

Restriction Fragment Length Polymorphisms of Virulence Plasmids in *Rhodococcus equi*

SHINJI TAKAI,^{1*} MASATO SHODA,¹ YUKAKO SASAKI,¹ SHIRO TSUBAKI,¹ GUILLAUME FORTIER,² STEPHANE PRONOST,² KARIM RAHAL,³ TEOTIMO BECU,⁴ ANGELA BEGG,⁵ GLENN BROWNING,⁶ VIVIAN M. NICHOLSON,⁷ AND JOHN F. PRESCOTT⁷

*Kitasato University, Towada, Aomori 034-8628, Japan*¹; *Laboratoire Frank Duncombe, Caen, Calvados*,² and *École Nationale Vétérinaire de Lyon*,³ France; *Clínica Equina S.R.L., Capitán Sarmiento, Provincia de Buenos Aires, Argentina*⁴; *Scone Veterinary Diagnostic Laboratory, Scone*,⁵ and *Veterinary Preclinical Center, The University of Melbourne, Parkville, Victoria 3052*,⁶ Australia; and *Ontario Veterinary College, University of Guelph, Ontario, Canada*⁷

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Virulent *Rhodococcus equi*, which is a well-known cause of pyogranulomatous pneumonia in foals, possesses a large plasmid encoding virulence-associated 15- to 17-kDa antigens. Foal and soil isolates from five countries—Argentina, Australia, Canada, France, and Japan—were investigated for the presence of 15- to 17-kDa antigens by colony blotting, using the monoclonal antibody 10G5, and the gene coding for 15- to 17-kDa antigens by PCR. Plasmid DNAs extracted from positive isolates were digested with restriction endonucleases *Bam*HI, *Eco*RI, *Eco*T22I, and *Hind*III, and the digestion patterns that resulted divided the plasmids of virulent isolates into five closely related types. Three of the five types had already been reported in Canadian and Japanese isolates, and the two new types had been found in French and Japanese isolates. Therefore, we tentatively designated these five types 85-kb type I (pREAT701), 85-kb type II (a new type), 87-kb type I (*Eco*RI and *Bam*HI type 2 [V. M. Nicholson and J. F. Prescott, *J. Clin. Microbiol.* 35:738–740, 1997]), 87-kb type II (a new type), and 90-kb (pREL1) plasmids. The 85-kb type I plasmid was found in isolates from Argentina, Australia, Canada, and France. Plasmid 87-kb type I was isolated in specimens from Argentina, Canada, and France. The 85-kb type II plasmid appeared in isolates from France. On the other hand, plasmids 87-kb type II and 90-kb were found only in isolates from Japan. These results revealed geographic differences in the distribution of the virulence plasmids found in the five countries and suggested that the restriction fragment length polymorphism of virulence plasmids might be useful to elucidate the molecular epidemiology of virulent *R. equi* in the world.

Rhodococcus equi is one of the most important bacterial pathogens in 1- to 6-month-old foals. Infections caused by this organism are characterized by chronic, suppurative bronchopneumonia and enteritis associated with a high mortality rate (1, 6, 16). We have recently reported that the 15- to 17-kDa antigens of *R. equi* are associated with virulence in mice (13, 17) and that the presence of an 85-kb or 90-kb plasmid is essential for virulence and the expression of 15- to 17-kDa antigens (7, 20–22). These virulence-associated antigens and virulence plasmids have been used as epidemiological markers to identify virulent *R. equi* isolates from horses and their environment by Western blot (immunoblot) assay using a monoclonal antibody and plasmid profiles (9, 10, 14, 15, 18). Recent studies have shown that only virulent strains of *R. equi* harboring a virulence plasmid of 85 to 90 kb can cause the disease in foals and that farms with endemic disease are more contaminated with virulent *R. equi* than are those without the disease (8, 9, 20). Amplification of DNA of a virulence-associated antigen gene by the PCR technique reportedly provides a means both to identify virulent *R. equi* in tracheal aspirates of foals and to diagnose *R. equi* pneumonia in foals (3, 12, 19).

More recently, Nicholson and Prescott (5) examined restriction enzyme digestion patterns of virulence plasmids in human and foal isolates using *Eco*RI and *Bam*HI. They showed the

presence of three major types of virulence plasmids (*Eco*RI and *Bam*HI types 1 to 3) in the isolates and demonstrated the following geographic differences in their distribution. Foal isolates with the *Eco*RI type 1 plasmid digestion pattern tended to come mostly from the United States, Canada, and European countries; foal isolates with the *Eco*RI type 2 pattern appeared mainly in specimens from Latin American countries; and the only *Eco*RI and *Bam*HI type 3 plasmid found so far came from a Japanese foal isolate. The purposes of this study were to investigate genotypic variation among virulence plasmids of *R. equi* from foals and their environment from different geographic sources and to evaluate whether these plasmids are useful as epidemiological markers in global surveillance of *R. equi* infection in foals.

The bacterial strains used as reference strains in this study were *R. equi* ATCC 33701 (pREAT701, 85 kb) and L1 (pREL1, 90 kb) (17). In addition, we used 29 isolates obtained from lung lesions of infected foals and from soil on horse-breeding farms in Caen, Calvados, France; 26 isolates from lung lesions of infected foals and soil on horse-breeding farms in Lyon, France; 26 isolates from lung lesions of infected foals in Argentina; 7 isolates from lung lesions of infected foals in Australia; 2 isolates from lung lesions of infected foals in Canada; 399 isolates from feces of healthy foals; and 54 isolates from lung lesions of infected foals in Japan. In this study, the laboratory number of isolates designated at each institution was used as the name of the strain.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting analysis were performed as

* Corresponding author. Mailing address: Department of Animal Hygiene, School of Veterinary Medicine and Animal Sciences, Kitasato University, Towada, Aomori 034-8628, Japan. Phone: 81-167-23-4371. Fax: 81-176-23-8703. E-mail: takai@vmas.kitasato-u.ac.jp.

TABLE 1. Virulence-associated antigens, plasmids, pathogenicities, and restriction enzyme digestion patterns of the five representative strains^a

Strain	Expression of 15- to 17-kDa antigens	PCR result (an approx 564-bp product)	Mouse pathogenicity (LD ₅₀)	<i>Bam</i> HI digestion pattern	<i>Eco</i> RI digestion pattern	<i>Eco</i> T22 digestion pattern	<i>Hind</i> III digestion pattern	Designation of plasmid
33701	+	+	1.2 × 10 ⁶	1	1	1	1	85-kb type I
96E35	+	+	1.5 × 10 ⁶	2	1	1	2	85-kb type II
222	+	+	1.5 × 10 ⁶	3	2	2	1	87-kb type I
96B6	+	+	1.5 × 10 ⁶	4	3	3	3	87-kb type II
L1	+	+	1.7 × 10 ⁶	5	4	1	4	90-kb

^a +, positive result.

described previously (15, 17). A monoclonal antibody against 15- to 17-kDa antigens (10G5) was used for the immunoblotting procedures (11, 14).

Virulence of the clinical isolates was examined by determining the 50% lethal dose (LD₅₀) by the probit method on groups of five mice, as described previously (15, 17). Three groups of five mice each for each strain were tested at three inoculum levels (approximately 10⁵, 10⁶, and 10⁷ cells). Next, 20- to 23-g ddY outbred mice were each given 0.2 ml of the bacterial suspension intravenously and were then observed for 10 days.

The target DNA for PCR amplification was the published sequence of the 15- to 17-kDa antigens gene of virulence plasmid DNA from *R. equi* ATCC 33701 (7). Primer 1 (5'-GACTCTTCACAAGACGGT-3') corresponded to the sense strand at positions 6 to 23 and primer 2 (5'-TAGGCGTTGTGCCAGCTA-3') corresponded to the antisense strand at positions 569 to 552 in the sequence of the 15- to 17-kDa antigen gene (12).

PCR amplification was performed with 10 ml of DNA preparation in a 50-μl reaction containing 10 mM Tris-HCl (pH 8.3 at 25°C), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM (each) deoxynucleotide triphosphate, 1 μM each primer, and 2.5 U of *Taq* DNA polymerase (Takara, Tokyo, Japan). The samples were subjected to 30 cycles of amplification in a GeneAmp PCR system 2400 (Perkin-Elmer, Norwalk, Conn.). The cycling conditions were as follows: denaturation, 90 s at 94°C; primer annealing, 1 min at 55°C; and extension, 2 min at 72°C (12).

Plasmid DNA was isolated from *R. equi* by the alkaline lysis method (2), with some modifications, as described previously (12). Plasmid DNAs were analyzed by digestion with restriction endonucleases *Bam*HI, *Eco*RI, *Eco*T22I, and *Hind*III for detailed comparison and estimation of the plasmid size. Samples of the plasmid preparations were separated in 0.7 or 1.0% agarose gels at approximately 5 V/cm for 2 h.

The 15- to 17-kDa antigen genes on fragments of plasmid DNAs were detected by Southern hybridization (12). Digested fragments of plasmid DNAs were transferred from gels to a sheet of nylon membrane (Hybond N; Amersham Japan Corp.,

Tokyo, Japan) by using the vacuum transfer method with VacuGene (Pharmacia-LKB Biotechnology AB, Uppsala, Sweden) and without depurination, following the manufacturer's instructions. Hybridization was carried out at 68°C for at least 6 h in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.0]) containing 0.1% sodium *N*-lauroyl sarcosine, 0.02% SDS, and 1% blocking reagent. After hybridization, the sheets were washed twice at room temperature for 5 min in 2× SSC containing 0.1% SDS and washed twice again for 15 min at 68°C in 0.1× SSC containing 0.1% SDS. Immunological detection of hybridized digoxigenin-labeled probe was performed with a commercial kit (Boehringer Mannheim-Yamanouchi, Tokyo, Japan). For Southern analysis, a digoxigenin-11-dUTP-labeled probe was amplified with the primers by PCR as described above (12).

During the screening of restriction fragment length polymorphisms of plasmid DNAs from 487 isolates obtained from the six different institutes in five countries, five different digestion patterns of plasmid DNAs were identified. Also, five isolates (ATCC 33701, 96E35, 96B6, 222, and L1), each carrying a distinct plasmid, were characterized. Plasmid and protein profiles of five representative isolates were investigated by agarose gel electrophoresis and immunoblotting. The representative strains contained a large plasmid and expressed 15- to 17-kDa antigens. These isolates gave an approximately 564-bp gene product of 15- to 17-kDa antigens in the PCR amplification (data not shown). The pathogenicities of the five isolates were tested in mice and found to be virulent (LD₅₀s ranged from 1.2 × 10⁶ to 1.7 × 10⁶) (Table 1).

Plasmid DNA preparations of the five isolates were analyzed further by restriction enzyme digestion with endonucleases *Eco*RI, *Bam*HI, *Eco*T22I, and *Hind*III (Fig. 1A). These results divided the plasmids of the representative isolates into five closely related types (Table 1), including one type that has been reported recently by Nicholson and Prescott (5). Plasmid DNAs from the five isolates digested with *Eco*RI, *Bam*HI, *Eco*T22I, and *Hind*III were examined by Southern analysis with the PCR probes (Fig. 1B). PCR products labeled with

TABLE 2. Distribution of virulence plasmids in *R. equi* isolates collected from five countries

Country	Sample source	No. of 15- to 17-kDa antigen-positive isolates	No. of isolates with plasmids that were:					Source
			85 kb, type I	85 kb, type II	87 kb, type I	87 kb, type II	90 kb	
France	Foals and soil	29	15	1	13	0	0	Fortier and Pronost
France	Foals and soil	26	7	4	15	0	0	Rahal
Argentina	Foals	26	10	0	15	0	0	Becu
Australia	Foals	7	7	0	0	0	0	Begg and Browning
Canada	Foals	2	1	0	1	0	0	Prescott
Japan	Foals	54	0	0	0	46	8	Takai
Japan	Feces of foals	399	0	0	0	323	76	Takai

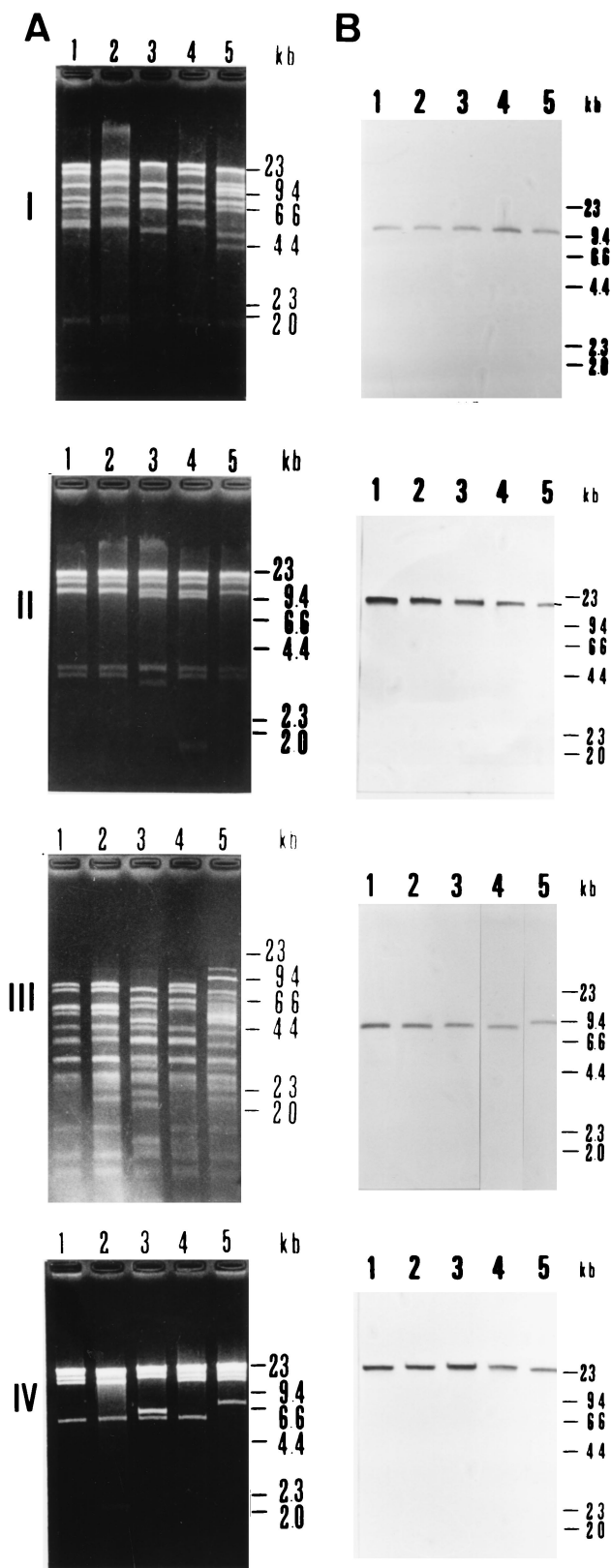


FIG. 1. (A) *EcoRI* (I), *EcoT22I* (II), *BamHI* (III), and *HindIII* (IV) restriction fragments of the plasmids of five representative isolates of *R. equi*; (B) Southern hybridization of digoxigenin-labeled PCR product probes to the *EcoRI* (I), *EcoT22I* (II), *BamHI* (III) and *HindIII* (IV) restriction fragments of the plasmids. Lanes: 1, ATCC 33701; 2, 96E35; 3, 96B6; 4, 2, 222; 5, L1.

digoxigenin-11-dUTP hybridized with one of the fragments of each plasmid DNA.

According to the above results, we tentatively designated these five types 85-kb type I (ATCC 33701), 85-kb type II (a new type), 87-kb type I (strain 222), 87-kb type II (a new type), and 90-kb (strain L1) plasmids (Table 1).

Five hundred forty-three isolates of *R. equi* from five countries were examined by plasmid profiles and endonuclease digestion analysis (Table 2). Of the isolates, those from Canada and Argentina contained either an 85-kb type I or an 87-kb type I plasmid; isolates from Australia contained an 85-kb type I plasmid; and isolates from France possessed either an 85-kb type I, an 87-kb type I, or an 85-kb type II plasmid. On the other hand, the Japanese isolates did not contain any of those three types of plasmids. Instead, they revealed an 87-kb type II or a 90-kb plasmid. Notably, there were differences in the geographic distribution of the five virulence plasmids obtained.

The present investigation revealed the presence of at least five distinct but closely related plasmids of 85 to 90 kb in isolates of *R. equi* from foals and their environments from the five countries tested, and these were associated with the expression of 15- to 17-kDa antigens. Three of the five plasmids were found in North and South America and Europe, but 87-kb type II and 90-kb plasmids were found only in the isolates from Japan. These results support the previous observation by Prescott and Nicholson (5) of the geographic differences in distribution of virulence plasmids, and they suggest that the restriction fragment length polymorphism of virulence plasmids might be useful for global surveillance of virulent *R. equi* in equids and their environment.

Previously, we demonstrated the relation in DNA homology between the 85-kb type I and 90-kb virulence plasmids obtained by restriction enzyme and by Southern blot analyses (20). However, the present study showed that the 85-kb type I plasmid was related more closely to the 85-kb type II plasmid by restriction fragment length polymorphism than to the 90-kb plasmid, since the *EcoRI* and *EcoT22I* digestion patterns of these two plasmids were identical. Although there are differences in the plasmid DNA fragments produced by digestion with restriction enzymes among the five plasmids, Southern blot analysis of these plasmids showed large regions of DNA homology among them and supported the finding of their common origin (5, 20).

In our epidemiological surveillance studies, a colony blot enzyme-linked immunosorbent assay with the monoclonal antibody 10G5 was used as a screening test to identify virulent *R. equi* (14, 15). Positive isolates showing 15- to 17-kDa antigens were virulent for mice ($LD_{50} = 1 \times 10^6$ to 2×10^6), whereas none of the mice injected with a maximum of 10^8 cells of negative isolates that do not produce the antigens died (13). Plasmid preparations from positive isolates were then separated along with the plasmids of *R. equi* ATCC 33701 (pREAT701; 85 kb) and L1 (pREL1; 90 kb) in agarose gel. Since the number of samples was so large, we were unable to characterize the plasmid profiles of these isolates in detail. In this study, we found a new plasmid of 87 kb type II, and then we reexamined all of the isolates considered to possess an 85-kb type I plasmid. The isolates showed an identical pattern of the same restriction enzyme fragments found in 87-kb type II. This finding differs from the data we reported in previous papers (9, 10, 14, 18).

In Japan, more than 100,000 horses have been bred and the majority of them are used for horse racing. Less than 3,000 horses are Japanese domestic horses (Hokkaido, Kiso, Tokara, Taishuh, Miyako, Misaki, and Yonaguni), which are inferior in size and strength to Western horses (4). We considered that

the majority of Japanese isolates of virulent *R. equi* were probably imported from European countries, since for the last 100 years, Japan has imported European horses for crossbreeding to improve Japanese stock for racing and military purposes. Patterns of restriction enzyme fragments of plasmid DNAs of Japanese isolates from thoroughbred and Anglo-Arab breeds revealed the existence of an 87-kb type II or a 90-kb plasmid, which was found only in the Japanese isolates. However, Australian isolates were likely imported, since no equids were in Australia before 1788, when seven horses arrived in the new land (4). We are now investigating virulence plasmids present in Japanese domestic horses.

A new plasmid, 85-kb type II, was found in French isolates collected from horses at different sites (Caen and Lyon) in France. These results suggest that virulent *R. equi* possessing an 85-kb type II might be widespread in France. Further studies are needed on the characteristics of virulence plasmids of *R. equi* and the molecular epidemiology of virulent *R. equi* throughout the world. Results from these investigations might reveal information on the evolution of horses in the world as well as the transmission of virulent *R. equi* using plasmid profiles.

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REFERENCES

- Barton, M. D., and K. L. Hughes. 1980. *Corynebacterium equi*: a review. *Vet. Bull.* **50**:65–80.
- Birnboim, H. C., and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* **7**:1513–1523.
- Haites, R. E., G. Muscatello, A. P. Begg, and G. F. Browning. 1997. Prevalence of the virulence-associated gene of *Rhodococcus equi* in isolates from infected foals. *J. Clin. Microbiol.* **35**:1642–1644.
- Hendericks, B. L. 1995. International encyclopedia of horse breeds, p. 51–53. University of Oklahoma Press, Norman.
- Nicholson, V. M., and J. F. Prescott. 1997. Restriction enzyme analysis of the virulence plasmids of VapA-positive *Rhodococcus equi* strains isolated from humans and horses. *J. Clin. Microbiol.* **35**:738–740.
- Prescott, J. F. 1991. *Rhodococcus equi*: an animal and human pathogen. *Clin. Microbiol. Rev.* **4**:20–34.
- Sekizaki, T., S. Takai, Y. Egawa, T. Ikeda, H. Ito, and S. Tsubaki. 1995. Cloning and nucleotide sequence of *Rhodococcus equi* virulence-associated 15- to 17-kilodalton antigens gene. *Gene* **155**:135–136.
- Takai, S. 1997. Epidemiology of *Rhodococcus equi* infections: a review. *Vet. Microbiol.* **56**:167–176.
- Takai, S., T. Anzai, Y. Sasaki, S. Tsubaki, and M. Kamada. 1993. Virulence of *Rhodococcus equi* isolates from lesions of infected foals. *Bull. Equine Res. Inst.* **30**:9–14.
- Takai, S., T. Anzai, K. Yamaguchi, S. Kakizaki, J. Takahagi, Y. Sato, F. Takehara, Y. Tamada, S. Matsukura, A. Tani, M. Kato, N. Seno, Y. Sasaki, S. Tsubaki, and M. Kamada. 1994. Prevalence of virulence plasmids in environmental isolates of *Rhodococcus equi* from horse-breeding farms in Hokkaido. *J. Equine Sci.* **5**:21–25.
- Takai, S., M. Iie, C. Kobayashi, T. Morishita, T. Nishio, T. Ishida, T. Fijimura, Y. Sasaki, and S. Tsubaki. 1993. Monoclonal antibody specific to virulence-associated 15- to 17-kilodalton antigens of *Rhodococcus equi*. *J. Clin. Microbiol.* **31**:2780–2782.
- Takai, S., T. Ikeda, Y. Sasaki, Y. Watanabe, T. Ozawa, S. Tsubaki, and T. Sekizaki. 1995. Identification of virulent *Rhodococcus equi* by amplification of gene coding for 15- to 17-kilodalton antigens. *J. Clin. Microbiol.* **33**:1624–1627.
- 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.
- Takai, S., T. Morishita, Y. Nishio, Y. Sasaki, S. Tsubaki, T. Higuchi, S. Hagiwara, H. Senba, M. Kato, N. Seno, T. Anzai, and M. Kamada. 1994. Evaluation of a monoclonal antibody-based colony blot test for rapid identification of virulent *Rhodococcus equi*. *J. Vet. Med. Sci.* **56**:681–684.
- Takai, S., S. Ohbushi, K. Koike, S. Tsubaki, H. Oishi, and M. Kamada. 1991. Prevalence of virulent *Rhodococcus equi* in isolates from soil and feces of horses from horse-breeding farms with and without endemic infections. *J. Clin. Microbiol.* **29**:2887–2889.
- Takai, S., Y. Sasaki, and S. Tsubaki. 1995. *Rhodococcus equi* infection in foals: current concepts and implication for future research. *J. Equine Sci.* **6**:105–119.
- 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.
- Takai, S., J. Takahagi, Y. Sato, K. Yamaguchi, S. Kakizaki, S. Takehara, S. Matsukura, Y. Tamada, A. Tani, Y. Sasaki, S. Tsubaki, and M. Kamada. 1994. Molecular epidemiology of virulent *Rhodococcus equi* in horses and their environments, p. 183–187. In H. Nakajima and P. D. Rossdale (ed.), *Equine infectious diseases VII*. R & W Publications (Newmarket) Limited, Newmarket, Suffolk, United Kingdom.
- Takai, S., G. Vigo, H. Ikushima, T. Higuchi, S. Hagiwara, S. Hashikura, Y. Sasaki, S. Tsubaki, T. Anzai, and M. Kamada. 1998. Detection of virulent *Rhodococcus equi* in tracheal aspirate samples by polymerase chain reaction for rapid diagnosis of *R. equi* pneumonia in foals. *Vet. Microbiol.* **61**:59–69.
- Takai, S., Y. Watanabe, T. Ikeda, S. Tsubaki, T. Ozawa, S. Matsukura, Y. Tamada, and T. Sekizaki. 1993. Virulence-associated plasmids in *Rhodococcus equi*. *J. Clin. Microbiol.* **31**:1726–1729.
- Tan, C., J. F. Prescott, M. C. Patterson, and V. M. Nicholson. 1995. Molecular characterization of a lipid-modified virulence-associated protein of *Rhodococcus equi* and its potential in protective immunity. *Can. J. Vet. Res.* **59**:51–59.
- Tkachuk-Saad, O., and J. F. Prescott. 1991. *Rhodococcus equi* plasmids: isolation and partial characterization. *J. Clin. Microbiol.* **29**:2696–2700.