence differences have also been reported to be linked to differences in cell wall mycolic acid content (9). The PLP-producing bacteria grew faster and also gave higher CFU values at stationary phase. This may partially explain the high virulence of PLP-producing bacteria. Such growth advantages have been described for *E. coli* lambda lysogens and Mu, P1, and P2 lysogens, which also exhibit altered outer membrane protein patterns (5, 6, 15).

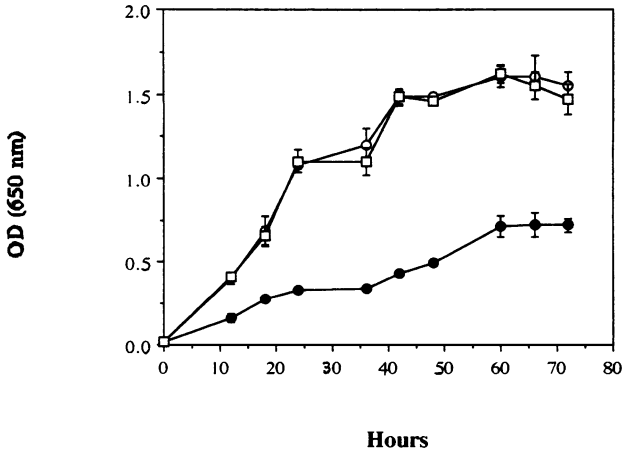

FIG. 6. Growth curves of three isolates of *R. equi* β-Lactam-resistant PN1003 R clinical isolate, (○), β-lactam-susceptible PN1003 S clinical isolate (●), and in vitro β-lactam-resistant PN1003 M1 mutant (□) were tested. Cultures were performed at 37°C in LB in a shaking water bath, and growth was measured by densitometry at 650 nm over 72 h. Both β-lactam-resistant strains harbored cell-surface-associated appendages and produced PLP. OD (650 nm), optical density at 650 nm.

Several conclusions could be drawn from the comparison of *R. equi* reference animal strain and *R. equi* human isolates. (i) *R. equi* ATCC 33701 and ATCC 33705, which were β-lactam resistant, harbored cell-surface-associated appendages, produced PLP induced chronic infection in nude mice, and grew quickly in vitro, were like the β-lactam-resistant human isolates. In contrast, *R. equi* ATCC 33703 was similar to the clinically derived susceptible isolates, in that it was β-lactam susceptible, did not harbor cell-surface-associated appendages, did not produce PLP, did not induce chronic infection in nude mice, and grew slowly in vitro. *R. equi* ATCC 33702 is an exception and appears to represent a third phenotype of *R. equi*. It was β-lactam resistant but possessed the described characteristics of the β-lactam-susceptible human isolates and did not produce PLP or appendages (resembling the in vitro β-lactam-resistant mutants PN1003 M3 to M10). While not ruling out their PBP affinity, β-lactam resistance remains unknown in these latter strains. (ii) It is known that, in *R. equi* animal reference strains, 15- to 17-kDa antigens which are cell surface associated are associated with the presence of an 85-kb plasmid and to virulence in mice (32, 33). Examination of the same animal reference strains gave a good correlation between PLP presence and virulence in mice. Surprisingly, the 85-kb plasmid found in virulent animal strains was not detected in any of the *R. equi* human virulent strains. Further work should focus on probing the 85-kb virulence plasmid with chromosomal DNA of *R. equi* human isolates or with their phages, once isolated.

Because *R. equi* β-lactam-resistant mutants were obtained at a high frequency, β-lactams (except carbapenems [20]) should not be used to treat *R. equi*-infected patients. As indicated by the virulence results, in vivo selection of β-lactam-resistant mutants may favor *R. equi* persistence in immunocompromised patients. Finally, this work indicates a correlation between antibiotic resistance and virulence in *Rhodococcus* species, which has very rarely been reported for bacteria.

ACKNOWLEDGMENTS

We are grateful to W. Arber for stimulating discussions of this work. We thank C. Nauciel, L. Gutmann, and J. F. Acar who also provided facilities; E. Kellenberger and M. Wurtz for help in the design of electron microscopy study; and E. R. Dabbs who kindly sent us a nonlysogenic *R. erythropolis* strain.

This work was funded by the Agence Nationale de Recherches sur le SIDA (92-DRS-V25), the Fondation pour la Recherche Médicale, Paris, France.

REFERENCES

1. **Barondess, J. J., and J. Beckwith.** 1990. A bacterial virulence determinant encoded by lysogenic coliphage λ. Nature (London) **346:**871–872.
2. **Barton, M. D., and K. L. Hughes.** 1980. *Corynebacterium equi*: a review. Vet. Bull. **50:**65–80.
3. **Dabbs, E. R.** 1987. A generalised transducing bacteriophage for *Rhodococcus erythropolis*. Mol. Gen. Genet. **206:**116–120.
4. **Desomer, J., D. Vereecke, M. Crespi, and M. Van Montaigu.** 1992. The plasmid-encoded chloramphenicol-resistance protein of *Rhodococcus fascians* is homologous to the transmembrane tetracycline efflux proteins. Mol. Microbiol. **6:**2377–2385.
5. **Edlin, G.** 1975. Lambda lysogens of *Escherichia coli* reproduced more rapidly than non-lysogens. Nature (London) **255:**735–737.
6. **Edlin, G., L. Lin, and R. Bitner.** 1977. Reproductive fitness of P1, P2, and Mu lysogens of *Escherichia coli*. J. Virol. **21:**560–564.
7. **Emmons, W., B. Reichwein, and D. L. Winslow.** 1991. *Rhodococcus equi* infection in the patient with AIDS literature review and report of an unusual case. Rev. Infect. Dis. **13:**91–96.
8. **Goodfellow, M.** 1987. The taxonomic status of *Rhodococcus equi*. Vet. Microbiol. **14:**205–209.
9. **Gotoh, K., M. Mitsuyama, S. Imaizumi, I. Kawamura, and I. Yano.** 1991. Mycolic acid-containing glycolipid as a possible virulence factor of *Rhodococcus equi* for mice. Microbiol. Immunol. **35:**175–185.
10. **Harvey, R. L., and J. C. Sunstrum.** 1991. *Rhodococcus equi* infection in patients with and without human immunodeficiency virus infection. Rev. Infect. Dis. **13:**139–145.
11. **Hiddema, R., M. D. Curran, N. P. Ferreira, J. N. Coetzee, and G. Lecatsas.** 1985. Characterization of phages derived from strains of *Rhodococcus australis* and *R. equii*. Intervirology **23:**109–111.
12. **Hietala, S. K., and A. A. Ardans.** 1987. Interaction of *Rhodococcus equi* with phagocytic cells from *Rhodococcus equi*-exposed and nonexposed foals. Vet. Microbiol. **14:**307–320.
13. **Kanaly, S. T., S. A. Hines, and G. H. Palmer.** 1993. Failure of pulmonary clearance of *Rhodococcus equi* infection in $CD4^+$ T-lymphocyte-deficient transgenic mice. Infect. Immun. **61:**4929–4932.

13a. **Kellenberger, A.** Personal communication.

14. **Laemmli, U. K.** 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
15. **Lin, L., R. Bitner, and G. Edlin.** 1977. Increased reproductive fitness of *Escherichia coli* lysogens. J. Virol. **21:**554–559.
16. **Magnusson, H.** 1923. Spezifische Infektiose Pneumonie beim Fohlen. Ein neuer Eitererrage beim Pferde. Arch. Wiss. Prakt. Tierheilk. **50:**22–38.
17. **Matthews, R. E. F.** 1982. Classification and nomenclature of viruses. Fourth report of the International Committee on Taxonomy of Viruses. Intervirology **17:**1–199.
18. **McNeil, M. M., J. M. Brown, and B. A. Lasker.** 1991. Evaluation of beta-lactam resistance among clinical isolates of *Rhodococcus equi*, abstr. 1009. Program Abstr. 31st Intersci. Conf. Antimicrob. Agents Chemother. American Society for Microbiology, Washington, D.C.
19. **National Committee for Clinical Laboratory Standards.** 1990. Methods for dilution antimicrobial susceptibility testing for bacteria that grow aerobically. Approved standard M7-A2. National Committee for Clinical Laboratory Standards, Villanova, Pa.
20. **Nordmann, P., J. J. Kerestedjian, and E. Ronco.** 1992. Therapy of *Rhodococcus equi* disseminated infections in nude mice. Antimicrob. Agents Chemother. **36:**1244–1248.
21. **Nordmann, P., M. H. Nicolas, and L. Gutmann.** 1993. Penicillin-binding proteins of *Rhodococcus equi*. Antimicrob. Agents Chemother. **37:**1406–1409.
22. **Nordmann, P., and E. Ronco.** 1992. In-vitro antimicrobial susceptibility of *Rhodococcus equi*. J. Antimicrob. Chemother. **29:**383–393.
23. **Nordmann, P., E. Ronco, and C. Nauciel.** 1992. Role of T-lymphocyte subsets in *Rhodococcus equi* infection. Infect. Immun. **60:**2748–2752.
24. **Nordmann, P., E. Rouveix, M. Guenounou, and M. H. Nicolas.** 1992. Pulmonary abscess due to rifampin and fluoroquinolone resistant *Rhodococcus equi* strain in an HIV-infected patient. Eur. J. Clin. Microbiol. Infect. Dis. **11:**557–558.
25. **Prescott, J. F.** 1991. *Rhodococcus equi*: an animal and human pathogen. Clin. Microbiol. Rev. **4:**20–34.
26. **Samies, J. H., B. N. Hathaway, R. M. Echols, J. M. Veazy, Jr., and V. A. Pilon.** 1986. Lung abscess due to *Corynebacterium equi* report of the first case in a patient with acquired immune deficiency syndrome. Am. J. Med. **80:**685–688.

26a. **SAS Institute Inc.** 1989. SAS/stat manual. SAS Institute Inc., Cary, N.C.

27. **Seeberg, A. H., R. M. Tolxdorff-Neutzling, and B. Wiedemann.** 1983. Chromosomal β-lactamases of *Enterobacter cloacae* are responsible for resistance to third-generation cephalosporins. Antimicrob. Agents Chemother. **23:**918–923.
28. **Smith, H. W.** 1972. Ampicillin resistance in *Escherichia coli* by phage infection. Nature (London) New Biol. **238:**205–206.
29. **Takai, S., M. Iie, Y. Watanabe, S. Tsubaki, and T. Sekizaki.** 1992. Virulence-associated 15- to 17-kilodalton antigens in *Rhodococcus equi*: temperature-dependent expression and location of the antigens. Infect. Immun. **60:**2995–2997.
30. **Takai, S., K. Koike, S. Ohbushi, C. Izumi, and S. Tsubaki.** 1991. Identification of 15- to 17-kilodalton antigens associated with virulent *Rhodococcus equi*. J. Clin. Microbiol. **29:**439–443.
31. **Takai, S., T. Michizoe, K. Matsumara, M. Nagai, H. Sato, and S. Tsubaki.** 1985. Correlation of in vitro properties of *Rhodococcus* (*Corynebacterium*) *equi* with virulence for mice. Microbiol. Immunol. **29:**1175–1184.
32. **Takai, S., T. Sekizaki, T. Ozawa, T. Sugawara, Y. Watanabe, and S. Tsubaki.** 1991. Association between a large plasmid and 15- to 17-kilodalton antigens in virulent *Rhodococcus equi*. Infect. Immun. **59:**4056–4060.
33. **Tkachuk-Saad, O., and J. Prescott.** 1991. *Rhodococcus equi* plasmids: isolation and partial characterization. J. Clin. Microbiol. **29:**2696–2700.
34. **Trias, J., V. Jarlier, and R. Benz.** 1992. Porins in the cell wall of mycobacteria. Science **258:**1479–1481.
35. **Van Etta, L. L., G. A. Filice, R. M. Ferguson, and D. N. Gerding.** 1983. *Corynebacterium equi*: a review of 12 cases of human infection. Rev. Infect. Dis. **5:**1012–1018.
36. **Wurtz, M., J. Kistler, and T. Hohn.** 1976. Surface structure of in vitro assembled bacteriophage lambda polyheads. J. Mol. Biol. **101:**39–56.
37. **Yanagawa, R., and E. Honda.** 1976. Presence of pili in species of human and animal parasites and pathogens of the genus *Corynebacterium*. Infect. Immun. **13:**1239–1295.