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Actinobacillus suis Strains Isolated from Healthy and Diseased Swine Are Clonal and Carry *apxICABD*$_{\text{var. suis}}$ and *apxIICA*$_{\text{var. suis}}$ Toxin Genes

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***Actinobacillus suis* isolates recovered from both healthy and diseased pigs were characterized by biochemical testing, serotyping, restriction endonuclease fingerprinting, and *apx* toxin gene typing. The clinical isolates analyzed were collected over a 10-year period from approximately 40 different locations in southwestern Ontario, Canada. Little variation in the biochemical profiles of these isolates was seen, and all isolates reacted strongly with rabbit antisera prepared against one of the strains. Similarly, by using *Bam*HI and *Bgl*II for restriction endonuclease fingerprinting (REF) analysis, all isolates were found to belong to a single REF group. Minor variations could be detected, especially in the *Bgl*II fingerprints, but overall the patterns were remarkably similar. Sequences that could be amplified by PCR with primers to the *apxICA* and *apxIICA* genes of *Actinobacillus pleuropneumoniae* were detected in all strains. Although no amplification was obtained with primers to the *A. pleuropneumoniae apxIBD* genes, sequences with homology to *apxIBD* were detected by hybridization. There was no evidence of *apxIII* homologs. Taken together, these data suggest that *A. suis* isolates are genotypically and phenotypically very similar, regardless of their source, and that they contain genes similar to, but not identical to, the *apxICABD* and *apxIICA* genes of *A. pleuropneumoniae*.**

The gram-negative bacterium *Actinobacillus suis* is a common commensal organism in conventionally reared swine and can be an opportunistic pathogen, most frequently in high-health-status herds (20, 21, 26, 34, 36, 37). *A. suis* infection can take place via the aerosol route, close contact, or breaks in the skin (20, 36). In very young animals, *A. suis* can cause an acute and rapidly fatal septicemia. These animals may show signs of cyanosis, respiratory distress, neurological disturbances, or arthritis (34, 36). In older animals, the disease is typically less severe and is characterized by fever, anorexia, and cough (34, 36). *A. suis* infection has also been associated with erysipelas-like lesions, abortion, metritis, and meningitis in mature animals (34, 36, 37). Most *A. suis* isolates are sensitive to a wide range of antibiotics including ampicillin, oxytetracycline, and streptomycin, but the rapid onset can make treatment difficult (23, 34). Autogenous bacterins have been used in herds with recurrent *A. suis* problems, but their efficacy has not been critically evaluated (34).

In the diagnostic laboratory, *A. suis* isolates may be identified on the basis of their hemolytic phenotype and their ability to grow on MacConkey agar; hydrolyze esculin; produce catalase, oxidase, and urease; and produce acid from arabinose, cellobiose, dextrose, lactose, melibiose, salicin, sucrose, and trehalose but not from mannitol or sorbitol (4, 9, 23, 27, 32). To date, no attempt has been made to serotype *A. suis*, and it is known that *A. suis* possesses some cross-reactive antigens including enterobacterial common antigen (6), outer membrane proteins (19), somatic antigens (29, 31), and exotoxins (7, 8, 16).

Little is known about the pathogenesis of *A. suis*, although it is thought to share many of the virulence factors of *Actinobacillus pleuropneumoniae* and other gram-negative organisms such as capsule (slime) and lipopolysaccharide (23, 36). There are several lines of evidence that *A. suis* also carries RTX (repeats in toxin) toxins that are related to the Apx toxins of *A. pleuropneumoniae* (7, 8, 16). RTX toxins are pore-forming protein exotoxins with hemolytic and/or cytotoxic activity, so-named because of the presence of characteristic glycine-rich repeats (25, 39). Most RTX operons have an A gene that encodes the toxin (which typically has hemolytic and/or cytotoxic activity), a C gene that encodes a protein required for the modification and activation of the structural A protein, and B and D genes that encode the proteins required for signal sequence-independent transport. Using the leukotoxin (*lktA*) determinant from *Pasteurella haemolytica*, Burrows and Lo (7) reported the cloning and sequencing of genes with a high degree of sequence homology to the *apxIIC* and *apxIIA* genes of *A. pleuropneumoniae*. They designated these genes *ashC* and *ashA*, respectively, and noted that there were no linked transport (BD) gene homologs. Two immunological studies also supported the notion that *A. suis* isolates could produce an Apx-like toxin. In 1989, Devenish et al. (8) showed that convalescent-phase serum from an *A. suis*-infected pig could recognize a 104-kDa hemolysin from *A. pleuropneumoniae* and, conversely, that antisera to the *A. pleuropneumoniae* hemolysin cross-reacted with a high-molecular-weight protein from the supernatant of *A. suis* cultures. Later, Kamp et al. (16) reported that culture filtrates of *A. suis* were strongly cytotoxic and hemolytic and that monoclonal antibodies made to the ApxI and ApxII toxins from *A. pleuropneumoniae* reacted strongly with these filtrates in dot blot experiments.

At present, there are no serodiagnostic tests or commercially available vaccines for *A. suis*. Furthermore, it is not known if all strains have equal pathogenic potential or if some are more likely to be associated with a particular clinical condition than others. Recently, there has been a renewed interest in raising

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very high health status swine in specific-pathogen-free units or by using segregated early weaning methods. In both these management systems, *A. suis* can cause significant losses (35). In order to determine if there is a difference between *A. suis* strains from healthy swine versus those from diseased swine, a total of 66 isolates from animals with disease and 11 independent strains isolated from apparently healthy animals were analyzed by biochemical testing, serotyping, restriction endonuclease fingerprinting (REF), and toxin typing by PCR and dot blot hybridization.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The sources of the strains used in this study are listed in Tables 1 and 2. The reference strain, *A. suis* ATCC 15557, was included for comparison (40). In the PCR analysis and hybridization experiments, the serotype 1 *A. pleuropneumoniae* type strain Shope 4074 (which is known to carry *apxICABD* and *apxIICA*) and the serotype 2 reference strain 1536 (which is known to carry *apxIBD*, *apxIICA*, and *apxIIICABD*) were used as controls (10). Bacteria were routinely grown overnight at 37°C in an atmosphere of 5% CO_2 on chocolate blood agar. Clinical isolates were plated initially on blood agar and MacConkey agar. Bacteria from healthy animals were obtained from tonsillar swabs of pigs from a nearby slaughterhouse. These swabs were plated onto a selective medium (13), and colonies resembling *A. suis* were streaked for pure culture and were analyzed further. Eleven independent *A. suis* isolates were recovered from 170 animals.

Measurement of hemolytic activity. The hemolytic activities of *A. pleuropneumoniae* Shope 4074^T and *A. suis* ATCC 15557, grown in Columbia broth with 0.02% (wt/vol) NAD and 10 mM Ca^{2+}, were measured by the method of Rosendal et al. (30).

Preparation of DNA for REF analysis. DNAs were prepared by a phenol-chloroform method described previously (5, 18). Briefly, cells were harvested from one 10-cm plate, pelleted, washed, and suspended in phosphate-buffered saline (pH 7.4). The cells were lysed by the addition of proteinase K (0.5 mg/ml) and sodium dodecyl sulfate (SDS; 1.7% [wt/vol]). The lysates were then phenol-chloroform extracted, ethanol precipitated, and suspended in 10 mM Tris (pH 7.4)–0.1 mM EDTA. For restriction digests, DNAs were incubated for 2 to 4 h in a fourfold excess of enzyme in the buffer supplied by the manufacturer [Pharmacia (Canada) Ltd., Baie d'Urfe, Quebec, Canada]. Ten micrograms of RNase A was added to each 10 μg of digest. The digests were checked for completeness by running 1 μg of DNA on an agarose gel, with a 1-kbp ladder used as the molecular size marker (Bio-Rad Laboratories, Richmond, Calif.).

Analysis of restriction endonuclease fragments by SDS-PAGE. The SDS-polyacrylamide gel electrophoresis (PAGE) procedure used was based on a modification of the method of McClenaghan et al. (24). Approximately 2.5 μg of digested DNA was loaded per lane, and the fragments were resolved by electrophoresis at 200 V for 1.7 h with cooling in an SDS–7.5% polyacrylamide gel by using a minigel apparatus (Hoefer Scientific Instruments, San Francisco, Calif.). The bands were visualized by silver staining as described previously (33). Briefly, gels were fixed in ethanol-acetic acid, soaked in 0.19% (wt/vol) $AgNO_3$, developed in 0.3% formaldehyde in 0.75 N NaOH, and then incubated in enhancer (0.75% [wt/vol] Na_2CO_3). Comparisons of REF profiles were always made within rather than between gels (22), and at least three independent gels were used to confirm the assignment of the REF patterns.

Biochemical testing. Biochemical tests were done with the Sensititre Autoidentification System with AP80 plates (Radiometer America Inc., Westlake, Ohio). The plates were inoculated automatically with a suspension (with a turbidity equivalent to that of a McFarland 0.5 standard) of pure culture grown overnight on blood agar. The plates were incubated for 24 h at 37°C in the presence of 5% CO_2 and were evaluated with the Sensititre Obturator (Radiometer America Inc.). As described previously, antibiotic resistance data (reported by the receiving laboratory) were obtained by the disk diffusion method (18).

Serotyping. Strains were serotyped by the slide agglutination method with hyperimmune rabbit antisera prepared against washed whole cells of a representative *A. suis* strain, strain S04 (28, 29). As a control, all isolates were tested against normal rabbit serum. A scale of 0 (no agglutination) to 3+ (complete agglutination within 30 s) was used (40).

Analysis of *apx* genes. The presence of *apx* toxin genes was analyzed by dot blot hybridization of genomic DNA from *A. suis* by using probes for each of the individual toxin genes: *apxIC*, *apxIA*, *apxIB*, *apxID*, *apxIIC*, *apxIIA*, *apxIIIC*, *apxIIIA*, *apxIIIB*, and *apxIIID* (3). In addition, specific oligonucleotide primer pairs for the amplification of *apxICA*, *apxIICA*, *apxIIICA*, *apxIBD*, and *apxIIIBD* were prepared as described previously (11). The PCRs were done with a DNA thermal cycler (Gene Amp 9600; Perkin-Elmer Cetus, Norwalk, Conn.). Two microliters of lysed cells was added to 48 μl of a *Taq*-PCR mixture (10 mM Tris-HCl [pH 8.3], 1.5 mM $MgCl_2$, 50 mM KCl, 0.005% Tween 20, 0.005% Nonidet P-40, 170 μM [each] deoxynucleoside triphosphate, 0.25 μM [each] oligonucleotide primer, and 0.5 U of *Taq* polymerase [Boehringer Mannheim, Mannheim, Germany]). The samples (50 μl) were subjected to 35 cycles of amplification (30 s at 95°C, 30 s at 55°C, and 2 min at 72°C). Ten microliters of each of the PCR products was resolved on a 0.7% agarose gel and visualized by ethidium bromide staining (1).

Southern blotting. Genomic DNA of *A. suis* ATCC 15557 was digested singly or in pairs with *Eco*RI, *Cla*I, *Hin*dIII, *Bam*HI, *Xho*I, *Pst*I, *Bgl*II, *Sal*I, *Spe*I, or *Eco*RV, separated by electrophoresis on a 0.7% agarose gel, transferred onto a positively charged nylon membrane (Boehringer Mannheim), and denatured (1). The membranes were preincubated with 20 ml of hybridization buffer (5× SSC [1× SSC is 150 mM NaCl plus 15 mM sodium citrate; pH 7.7], 0.1% *N*-lauroylsarcosine, 0.02% SDS [wt/vol], and 1% [wt/vol] blocking reagent [Boehringer Mannheim]) per 100-cm^2 membrane at 68°C for 2 h. Following preincubation, the blots were incubated in 2.5 ml of hybridization buffer per 100 cm^2 containing 1 μg of probe for the individual *apx* toxin genes labelled with digoxigenin-11-dUTP (3) for 18 h at 68°C. The membranes were washed twice for 5 min each time with 2× SSC containing 0.1% SDS at room temperature and twice for 15 min each time with 0.2× SSC containing 0.1% SDS at 68°C. The digoxigenin-labelled probe was detected by using phosphatase-labelled antidigoxigenin antibodies (Boehringer Mannheim) according to the manufacturer's instructions.

RESULTS

A. suis isolates, obtained from clinical material submitted to the Huron Park and Guelph Veterinary Laboratory facilities of the Ontario Ministry of Agriculture, Food, and Rural Affairs, were collected over a 10-year period. The samples originated from approximately 40 different locations in southwestern Ontario. For the animals for which a diagnosis was made, 27% (15 of 56) of the *A. suis* isolates were associated with septicemia, 20% (11 of 56) were associated with pneumonia, and 20% (11 of 56) were associated with enteritis, while the remaining isolates were associated with a wide variety of conditions. It should be noted that although the *A. suis* isolates examined in this study were present in the clinical samples in significant numbers, they were not always the sole organism present, nor was it always possible to determine if they were the causative agent of the diagnosed condition.

Biochemical testing, serotyping, antibiotic resistance profiles, and hemolytic activity. The cumulative results of the biochemical testing of the isolates are presented in Table 3. The results obtained with the Sensititre system were comparable to those obtained by more traditional test methods, as reported previously (4, 9, 23, 27, 32). The biotypes of the *A. suis* isolates from healthy animals were virtually identical to those of clinical isolates, with the exception of esculin hydrolysis and the ability to produce acid from xylose. The reference strain (ATCC 15557), 61 of the 66 *A. suis* clinical isolates, and 11 of 11 isolates from healthy swine agglutinated completely and rapidly (3+) with rabbit antisera prepared against strain S04, while none reacted with normal rabbit serum. Five of the clinical isolates (isolates H93-1284, VSB 3714, 371, H90-0122, and H91-0084) agglutinated less completely (2+). All but 3 of the 50 clinical isolates tested were sensitive to ampicillin, carbenicillin, cephalothin, gentamicin, kanamycin, neomycin, penicillin, polymyxin, tetracycline, and trimethoprim-sulfamethoxazole. Strain H93-0073 was resistant to ampicillin and tetracycline, strain H90-2662 was resistant to penicillin and tetracycline, while strain H91-0062 was resistant to ampicillin, carbenicillin, kanamycin, neomycin, penicillin, and tetracycline. The hemolytic activities of culture supernatants of *A. suis* ATCC 15557 and *A. pleuropneumoniae* Shope 4074^T at the end of exponential growth were 25 ± 5 and 200 ± 25 hemolytic units, respectively.

REF. *Bam*HI digests, which produced 12 clearly resolved fragments in the 500- to 3,500-bp range, were most convenient for grouping related strains (Fig. 1A). With *Bam*HI, the DNAs of all of the isolates from both healthy animals and animals with clinical cases of infection produced profiles that were identical except for a single band difference in 2,800-bp region. Consistent with the criterion of Kristiansen et al. (17), strains

TABLE 1. Disease caused by clinical *A. suis* isolates, date and location of isolation, and REF profiles of clinical *A. suis* isolates

Strain	Date (mo-yr)	Location[a]	Disease	REF profile obtained by digestion with the following enzyme:	
				*Bam*HI	*Bgl*II
ATCC 15557[b]				1a	1a
H93-1284	June-1993	Shakespeare	Enteritis	1a	1a
H93-1042	May-1993	Stratford	Septicemia	1a	1a
H91-0151	Jan-1991	Sebringville	Other	1a	1a
H90-1984	Aug-1990	Tillsonburg	Septicemia	1a	1a
H90-1857	Jul-1990	Sebringville	Septicemia	1a	1a
H90-0971	Apr-1990	Kippen	NR[c]	1a	1a
H90-0039	Jan-1990	Tillsonburg	Septicemia	1a	1a
H88-0778	Mar-1988	Varna	Septicemia	1a	1a
H93-0073	Jan-1993	Brussels	Other	1a	1b
068	Jan-1991	NR	NR	1a	1b
H90-1567	Jun-1990	Thedford	Pneumonia	1a	1b
H90-0914	Apr-1990	Dublin	Pneumonia	1a	1b
S04	Oct-1983	Arkell	NR	1a	1b
H93-0958	May-1993	Brussels	Enteritis	1a	1c
H93-0979	May-1993	Brussels	Enteritis	1a	1c
H92-2596	Nov-1992	Dashwood	Other	1a	1c
H90-3054	Dec-1990	Zurich	Pneumonia	1a	1c
H90-3018	Dec-1990	Crediton	Enteritis	1a	1c
H90-2662	Nov-1990	Crediton	Arthritis	1a	1c
H93-0984	May-1993	Brussels	Other	1a	1d
H90-2385	Oct-1990	Arkona	Enteritis	1a	1d
H90-1048	Apr-1990	Monkton	Arthritis	1a	1d
H90-0969	Apr-1990	Lucan	Septicemia	1a	1d
H90-0155	Jan-1990	St. Pauls	Septicemia	1a	1d
ACT 1610	Mar-1984	NR	NR	1a	1d
H93-0055	Jan-1993	Salford	Enteritis	1a	1e
H90-0453	Feb-1990	Mitchell	NR	1a	1f
H91-2020	Oct-1991	St. Mary's	NR	1a	1g
H90-2269	Sep-1990	Kirkton	Septicemia	1a	1g
H90-1256	May-1990	Seaforth	Other	1a	1g
H91-0062	Jan-1991	Palmerston	Arthritis	1a	1h
H90-0939	Apr-1990	Dashwood	Pneumonia	1a	1h
H93-0127	Jan-1993	Brussels	Other	1a	1i
H90-2412	Oct-1990	Arkona	Septicemia	1a	1i
ACT 2699	May-1984	NR	NR	1a	1i
H93-1250	Jun-1993	Tavistock	Septicemia	1a	1j
H93-0902	Apr-1993	Thedford	Enteritis	1a	1k
H90-2324	Sep-1990	Tillsonburg	Arthritis	1a	1l
H90-1018	Apr-1990	Crediton	Enteritis	1a	1m
VSB 3714	Jul-1989	NR	NR	1a	1n
ACT 4187	Jul-1984	NR	NR	1a	1o
H88-0777	Mar-1988	Woodham	Septicemia	1b	1p
H91-0406	Feb-1991	St. Mary's	Pneumonia	1b	1p
H91-0380	Feb-1991	St. Mary's	Septicemia	1b	1p
371	Jan-1991	Arkell	Other	1b	1p
042	Jan-1991	Arkell	Other	1b	1p
VTH 3325	Oct-1990	Arkell	NR	1b	1p
H90-0415	Feb-1990	Komaka	Septicemia	1b	1p
H90-0122	Jan-1990	Lakeside	Pneumonia	1b	1p
193	Jan-1990	Fergus	Other	1b	1p
H89-2586	Sep-1989	Fullarton	Septicemia	1b	1p
H89-1173	Apr-1989	Ailsa Craig	Pneumonia	1b	1p
H89-1125	Apr-1989	Woodham	Pneumonia	1b	1p
H92-2146	Oct-1992	Goderich	Enteritis	1b	1q
H90-0251	Jan-1990	Holyrood	Pneumonia	1b	1q
H91-0149	Jan-1991	Delaware	Other	1b	1r
H91-0084	Jan-1991	Glencoe	Septicemia	1b	1s
H90-2521	Oct-1990	Mitchell	Other	1b	1t
H90-2526	Oct-1990	Mitchell	Arthritis	1b	1u
H90-0252	Jan-1990	Holyrood	Pneumonia	1b	1v
H90-2504	Oct-1990	Ailsa Craig	Other	1c	1e
H91-0169	Jan-1991	St. Mary's	Enteritis	1c	1f
H91-0092	Jan-1991	Zurich	Other	1c	1f
H90-0641	Mar-1990	Londesborough	Pneumonia	1c	1f
H90-2621	Nov-1990	Stratford	Enteritis	1c	1e
H90-1492	Jun-1990	Wallenstein	Other	1d	1h

[a] All locations are in Ontario, Canada.
[b] From irradiated swine (40).
[c] NR, not recorded.

TABLE 2. Date of isolation and REF profiles of *A. suis* isolates from healthy swine

Strain	Date	REF profile obtained by digestion with the following enzyme:	
		*Bam*HI	*Bgl*II
B49	May-1994	1a	1a
B04	May-1994	1a	1a
C36	Jun-1994	1a	1b
B28	May-1994	1a	1b
C46	Jun-1994	1a	1c
B44	May-1994	1a	1d
C84	Jun-1994	1a	1w
C91	Jun-1994	1a	1w
C06	Jun-1994	1a	1x
A08	May-1994	1a	1y
B50	May-1994	1c	1e

with profiles in which no more than 10% of the bands differed were considered subtypes or variants. These subtypes were designated 1a to 1d (Tables 1 and 2). Results for representatives of the three main *Bam*HI REF groups, groups 1a, 1b, and 1c, are presented in Fig. 1A (lanes 1 to 3, 4 and 5, and 6 and 7, respectively). The *Bgl*II digests, in which 60 fragments in the 500- to 2,500-bp range could be resolved, were more heterogeneous (Fig. 1B). These variants, which differed by no more than four bands, were designated 1a through 1y. The same REF profiles could be detected over many years (Tables 1 and 2). For example, strains with a *Bam*HI 1a and *Bgl*II 1a profile were detected in Ontario in 1988, 1990, 1991, and 1993. This REF profile was also shared by the reference strain ATCC 15557, which had been isolated in the United States in the early 1960s (39). Similarly, *A. suis* isolates from both healthy and diseased animals shared the same REF profile. Except for isolates C84, C91, C06, and A08, all of the isolates from healthy animals shared REF profiles with isolates from diseased animals. For example, strains B49 and B04 had a *Bam*HI 1a and *Bgl*II 1a, profile as did strain H93-1042 and four other isolates from animals with septicemia (Tables 1 and 2).

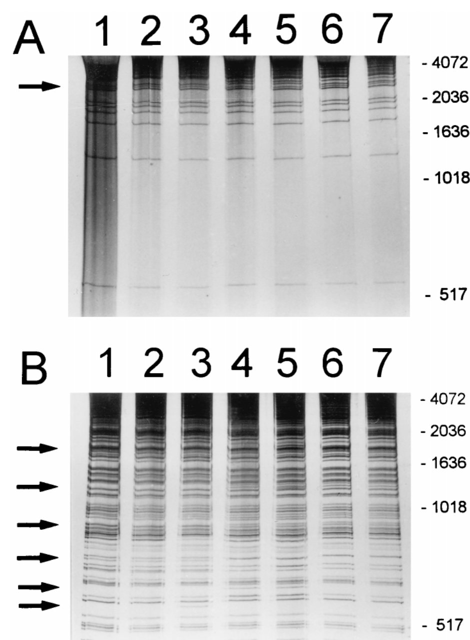

FIG. 1. REF profiles of *A. suis* DNAs-digested with *Bam*HI (A) or *Bgl*II (B). Strains ATCC 15557 (lane 1), C36 (lane 2), H90-3054 (lane 3), H90-0415 (lane 4), H92-2146 (lane 5), B50 (lane 6), and H91-0092 (lane 7) were tested. The sizes (in base pairs) and positions of the molecular size markers are indicated on the right. The positions where there are differences in the patterns are marked with arrows.

TABLE 3. Biochemical reactions of *A. suis* isolates from healthy and diseased swine

Test[a]	% Positive	
	Clinical specimens	Healthy animals
FR12: 4MU 2-acetamido-2-deoxyglycopyranoside and α-L-arabinopyranoside	100	100
FR8: 4MU bisphosphate	100	100
FR6: 4MU α-D-galactopyranoside	100	100
FR10: 4MU β-D-galactopyranoside	100	100
FR3: 4MU phosphate	100	100
Urea	100	100
Arabinose	93	91
Cellobiose	91	100
Esculin	85	100
Fructose	96	100
FR1: lysine 7AMC	82	91
Maltose	96	100
Raffinose	96	100
Sucrose	96	100
Trehalose	97	100
Xylose	99	64
Agmatine	0	0
Arginine	0	0
Arabitol	0	0
Citrate	0	0
FR4: 4MU α-D-glucopyranoside	0	0
FR7: 4MU γ-glutamine	0	0
Inositol	0	0
Lysine	0	0
Malonate	0	0
Mannitol	0	0
Ornithine	0	0
FR5: proline 7AMC	0	0
TDA tryptophan deaminase	0	0
FR9: 4MU β-D-glucuronide	0	9
Pyruvate	0	9
Sorbitol	3	9

[a] FR, fluorescent reagent; 4MU, 4-methylumbelliferone; 7AMC, 7-amino-4-methylcoumarin.

Presence of *apx* homologs in *A. suis*. The genomic DNA of *A. suis* ATCC 15557 hybridized strongly with the *apxIC*, *apxIA*, *apxIB*, and *apxID* probes, as well as with the *apxIIC* and *apxIIA* probes, in dot blots (Fig. 2). The strengths of the signals obtained with all of these probes were comparable to those seen with *A. pleuropneumoniae* serotype 1 strain Shope 4074^T. No hybridization was detected with the probes to *apxIIIC*, *apxIIIA*, *apxIIIB*, and *apxIIID*. As expected, the *A. pleuropneumoniae* serotype 2 strain 1536 hybridized strongly with all of the *apxII* and *apxIII* gene probes (Fig. 2).

The restriction map of the operon analogous to *apxI* was determined by Southern blotting (Fig. 3). In *Pst*I-digested genomic DNA from *A. suis* ATCC 15557, a 13.5-kbp band hybridized with the *apxIC*, *apxIA*, *apxIB*, and *apxID* probes. When DNA was digested with *Pst*I and *Hin*dIII, a 4.5-kbp band hybridized with the *apxIC* and *apxIA* probes, while a 9-kbp band hybridized with the *apxIA*, *apxIB*, and *apxID* probes. Following *Pst*I and *Eco*RV digestion, we detected a 9.5-kbp

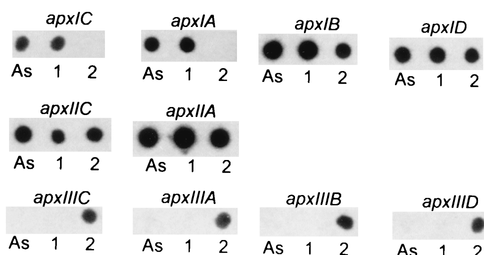

FIG. 2. Dot blot of chromosomal DNAs from *A. suis* ATCC 15557 (dots labelled As), *A. pleuropneumoniae* serotype 1 strain Shope 4074^T (dots labelled 1), and *A. pleuropneumoniae* serotype 2 strain 1536 (dots labelled 2) hybridized with toxin genes *apxIC*, *apxIA*, *apxIB*, *apxID*, *apxIIC*, *apxIIA*, *apxIIIC*, *apxIIIA*, *apxIIIB*, and *apxIIID* as described previously (3).

band that hybridized with all four probes. In addition, there was a 4.2-kbp band that hybridized only with the *apxID* probe. *Pst*I and *Cla*I digests resulted in a 10.5-kbp band that hybridized with the *apxIA*, *apxIB*, and *apxID* probes and a 900-bp band that hybridized with *apxIC*. The same 900-bp band was also seen when the DNA was digested with *Cla*I alone, while the other probes hybridized with bands larger than 15 kbp. *Bgl*II- and *Pst*I-digested DNA produced a 6-kbp band which hybridized with the *apxIC* and *apxIA* and *apxIB* probes. Also, two bands of 500 bp and 4.5 kbp hybridized with the *apxID* probe. *Bgl*II digestion alone resulted in the same bands obtained by double digestion with *Pst*I, except that the 4.5-kbp band, which hybridized with *apxID*, was missing and a fragment larger than 15 kbp was seen. Finally, the *Xho*I sites were mapped by a *Pst*I-*Xho*I double digestion, resulting in a 3.0-kbp band that hybridized with the *apxIC* probe and a 10.5-kbp band which hybridized with all four probes. A few triple digestions were used to verify the results.

PCR analysis was then done to rapidly screen the field strains examined in this study. Sequences which amplified with primers to *apxICA* with the predicted size of 2,420 bp and with primers to *apxIICA* with the predicted size of 2,088 bp were detected in 41 of 41 of the DNAs of clinical isolates and 11 of 11 of the DNAs of *A. suis* isolates from healthy animals. No amplification of *A. suis* DNA was detected with primers to *apxIBD*, *apxIIICA*, or *apxIIIBD*, although bands with the predicted sizes of 1,447 and 968 bp were amplified in the *A. pleuropneumoniae* control DNAs. Sequences with homology to the *apxIBD* genes could be detected by dot blotting, however, in all but one of the *A. suis* DNAs tested (isolate VSB 3714).

DISCUSSION

In order to design effective diagnostic tests and treatment strategies for *A. suis* (and other veterinary pathogens), it is necessary to understand the population structure and to identify important and/or unique factors, especially those associated with virulence. In this study we have shown that *A. suis* isolates recovered from both healthy and diseased swine (with a wide range of conditions) are very homogeneous. All of the strains tested had very similar REF types and biotypes, and on the basis of agglutination test results, all strains appeared to share the same major surface antigens (Fig. 1 and 2; Tables 1 to 3). In contrast, in an analysis of another opportunistic swine pathogen, *Haemophilus parasuis*, considerable heterogeneity was detected and none of the strains from animals with disease shared REF profiles with isolates from healthy animals (38). Porcine serotype 2 isolates of *Streptococcus suis* are also quite heterogeneous (2). The REF profiles of isolates from diseased animals are different than those of isolates from healthy animals, and it can be further demonstrated that isolates from animals with pneumonia typically have a *Bam*HI A and *Bgl*II A pattern, whereas strains from animals with septicemia have a *Bam*HI B and *Bgl*II B pattern (2). The absence of any obvious genotypic or phenotypic heterogeneity suggests either that all *A. suis* isolates have essentially equal pathogenic potential or that additional tests will have to be developed to discriminate between virulent and avirulent strains.

In *A. pleuropneumoniae*, the Apx toxins have been shown to play a very important role in virulence, and PCR-based tests of these genes are being developed for the rapid diagnosis of *A. pleuropneumoniae* (10, 11). Apx homologs were detected in all *A. suis* isolates examined in this study, regardless of whether they came from healthy or diseased animals. All of the strains had *apxICA* and *apxIICA* (*ashCA*) homologs that could be amplified in PCRs with primers designed to amplify *apxICA* and *apxIICA* genes in *A. pleuropneumoniae*. Genes corresponding to the *apxIBD* genes, which could be expected to be present on the basis of the organization seen in *A. pleuropneumoniae*, were not amplified in PCRs with *apxIBD* primers, but sequences with homology to the *A. pleuropneumoniae apxIBD* genes were detected by dot blotting in all but one strain (Fig. 2). Southern blot analysis revealed that the *apxICABD* genes in *A. suis* ATCC 15557 were contiguous and expected to form an operon (Fig. 3). To our knowledge, this is the first report of genetic evidence of *apxI* gene homologs, although their existence has been postulated.

Comparison of the restriction map of the *A. suis apxICABD* operon (Fig. 3) with that of *A. pleuropneumoniae*, as deduced from the nucleotide sequence, revealed differences in all four

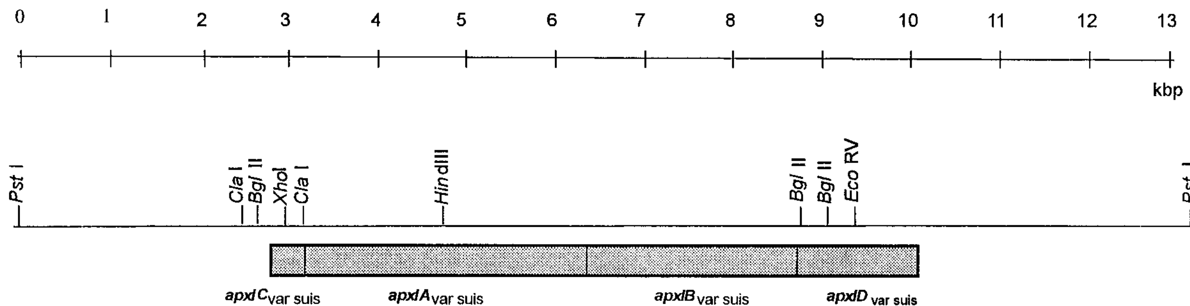

FIG. 3. Restriction map of the *apxICABD* homolog in *A. suis*. The $apxICABD_{var.suis}$ map is based on Southern blotting data described in detail in the Results section. Note that the precise locations and sizes of the genes are not known.

genes (12, 15). In addition, further differences were detected in the *apxBD* homologs since the PCR designed to amplify *apxIBD* of *A. pleuropneumoniae* (11) failed to amplify the analogous genes in *A. suis*. Due to these distinct differences on one hand and the high similarity of the *A. suis apxICABD* genes to those found in *A. pleuropneumoniae* on the other, we suggest the use of the designations $apxIC_{\text{var.suis}}$, $apxIA_{\text{var.suis}}$, $apxIB_{\text{var.suis}}$, and $apxID_{\text{var.suis}}$ to reflect these facts.

Differences in the *apxIB* and *apxID* genes may explain why lower amounts of ApxI and ApxII are present in culture supernatants and why *A. suis*, in general, seems to cause acute disease less frequently than *A. pleuropneumoniae*. It is possible that the *apxIBD* homologs of *A. suis* may have a function similar to that of the *lktBD* gene products in *Actinobacillus actinomycetemcomitans*, which are responsible for the transport of the toxin to the cell membrane but not for its excretion, but the significance of these differences remains to be established (14).

In summary, these data suggest that *A. suis* isolates from healthy swine and from animals with a variety of different clinical conditions are very similar. Thus, all *A. suis* isolates should be considered to have pathogenic potential. The fact that there appears to be a single clonal population of *A. suis* means that it should be possible to develop a diagnostic test for *A. suis* infection that would recognize all isolates. Similarly, vaccine development should be simplified by the presence of a single clone. Finally, the analysis of *apx* homologs in *A. suis* indicates that current *apxICA* or *apxIICA* PCR tests for the detection of *A. pleuropneumoniae* will give rise to false-positive results if *A. suis* is present. It should, however, be possible to use differences in the $apxBD_{\text{var.suis}}$ genes to create unique primers to confirm the presence of *A. suis.*

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