Journal of
Clinical Microbiology

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Rhodococcus equi Plasmids: Isolation and Partial Characterization

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Fifty-four strains of *Rhodococcus equi* from different clinical sources (mainly horses and pigs) were examined for their plasmid content by two screening methods. Plasmids were detected in 49 of 54 strains. A plasmid of approximately 80 kb was isolated from 21 of 22 isolates from horses and 20 of 28 isolates from pigs, and a 105-kb plasmid was isolated from 7 of 28 isolates from pigs. The 80-kb plasmid was significantly associated with strains of equine rather than porcine origin, and the 105-kb plasmid was significantly associated with strains of porcine origin. The type strain, ATCC 6939, consistently failed to yield a plasmid. Restriction enzyme analysis of purified plasmid DNA confirmed the relatedness of the 80-kb plasmids isolated from strains of equine and porcine origin. More differences between the restriction patterns of plasmids from strains isolated from horses and from pigs than among strains from either species were observed. Restriction enzyme analysis also showed relatedness of the 105-kb plasmid to the 80-kb plasmid. Three strains shown by others to be virulent in horses or mice possessed the 80-kb plasmid, whereas three other strains not virulent for horses or mice lacked the plasmid, although one had the 105-kb plasmid. There was a significant but not perfect association between the presence of the 80-kb plasmid and production of a diffuse 17.5-kDa thermoregulated, virulence-associated protein. Further study is needed to determine whether this plasmid is associated with virulence in *R. equi*.

Rhodococcus equi is an important pathogen of foals less than 4 months old (1, 17). Characteristically, it causes a purulent bronchopneumonia with variable morbidity and mortality. Rarely, it is an opportunistic pathogen of other mammals, including humans, in which infection generally follows an immunosuppressive event. It is increasingly isolated from patients with AIDS (10, 11). The natural habitat of *R. equi* is thought to be soil, particularly that contaminated by herbivore manure, which favors the growth of the organism in high environmental temperatures, but *R. equi* also multiplies in the intestines of young foals (17).

R. equi is a facultative intracellular pathogen. It survives inside alveolar macrophages of nonimmune foals, producing pyogranulomatous, macrophage-rich lesions in the lung. The basis of its pathogenicity appears to reside in its ability to prevent phagosome-lysosome fusion within the macrophage (27). Differences in virulence for foals, mice, or phagocytes between isolates from clinical specimens and those from the environment have been described previously (2, 13, 26), but the basis of this difference was not determined. Recent studies have, however, identified a diffuse 15- to 17-kDa protein in *R. equi* as a marker for virulence for mice (25). This protein, which we describe as a diffuse 17.5-kDa protein, is expressed only at 30°C or above (4, 5). Similarly, Congo red-binding proteins in *R. equi* with which this protein appears to be linked are also expressed at 30°C or above (4). Other pathogens with environmental life cycles, including *Shigella* spp. and *Yersinia* spp., produce plasmid-mediated and virulence-associated thermoregulated Congo red-binding proteins (3, 15, 19, 24).

The purposes of the study described here were to screen clinical isolates of *R. equi* for plasmids, to compare plasmid contents of isolates from different sources, to compare representative *R. equi* plasmids by restriction enzyme analysis (REA), and to assess the relationship between carriage of plasmids by selected strains with known virulence for mice or horses and production of the virulence-associated diffuse 17.5-kDa protein.

MATERIALS AND METHODS

Bacterial strains and media. The reference numbers and species of origin of the 54 *R. equi* strains examined are shown in Table 1 (16). All isolates were from clinical material, usually from lungs for horses and from lymph nodes for pigs, and, except for isolates from pigs, were from independent sources (16). They included representatives of capsular serotypes 1 to 6 (16). The isolates from pigs were made from cervical lymph nodes obtained after slaughter, but the relatedness of the pigs was unknown. The culture collection was maintained at 4°C on tryptic soy agar (TSA) (Difco Laboratories, Detroit, Mich.) slants or in a lyophilized state. Isolates on slants had not been subcultured more than three times since their collection between 1977 and 1979. Strains were routinely grown on blood agar (TSA with 5% citrated bovine blood) and were incubated aerobically for 48 h at 37°C after subculture from TSA. Strains described as plasmid negative following culture from slants were reexamined after recovery from lyophilized cultures, which had been made immediately after isolation.

Plasmid isolation procedures. Two methods were used to screen strains for plasmids. The alkaline-detergent lysis method was a modification of the techniques described by Dillon et al. (9) and Schiller et al. (21). Isolates were streaked on three TSA plates and incubated aerobically for 48 h at 37°C. Colonies were scraped from the plates after each was flooded with 5 ml of tryptic soy broth (TSB) (Difco). The bacterial suspension was incubated aerobically for 2 h at 37°C with shaking (150 rpm), 120 μg of penicillin G was then added per ml, and the cultures were reincubated for an additional 2 h. Bacteria were recovered by centrifugation and washed in 10 ml of 10 mM Tris, pH 8.0, and then they were resuspended in 5 ml of 10 mM Tris, pH 8.0, containing

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TABLE 1. Characteristics of plasmids of 54 *R. equi* strains

Plasmid size(s) (kb)	Source	No. of strains	Strain(s)
80	Horse	18	ATCC 33701; 2 to 4, 6, 7, 9, 10, 20, 26, 49, 51 to 53, 99, 103, 104
	Pig	15	ATCC 7699, ATCC 33704; 13, 14, 19, 27, 28, 34, 36, 37, 42, 44, 46, 48
	Cat	1	102
75	Horse	1	21
	Pig	4	ATCC 33705; 31 to 33
85	Horse	1	50
	Pig	1	47
105	Pig	5	ATCC 33703; 29, 30, 32, 33
80, 105	Pig	1	40
	Cat	1	23
65, 105	Pig	1	35
60, 80	Horse	1	101
None (no plasmid detected	Horse	1	ATCC 6939
	Pig	1	12, 15
	Dog	2	ATCC 33702; 5

0.5 M sucrose and 5 mg of lysozyme per ml. After incubation for 1 h at 37°C, cells were centrifuged and were washed with 10 ml of 50 mM Tris–20 mM disodium EDTA, pH 8.0 (TE buffer), and then they were resuspended in 0.5 ml of the same buffer. Cells were lysed by the addition of 9.5 ml of 2% (wt/vol) sodium dodecyl sulfate (SDS) in TE buffer adjusted to pH 12.45 with 3 N sodium hydroxide (NaOH). To enhance lysis, the tubes were inverted gently 10 times and incubated for 30 min at 37°C, and then the solution was neutralized by the addition of 0.8 ml of 2 M Tris, pH 7.4.

Chromosomal DNA and protein-SDS complexes were precipitated by the addition of 1 ml of 5 M sodium chloride (NaCl) to the lysate, with gentle mixing by inversion, and storage on ice for 4 h or overnight at 4°C. The supernatant was collected by centrifugation, transferred to polypropylene tubes, and extracted with phenol. The aqueous upper phase was recovered and plasmid DNA was precipitated by the addition of 0.1 volume of 3 M sodium acetate (pH 4.8) and 1 volume of cold isopropanol, and samples were stored at −20°C overnight. Precipitated DNA was recovered by centrifugation (35,000 × g, 30 min, 4°C), the supernatant was decanted, and the DNA pellet was air dried and then dissolved in 100 μl of 10 mM Tris–1 mM EDTA, pH 8.0. Samples were stored at 4°C for agarose gel electrophoresis.

The second screening approach was an SDS lysis method based on that reported by Serwold-Davis and Groman (22). Briefly, 5 ml of TSB was inoculated with *R. equi* and incubated for 24 h at 37°C. This culture was used to inoculate 200 ml of TSB in a 500-ml Erlenmeyer flask, which was incubated at 37°C for 60 to 72 h with shaking at 180 rpm. Bacteria were harvested by centrifugation, washed in water, and suspended in 12.5 ml of freshly prepared lysozyme solution (5 mg of lysozyme per ml in 50 mM Tris–2.5 mM EDTA). The suspension was incubated at 37°C for 2 h with shaking (180 rpm). Cells were then lysed by the addition of 1.25 ml of 50 mM Tris–0.25 M EDTA (pH 8.0) and 0.8 ml of

20% (wt/vol) SDS. After gentle inversion 20 times, the suspension was incubated at 55°C for 30 min. Chromosomal DNA was then denatured by the addition of 0.8 ml of 3 N NaOH, and the tubes were inverted intermittently for 10 min. The lysate was neutralized with 1.3 ml of 2 M Tris (pH 7.0), and the tubes were gently inverted for 5 min. Following the addition of 1.9 ml of 5 M NaCl, recovery of plasmid DNA followed the steps described for the first method. In some cases, a second phenol extraction was required in order to obtain a clear aqueous phase.

CsCl-EtBr density gradient centrifugation. For purification of plasmid DNA, strains 10, 99, 101, 103, and ATCC 6939 were cultured as described for the SDS lysis procedure, except that 2.4 liters of TSB (12 × 200 ml) was used for each strain. Plasmid DNA was extracted by the SDS lysis method for each strain and purified twice by cesium chloride (CsCl)-ethidium bromide (EtBr) density gradient centrifugation (18) with a Beckman VTi.65.2 rotor (Beckman L5-75 ultracentrifuge) at 327,802 × g for 18 h at 20°C. The EtBr was then removed, and plasmid DNA was recovered by ethanol precipitation (18).

Agarose gel electrophoresis. Agarose gel electrophoresis of plasmid DNA was carried out on horizontal gels by using 0.7 and 0.4% (wt/vol) agarose with Tris-borate EDTA (TBE) buffer (89 mM Tris, 89 mM boric acid, 2.5 mM disodium EDTA) before staining with EtBr and photography (18). The sizes of covalently closed circular (CCC) plasmids were estimated by measuring their relative mobilities in comparison with those of plasmids of known molecular sizes by using gel electrophoresis. The CCC DNA molecular size marker plasmids were extracted from reference strains *Escherichia coli*(pDT285), *E. coli* V517, *E. coli*(pDT369), and *Salmonella typhimurium*(pSLT2) by standard methods (6).

REA. Purified plasmid DNAs recovered from the selected strains described above and plasmid DNAs isolated from strains ATCC 33703 (105-kb plasmid) and 13 and 19 (80-kb plasmid) were digested to completion with the restriction enzyme *Eco*RI (Bethesda Research Laboratories, Inc. [BRL], Gaithersburg, Md.) and used according to the manufacturer's instructions. Purified plasmid DNAs from strains 10 and 103 were also digested with *Bgl*II and *Hin*dIII (BRL). A 20- to 40-μl volume of digested DNA was electrophoresed in TBE buffer at 25 V for 18 h in 0.4% agarose gels. Bacteriophage lambda and high-molecular-weight DNA size markers (BRL) were included to estimate restriction fragment sizes (20).

Production of diffuse 17.5-kDa virulence-associated protein. Five plasmid-negative strains (ATCC 6939 and 33702 and strains 5, 12, and 15) and 15 strains carrying the 80-kb plasmid (ATCC 33701 and strains 2 to 4, 6, 13, 14, 16, 19, 20 to 22, 24, 26, and 27) were grown in 500 ml of nutrient broth (Difco) shaken at 90 rpm for 72 h at 37°C. Cells were harvested by centrifugation, washed twice in phosphate-buffered saline (pH 7.2), resuspended in 0.5 ml of distilled water (giving about 10^{11} bacteria per ml), and frozen at −70°C until used. If strains were classified as negative for the diffuse 17.5-kDa protein, they were recultured and examined in an identical manner before being described as negative for this protein. Bacterial samples in distilled water were boiled in SDS sample buffer for 10 min and briefly centrifuged, and 10 to 40 μl of sample was added to each well of a 4% polyacrylamide stacking gel. Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) by using a 15% separating gel and were stained with Coomassie blue.

FIG. 1. Agarose gel separation of plasmid DNA isolated from various *R. equi* strains. The molecular size markers used (in kilobases) are as follows: lane A, 144; lane B, 90; lane C, 54; and lane D, 35. *R. equi* plasmids were used in lanes E through O. Lane E, strain 101, two plasmid bands (80 and 60 kb) purified from CsCl gradient and used as a control; lane F, also strain 101, prominent 60-kb plasmid; lanes G through K, strains 10, 16, 19, 20, and 22, respectively (80-kb plasmid); lane L, strain 23 (>144-kb open circular form and 105- and 80-kb plasmids); lane M, strain 30 (no plasmid detected in this preparation); lanes N and O, strains 37 and 104, respectively (144-kb open circular form, 80-kb plasmid). PL, plasmid band; CHR, chromosomal band.

RESULTS

Isolation of plasmids from *R. equi* strains. The alkaline-detergent method detected plasmids in 34 of 54 strains, and the SDS method did the same in 36 of 54 strains, with plasmids recognized by the two methods in 40 of 54 strains. The 14 plasmid-negative strains were further examined from lyophilized cultures rather than slant cultures, and 9 were found to contain plasmids when examined by the SDS lysis procedure. The sizes of plasmids detected are summarized in Table 1, and examples are shown in Fig. 1. Of 54 strains, 43 had plasmids of estimated 75 to 85 kb, with the majority being 80 kb. A second band of >144 kb observed with many of the 80-kb plasmids was regarded as an open circular form. Of 22 horse-derived isolates, 21 contained the 75- to 85-kb plasmids, compared with 20 of 28 pig-derived isolates ($P <$ 0.05, chi-square test). No horse-derived isolate had the 105-kb plasmid, compared with 7 of 28 pig-derived isolates ($P <$ 0.05, chi-square test). Only two isolates (one of cat and one of pig origin) contained both the 80- and the 105-kb plasmids. No relationship between plasmid carriage and capsular serotype was apparent.

REA of selected plasmids. Plasmid DNA was purified by CsCl-EtBr density centrifugation of large-scale preparations of strains 10, 99, 101, and 103 isolated from horses. Strain ATCC 6939 failed to yield a plasmid. The sizes of fragments produced by digestion with *Eco*RI of purified plasmid DNA are shown in Table 2. Plasmids were sized as 80.4 kb. Digestion of purified plasmid from strain 10 or 103 with *Bgl*II yielded fragments of mean sizes 36.8, 21.1, 10.2, 5.3, and 4.3 kb, for a mean plasmid size estimate of 77.7 kb. With *Hind*III digestion, mean fragment sizes of plasmid purified from strain 10 or 103 were 40.6, 16.4, 13.7, 6.4, and 5.5 kb, for a mean plasmid size estimate of 82.6 kb. The mean size of plasmids obtained from these strains by REA with all enzymes used was 80.4 kb.

Digestion of plasmids isolated (but not purified) from strain ATCC 33703 and strains 13 and 19, all recovered from pigs, showed restriction fragments in common with *Eco*RI-digested plasmids purified from the four horse-derived isolates (Table 2). Of particular note was the presence of fragments shared by the 105-kb plasmid of strain ATCC

33703 (97.7 kb by *Eco*RI restriction enzyme sizing) and the 80-kb plasmids isolated from horse- and pig-derived strains.

Association of the 80-kb plasmid with production of virulence-associated diffuse 17.5-kDa protein. Different strains produced different quantities of the diffuse 17.5-kDa protein, so that in some cases it was necessary to load large (40-µl) quantities of material and in three cases it was necessary to reculture strains that were classified as weak or questionably positive. Of five strains classified as plasmid negative, three (ATCC 6939 and 33702 and strain 5) did not produce the protein (Fig. 2), whereas two (strains 12 and 15) did. Of 15 strains classified as plasmid positive, 13 produced the protein but two (strains 4 and 20) did not. The association between presence of the 80-kb plasmid and production of the 17.5-kDa protein was significant ($P <$ 0.05, chi-square test).

DISCUSSION

This is the first description of plasmids in *R. equi*, although plasmids have been described for other *Rhodococcus* spp. (7, 8, 23), including the plant pathogen *R. fascians*, in which they may be associated with virulence (12, 14). The two methods used to screen *R. equi* for plasmids were complementary, with the SDS lysis method being marginally more

TABLE 2. Common patterns of *Eco*RI restriction fragments of the 80-kb[a] and 105-kb[b] plasmids isolated from selected *R. equi* strains

Mean fragment size (kb)	Result for:						
	Horse-derived strain				Pig-derived strain		
	10	99	101	103	13	19	33703
27.5	+	+	+	+	+	+	+
21.0	−	−	−	−	+	+	+
16.5	+	+	+	+	+	+	+
14.1	−	−	−	−	−	−	+
10.7	+	+	+	+	−	−	−
8.0	+	+	+	+	−	+	+
7.4	−	−	−	−	−	+	−
7.1	+	+	+	+	−	−	−
5.5	+	+	+	+	+	−	+
5.1	+	+	+	+	+	−	+
Total	80.4	80.4	80.4	80.4	75.6	80.4	97.7

[a] Contained in strains 10, 13, 19, 99, 101, and 103.
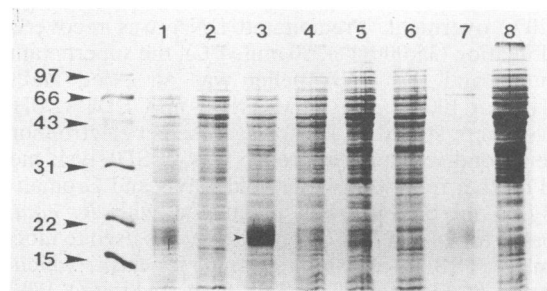[b] Contained in strain ATCC 33703.

FIG. 2. Coomassie blue-stained SDS-PAGE profile of whole-cell proteins from eight *R. equi* strains cultured in nutrient broth. Leftmost lane, molecular weight markers; lanes 1 through 8, strains 2, 4, 3, 8, 12, 20, and 22 and ATCC 6939, respectively. Lanes 1, 3, 5, and 7 show the diffuse 17.5-kDa virulence-associated protein of *R. equi*, readily seen in lane 3 (arrow) but barely visible in lane 5. Lanes 2, 4, 6, and 8 do not show this protein.

sensitive. This method, which omitted the use of penicillin to weaken the cell wall, used larger amounts of bacterial mass than did the alkaline-SDS lysis method. Of 14 strains originally classified as negative by both screening methods, 9 were positive by the SDS lysis method when the strains were obtained from lyophilized cultures. The cultures from slants may have lost a plasmid on subculture, even though strains had not been subcultured more than three times since the original isolation. Alternatively, because of the low copy number of large plasmids, it may be difficult to isolate the 80- and 105-kb plasmids from *R. equi*, so repeated attempts are necessary. Because of a low copy number, it may be that some strains classified as plasmid negative by the isolation techniques employed here contain plasmids. However, the avirulent type strain, ATCC 6939, is unlikely to contain plasmids, since attempts to isolate plasmids from large quantities of the organism were unsuccessful.

Most strains examined carried a plasmid sized at 80 kb by gel electrophoresis with CCC plasmid DNA markers. The few plasmids sized as 75 or 85 kb rather than as 80 kb may have represented 80-kb plasmids in which minor differences in purity resulted in minor differences in apparent size. This question could be resolved by sizing of fragments following restriction enzyme digestion. The mean plasmid size by *Eco*RI digestion was 80.4 kb for five of six plasmids sized as 80 kb by CCC DNA markers. By restriction enzyme fragment sizing, plasmids from strain 13 (75.6 kb) and strain ATCC 33703 (97.7 kb) were determined to be smaller than they were by CCC DNA sizing (80 and 105 kb, respectively) and might therefore possess unrecognized comigrating or small fragments that have run from the gel. Their sizes need to be confirmed by further REA.

Strains tended to possess either the 80- or the 105-kb plasmid, and only two strains had both. The demonstration by REA that these two plasmids were related (Table 2) indicates that these plasmids contain common genes. The significance of the isolation of the 105-kb plasmid only from strains recovered from species other than horses is unclear. Similarly, the differences in restriction patterns between plasmids isolated from horses and pigs (Table 2) is of interest, but the sample size was too small to make conclusions about this. The remarkably close relationship in restriction patterns of the 80-kb plasmids isolated from four unrelated horse strains of three different serotypes suggests that highly conserved regions of plasmid DNA exist in clinical isolates from horses. This needs to be further investigated by DNA hybridization techniques.

An interesting observation, which deserves further study, was the relationship of the possession of the 80-kb plasmid to virulence for mice or horses. Three strains containing the 80-kb plasmid (ATCC 33701, 33704, and 33705) had been shown earlier by Takai and coworkers to be virulent for mice (26), whereas three strains not possessing this plasmid (ATCC 6939, 33702, and 33703) were shown to be not virulent for mice (26). Strain ATCC 6939 is avirulent for horses (13). Strain ATCC 33703 nevertheless had the 105-kb plasmid. All horse isolates other than ATCC 6939 possessed the 80-kb plasmid. This association of plasmid presence with virulence for mice or horses was based on a small sample size and on work done in another laboratory which, since plasmids appeared to be unstable on storage in slants, may be questionable. We examined a possible relationship with virulence, using the diffuse 17.5-kDa protein as an indirect measure. There was a statistically significant, but not perfect, association between the production of this protein and the presence or absence of the 80-kb plasmid. More sensitive

methods for detection of both the plasmid (large-scale preparations and DNA hybridization) and the protein (immunoblotting) may show that strains classified as negative by the isolation techniques used here might be positive and may show better correlation between these two properties. Therefore, before conclusions can be made about the role of the 80-kb plasmid, further study, such as the examination of isogeneic isolates with and without the plasmid for production of the 17.5-kDa protein and for virulence, is needed.

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