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Immunoblot Reactivity of Polyclonal and Monoclonal Antibodies with Periplasmic Flagellar Proteins FlaA1 and FlaB of Porcine *Serpulina* Species†

L. N. FISHER,[1]‡ G. E. DUHAMEL,[1]* R. B. WESTERMAN,[2]§ AND M. R. MATHIESEN[1]

Department of Veterinary and Biomedical Sciences, University of Nebraska—Lincoln, Lincoln, Nebraska 68583-0905,[1] *and Department of Veterinary Diagnostic Investigation, College of Veterinary Medicine, Kansas State University, Manhattan, Kansas 66506*[2]

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The periplasmic-flagellum (PF) proteins of Triton X-100-soluble and Triton X-100-insoluble sodium dodecyl sulfate-treated fractions from reference and field strains of *Serpulina hyodysenteriae*, *Serpulina innocens*, and *Serpulina pilosicoli* were characterized by Western blotting with a rabbit polyclonal antibody (PAb) specific for the 44-kDa PF sheath protein of *S. hyodysenteriae* (Z. Li, F. Dumas, D. Dubreuil, and M. Jacques, J. Bacteriol. 175:8000–8007, 1993) and a murine monoclonal antibody (MAb), designated 7G2, specific for the PF core FlaB proteins of *S. hyodysenteriae*. The MAb 7G2 reacted with a conserved epitope present in the 37-, 34-, and 32-kDa PF core FlaB proteins of all *Serpulina* species. This suggested that the core FlaB proteins are conserved among porcine *Serpulina* species. An immunoreactive band of approximately 44 kDa was present with all *S. hyodysenteriae*, *S. innocens*, and *S. pilosicoli* strains that were reacted with the PAb. The specificities of the PAb and the MAb for the FlaA1 and FlaB proteins of *Serpulina* species were confirmed by N-terminal amino acid sequencing of 44- and 37-kDa proteins, respectively, of *S. hyodysenteriae* and *S. pilosicoli*. Results from this study provide further evidence that the 44-kDa protein FlaA1 and the 37-, 34-, and 32-kDa FlaB proteins are conserved among porcine *Serpulina* species.

The genus *Serpulina* of intestinal spirochetes consists of at least three species. The first two species, originally classified as *Treponema*, have been reclassified based on DNA:DNA reassociation and 16S rRNA sequencing as a unique genus, *Serpulina* (3, 32, 34). The first species identified was *Serpulina hyodysenteriae*, the etiologic agent of swine dysentery (11), a diarrheal disease of growing and finishing pigs that has worldwide economic importance (27). The spirochete colonizes the cecum and the colon, resulting in severe mucohemorrhagic typhlocolitis, which can lead to dehydration and death if the pigs are left untreated. The second species of intestinal spirochetes is morphologically similar to *S. hyodysenteriae* but produces a weak beta-hemolysis when cultured anaerobically on agar medium containing blood. Because it is found in the colons of healthy swine, it was given the name *Serpulina innocens* (15). The third species of intestinal spirochetes, which is also weakly hemolytic, was recently described as *Serpulina pilosicoli* (34). Strains of *S. pilosicoli* are associated with colonic spirochetal infections of humans, pigs, and dogs (3, 18, 34).

S. hyodysenteriae has been classified into serogroups based on the reactivity of lipooligosaccharide antigens with antisera raised against whole cells (1, 6–9, 22, 24). At least nine serogroups, designated A through I, with unique serovars within the serogroups, have been proposed (6, 7, 9). Serogroups A through D are represented by *S. hyodysenteriae* strains which have been identified by Baum and Joens (1) as type strains for serotypes 1 through 4. The serotyping system of Baum and Joens (1), which is also based on lipooligosaccharide antigens, was expanded to include serotypes 5 through 9 by cross-adsorption of antisera to differentiate cross-reactive serotypes (22, 24). More recently, Pettersson and coworkers proposed a classification of porcine intestinal spirochetes based on phylogenetic analysis of the 16S rRNA sequence (28). At least four phenotypes within three phylogenetic clusters were identified. One cluster included phenotype I, representing the strongly beta-hemolytic, pathogenic *S. hyodysenteriae*, whereas phenotype II comprised weakly beta-hemolytic variants that were biochemically similar to *S. hyodysenteriae*. A second cluster, represented by phenotype III, was the most variable, with three subgroups, designated IIIa, IIIb, and IIIc, of *S. innocens* strains. The third cluster consisted of phenotype IV and included weakly beta-hemolytic spirochetes similar to strain P43/6/78, the type strain for *S. pilosicoli* (28, 34).

A characteristic morphologic feature of spirochetes is the presence of flagella in the periplasmic space. The periplasmic flagella (PF) are responsible for motility and thus are important virulence attributes of pathogenic spirochetes. The motility of *S. hyodysenteriae* allows penetration of the colonic mucus layer and establishment of the spirochete in the crypts of Lieberkühn and the surface epithelium (13). The PF of *S. hyodysenteriae* are composed of five proteins: two outer-sheath proteins with molecular masses of 44 and 35 kDa and three core proteins with molecular masses of 37, 34, and 32 kDa (14, 16). The genes encoding the 37- and 34-kDa core proteins have been designated *flaB1* and *flaB2* (5, 19). Similarly, the gene encoding the 44-kDa sheath protein, designated FlaA1, has been cloned and sequenced (17). Single FlaA1 and FlaB1 and dual FlaA1 FlaB1 mutants have altered motility compared to

* Corresponding author. Mailing address: Room 147, Veterinary Basic Science Bldg., Department of Veterinary and Biomedical Sciences, University of Nebraska—Lincoln, Lincoln, NE 68583-0905. Phone: (402) 472-3862. Fax: (402) 472-9690. E-mail: Vets041@unlvm.unl.edu.

† Paper 11754, Agricultural Research Division, Institute of Agriculture and Natural Resources, University of Nebraska—Lincoln, Lincoln.

‡ Present address: Pfizer Animal Health, Lincoln, NE 68501.

§ Present address: Route 1, Piqua, KS 66761.

TABLE 1. Strains of *Serpulina* species investigated in this study

Species and strain	Phenotype/serogroup[a]	Origin	Reference(s)	Source[b]
S. hyodysenteriae				
B78[c] (ATCC 27164)	I/A	United States	1, 8, 15	1
B204	I/B	United States	1, 8	1
B169	I/C	Canada	1, 9	1
A1	I/D	United Kingdom	1, 9	1
WA-6	I/E	Australia	9	2
Q16	I/G	Australia	6	2
VIC2	I/H	Australia	6	2
NSW-1	I/I	Australia	6	2
S. innocens				
C301	IIIa/ND	Sweden	28	3
C378	IIIa/ND	Sweden	28	3
C336	IIIb/ND	Sweden	28	3
B256[c] (ATCC 29796)	IIIc/ND	United States	15	4
4/71	ND/ND	United Kingdom	15	5
S. pilosicoli				
P43/6/78[c] (ATCC 49776)	IV/ND	United Kingdom	34	5
UNL-3	ND/ND	United States	26, 29	6
UNL-5	ND/ND	United States	26, 29	6
UNL-8	ND/ND	United States	26	6
D9201243A	ND/ND	United States	2, 26	7
T9300098	ND/ND	United States	2, 26	7
T9301604B	ND/ND	United States	2, 26	7
B359	ND/ND	United States	15, 26	1
B1555a	ND/ND	United States	15, 26	1

[a] Phenotype as reported by Pettersson and coworkers (28) and serogroup as reported by Hampson and coworkers (6–9). ND, not determined.
[b] 1, J. M. Kinyon, College of Veterinary Medicine, Iowa State University, Ames; 2, D. J. Hampson, School of Veterinary Studies, Murdoch University, Murdoch, Western Australia, Australia; 3, C. Fellstrom, Department of Medicine and Surgery, Faculty of Veterinary Medicine, Swedish University of Agricultural Sciences, Uppsala, Sweden; 4, American Type Tissue Culture Collection, Rockville, Md.; 5, T. B. Stanton, National Animal Disease Center, Ames, Iowa; 6, Veterinary Diagnostic Laboratory, University of Nebraska—Lincoln, Lincoln; 7, R. L. Walker, California Veterinary Diagnostic Laboratory System, University of California, Davis.
[c] Type strain for the species.

the wild type and had attenuated virulence in a murine model of swine dysentery (30, 31).

Genetic analyses of the PF genes and proteins of *Serpulina* species have shown that the core proteins are highly conserved among spirochetes, whereas the sheath proteins may be more diverse (5, 18, 23). Using hyperimmune rabbit serum produced against the 44-kDa antigen of *S. hyodysenteriae*, Li and coworkers (23) investigated *S. hyodysenteriae*, *S. innocens*, and some other uncharacterized weakly beta-hemolytic porcine intestinal spirochetes and suggested that the FlaA1 sheath protein was unique to *S. hyodysenteriae*. This result may provide a molecular basis for the development of serologic methods for detection of swine exposed to *S. hyodysenteriae*. However, after the work of Li and coworkers, Koopman and coworkers reported that the DNA of *S. innocens* reacted with a *flaA1* gene probe prepared from *S. hyodysenteriae* (18). These conflicting reports indicate the need for further evaluation of the 44-kDa PF sheath protein, FlaA1, of porcine *Serpulina* species as a potential antigen for detection by serology of swine exposed to *S. hyodysenteriae*. We examined the specificity of the rabbit polyclonal antibody (PAb) to the 44-kDa PF sheath protein, FlaA1, of *S. hyodysenteriae* by using well-characterized reference and field strains of *S. hyodysenteriae*, *S. innocens*, and *S. pilosicoli*. Additionally, a mouse monoclonal antibody (MAb) specific for a conserved epitope of the PF core FlaB proteins of *Serpulina* species was characterized.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains of *Serpulina* species used for characterization of the PAb and the MAb are described in Table 1. The spirochetes were propagated in prereduced anaerobically sterilized Trypticase soy broth as previously described (20). Cultures were grown to late log phase (approximately 10^8 cells per ml) in 5-ml volumes in Hungate tubes and were stirred constantly at 37°C under an atmosphere of 10% hydrogen, 10% carbon dioxide, and 80% nitrogen.

Detergent extractions. Late-log-phase cultures of spirochetes were harvested (1,800 × *g*, 20 min), washed three times in Tris-buffered saline (TBS; 10 mM Tris-HCl, 150 mM NaCl [pH 7.4]), resuspended in 1% Triton X-100 (vol/vol; Pierce, Rockford, Ill.) in TBS, and incubated for 18 to 20 h at 5°C with gentle rocking. After centrifugation (15,000 × *g*, 5 min), the Triton X-100-soluble (TXS) fraction was harvested and the pellet was solubilized in sodium dodecyl sulfate (SDS) buffer (125 mM Tris-HCl, 1% SDS, 15% glycerol, and 5% β-mercaptoethanol; Integrated Separation Systems, Natick, Mass.) and heated at 100°C for 10 min. The TXS and Triton X-100-insoluble SDS-treated (TXI) fractions were kept at −20°C until needed.

Preparation of purified PF. The PF of *S. hyodysenteriae* B204 were isolated by a modification of a previously described method (25). After a washing with distilled water (95,000 × *g*, 60 min), the PF were resuspended in a small volume of distilled water and were stored at −70°C.

Mouse MAb. A mouse MAb was produced by using whole-cell suspensions of killed *S. hyodysenteriae* B234 as previously described (36). Supernatants from hybridomas were screened for antibody production against whole-cell lysates of *S. hyodysenteriae* B234 and *S. innocens* B256 by an enzyme-linked immunosorbent assay (36). One hybridoma secreting monoclonal immunoglobulin G antibodies reacted with *S. hyodysenteriae* B234 and *S. innocens* B256. The MAb, designated 7G2, produced a banding pattern by immunoblot against whole-cell lysate and purified PF (35, 36) that was suggestive of reactivity with the 37-, 34-, and 32-kDa FlaB proteins of *S. hyodysenteriae* B204.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Detergent extracts (2,500 ng) in sample buffer (125 mM Tris-HCl, 1% SDS, 5% β-mercaptoethanol, and 15% glycerol; Integrated Separation Systems) were heated for 5 min at 100°C, loaded in each lane of 10-to-18% gradient polyacrylamide gels (Integrated Separation Systems), and electrophoresed in 25 mM Tris–192 mM glycine–

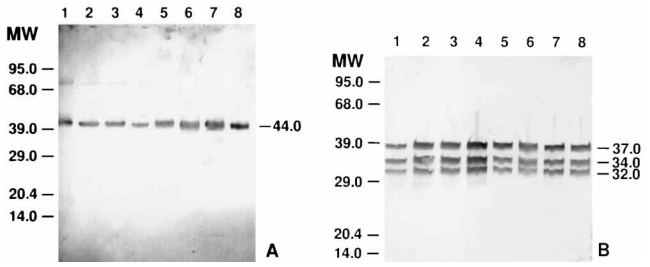

FIG. 1. Results of SDS-PAGE and immunoblotting of *S. hyodysenteriae* strains. A 44-kDa-protein-specific rabbit PAb was reacted with TXI fractions (A), and mouse MAb 7G2 was reacted with TXS fractions (B). Lanes: 1, B78; 2, B204; 3, B169; 4, A1; 5, WA-6; 6, Q16; 7, VIC2; 8, NSW-1. MW, molecular weight (in thousands).

0.1% SDS, pH 8.5 (Tris-Gly-SDS Seprabuf; Integrated Separation Systems), at 30 mA of constant current per gel until the dye marker was within 2 to 3 mm of the bottom of the gel (21). After separation, proteins were electrotransferred to nitrocellulose (0.2-μm pore size; Pharmacia, Uppsala, Sweden) in 25 mM glycine–192 mM Tris-HCl–20% methanol with graphite electrodes (NovaBlot; Pharmacia) at a constant current of 0.8 mA/cm^2 for 1 h (33). Duplicate gels were stained with Coomassie blue R250. The gels were dried between sheets of cellophane (gel drying system; Novex, San Diego, Calif.).

Western blotting (immunoblotting). The immunoblots were completed as previously described (23). Briefly, the membranes were blocked with 2% nonfat dry milk in TBS (MTBS) for 2 h at 4°C. The MTBS was removed, and a 44-kDa-specific rabbit PAb (kindly provided by M. Jacques, Faculté de Médecine Vetérinaire, Université de Montréal, Québec, Canada) (23) or MAb 7G2 in MTBS was added. The PAb was incubated for 16 to 18 h at 4°C with rocking. The MAb was incubated for 2 h at room temperature with rocking. Following incubation, the membranes were washed four times with TBS, and alkaline-phosphatase-conjugated goat anti-rabbit or goat anti-mouse antiserum (Kirkegaard and Perry, Gaithersburg, Md.) in MTBS was added to the membranes and incubated for 2 h at room temperature with rocking. The membranes were washed four times with TBS, and the bands were visualized with 5-bromo-4-chloro-3-indolyl-phosphate (BCIP)–nitroblue tetrazolium substrate (Kirkegaard and Perry). The dried gels and membranes were analyzed by laser densitometry (BioImage scanner; Waters, Ann Arbor, Mich.).

N-terminal amino acid sequencing. The TXI fractions from *S. hyodysenteriae* B78 and *S. pilosicoli* P43/6/78 were separated by SDS-PAGE with 10% Sepragels (Integrated Separations Systems) and immobilized on polyvinylidene difluoride (Problott; Applied Biosystems, Foster City, Calif.). After Coomassie blue R250 staining, visible bands at 37 and 44 kDa were excised with sterile razor blades. The N-terminal amino acid sequences were determined with a model 477A Applied Biosystems protein sequencer by the Protein Structure Core Facility at the University of Nebraska Medical Center, Omaha.

RESULTS

Immunoblotting of *S. hyodysenteriae*. Immunoblotting of the purified PF from strain B204 with the PAb revealed a single band of approximately 44 kDa (data not shown). Immunoblotting of the TXI fractions of reference strains of *S. hyodysenteriae* serogroups with the PAb revealed a band of approximately 44 kDa for all the strains (Fig. 1A). The 44-kDa immunoreactive band was present in the TXS fractions of *S. hyodysenteriae* B78, B204, B169, and A1 but not in the Australian strains, WA-6, Q16, VIC2, and NSW-1 (data not shown). A weakly immunoreactive band of unknown significance also was present at approximately 75 kDa in the TXI fractions of strains B78, B204, and B169 (Fig. 1A). Immunoblotting of purified PF from strain B204 and of TXS extracts from reference strains of *S. hyodysenteriae* serogroups with MAb 7G2 revealed three bands corresponding to the core flagellar proteins FlaB1 (37 kDa) and FlaB2 (34 kDa) and the 32-kDa FlaB protein (Fig. 1B).

Immunoblotting of *S. innocens*. Immunoblotting of TXI fractions of *S. innocens* strains with the PAb revealed an immunoreactive band of approximately 44 kDa in all the strains (Fig. 2A). While strains B256 and C301 appeared to have a slightly slower-migrating band, strain C378 had a faster-migrating band, and strains 4/71 and C336 had a mixture of both. Additionally, a weakly immunoreactive band of unknown significance was present at approximately 75 kDa in the TXI fractions of strains B256, 4/71, C336, and C301. Immunoblotting of the TXS extracts from the reference *S. innocens* strains with the MAb 7G2 revealed three bands corresponding to the core flagellar proteins FlaB1 (37 kDa) and FlaB2 (34 kDa) and the 32-kDa FlaB protein (Fig. 2B) seen in the reference *S. hyodysenteriae* strains and in the purified PF from *S. hyodysenteriae* B204.

Immunoblotting of *S. pilosicoli*. Although a constant protein content was loaded in each lane, the relative immunoreactivity of the 44- and 75-kDa bands varied among the TXI fractions of *S. pilosicoli* strains reacted with the PAb (Fig. 3A). While most strains appeared to have a faster-migrating 44-kDa band, strains P43/6/78, UNL-8, and B1555a had a slower-migrating 44-kDa band. Immunoblotting of TXS extracts from the reference strains and from field isolates of *S. pilosicoli* with MAb 7G2 revealed three bands corresponding to the core flagellar proteins FlaB1 (37 kDa) and FlaB2 (34 kDa) and the 32-kDa FlaB protein (Fig. 3B) seen in the reference *S. hyodysenteriae* strain B78 and in *S. innocens* B256.

N-terminal amino acid sequencing. To confirm the identity of the immunoreactive bands, the N-terminal amino acid sequences of the 37- and 44-kDa bands from the TXI fractions of *S. hyodysenteriae* B78, reacted with MAb 7G2 and the PAb, respectively, and the 44-kDa band from the TXI fraction of *S. pilosicoli* P43/6/78, reacted with the PAb, were sequenced through the first 10 amino acid residues. The resulting amino acid sequences were compared with available sequences for the FlaA1 and the FlaB1 proteins of *S. hyodysenteriae* C5 (Table 2) (16). The 44-kDa protein of *S. hyodysenteriae* B78 had 9 of 10 amino acid residues identical to those of the FlaA1 protein of *S. hyodysenteriae* C5 (90% identity) (16). The 44-kDa protein of *S. pilosicoli* P43/6/78 had 8 of 10 residues identical to those of the FlaA1 protein of *S. hyodysenteriae* C5 (80% identity). This confirmed that the PAb was specific for the PF sheath protein FlaA1 and that the FlaA1 protein was conserved among porcine *Serpulina* species. The 37-kDa protein from *S. hyodysenteriae* B78 had 10 of 10 amino acid residues identical to those of the PF core protein FlaB1 of *S. hyodysenteriae* C5 (16). This confirmed that MAb 7G2 reacted with the core FlaB proteins.

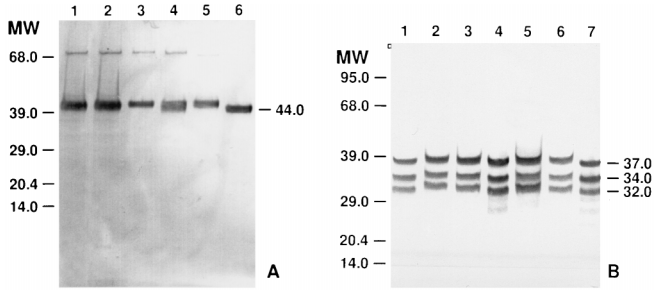

FIG. 2. Results of SDS-PAGE and immunoblotting of *Serpulina* species. A 44-kDa-protein-specific rabbit PAb was reacted with TXI fractions (A) and mouse MAb 7G2 was reacted with TXS fractions (B) of *S. hyodysenteriae* B78 (lane 1), *S. innocens* 4/71 (lane 2), B256 (lane 3), C336 (lane 4), C301 (lane 5), and C378 (lane 6), and purified PF of *S. hyodysenteriae* B204 (lane 7). MW, molecular weight (in thousands).

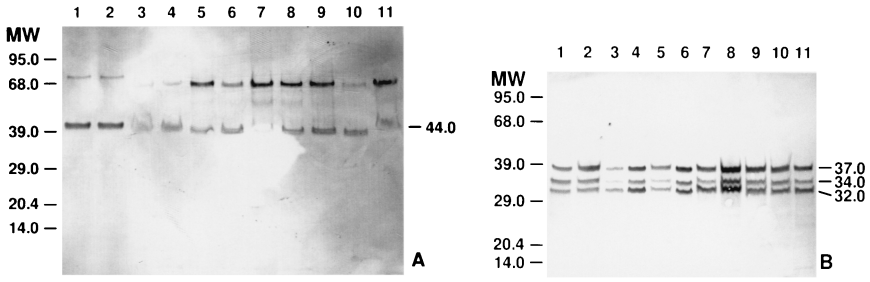

FIG. 3. Results of SDS-PAGE and immunoblotting of *Serpulina* species. A 44-kDa-protein-specific rabbit PAb was reacted with TXI fractions (A) and mouse MAb 7G2 was reacted with TXS fractions (B) of *S. hyodysenteriae* B78 (lane 1), *S. innocens* B256 (lane 2), and *S. pilosicoli* P43/6/78 (lane 3), UNL-8 (lane 4), UNL-5 (lane 5), T9300098 (lane 6), UNL-3 (lane 7), T9301604B (lane 8), D9201243A (lane 9), B359 (lane 10), and B1555a (lane 11). MW, molecular weight (in thousands).

DISCUSSION

The banding patterns of the immunoreactive bands from the TXS fractions of porcine *Serpulina* species which were immunoblotted with the MAb 7G2 corresponded to those of the PF core proteins, the FlaB proteins, of *S. hyodysenteriae*. Comparisons of the N-terminal amino acids of the PF core proteins FlaB1 and FlaB2 of *S. hyodysenteriae* with those of the PF core proteins of *Treponema pallidum*, *Treponema phagedenis*, *Spirochaeta aurantia*, *Borrelia burgdorferi*, and *Leptospira borgpetersenii* have shown a high degree of sequence homology (16). The immunoblot reactivity of MAb 7G2 provided further evidence from strains obtained from pigs in North America, Europe, and Australia for the universal conservation of the PF core proteins among porcine intestinal spirochetes belonging to *Serpulina* species.

The reactivity of the 44-kDa-protein-specific rabbit PAb with *S. hyodysenteriae*, *S. innocens* B256, and three uncharacterized weakly beta-hemolytic porcine intestinal spirochetes suggested that the PF sheath protein FlaA1 was unique to *S. hyodysenteriae* (23). This was consistent with a report indicating that the FlaA1 protein has the highest degree of variation among PF proteins, suggesting less functional constraint for the sheath protein (16). In this study, we examined reference strains for the *S. hyodysenteriae* serogroups proposed by Hampson, with the exception of strain VIC1 of serogroup F, which was not available. The reactivity of the PAb with the TXS fractions of *S. hyodysenteriae* B78, B204, B169, and A1 was consistent with previous observations made by Li and coworkers (23) using the same antiserum reacted with Triton X-114 aqueous and detergent-soluble extracts. However, when the Australian *S. hyodysenteriae* strains WA-6, Q16, VIC2, and NSW-1 were immunoblotted with the PAb, a band of approximately 44 kDa, presumably the FlaA1 protein, was present in the TXI fraction but not in the TXS fraction, as was seen in the reference *S. hyodysenteriae* serotypes 1 through 4. Initial experiments suggested that the Triton X-100 detergent provided a more consistent solubilization of the PF proteins, but further evaluation with the TXI fractions indicated variations in the solubilities of the FlaA1 proteins among porcine *Serpulina* species. Efforts to solubilize these proteins with Triton X-114 resulted in even more variable separation of the FlaA1 between the detergent and the aqueous phase (data not shown). The differences in solubility of the FlaA1 protein in Triton X-100 and Triton X-114 and the minor variations in the mobility of the 44-kDa immunoreactive band among *Serpulina* strains remain unexplained at this time. Nevertheless, we found that the reactivity of the PAb with the TXI fractions of *S. innocens* strains was similar to that seen with *S. hyodysenteriae* strains. This discrepancy with the data of Li and coworkers (23) may be attributable to variations in the solubility of the PF sheath protein FlaA1 among the porcine *Serpulina* species with Triton X-114 and Triton X-100 with or without SDS treatment.

The TXI fractions of some strains of *S. hyodysenteriae* and *S. innocens* that were reacted with the PAb had weakly immunoreactive bands of approximately 75 kDa, while the TXI fractions of five of the nine *S. pilosicoli* strains had 75-kDa immunoreactive bands that were stronger than the 44-kDa bands. Because the 75-kDa immunoreactive band was absent from purified PF but present in the TXI fraction of *S. hyodysenteriae* B204, we concluded that the origin of the 75-kDa antigen in detergent extracts was uncertain.

Currently, swine dysentery is diagnosed on the basis of results from bacteriologic culture of feces or mucosal scrapings or based on demonstration of *S. hyodysenteriae*-specific products after amplification of DNA sequences by PCR (4, 10). The PF of *B. burgdorferi* have been used as antigens for serologic diagnosis of Lyme disease in human beings; patients develop specific reactivity to the PF proteins early in the course of the infection (12, 37). The evaluation of additional strains of *Serpulina* species with a 44-kDa-protein-specific PAb provided evidence that this reagent reacted with the PF sheath protein FlaA1 and that, although it was more variable than the other PF proteins, FlaA1 was not unique to *S. hyodysenteriae* and was present in all porcine *Serpulina* species. The immunoblot reactivity of MAb 7G2 indicated that it was specific for the PF core FlaB proteins and further confirmed that these proteins were conserved among porcine *Serpulina* species. MAb 7G2 may be useful for the detection of spirochetes and in studies aimed at determining the role of FlaB in the pathogenesis of enteric diseases caused by porcine intestinal spirochetes.

TABLE 2. Comparison of the amino-terminal sequences of porcine *Serpulina* species

Sample	Sequence	Reference
S. hyodysenteriae C5, FlaA1	LSNSTLIDFA	16
S. hyodysenteriae B78, 44-kDa band	LXNSTLIDFA	This report
S. pilosicoli P43/6/78, 44-kDa band	XXNSTLIDFA	This report
S. hyodysenteriae C5, FlaB1	MVINNNISAI	16
S. hyodysenteriae B78, 37-kDa band	MVINNNISAI	This report

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the rabbit PAb specific for the 44-kDa PF sheath protein of *S. hyodysenteriae*.

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