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Virulence-Associated 15- to 17-Kilodalton Antigens in *Rhodococcus equi*: Temperature-Dependent Expression and Location of the Antigens

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Virulent *Rhodococcus equi* showing 15- to 17-kDa antigens, which is virulent in mice, was found to harbor an 85-kbp plasmid, and the 15- to 17-kDa antigens were found to be associated with possession of the 85-kbp plasmid of *R. equi* (S. Takai, T. Sekizaki, T. Ozawa, T. Sugawara, Y. Watanabe, and S. Tsubaki, Infect. Immun. 59:4056–4060, 1991). The expression of these antigens was temperature regulated: when cells were grown at a low temperature (25 to 32°C), they did not express them, whereas they expressed them in large amounts when the cells were grown at a higher temperature (34 to 41°C). The antigens were expressed on the cell surface, as evidenced by their susceptibility to proteolysis by a trypsin and by the biotin-avidin protein-blotting technique.

Rhodococcus equi is one of the most important bacterial pathogens in 1- to 3-month-old foals, and the infection is characterized by chronic, suppurative bronchopneumonia and enteritis associated with a high mortality rate (1, 13). *R. equi* is an inhabitant of both soil and the equine intestinal tract (1, 14, 17). Variation in the virulence of *R. equi* isolates has been identified with experimentally infected mice and foals (11, 13, 16), but little is known of the markers and factors associated with the virulence of *R. equi*.

Recently, we have shown that 15- to 17-kDa antigens of *R. equi* are associated with virulence for mice and that these antigens are present in epidemiologically important strains, making them potentially useful as virulence markers in an antigen detection assay (15). Furthermore, it has been shown that virulent *R. equi* contains a large plasmid of approximately 85 kbp and that curing of the plasmid coincides with a loss of detectable 15- to 17-kDa antigens and a dramatic decrease in lethality in mice (18). More recently, Tkachuk-Saad and Prescott (19) have isolated and partially characterized *R. equi* plasmids. They isolated six plasmids that were different in size. Comparison between the plasmid contents and the production of a 17.5-kDa antigen showed a significant but not perfect association between the presence of an 80-kbp plasmid and the production of virulence antigens (19). However, little is known about the regulation of expression, the cellular location, and the function of the 15- to 17-kDa antigens expressed in virulent *R. equi*. The results of our study indicate that expression of the 15- to 17-kDa antigens is temperature regulated and that these antigens are expressed on the cell surface.

R. equi ATCC 33701, L1, and their plasmid-cured derivatives ATCC 33701P$^-$ and L1P$^-$ (which were renamed ATCC 33701 17kDa$^-$ and L1 17kDa$^-$, respectively) were used (18). Both strains have been reported to be virulent (18). The strains are usually grown in brain heart infusion broth (Difco Laboratories, Detroit, Mich.). The cultures were incubated in a rotary shaker at 100 rpm at various temperatures as described in Results. Stock cultures grown at 30°C were maintained as suspensions of cells in 20% glycerol at −80°C.

Whole-cell antigens, which were prepared by harvesting bacteria grown at 38°C for 48 h from the broth and solubilized in sodium dodecyl sulfate (SDS) reducing buffer, were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) (8), electrotransfer of proteins to nitrocellulose sheets, and Western immunoblot, as described previously (15, 18). Serum from a foal naturally infected with *R. equi* was used for the immunoblotting procedures (15).

R. equi ATCC 33701 and L1 were grown at 38°C, washed in phosphate-buffered saline (PBS, pH 7.2), and suspended in PBS to 10^8 cells per ml. The suspension was incubated with 2.5 mg of trypsin (Sigma Chemical Co., St. Louis, Mo.) per ml at 37°C for 30 min. Bovine serum albumin at 0.1% was added to the incubation mixture to quench the reaction, and the culture was centrifuged and washed three times with PBS. The pellet was suspended in sample buffer for SDS-PAGE. The influence of the trypsin on the viability of the cell was determined by comparison with nontrypsinized controls. Survival of the cells was not affected by trypsin treatment, as determined by plating out cells at a range of appropriate dilutions.

R. equi ATCC 33701 and L1 were grown at 38°C, washed in PBS, and suspended in 0.1 M carbonate (pH 8.2) to an optical density at 550 nm of 1.0. A sample (12.5 μl) of biotin-*n*-hydroxysuccinimide (10 mg/ml in dimethyl sulfoxide; Sigma) was added to 1 ml of the bacterial suspension and incubated for 2 h at ambient temperature. The *R. equi* cells were then washed three times with PBS. The biotin-labeled cells were suspended in 40 μl of SDS reducing buffer and subjected to SDS-PAGE and electroblotting. Blotted nitrocellulose sheets were blocked in 2% bovine serum albumin–0.1% Triton X-100 for 30 min at 37°C. The sheets were incubated for 30 min at 37°C in avidin-horseradish peroxidase conjugate (Sigma) at a concentration of 3.5 μg/ml in PBS. The sheets were then washed three times with 0.1% bovine serum albumin and 0.05% Tween 20 in PBS for 5 min per wash at 37°C. Bound avidin-peroxidase was detected as

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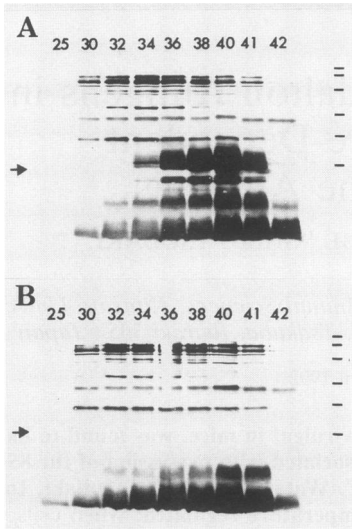

FIG. 1. Immunoblot profiles of *R. equi* ATCC 33701 (A) and ATCC 33701P$^-$ (B) incubated at different growth temperatures. Whole-cell preparations were analyzed by immunoblotting with serum from a naturally infected foal. The growth temperature (°C) is indicated above each lane. The molecular weight markers (M_r) were phosphorylase b (106,000), bovine serum albumin (80,000), ovalbumin (49,500), carbonic anhydrase (32,500), soybean trypsin inhibitor (27,500), and lysozyme (18,500) and are indicated by bars on the right. The arrow on the left indicates the band for the 15- to 17-kDa antigens.

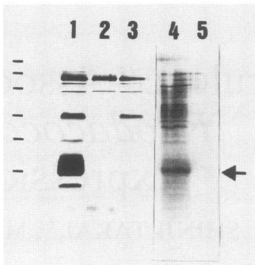

FIG. 2. Effect of trypsin treatment on expression of the 15- to 17-kDa antigens by *R. equi* ATCC 33701 and by biotin-labeled cells. Strain ATCC 33701 grown at 38°C was incubated at 37°C with trypsin (2.5 mg/ml). Biotinylation of strain ATCC 33701 was done as described in Materials and Methods. Whole-cell preparations were analyzed by immunoblotting with serum from a naturally infected foal (lanes 1 to 3) and with an avidin-peroxidase conjugate (lanes 4 and 5). Lanes: 1, ATCC 33701; 2, ATCC 33701 treated with trypsin; 3, ATCC 33701P$^-$; 4, biotin-labeled ATCC 33701; 5, biotin-labeled ATCC 33701 treated with trypsin. Molecular weight markers are indicated by bars on the left (see Fig. 1). Arrow, 15- to 17-kDa antigens.

follows. For each sheet, 5 ml of a freshly prepared solution containing 2.5 mg of diaminobenzidine tetrahydrochloride (Bio-Rad Laboratories, Richmond, Calif.), 10 mM Tris-hydrochloride (pH 7.5), and 0.84 mM cobalt chloride was incubated for 10 min on ice in the dark. Hydrogen peroxide, 7.5 μl of a 30% solution, was added and mixed, and the solution was immediately applied to the blot in a flat-bottomed tray. The developed sheet was rinsed well in distilled water and dried.

The effect of growth temperature on the expression of the 15- to 17-kDa antigens was analyzed by Western blots. Immunoblot profiles were examined with whole-cell extracts of strains ATCC 33701, L1, and their plasmid-cured derivatives grown at 25, 30, 32, 34, 36, 38, 40, 41, and 42°C. As shown in Fig. 1A and B, the 15- to 17-kDa antigens were detected by an infected foal serum in virulent strain ATCC 33701 grown at above 34°C but not in the parent strain grown at below 32°C or in the avirulent derivative, ATCC 33701P$^-$, cultivated at any temperature. In virulent strain L1 (data not shown), the expression of the antigens increased with increasing growth temperature, i.e., antigens in cells grown at 34°C displayed a weaker reaction, and those in cells grown at 40°C displayed the most intense reaction, in the same manner as strain ATCC 33701. In avirulent L1P$^-$ grown at from 25 to 42°C, the 15- to 17-kDa antigens were not detected. These results indicate that expression of the plasmid-mediated 15- to 17-kDa antigens was temperature regulated.

If indeed the 15- to 17-kDa antigens are exposed on the bacterial surface, they should be susceptible to digestion by exogenously added protease. Strains ATCC 33701 and L1 were cultured at 38°C. After being washed, the sedimented cells were suspended in PBS containing 2.5 mg of trypsin per ml and incubated for 30 min at 37°C. At the end of the incubation period, the samples were washed three times with PBS and subjected to SDS-PAGE, followed by immunoblotting. Figure 2 represents the results of a typical experiment with strain ATCC 33701. Figure 2, lanes 1 and 2, indicates that the 15- to 17-kDa antigens were susceptible to proteolytic digestion by the trypsin.

Biotinylation of intact cells of strains ATCC 33701 and L1 grown at 38°C, followed by SDS-PAGE and blotting of the antigens, showed that the 15- to 17-kDa antigens were accessible for labeling by the biotin labeling procedure (Fig. 2, lane 4). Biotinylated bacteria were also examined for the susceptibility of their antigens to proteolytic digestion (Fig. 2, lane 5). The results indicate that the 15- to 17-kDa antigens of *R. equi* were indeed surface localized as well as externally exposed on the cell surface.

Previous studies have shown that *R. equi* strains that demonstrate the 15- to 17-kDa antigens in immunoblots are virulent in mice (15) and that the 85-kbp plasmid present in virulent *R. equi* is associated with the 15- to 17-kDa antigens (18). However, little is known about the properties and functions of the 15- to 17-kDa antigens of *R. equi*. The results of the present study show that expression of the 15- to 17-kDa antigens is temperature regulated and that the antigens are located on the externally exposed surface of the cell.

It is well known that many pathogens regulate the expression of virulence factors in a coordinated manner as a response to changes in their environment (3, 9). These changes are seen when the pathogen first enters the host from an outside environment (3, 9). Growth temperature, one of the environmental changes, appears to influence several virulence functions of pathogenic bacteria, e.g., production of K1 capsular antigen (2) and adherence factors mediating colonization by enteropathogenic *Escherichia coli* (4, 5) or *Salmonella typhimurium* (7), as well as expression of invasive virulence by pathogenic *Shigella* (10) and *Yersinia* (9, 22) spp. *R. equi* grows well at temperatures ranging from 10 to 40°C (1). The 15- to 17-kDa antigens are expressed during growth at 34 to 41°C but not below 32°C. From a consideration of the epidemiology of *R. equi* infections (1, 20), it is assumed that organisms which are inhaled and ingested from contaminated soil are most probably the

progeny of low-temperature-grown cells and hence lack the 15- to 17-kDa antigens (which are not expressed at a low temperature). Since infected foal sera revealed these antigens intensely in Western blots (15), these antigens must have been synthesized during resistance against the invading cells, or their offspring, in the body of their host. Thus, they may well be involved in the infection process (15). However, the function of the 15- to 17-kDa antigens is presently unclear.

The present study demonstrates that these antigens are located, at least in part, on the externally exposed surface of cells. This conclusion is based on the following observations: (i) the antigens associated with intact cells are susceptible to digestion by exogenously added protease; (ii) in the whole-cell biotinylation procedure, labeled 15- to 17-kDa antigens were detected with an avidin-peroxidase conjugate and color indicator. Bacterial proteins that play a role in virulence are often associated with the outer membrane, i.e., capsule, lipopolysaccharides, and cell appendages, including fimbriae (pili) and flagella, play a role in bacterial pathogenicity and in the ability of strains to survive in the environment (6). *R. equi* is a gram-positive, pleomorphic bacillus that bears a polysaccharide capsule. Epidemiological studies have shown that a capsular antigen is unlikely to be associated with virulence (12, 15). *R. equi* is a nonmotile organism without flagella (1). One report noted the presence of scant pili on the surface of the organism (21), but the significance of these structures is unknown. It will be very interesting to identify whether the 15- to 17-kDa antigens of virulent *R. equi* are associated with these structures or not. We are now preparing monoclonal antibodies specific for the antigens and plan to use immunogold electron microscopy to reveal the surface structure of the antigens.

It is likely but not proven that the structural gene of the 15- to 17-kDa antigens is located on the virulence plasmid (18). Thus, it is presently unclear that temperature-regulated and plasmid-associated expression of the 15- to 17-kDa antigens in *R. equi* is controlled at the transcriptional or translational level. To find out more about the function and regulation of expression, we are now in the process of cloning the genes for the antigens from the 85-kbp virulence plasmid of *R. equi*.

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