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Discrimination of Virulent and Avirulent *Streptococcus suis* Capsular Type 2 Isolates from Different Geographical Origins

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In an effort to relate the protein profile to virulence, proteins from the cellular fractions and from culture supernatants of *Streptococcus suis* capsular type 2 strains from different geographical origins were compared by using Western blots (immunoblots). The protein profiles of the cellular fractions were similar for the majority of virulent and avirulent isolates studied, with the exception of three virulent Canadian strains for which a 135-kDa protein was not detected. Examination of the culture supernatants revealed the presence of a 135-kDa protein in all strains except the same three virulent Canadian isolates. In addition, a 110-kDa protein was present in 14 of 16 virulent strains and not in avirulent isolates. When injected into mice, the 110-kDa protein induced an immunoglobulin G response and protected against infection with homologous and heterologous virulent strains. Four strains (1330, 0891, TD10, and R75/S2) that were avirulent in the mouse model of infection and four other strains (1591, 999, JL590, and AAH4) that were virulent in the mouse model were injected into pigs. All virulent strains reproduced the disease, and all avirulent strains failed to reproduce the disease (with the exception of transient lameness in one case and fever in another case). The 110-kDa protein therefore appears to be a reliable virulence marker and a good candidate for a subunit vaccine.

Streptococcus suis capsular type 2 is an important worldwide cause of septicemia and meningitis in swine (1). It can also induce clinical manifestations in humans (2). Little is known about the pathogenesis of the infection. Williams (16) has reported that virulent strains could survive within macrophages. Vecht et al. (15) have described for European isolates a 110-kDa extracellular factor (EF) and a 136-kDa cell wall protein previously known as the muraminidase-released protein (MRP). These proteins were present only in strains virulent for pigs and therefore were reported to be virulence markers. On the basis of the presence of MRP and EF in the culture supernatants of the strains, three phenotypes were described: MRP^+ EF^+, virulent strains; MRP^+ EF^-, strains associated with slight pathological changes; and MRP^- EF^-, avirulent strains (15). The same authors subsequently developed a double-antibody sandwich enzyme-linked immunosorbent assay with monoclonal antibodies directed against those two virulence markers to discriminate virulent and avirulent isolates (14).

Healthy carrier pigs are thought to play an important role in the epidemiology of the *S. suis* capsular type 2 infections (1). Since vaccination often leads to equivocal results, the detection of animals carrying virulent strains could be very helpful in the prevention of the infection (1). Furthermore, the characterization of virulent and avirulent isolates, the identification of virulence determinants, and the development of an experimental model of infection are important steps towards understanding of the pathogenesis of the infection (10). Mouse models have proven to be an important tool in studying *S. suis* capsular type 2 infections, allowing the evaluation of bacterial virulence (3, 8, 17).

In this study, we have compared, using Western blots (immunoblots), the immunogenicities of proteins from the cellular fractions and the culture supernatants of *S. suis* serotype 2 strains from different geographical origins with the aim of relating the protein profile to the virulence of the strains. We identified a virulence marker and evaluated its protective effect by using the experimental mouse model of infection.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Twenty *S. suis* capsular type 2 isolates were used in this study. The serotype 2 reference strain (735), isolated in Denmark, was provided by J. Henrichsen, Statens Seruminstitut, Copenhagen, Den-

TABLE 1. Evaluation of the virulence of *S. suis* capsular type 2 strains from different origins in an experimental murine model of infection

Strain	Origin (country/species)	No. of dead mice[a]	Degree of virulence[b]
1591	Canada/pig	9	HV
999	Canada/pig	9	HV
JL590	Mexico/pig	9	HV
559	Canada/pig	8.5	HV
4/3 H1	Canada/pig	8.5	HV
4/39 H1	Canada/pig	8.5	HV
4/40 H2	Canada/pig	8	HV
735	Denmark/pig	7	HV
AAH4	United States/pig	7	HV
614	United States/pig	6.5	HV
JL819	Mexico/pig	5.5	MV
AR770357	Netherlands/human	5	MV
6891	Canada/pig	4	MV
4223	Canada/pig	3.5	MV
AR770297	Netherlands/human	3.5	MV
0891	Canada/pig	1	AV
TD10	United Kingdom/pig	1	AV
R75/S2	United Kingdom/pig	0	AV
1330	Canada/pig	0	AV

[a] Means of two separate experiments. Ten mice were tested.
[b] HV, highly virulent (seven or more deaths); MV, moderately virulent (three to six deaths); AV, avirulent (fewer than three deaths) (3).

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TABLE 2. Experimental infection of pigs with *S. suis* serotype 2 strains

Strain	Virulence in mice[a]	Pig[b]	Clinical sign(s)[c]	Pathological finding[d]
1591	HV	1	Found dead	Meningitis
		2	A, F, LD, N (Eu[e])	Meningitis
		3	A, F, N	—
999	HV	1	A, F, LD (Eu)	Pericarditis
		2	—	—
		3	A, F, LA, VD (Eu)	Septicemia
AAH4	HV	1	A, F, N	—
		2	A, F, LD (Eu)	Septicemia
		3	A, F, LA, VD (Eu)	Polyarthritis
JL590	HV	1	A, F, LA	—
		2	—	—
		3	A, F, VD (Eu)	Meningitis
1330	NV	1	—	—
		2	LA	—
		3	—	—
TD10	NV	1	—	—
		2	—	—
		3	—	—
0891	NV	1	—	—
		2	A, F	—
		3	—	—
R75/S2	NV	1	—	—
		2	—	—
		3	—	—

[a] See Table 1, footnote *b*.

[b] Three pigs were injected intraveneously with 3×10^8 CFU of each *S. suis* strain (see Materials and Methods). The data represent the first of two separate experiments, which had similar results.

[c] As recorded during the 10-day experiment. A, anorexia; F, fever; LA, lameness; LD, persistent lateral decubitus; N, nervous signs; VD, persistant ventral decubitus; —, no signs.

[d] Main pathological lesions.

[e] Eu, animal showed persistant decubitus for more than 12 h and was euthanized.

mark. Strains R75/S2 and TD10, from the United Kingdom, were provided by T. J. L. Alexander, Department of Clinical Veterinary Medicine, University of Cambridge, Cambridge, United Kingdom. Isolates from the United States, AAH4 and 614, were provided by Brad Fenwick, Kansas State University, Manhattan. Mexican isolates, JL590 and JL819, were provided by Jose Luis Monter Flores, University of Toluca, Toluca, Mexico. Isolates from The Netherlands, AR770297 and AR770357, were provided by J. P. Arends, Groningen, The Netherlands. The other 11 isolates were from the Faculty of Veterinary Medicine, University of Montreal, St-Hyacinthe, Canada. With the exceptions of strains 4/40 H2, 4/3 H1, and 4/39 H1 (from healthy pigs), AR770297 and AR770357 (from human meningitis cases), and 741 (from bovine abortion), all strains originated from diseased pigs.

For each strain, four colonies from a 24-h culture on 5% bovine blood agar plates were inoculated in 200 ml of Todd-Hewitt broth and incubated overnight in an aerobic atmosphere at 37°C. Cells were harvested by centrifugation, washed with a sterile saline solution, and resuspended in 3 ml of K_2HPO_4 (0.1 M, pH 7.0). Culture supernatants were collected and concentrated 100 times by ultrafiltration (type YM 30 filters; Amicon Corp., Danvers, Mass.).

Evaluation of the virulence of *S. suis* isolates. The virulence of the non-Canadian isolates (except strains 735 and AR770297) was estimated by using a mouse model already described (3). The virulence of the Canadian isolates (except strain 0891) had already been evaluated with that model (3). Briefly, strains were growth in Todd-Hewitt broth supplemented with inactivated bovine serum to an optical density of 0.1 (at 540 nm), 1 ml of the suspension was injected intraperitonealy into groups of 10 28-day-old CF1 mice, and mortality was recorded for 1 week. In order to detect any toxic effect of the culture supernatant, groups of 10 mice were also injected with 100-fold-concentrated supernatants of overnight cultures of strains 1591 and 735. To demonstrate the relevance of the model of infection in the natural host, four virulent isolates (1591, 999, AAH4, and JL590) and four avirulent isolates (1330, TD10, R75/S2, and 0891) were injected into groups of three 6-week-old cross-bred pigs by the same protocol but by the intravenous route. Pigs had previously been tested by using the Western blot technique to detect antibodies against *S. suis* and were monitored twice a day for 10 days following the experimental infection.

Production of antisera. Adult New Zealand rabbits were injected once a week for 4 weeks intramuscularly with a formalin-killed culture (0.5% [vol/vol] formalin was added to an overnight culture in Todd-Hewitt broth) of the reference strain. In order to obtain antisera from specific fractions of the cellular protein profile, other rabbits were injected once a week with polyacrylamide gel bands of 128 and 135 kDa from the cellular protein profile (see below) mixed with incomplete Freund's adjuvant. Bands corresponding to the 128-kDa cell fraction were collected from the processed strain 1591 culture, while the cellular fraction bands of 135 kDa were collected from the reference strain. Monoclonal antibodies raised against the 136-kDa MRP and the 110-kDa extracellular protein (15) were kindly provided by U. Vecht (DLO-Central Veterinary Institute, Lelystad, The Netherlands).

Western blots. Cells from the different *S. suis* strains were processed in a French press cell (20,000 lb/in^2, three times), treated with lysozyme (Sigma Chemical Co., St. Louis, Mo.) (5 mg/ml) for 4 h at 37°C, and centrifuged (12,500 $\times$ *g*, 20 min). Supernatants were harvested, and the protein content was evaluated by a Bradford colorimetric assay (Bio-Rad, Hercules, Calif.). This solution, as well as the concentrated culture supernatants, was mixed with an equal volume of solubilization buffer, boiled for 4 min, and processed in 5 and 7.5% polyacrylamide vertical slab gels (with a 4.5% stacking gel) as described previously (9). Following sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), material was transferred from the slab gel to the nitrocellulose membrane by the methanol-Tris-glycine system (12). Electroblotting was done in a Transblot apparatus (Hoefer Scientific Instruments, San Francisco, Calif.) for 18 h at 60 V. Casein (2%, wt/vol) was then used to block unreacted sites, and the nitrocellulose membrane was incubated for 2 h with 1:200 (vol/vol) dilutions of

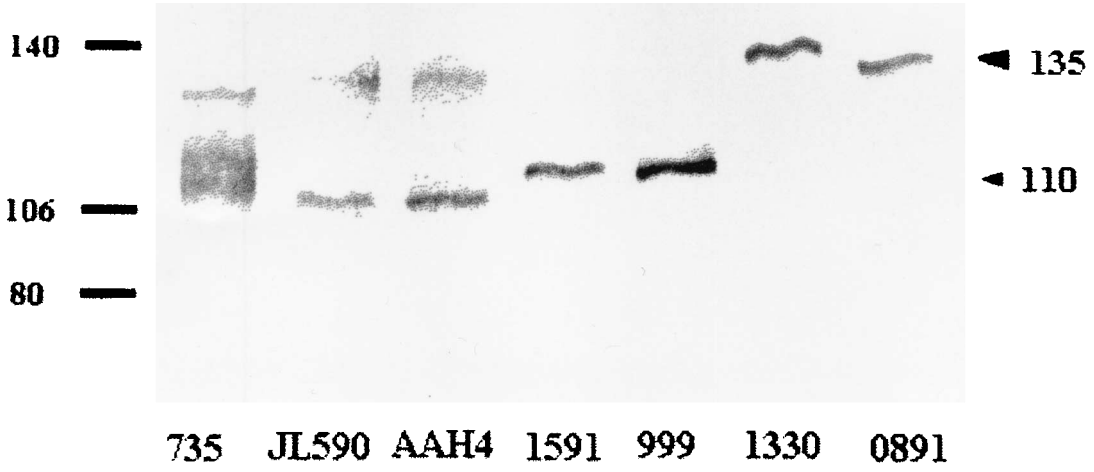

FIG. 1. Western blots of different *S. suis* capsular type 2 strains. SDS-PAGE (5.0%) was performed with culture supernatants. Protein profiles were revealed with rabbit antiserum raised against the reference strain of serotype 2. Left, molecular mass markers (in kilodaltons). Bottom, strain identification numbers. Right, large arrowhead, 135-kDa protein; small arrowhead, 110-kDa protein.

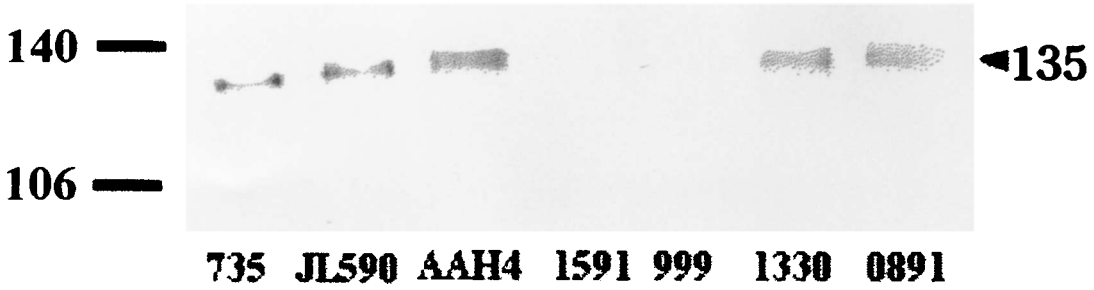

FIG. 2. Western blots of different *S. suis* capsular type 2 strains. SDS-PAGE (5.0%) was performed with culture supernatants. Protein profiles were revealed with rabbit antiserum raised against the 135-kDa protein. Left, molecular mass markers (in kilodaltons). Bottom, strain identification numbers. Arrowhead, 135-kDa protein.

the different antisera. After four washings in Tris-NaCl, sheets were incubated with a peroxidase-labeled immunoglobulin G (IgG) fraction of goat antiserum raised against rabbit IgGs (Bio-Rad) for 60 min at a dilution of 1:5,000 in a 2% casein in Tris-NaCl. After repeated washings, the presence of bound antigens was visualized by reacting the nitrocellulose membrane with 0.06% 4-chloro-1-naphthol (Sigma) in cold methanol mixed with 0.02% H_2O_2 in Tris-HCl. Apparent molecular masses were calculated by comparison with standards of known molecular mass.

Immunization assays with the 110-kDa fraction. The concentrated culture supernatant of strain 1591 was processed on a polyacrylamide gel and stained with Coomassie blue. The 110-kDa band was excised from the gel, mixed with Freund's incomplete adjuvant as previously described (6), and injected into a group of 13 mice once a week for 3 weeks, with each mouse receiving approximately 30 μg of protein at each injection. On day 21, three mice were euthanized, and their blood was collected. The IgG response to the protein was monitored by Western blot, and this specific antiserum was used to detect the 110-kDa protein in the different strains. The other 10 mice were experimentally infected with the reference strain 735 (5 mice) and strain 1591 (5 mice).

RESULTS

By using the mouse experimental model of infection, *S. suis* strains were classified as highly virulent, moderately virulent, or avirulent (Table 1). The three most virulent isolates were strains 1591 and 999 (Canadian isolates) and JL590 (a Mexican isolate), while four other strains were found to be avirulent (strains 0891 and 1330 [Canada] and TD10 and R75/S2 [United Kingdom]). When the four avirulent strains and four virulent strains, as estimated with the mouse model of infection, were injected into pigs, all virulent strains reproduced the disease and all avirulent strains failed to reproduce the disease (with the exception of a transient lameness in one case and anorexia and fever in another one) (Table 2). *S. suis* was recovered from lesions of all but one of the diseased pigs. The

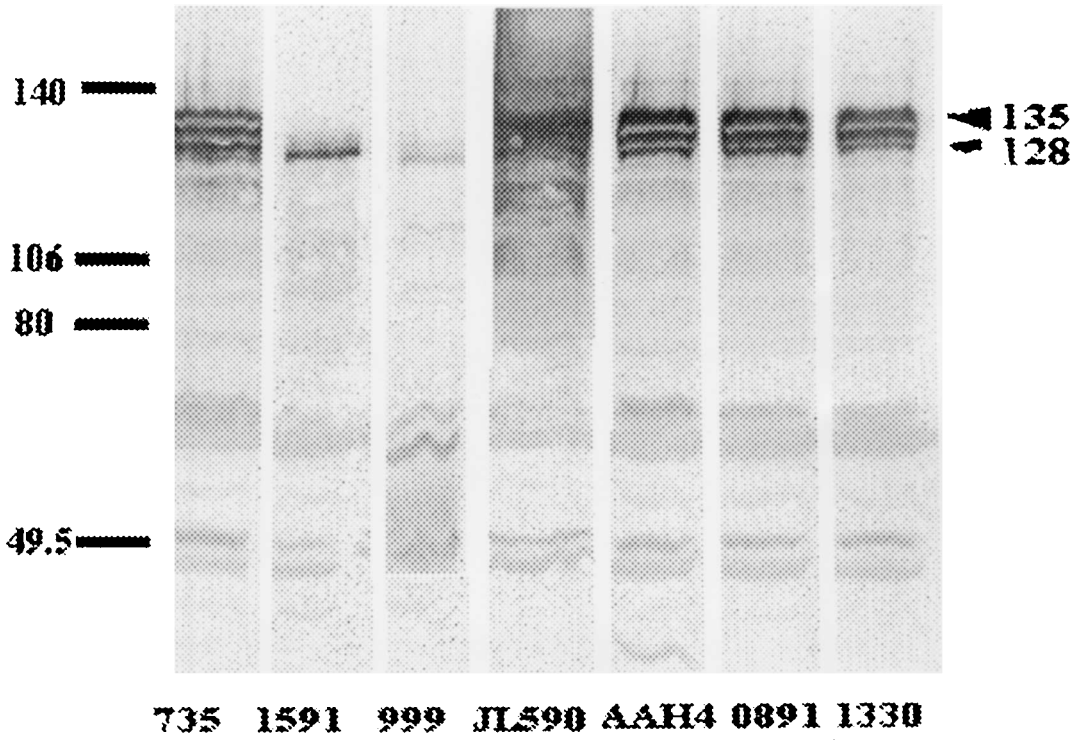

FIG. 3. Western blots of different *S. suis* capsular type 2 strains. SDS-PAGE (7.5%) was performed with cellular fractions. Protein profiles were revealed with rabbit antiserum raised against the reference strain. Left, molecular mass markers (in kilodaltons). Bottom, strain identification numbers. Right, large arrowhead, 135-kDa protein; small arrowhead, 128-kDa protein.

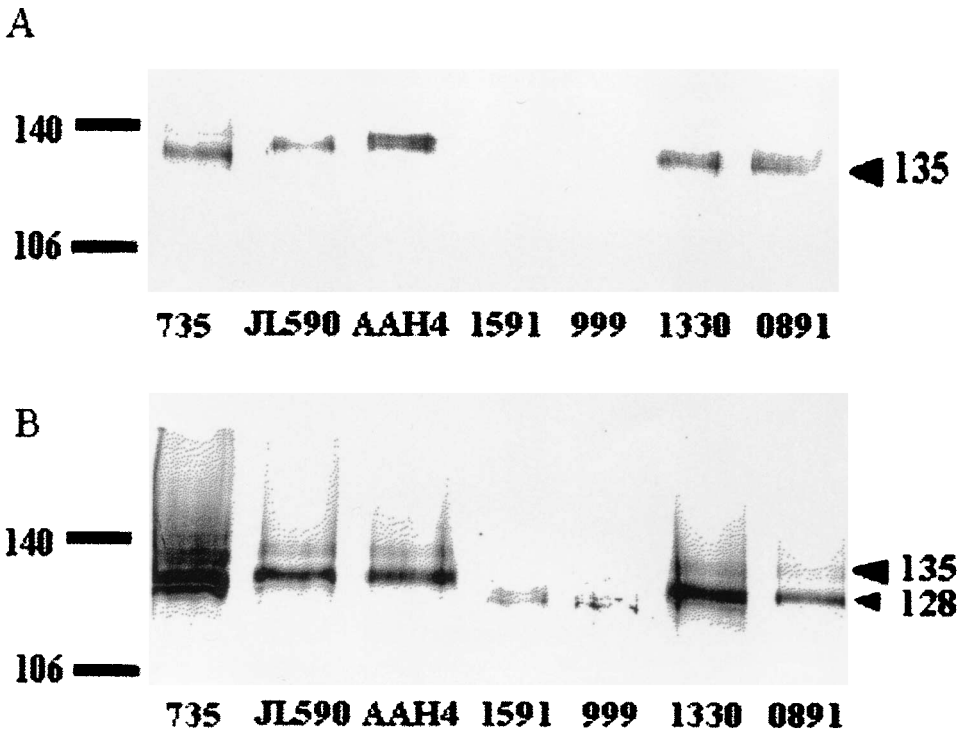

FIG. 4. Western blots of different *S. suis* capsular type 2 strains. SDS-PAGE (7.5%) was performed with cellular fractions. Protein profiles were revealed with rabbit antiserum raised against the 135-kDa protein (A) and the 128-kDa protein (B). Left, molecular mass markers (in kilodaltons). Bottom, strain identification numbers. Right, large arrowheads, 135-kDa protein; small arrowhead, 128-kDa protein.

injection of concentrated supernatants from strains 1591 and 735 failed to induce any clinical signs in mice.

When the protein profiles of the culture supernatants were compared, a protein of about 110 kDa was found to be present in all moderately and highly virulent strains (except the human isolates AR770357 and AR770297, from The Netherlands) and to be absent in all avirulent isolates (Fig. 1). Variation in the molecular mass of this protein was noted. Western blotting performed with antisera of mice immunized against the 110-kDa supernatant protein from strain 1591 recognized the different variants. A protein of about 135 kDa was detected in all virulent and avirulent strains except strains 1591 and 999 (Fig. 1) and 6891. Furthermore, the rabbit antiserum produced against the 135-kDa band did not detect any protein in the culture supernatants of strains 1591 and 999 (Fig. 2) and 6891, while it did in other strains.

When the cellular protein profiles of the different isolates were compared by using Western blots and rabbit antisera, the protein profiles of the highly and moderately virulent strains of different origins were similar, with the exception of three Canadian isolates, strains 1591 and 999 (Fig. 3) and 6891, in which a 135-kDa protein was not detected. The 135-kDa protein was present in all avirulent strains tested. Western blots of the cell protein fraction with antiserum produced against the 135-kDa protein did not detect this protein in the cellular fractions of strains 1591 and 999 (Fig. 4A) and 6891 but detected it in all the other strains tested. When antiserum raised against the 128-kDa fraction was used, Western blots showed that this antiserum could recognize the 128-kDa protein and to a lesser extent the 135-kDa protein in all strains except strains 1591 and 999 (Fig. 4B) and 6891, in which only the 128-kDa protein was detected.

With monoclonal antibodies raised against the 136-kDa MRP, results were similar to those with polyclonal antibodies raised against the 135-kDa protein. We did not detect the 136-kDa protein in the supernatants of strains 1591, 999, and 6891 even when the supernatant was 100-fold concentrated. The monoclonal antibodies raised against the 110-kDa EF did not recognize the 110-kDa protein of the strains used in this study.

Immunization assays with the 110-kDa protein collected from the culture supernatant of strain 1591 showed that this protein induced an IgG response and protected mice against infection with the homologous strain and even with another virulent strain (735) (Table 3).

TABLE 3. Protective effect of a 110-kDa protein of *S. suis* capsular type 2 against experimental infection in mice

Injection[a]	Challenge strain[b]	Presence of 110-kDa band on Western blot (no. positive mice/no. tested)	No. sick/no. tested[c]	No. dead/no. tested
110-kDa protein	1591	5/6	1/5	0/5
	735		0/5	0/5
PBS	1591	0/3	5/5	5/5
	735		5/5	4/5

[a] Mice were injected with the 110-kDa protein or phosphate-buffered saline (PBS), each in Freund's incomplete adjuvant.
[b] 3×10^8 cells.
[c] Means of two separate experiments with similar results.

DISCUSSION

The identification and characterization of virulence determinants can be very important for the understanding of the pathogenesis of an infection. Vecht et al. (15) reported that both a membrane protein of 136 kDa (MRP) with homology to a *Staphylococcus aureus* fibronectin-binding protein (11) and a 110-kDa EF were virulence markers for *S. suis* capsular type 2 isolates. In this study, a 135-kDa protein, or a variant of about this molecular mass, has been found to be present in the majority of virulent isolates but also in avirulent isolates. On the other hand, an EF of about 110 kDa has been found to be present in all virulent strains, with the exception of two human isolates, and absent in the avirulent isolates. However, the 110-kDa protein found in this study was antigenically different from the EF previously reported (15), as shown by the use of monoclonal antibodies. Considerable genetic diversity has been found among isolates of the same *S. suis* serotype (5), and it is thus possible that phenotypic variants of the 110-kDa protein might exist. We are currently investigating the relatedness of these two proteins. Variability in the molecular mass of the 110-kDa protein was found in this study. The use of specific antisera showed that these proteins were related. The fact that strains 1591 and AAH4, with 110-kDa proteins showing variation in molecular mass, both produced disease in pigs tends to show that both forms of the 110-kDa protein found as a virulence marker in this study can be recovered from virulent strains. The possible role of this protein is not yet known, but the fact that mice injected with concentrated supernatant did not show any clinical signs would indicate that it is not by direct activity that this protein would play a role in the infection.

By using a rabbit antiserum raised against the reference strain, the 135-kDa protein was not detected in two highly virulent Canadian strains. However, in those two strains, a 128-kDa protein was present, and the use of antiserum produced against that protein revealed the presence of cross-reactivity with the 135-kDa band. This suggests that these proteins could be antigenically related to one another. However, the 135-kDa fraction was not detected in the cellular fractions or in the culture supernatants of these two highly virulent strains by using a 135-kDa-specific antiserum or an antiserum raised against the whole bacterial cell. The existence of highly virulent strains that do not possess the 135-kDa protein raised some questions about the role of this protein in *S. suis* infections.

Differences in the virulence of *S. suis* strains in the experimental model of infection (3, 8, 17) as well as in the natural host (13) have been noted. The TD10 and R75/S2 isolates were previously shown to possess a low level of virulence (6) and were found to be avirulent in mice in the present study. In reproducing the disease in the natural host, some authors (15) had to use preinfection with *Bordetella* spp. even for strains reported to be virulent. Experiments carried out with pigs in the present study have confirmed the presence of differences in the virulence of strains; they have also demonstrated the value of the mouse experimental model used (3). Finally, in accordance with the results of Iglesias et al. (7), it has been shown that highly virulent isolates could experimentally produce the disease in the natural host without the need for a preinfection.

Successful passive immunizations in mice with rabbit antisera directed against cell wall protein have been reported (4, 6). The active immunization experiment reported here with a 110-kDa culture supernatant protein in mice showed that this protein could induce an IgG response and adequately protect against experimental infection with virulent homologous and heterologous *S. suis* strains. Other experiments with the natural host are needed to confirm the protective potential of this fraction, but the 110-kDa protein might eventually be a good candidate for a subunit vaccine.

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