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The Phylogeny of Intestinal Porcine Spirochetes (*Serpulina* Species) Based on Sequence Analysis of the 16S rRNA Gene

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Four type or reference strains and twenty-two field strains of intestinal spirochetes isolated from Swedish pig herds were subjected to phylogenetic analysis based on 16S rRNA sequences. Almost complete (>95%) 16S rRNA sequences were obtained by solid-phase DNA sequencing of in vitro-amplified rRNA genes. The genotypic patterns were compared with a previously proposed biochemical classification scheme, comprising beta-hemolysis, indole production, hippurate hydrolysis, and α-galactosidase, α-glucosidase, and β-glucosidase activities. Comparison of the small-subunit rRNA sequences showed that the strains of the genus *Serpulina* were closely related. Phylogenetic trees were constructed, and three clusters were observed. This was also confirmed by signature nucleotide analysis of the serpulinas. The indole-producing strains, including the strains of *S. hyodysenteriae* and some weakly beta-hemolytic *Serpulina* strains, formed one cluster. A second cluster comprised weakly beta-hemolytic strains that showed β-galactosidase activity but lacked indole production and hippurate-hydrolyzing capacity. The second cluster contained two subclusters with similar phenotypic profiles. A third cluster involved strains that possessed a hippurate-hydrolyzing capacity which was distinct from that of the former two clusters, because of 17 unique nucleotide positions of the 16S rRNA gene. Interestingly, the strains of this third cluster were found likely to have a 16S rRNA structure in the V2 region of the molecule different from that of the serpulinas belonging to the other clusters. As a consequence of these findings, we propose that the intestinal spirochetes of this phenotype (i.e., P43/6/78-like strains) should be regarded as a separate *Serpulina* species. Furthermore, this cluster was found to be by far the most homogeneous one. In conclusion, the biochemical classification of porcine intestinal spirochetes was comparable to that by phylogenetic analysis based on 16S rRNA sequences.

In the early 1970s, a strongly hemolytic spirochete was found to be the causative agent of swine dysentery, a severe mucohemorrhagic colitis of swine (16, 56, 59). The disease was originally assumed to be caused by a vibrio-like organism (34) but in 1972 was found to be etiologically associated to a treponeme, *Treponema hyodysenteriae* (16). However, not all intestinal spirochetes caused swine dysentery (20, 29). The discovery of nonpathogenic weakly beta-hemolytic strains led to the designation of a new species, *Treponema innocens* (27). *T. hyodysenteriae* and *T. innocens* not only were found to be two different species by DNA-DNA reassociation studies but also could be distinguished from other treponemas by a significantly lower G+C content (40, 41). Recently *T. hyodysenteriae* and *T. innocens* were classified in a new genus, *Serpulina*, and renamed *Serpulina hyodysenteriae* and *Serpulina innocens* (54, 55). The reclassification was supported by electrophoretic analysis of cell proteins, DNA-DNA reassociation studies, restriction endonuclease analysis, and 16S rRNA sequence analysis. Furthermore, 16S rRNA sequence analysis of selected spirochetes showed a distinct and tight clustering of *S. hyodysenteriae* and *S. innocens* (45, 55).

Certain strains of weakly beta-hemolytic intestinal spirochetes (WBHIS) different from *S. innocens* have been described (5, 7, 30, 32, 57). WBHIS have also been reported to cause enteric disease (52, 57), but the spirochetal role of gastrointestinal disorders is poorly understood. This is partly because the techniques that are used for typing and classification are not sufficiently specific. Serotyping of *S. hyodysenteriae* based on antibodies to lipopolysaccharides has been used for differentiation (1, 15, 38). Multilocus enzyme electrophoresis (MEE) was used for discrimination between strains of *S. hyodysenteriae* (31, 35) and *S. innocens* (32). These studies showed that *S. innocens* is by far the most heterogeneous species, and new species names have been proposed: "*Serpulina intermedius*" has been proposed for the weakly beta-hemolytic and indole-producing *Serpulina* sp. strains (32) and "*Serpulina murdochii*" has been proposed for weakly beta-hemolytic strains without α-galactosidase activity (30). WBHIS that cause intestinal spirochetosis have been found to differ from other serpulinas both genetically and phenetically (7, 9, 10, 19, 30, 32, 49, 57). The reference strain for these spirochetes is *Serpulina* strain P43/6/78 (57). Proposals have been made to classify strains which are similar to P43/6/78 into a new species, on the basis of DNA-DNA reassociation studies (7), and into a new genus, "*Anguillina*" gen. nov., on the basis of MEE patterns (30, 32). Two main drawbacks with MEE analysis are that environmental and cultivation factors can alter the expected enzyme profile. Furthermore, enzyme analysis of new *Serpulina* strains can give profiles which do not necessarily reflect true phylogenetic relations. Attempts to identify and classify intestinal spirochetes have been performed by restriction enzyme analysis of genomic DNA and hybridization with DNA probes, specific for different genes (4, 24, 29, 42, 58).

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TABLE 1. *Serpulina* strains investigated in this study

Species	Strain	Phenotype[a]	Animal of origin or characteristic(s)	Country of origin	Accession no.[b]	Reference
S. hyodysenteriae	B78^T	I	Type strain; serotype 1	U.S.A.[c,d]	**U14930**; M57743	This study; references 16 and 55
	B204	I	Serotype 2	U.S.A.[c]	**U14932**; M57741	This study; references 28 and 55
	AN174:92	I	Dysenteric pig	Sweden	**U14931**	This study
	A1	ND[e]	Dysenteric pig; serotype 4	U.K.[f]	M57742	55, 56
Serpulina sp.	AN26:93	II	Diarrheic pig	Sweden	**U14916**	This study
	AN520:93	II	Diarrheic pig	Sweden	**U14934**	This study
	AN983:90	II	Diarrheic pig	Sweden	**U14933**	This study
S. innocens	C301	IIIa	Nondiarrheic pig and herd	Sweden	**U14917**	This study
	C378	IIIa	Nondiarrheic pig and herd	Sweden	**U14918**	This study
	C320[g]	IIIb	Nondiarrheic pig and herd	Sweden	**U14919**	This study
	C336[g]	IIIb	Nondiarrheic pig and herd	Sweden	ID to **U14919**	This study
	C555[g]	IIIb	Nondiarrheic pig and herd	Sweden	**U14926**	This study
	C562[g]	IIIb	Nondiarrheic pig and herd	Sweden	ID to **U14926**	This study
	C567[g]	IIIb	Nondiarrheic pig and herd	Sweden	ID to **U14926**	This study
	B256^T	IIIc	Type strain	U.S.A.[c]	**U14920**; M57744	This study; references 27 and 55
	AN1004:90	IIIc	Diarrheic pig	Sweden	**U14924**	This study
	AN1023:90	IIIc	Nondiarrheic pig and herd	Sweden	**U14915**	This study
	AN3706:90	IIIc	Diarrheic pig	Sweden	**U14925**	This study
	C109	IIIc	Nondiarrheic pig and herd	Sweden	**U14922**	This study
	C173	IIIc	Nondiarrheic pig and herd	Sweden	**U14921**	This study
	4/71	ND	Nondiarrheic pig	U.K.	M57745	20, 55
Serpulina sp.	P43/6/78	IV	Diarrheic pig	U.K.[c]	**U14927**	This study; reference 57
	AN76:92	IV	Diarrheic pig	Sweden	ID to **U14927**	This study
	AN497:93	IV	Diarrheic pig	Sweden	ID to **U14927**	This study
	AN914:90	IV	Diarrheic pig	Sweden	ID to **U14927**	This study
	AN916:90	IV	Diarrheic pig	Sweden	**U14929**	This study
	C62	IV	Nondiarrheic pig and diarrheic herd	Sweden	ID to **U14927**	This study
	C162	IV	Nondiarrheic pig and herd	Sweden	**U14928**	This study

[a] Biochemical groups as determined and defined by Fellström and Gunnarsson (9). See Table 2 for descriptions.
[b] Boldface type indicates accession numbers of nucleotide sequences determined in this work. ID, identical.
[c] Obtained from Hans Rønne, Copenhagen, Denmark.
[d] U.S.A., United States of America.
[e] ND, not determined in this study.
[f] U.K., United Kingdom.
[g] Strains C320 and C336 and strains C555, C562, and C567 were isolated from the same herds.

Sequence comparison of rRNA genes of the small subunit has been shown to be a powerful tool for phylogenetic analysis of bacterial species (43, 61). Attempts to investigate the spirochetal phyla by the oligonucleotide rRNA cataloging method (46) resulted in a quintet of subclasses. This taxonomical finding was further refined by the more accurate 16S rRNA sequencing method, indicating a coherent taxon composed of six main clusters (45). The branch of the genus *Serpulina* showed a descent line and, furthermore, a high interspecies homology (>99%) between *S. hyodysenteriae* and *S. innocens* (45, 55). A partial 16S rRNA sequence (532 nucleotides) alignment was reported from 21 *Serpulina* strains, isolated from Swedish swine herds with or without history of dysentery (10). The sequence analysis was used for verification of a biochemical classification scheme, recently proposed by Fellström and Gunnarsson (9). Four unique nucleotide positions correlated to the six groups derived from the biochemical typing, except for the weakly hemolytic strains with the ability to produce indole. The nucleotide sequences of these WBHIS were identical to the corresponding regions of *S. hyodysenteriae* (strains B78^T and B204). In this work we report nearly full-length sequences (1,435 bases) of the 16S rRNA genes obtained by semiauto-

TABLE 2. Biochemical reaction scheme for *Serpulina* phenotypes (9)[a]

Phenotype	Hemolysis	Indole production	Hippurate hydrolysis	α-Galactosidase activity	α-Glucosidase activity	β-Galactosidase activity
I	Strong	+	−	−	+	+
II	Weak	+	−	−	+	+
IIIa	Weak	−	−	−	−	+
IIIb	Weak	−	−	+	−	+
IIIc	Weak	−	−	+	+	+
IV	Weak	−	+	+	−	−

[a] Classifications are based on degrees of hemolysis and the presence (+) or absence (−) of the given characteristics and activities. Adapted from reference 10 with permission of the publisher.

TABLE 3. Primers used for PCR and DNA sequencing of *Serpulina* species

Primer[a]	Position[b]	Sequence[c,d]	Application
609	−25–−1 (11–35)	5′-USP-GTTTGATYCTGGCTYAGARCKAACG-3′	PCR
388	299–320 (334–356)	5′-USP-CCARACTCCTACGGRAGGCAGC-3′	PCR
390B	876–853 (939–917)	5′-CTTGTGCGGGYYCCCGTCAATTC-3′	PCR
594B	1457–1436 (1517–1496)	5′-CCSSTACGGMTACCTTGTTACG-3′	PCR
USP		5′-CGTTGTAAAACGACGGCCAG-3′	Sequencing
538F	740–758 (800–818)	5′-GTAGTCCACGCCGTAAACG-3′	Sequencing
390F	876–853 (939–917)	5′-CTTGTGCGGGYYCCCGTCAATTC-3′	Sequencing
597F	1114–1133 (1175–1193)	5′-GAGGAAGGTGGGGATGATG-3′	Sequencing
595F	1457–1436 (1517–1496)	5′-CCSSTACGGMTACCTTGTTACG-3′	Sequencing

[a] B and F indicate that a biotin or a fluoroscein isothiocyanate dye, respectively, is coupled to the oligonucleotide.
[b] Numbers are according to the *Serpulina* consensus sequence shown in Fig. 3. The numbers within parentheses are positions in the 16S rRNA gene of *E. coli*. Note that primers 609, 594B, and 595F anneal in the flanking regions of the consensus sequence.
[c] USP means that the primer has a handle at the 5′ end with the sequence of the universal sequencing primer.
[d] K, M, R, S, and Y denote degenerated positions according to the single-letter code suggested by the Nomenclature Committee of the International Union of Biochemistry.

mated solid-phase DNA sequencing (22, 23, 47, 60) of 4 *Serpulina* type and reference strains and 22 isolates collected from Swedish pig farms. The sequence information was used to infer the phylogeny of the genus *Serpulina*.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and template preparation for PCR. The *Serpulina* strains used in this study are listed in Table 1. Two strains, *S. hyodysenteriae* A1 and *S. innocens* 4/71, were not biochemically characterized or sequenced in this study (Table 1). The 16S rRNA sequences from these strains (55) were retrieved from GenBank (National Center for Biotechnology Information, Rockville, Md.). The bacteria were cultivated and stored as described previously (9). One milliliter of the bacterial suspension was lysed and stored at −20°C until used as template for in vitro amplification by PCR.

Biochemical analysis. The phenotypical characteristics based on beta-hemolysis, indole production, hippurate hydrolysis, and the API-ZYM profile for α-galactosidase, α-glucosidase, and β-glucosidase activities were determined as described by Fellström and Gunnarsson (9). Each biochemical group is defined in Table 2 and referred to by its roman numeral.

In vitro amplification of 16S rRNA genes. The 16S rRNA genes were amplified directly from the bacterial genome in a seminested fashion. The first amplification was performed with the primers 609 and 594B, which were positioned close to the termini of the 16S rRNA gene. The PCR products were diluted (10^4-fold), and 1 μl was used as the template in two subsequent and different reactions. The latter amplifications were performed with an inner primer (390B or 388) instead of one of the outer primers (609 or 594B). This resulted in two different PCR products. The primer pair 609 and 390B generated a fragment with the approximate length of 900 bp and the primers 388 and 594B gave a PCR product of about 1,200 bp. Thereby, these two products overlap each other by almost 600 nucleotides. Primer details are given in Table 3. In general, the reverse primers were biotinylated, which made the PCR products suitable for solid-phase DNA sequencing. The PCRs were performed with 5 pmol of each primer and 30 cycles as described (47). The profile for each cycle was denaturation at 96°C for 15 s and a combined annealing-extension step at 70°C for 90 s. Finally, a 10-min incubation was performed at 72°C.

Direct solid-phase DNA sequencing. Immobilization of the biotinylated PCR products, followed by strand separation and template preparation, was performed with superparamagnetic beads (Dynabeads M-280 Streptavidin; Dynal, Oslo, Norway). The nucleotide sequences of the 16S rRNA genes were determined in both directions by automated solid-phase DNA sequencing (22, 23, 47, 60) with the automated laser fluorescence system (Pharmacia Biotech, Uppsala, Sweden). The sequencing primers are listed in Table 3.

Phylogenetic analysis. The nucleotide sequences of *Serpulina* species and strains were aligned manually with spirochetal 16S rRNA sequences obtained from the Ribosomal Database Project (37). Secondary structures of the 16S rRNA molecules of three members of the family *Spirochaetales* (14) were used as guidelines in the alignment procedure. A secondary structure was also created for the *Serpulina* 16S rRNA molecule for proper identification of variable and conserved regions of the corresponding genes of the serpulinas. The sequences were aligned in two different ways. In the first alignment, for comparison with serpulinas of different phenotypes, the 16S rRNA sequences from a selection of spirochetal genera, according to Hookey et al. (17), were aligned with that of *Escherichia coli*. Hypervariable regions and nucleotide positions that could not be unambiguously aligned, were omitted (112 nucleotides similar to those omitted by Hookey et al. [17] and Paster et al. [45]) from the final alignment that

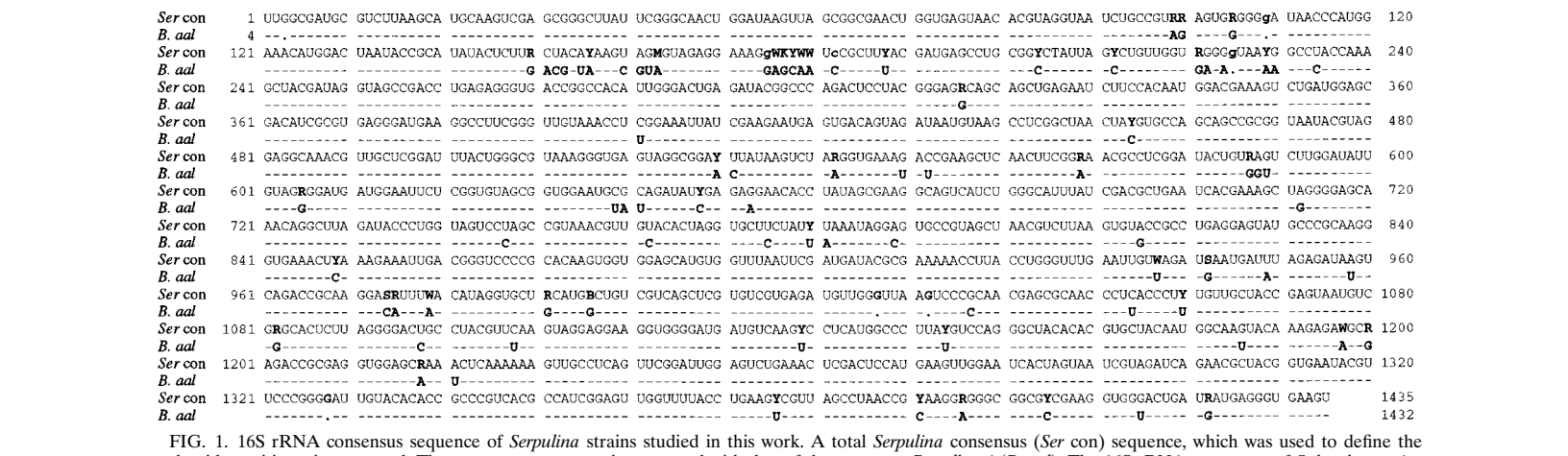

FIG. 1. 16S rRNA consensus sequence of *Serpulina* strains studied in this work. A total *Serpulina* consensus (*Ser* con) sequence, which was used to define the nucleotide positions, is presented. The consensus sequence is compared with that of the outgroup *B. aalborgi* (*B. aal*). The 16S rRNA sequences of *S. hyodysenteriae* A1 and *S. innocens* 4/71, which were obtained from the database, are not included (see text for details). All variable positions are indicated with the single-letter code suggested by the Nomenclature Committee of the International Union of Biochemistry (M = A or C, K = G or U, S = C or G, W = A or U, Y = C or U, R = A or G, and B = C, G, or U). A dot within the sequence represents a gap that was introduced for proper alignment, and a lowercase letter indicates that the position has a gap (indicated as g) or that the actual residue is missing (indicated as c) in one or more of the aligned sequences.

TABLE 4. Variable nucleotide positions in the 16S rRNA gene of the different *Serpulina* strains examined in this study

Serpulina strain (phenotype)	Nucleotide at position[a]:																								
	99	100	105	109	150	156	163	168	175	176	177	178	179	180	182	188	196	204	212	221	225	229	316	454	530
S. hyodysenteriae B204 (I)	A	G	G	.	G	**U**	A	A	G	A	G	C	A	A	C	U	G	C	C	G	.	C	G	C	C
S. hyodysenteriae B78 (I)	A	G	G	.	G	**U**	A	A	G	A	G	C	A	A	C	U	G	C	C	G	.	C	G	**U**	C
S. hyodysenteriae A1 (ND[b])	A	G	G	.	G	**U**	A	A	G	A	G	C	A	A	C	U	.	C	C	G	.	C	G	C	C
S. hyodysenteriae AN174:92 (I)	A	G	G	**G**	G	**U**	A	A	G	A	G	C	A	A	C	U	G	C	C	G	.	C	G	C	C
Serpulina sp. AN520:93 (II)	A	G	G	.	G	**U**	A	A	G	A	G	C	A	A	C	U	G	C	C	G	.	C	G	C	C
Serpulina sp. AN26:93 (II)	A	G	G	.	G	C	A	A	G	A	G	C	A	A	C	U	G	C	C	G	.	C	G	C	C
Serpulina sp. AN983:90 (II)	A	G	G	.	G	**U**	A	A	G	A	G	C	A	A	C	U	G	C	C	**A**	.	C	G	C	C
S. innocens C378 (IIIa)	A	G	**A**	.	G	C	**C**	A	G	A	G	**U**	A	A	C	U	G	C	**U**	G	.	C	G	C	C
S. innocens C301 (IIIa)	A	G	G	.	G	**U**	**C**	A	G	A	G	C	A	A	C	U	G	C	C	G	.	C	G	C	C
S. innocens B256 (IIIc)	A	G	G	.	G	C	A	A	G	A	G	C	A	A	C	U	G	**U**	C	G	.	C	G	C	C
S. innocens C555 (IIIb)	A	G	G	.	G	C	A	A	G	A	G	C	A	A	C	U	G	C	C	G	.	C	G	C	C
S. innocens AN1023:90 (IIIc)	A	G	G	.	G	C	A	A	G	A	G	C	A	A	C	U	G	C	C	G	.	C	**A**	C	C
S. innocens C173 (IIIc)	A	G	G	.	G	C	A	A	G	A	G	C	A	A	C	U	G	C	C	G	.	C	G	C	C
S. innocens AN3706:90 (IIIc)	A	G	G	.	**A**	C	A	A	G	A	G	C	A	A	C	U	G	C	C	G	.	C	G	C	C
S. innocens C320 (IIIb)	A	G	G	.	G	C	A	A	G	A	G	C	A	A	C	U	G	C	C	G	.	C	G	C	C
S. innocens 4/71 (ND)	A	G	G	.	G	C	A	**G**	G	A	G	C	A	A	C	U	.	C	C	G	.	C	G	C	C
S. innocens AN1004:90 (IIIc)	A	G	G	.	G	C	A	A	G	A	G	C	A	A	C	U	G	C	C	G	**G**	C	G	C	C
S. innocens C109 (IIIc)	A	G	G	.	G	C	A	A	G	A	G	C	A	A	C	U	G	C	C	G	**G**	C	G	C	C
Serpulina sp. P43/6/78 (IV)	**G**	**A**	G	.	G	**U**	A	A	.	**U**	**U**	**U**	**U**	**U**	.	**C**	G	C	C	**A**	.	**U**	G	C	**U**
Serpulina sp. C162 (IV)	**G**	**A**	G	.	G	**U**	A	A	.	**U**	**U**	**U**	**U**	**U**	.	**C**	G	C	C	**A**	.	**U**	G	C	**U**
Serpulina sp. AN916:90 (IV)	**G**	**A**	G	.	G	**U**	A	A	.	**U**	**U**	**U**	**U**	**U**	.	**C**	G	C	C	**A**	.	**U**	G	C	**U**

[a] Numbers are according to the *Serpulina* consensus sequence shown in Fig. 1. Boldface type indicates a residue different from that commonly found at the actual postion. Dots represent gaps introduced for proper alignment.
[b] ND, not determined.

comprised 1,384 nucleotides. The second alignment was performed with the 16S rRNA sequences of *Serpulina* strains determined in this study and sequences retrieved from databases (Table 1). *Brachyspira aalborgi* (17, 18) was used as an outgroup, and only the ambiguously aligned positions were omitted from the *Serpulina* alignment. The latter data set comprised 22 sequences of 1,385 nucleotides. The phylogenies were inferred by using DNADIST, contained in the phylogenetic inference package PHYLIP 3.51c (11). Evolutionary distances were calculated by the method of Jukes and Cantor (26). User trees based on distance matrices were computed by neighbor-joining, with the software NEIGHBOR (50), and by the Fitch-Margoliash method, with the software FITCH (12). Parsimony analysis was performed by using DNAPARS, and a maximum-likelihood phylogeny was constructed with the DNAML program. The results obtained by both DNADIST and DNAPARS were subjected to bootstrap analysis by using SEQBOOT in sets of 500 resamplings. Majority-rule consensus trees were computed from the user trees by using the CONSENSE program. Similarity matrices were corrected for multiple base changes at single locations by the method of Jukes and Cantor (26).

Nucleotide sequence accession numbers. The sequences of the 16S rRNA genes of the *Serpulina* species and strains studied in this work have been deposited in GenBank under the accession numbers listed in Table 1.

RESULTS AND DISCUSSION

Solid-phase DNA sequencing of the 16S rRNA gene. Almost (>95%)-complete nucleotide sequences were generated from the 16S rRNA genes of 26 intestinal spirochete strains listed in Table 1. The list consists of 22 strains found in Swedish pig herds with or without histories of diarrhea, two strains of *S. hyodysenteriae* (B78^T and B204), the type strain *S. innocens* B256^T, and the reference strain of spirochetal diarrhea, *Serpulina* strain P43/6/78 (57). The 16S rRNA sequences of *S. hyodysenteriae* A1 and *S. innocens* 4/71 (55) were retrieved from the database. The solid-phase DNA sequencing procedure re-

TABLE 4—*Continued*

Nucleotide at position[a]:																														
542	569	587	605	648	780	849	927	929	937	942	974	975	979	991	996	1060	1082	1139	1154	1197	1200	1213	1218	1376	1391	1396	1405	1422
A	**A**	A	G	C	U	C	U	U	A	G	C	G	U	G	**C**	U	G	U	U	A	A	G	A	U	C	G	C	G
A	**A**	A	G	C	U	C	U	U	A	G	C	G	U	G	G	U	G	U	U	A	A	G	A	U	C	G	C	G
A	**A**	A	G	C	U	C	**C**	.	A	G	C	G	U	G	**C**	U	G	U	U	A	A	**U**	A	U	C	G	C	ND
A	**A**	A	G	**U**	U	C	U	U	A	G	C	G	U	G	G	U	G	U	U	A	A	G	A	U	C	G	C	G
A	**A**	A	G	C	U	C	U	U	A	G	C	G	U	G	G	U	G	U	U	A	A	G	A	U	C	G	**U**	G
A	**A**	A	G	C	U	C	U	U	A	G	C	G	U	G	G	U	G	U	U	A	A	G	A	U	C	G	C	**A**
A	**A**	A	G	C	U	C	U	U	A	G	C	G	U	G	G	U	G	U	U	A	A	G	A	U	C	G	C	G
A	G	A	G	C	U	C	U	U	A	**C**	**G**	**A**	U	G	G	U	G	U	U	A	**G**	G	A	U	C	G	C	G
A	G	A	G	C	U	C	U	U	A	**C**	**G**	**A**	U	G	G	U	G	U	U	A	**G**	G	A	U	**U**	G	C	G
A	G	**G**	G	C	U	C	U	U	A	G	C	G	U	G	**C**	U	G	U	U	A	**G**	G	A	U	C	G	C	G
A	G	**G**	G	C	**C**	C	U	U	A	**C**	**G**	**A**	U	**A**	G	U	G	**C**	U	**U**	**G**	G	**G**	U	C	G	C	G
A	G	**G**	G	C	**C**	C	U	U	A	**C**	**G**	**A**	U	G	**U**	U	G	**C**	U	**U**	**G**	G	**G**	U	C	G	C	G
A	G	**G**	G	C	U	C	U	U	A	**C**	**G**	**A**	U	G	G	U	G	U	U	**U**	**G**	G	**G**	U	C	G	C	G
A	G	**G**	G	C	U	C	U	U	A	**C**	**G**	**A**	U	G	G	U	G	U	U	**U**	**G**	G	**G**	U	C	G	C	G
A	G	**G**	**A**	C	U	C	U	U	A	**C**	**G**	**A**	U	G	G	U	G	U	U	**U**	**G**	G	**G**	U	C	G	C	G
A	G	**G**	G	C	U	C	U	.	A	**C**	**G**	**A**	U	G	**C**	U	G	U	U	**U**	**G**	G	**G**	U	C	G	C	G
A	G	**G**	G	C	U	C	U	U	A	**C**	**G**	**A**	U	G	G	U	G	U	U	**U**	**G**	G	**G**	U	C	G	C	G
A	G	**G**	G	C	U	C	U	U	A	**C**	**G**	**A**	U	G	G	U	G	U	**C**	**U**	**G**	G	**G**	U	C	G	C	G
A	G	A	G	C	U	C	U	U	**U**	G	C	G	**A**	G	G	**C**	**A**	U	U	A	A	G	A	**C**	C	**A**	**U**	G
A	G	A	G	C	U	**U**	U	U	**U**	G	C	G	**A**	G	G	**C**	**A**	U	U	A	A	G	A	**C**	C	**A**	**U**	G
G	G	A	G	C	U	C	U	U	**U**	G	C	G	**A**	G	G	**C**	**A**	U	U	A	A	G	A	**C**	C	**A**	**U**	G

sulted in an unambiguous determination of 1,431 to 1,434 bases, depending on the strain. Internal primers were used to amplify the target gene, and the sequences are therefore incomplete in the 5′ and 3′ ends. Microheterogeneities due to nucleotide differences between the rRNA genes were not observed as previously reported for mycoplasmas (47, 48). A total consensus compiled from all 16S rRNA sequences of the *Serpulina* strains studied in this work aligned with the outgroup *B. aalborgi* is presented in Fig. 1. The numbering of all discussed nucleotide positions refers to this total consensus sequence unless otherwise stated. Because of the many ambiguities in the previously deposited 16S rRNA sequences of *S. hyodysenteriae* A1 and *S. innocens* 4/71, these strains were not included in the compilation shown in Fig. 1.

Comparison with previously published *Serpulina* sequences. Only a few nucleotide differences between the *Serpulina* strains were expected, and it was, therefore, considered important to determine the 16S rRNA sequences as accurately and completely as possible. Resequencing of the 16S rRNA genes of the strains *S. hyodysenteriae* B78^T, *S. hyodysenteriae* B204, and *S. innocens* B256^T resulted in clear-cut determinations of all the ambiguous nucleotide positions (31, 1, and 12 positions, respectively) in the deposited sequences (55). Furthermore, all three strains had an extra U residue in position 929 and in strains B78^T and B204 an additional G was found in position 196. Three nucleotide differences between the deposited sequences and the nucleotide data for the type strains, as determined in this study, were found. In *S. hyodysenteriae* B78^T, the C in position 454 was by the solid-phase sequencing procedure determined to be a U and in position 1362 an A should be replaced with a G. *S. innocens* B256^T was found to have a U in position 1132 in contrast to the deposited sequence which has a C. All strains were very closely related, but nevertheless, 21 strains out of 28 were found to have unique 16S rRNA se-

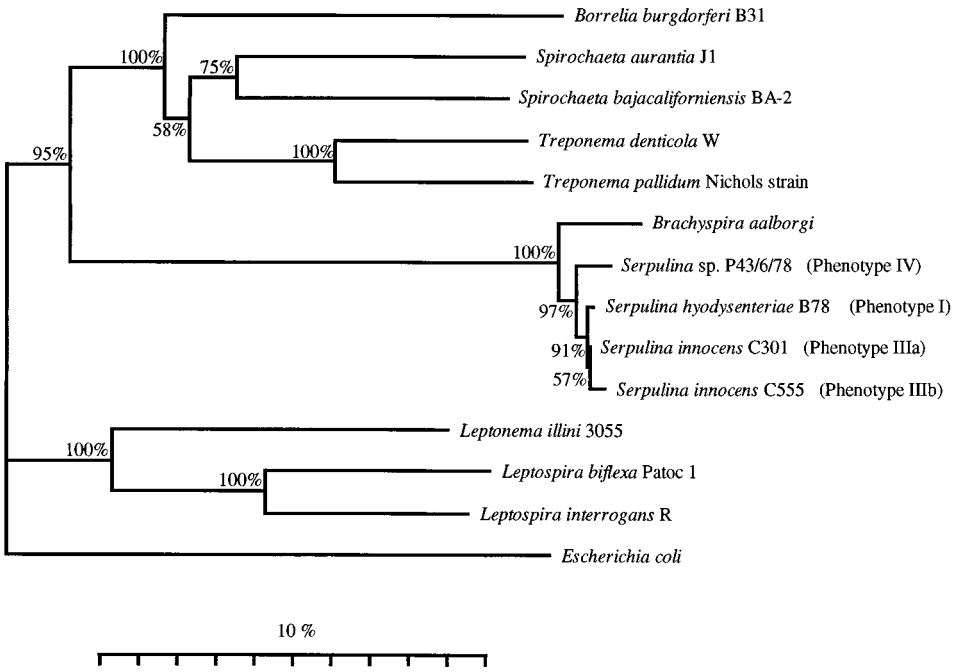

FIG. 2. Shown is the unrooted consensus distance tree derived from the similarity matrix corrected by the method of Jukes and Cantor (26) by using the neighbor-joining method of Saitou and Nei (50). The tree shows the distribution of a selection of biochemically different *Serpulina* strains according to Fellström and Gunnarsson (9) in relation to related spirochaetal genera with *E. coli* as the outgroup. Each bootstrap value is the percentage of times out of 500 replicates that a species or a strain to the right of the actual node occurred. The scale bar represents a 10% difference in nucleotide sequence. Roman numerals refer to the biochemical groups as indicated in Table 2.

quences (details are given in Table 4). Moreover, most strains showed strain-specific nucleotide positions; a compilation of all the variable nucleotide positions of the serpulinas is presented in Table 4. All individual 16S rRNA sequences of the *Serpulina* strains can be deduced from Fig. 1 in combination with Table 4.

Phylogeny of intestinal porcine spirochetes (*Serpulina* species). A sequence similarity matrix was calculated by the method of Jukes and Cantor (26) for the 16S rRNA sequences of selected members of the *Spirochaetales* family and intestinal spirochetes and of *E. coli* as an outgroup (not shown). The intestinal spirochetes were represented by sequence data from four different *Serpulina* phenotypes (*S. hyodysenteriae* B78^T [this study], *S. innocens* C301, *S. innocens* C555, and *Serpulina* sp. strain P43/6/78 for phenotypes I, IIIa, IIIb, and IV, respectively) and from the human isolate *B. aalborgi*. A dendrogram constructed from the similarity matrix by the neighbor-joining method is shown in Fig. 2, and the overall topology was consistent with previous reports (17, 45, 55). The robustness of the tree was supported by the relatively high bootstrap values and the conserved branching order when hypervariable regions were included in the analysis (data not shown). The intestinal spirochetes showed a distinct line of descent within the *Spirochaetales* family and a tight clustering with a ≥95.5% similarity (Table 5). *B. aalborgi* was found to branch earlier than the serpulinas in all trees constructed (Fig. 2). Furthermore, *B. aalborgi* was found to have 36 unique nucleotide positions compared with the serpulinas, and it was considered suitable as an outgroup in the second tree derived from 16S rRNA data of intestinal porcine spirochetes.

A sequence similarity matrix for *B. aalborgi* and 21 intestinal porcine spirochetes was determined. A dendrogram derived from the similarity data illustrating the phylogeny of the genus *Serpulina* is presented in Fig. 3. The majority of the *Serpulina* strains fall into three major clusters and two subclusters. Selected nodes have been furnished with the percentages from

TABLE 5. Similarity matrix of major *Serpulina* clusters observed by phylogenetic analysis[a]

Phylogenetic group	% Similarity to:					
	B. aalborgi	Cluster I/II	Subcluster IIIa	Subcluster IIIb/c	*S. innocens* B256^T	Cluster IV
B. aalborgi						
Cluster I/II	96.5–96.6	99.8–99.9				
Subcluster IIIa	96.2–96.4	99.1–99.4	99.6			
Subcluster IIIb/c	96.2–96.5	99.0–99.4	99.2–99.6	99.6–99.9		
S. innocens B256^T	96.5	99.5–99.6	99.3–99.4	99.3–99.5		
Cluster IV	95.7–95.8	98.4–98.7	98.1–98.3	97.8–98.1	98.3	99.9

[a] The values (intervals) show the lowest and the highest degrees of similarity of the strains belonging to a cluster compared with the strains of another cluster. The clusters are chosen according to the dendrogram in Fig. 3 except for cluster III, which is represented by *S. innocens* B256^T and its subclusters IIIa and IIIb/c. The similarity values are corrected for multiple base changes by the method of Jukes and Cantor (26). The similarity values underlined and shown in the diagonals refer to intracluster variations between the strains of the indicated clusters.

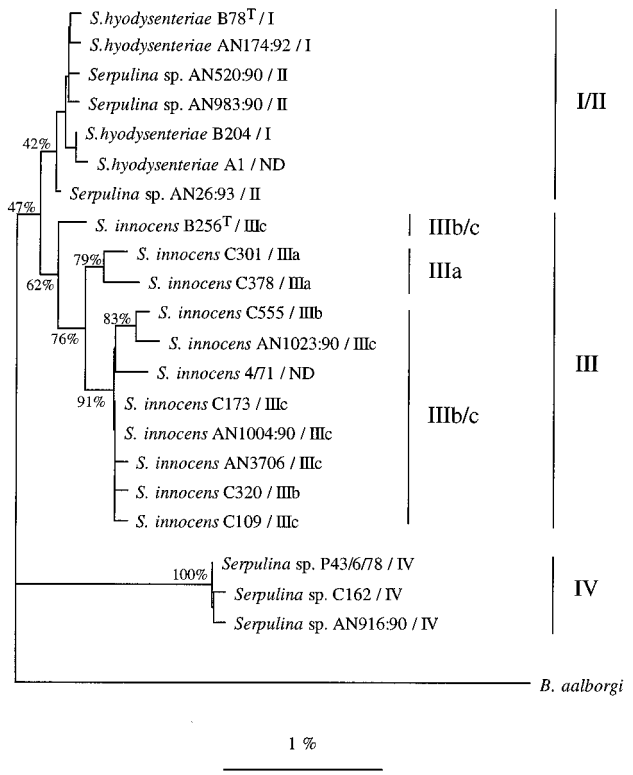

FIG. 3. Shown is the unrooted consensus distance tree based on 16S rRNA sequence comparison and derived from the similarity matrix corrected by the method of Jukes and Cantor (26) by the neighbor-joining method of Saitou and Nei (50), showing the relation between intestinal porcine spirochetes, biochemically grouped by Fellström and Gunnarsson (9), outgrouped to *B. aalborgi*. Each bootstrap value is the percentage of times out of 500 replicates that a cluster or a subcluster to the right of the actual node occurred. The scale bar represents a 1% difference in nucleotide sequence. Roman numerals refer to the biochemical groups as indicated in Table 2, and nd indicates that the biochemical profile was not determined in this study.

bootstrap analysis, which reflect the stability of the actual node. The numbers at the nodes denotes the percentage of times that the strains to the right of that node clustered together as shown by bootstrap analysis of the data set. All *Serpulina* strains showed high similarity values, and the matrix in Table 5 was therefore reduced to represent the minimum and the maximum similarities when the strains of one cluster are compared with strains of the other clusters and subclusters (Fig. 3). The values that are given on the diagonal are the intracluster strain similarity values for clusters that consist of two or more strains. Interestingly, the phylogeny based on 16S rRNA sequences was found to be in general agreement with the previously reported biochemical reaction scheme (9) in Table 2.

During the preparation of this work, six new 16S rRNA sequences from intestinal porcine spirochetes were deposited in GenBank. We have compared them with our data set by construction of phylogenetic trees without alteration of our findings (data not shown).

Signature nucleotide positions for the intestinal spirochetes are presented in Table 6. Signature nucleotides are positions within the sequence of *Serpulina* strains and *B. aalborgi* where the base, or base pair, differs from that found in other bacteria. Furthermore, the unique positions for the different phylogenetic clusters of intestinal porcine spirochetes are summarized in Table 7. The positions were extracted from Table 4 for a facilitated overview of their correlation to the different clusters. These positions can probably be utilized for the design of diagnostic methods based on the 16S rRNA gene.

Cluster I/II, including *S. hyodysenteriae.* Cluster I/II contains the strongly hemolytic species *S. hyodysenteriae*, the etiological agent of swine dysentery which belongs to biochemical group I. Furthermore, the cluster also comprises strains with weak hemolytic properties. The two phenotypes I and II (Table 2) are almost totally mixed up within the cluster (Fig. 3). Four strains of the species *S. hyodysenteriae* were subjected to 16S rRNA sequence analysis, and no discerning nucleotide pattern was found. Thus, it will not be possible to design a specific diagnostic system based on the 16S rRNA gene for the intestinal spirochetes of group I. This observation is consistent with a previous report (55). However, serpulinas of biochemical group I shared an A in position 569 (Fig. 1; Tables 4 and 7) with the weakly hemolytic *Serpulina* strains of biochemical group II. In other terms, position 569 seems to be a unique nucleotide position for indole-producing strains of the serpulinas since all the other strains have a G in this position. Previously, *Serpulina* strains belonging to group II were reported to be less virulent than *S. hyodysenteriae* (32) while pathogenicity among strains of this biochemical group has been suggested (2, 9). However, colitis was found impossible to induce by exposing susceptible pigs to these spirochetes (20, 39). Conversely, the occurrence of avirulent strains of *S. hyodysenteriae* has been reported (3, 21, 25, 31, 36). Since strains of the species *S. hyodysenteriae* are phenotypically different from group II only by a strong hemolysis (Table 2), they are not easily distinguished from the less virulent *Serpulina* strains of group II. These findings lead to the conclusion that a classification based entirely on pathogenicity will be misleading. Previously, Dupont et al. reported that the strong hemolysis could be irreversibly neutralized by $ZnSO_4$ treatment (8). It remains uncertain, though, if spirochetes of group II harbor the beta-hemolysin gene and fail to express the gene or if a dysfunctional beta-hemolysin is produced. A feature distinguishing between groups I and II was reported to be the ability of the group II spirochetes to ferment fructose (32). However, fructose fermentation between strains which are indole positive and weakly beta-hemolytic (2, 33) has been found to vary and is, therefore, not a unique biochemical feature for group II. A study based on MEE profiles by the method of Selander et al. (51) led to a proposal of two different species; *S. hyodysenteriae* and the weakly pathogenic strains were proposed to represent a new species, "*S. intermedius*" (32).

Genetic distances are sometimes overestimated by the MEE method. The comparison of 16S rRNA sequences performed in this study suggests that all strains within this cluster should be regarded only as variants of *S. hyodysenteriae* until further investigations (DNA-DNA reassociation studies, genome mapping, etc.) have been performed. This can also be justified from a clinical point of view, since both types are enteropathogenic (2, 9, 16).

Cluster III, including *S. innocens.* Cluster III is composed of two subclusters and contains strains generally recognized as *S. innocens*. All strains of the cluster III have four unique nucleotide positions in common (942, 974, 975, and 1200 [Table 7]). Only one exception from this rule was found, namely, the type strain *S. innocens* $B256^T$ of group IIIc, which shared only the G in position 1200 with all the other members of group III. This strain also formed an early branch of cluster III, and *S. innocens* $B256^T$ will, therefore, be discussed separately.

Subcluster IIIa comprised the two strains of *S. innocens* with the biochemical profile of group IIIa (Table 2). The strains from the biochemical group IIIa clustered together in all phylogenetic trees (Fig. 3), with bootstrap values of >70%. The

TABLE 6. Signature nucleotide positions of intestinal spirochetes

Position of base or base pair[a]	Base or base pair in:			
	Intestinal spirochetes[b]	Spirochetes[c]	Bacteria[d]	Exception(s)[e] (phylum, genus, or other phylogenetic group)
1 · 488 (36 · 548)	U · A	C · G	C · G	U · A (*Planctomyces*, *Myxobacteria*)
7 · 365 (42 · 400)	A · U	G · C	G · C	A · U (FCB [some], *Porphyromonas*)
404 (439)	A	U	U	A (*Deinococcus*, *Thermus*, *Geotoga*, *Petrotoga*)
405 (440)	A	U or A (*Lepto*)	C, G, or U	A (γ-Prp1 [some], Acetogenium assemblage *Deinococcus*, *Thermus*, *Gemmata*, *Geotoga*, *Petrotoga*)
595 · 691 (655 · 751)	G · C	A · U	A · U	G · C (*Thermotogales* group, *Fibrobacter*)
661 (721)	U	A or G	A, C, or G	U (*Mycoplasma* and related genera, *Campylobacter* [2], *Buchnera* [2], *Alicyclobacillus*)
664 (724)	A	G	G or U	A (FCB, *Epulopiscium*, *Flexistipes*, *Clostridium*, *Thermus*, *Peptostreptococcus*, *Planctomyces*)
727 (787)	C	A	A	C (*Propionibacterium* group, *Asteroleplasma*, *Auroebacterium*)
773 · 790 (833 · 853)	C · G	U · G	G · C, U · G	C · G (FCB [6], Prp1 [5])
806 (869)	C	A, G, or U	A, G, or U	C (Prp1 [8], *Fibrobacter* [2])
823 (886)	A	G	G	A (*Mycoplasma* and related genera, *Chlamydia*, *Erythrobacter*, *Holospora*)
932 (995)	A	C	C/U	A (FCB, *Myxobacteria*, *Chlorobium*)
933 · 983 (996 · 1045)	U · U	A · C	A · C, A · A	U · U (FCB [*Porphyromonas*, *Sphingobacterium*])
				U · A (FCB, *Polyangium*, *Asteroleplasma*)
1002 · 1132 (1064 · 1192)	G · U	G · C	G · C	G · U (*Cowdria*, *Ehrlichia*, *Coxiella*)

[a] Nucleotide positions according to the *Serpulina* consensus sequence in Fig. 1. *E. coli* numbering is shown in parentheses.
[b] Includes the human isolate *B. aalborgi.*
[c] Base or base pair commonly found in spirochetes (intestinal spirochetes not included). *Lepto*, *Leptospira* sp.
[d] Base or base pair commonly found in (eu)bacteria.
[e] Shown in brackets is the number of sequences in which the actual residue is found. Groups and subgroups are according to the Ribosome Database Project (38).
Abbreviations: FCB, *Flexibacter-Cytophaga-Bacteroides* phylum; Prp1, purple bacteria (*Proteobacteria*); γ, gamma subdivision (of purple bacteria).

biochemical subgroup IIIa differed from the other clusters and subclusters with respect to nucleotide position 163 of the 16S rRNA gene. Moreover, only four out of seven unique positions for group IIIb/c are shared (Table 7). A new species, "*S. murdochii*" sp. nov., has been proposed (30) for *Serpulina* strains with the biochemical profile of group IIIa. The proposal was due to a significant genetic distance as determined by MEE of 15 constitutive enzymes. In our analysis, the strains of IIIa show similarity values between 99.2 and 99.6% (Table 5) to other group III strains. A general rule is that similarity values of between 95 and 97% are expected for different species within a genus (6). DNA-DNA reassociation studies may, however, justify the classification into new species regardless of high-level 16S rRNA sequence similarities (13, 53). This fact implies that the *S. innocens* strains of the group IIIa should be regarded as merely a subspecies of *S. innocens*. The proposal to group the IIIa strains into a subspecies of *S. innocens* has to be regarded as tentative until further experiments have been performed since only two strains of the biochemical group IIIa were investigated.

Subcluster IIIb/c contained strains of *S. innocens* with the biochemical profiles IIIb and IIIc. Analysis was performed by using the 16S rRNA sequences from five strains of the former and six strains of the latter biochemical subgroup. The strains of group IIIb/c formed a phylogenetic subcluster with a relatively high bootstrap percentage of 91% (Fig. 3). Furthermore, the subcluster IIIb/c is also supported by the three unique nucleotide positions 587, 1197, and 1218 (Table 7). No clustering tendency or nucleotide pattern discerning between the subgroups IIIb and IIIc was found. Interestingly, serpulinas of the group IIIb that were isolated from the same herd showed identical 16S rRNA sequences, which indicates that sequence analysis of the 16S rRNA gene can be used for epidemiological studies. The strains C555, C562, and C567 isolated from the same herd were identical and therefore represented by the strain C555. Identity between the other two strains, C320 and C336, of subgroup IIIb was also found. These strains were isolated from another herd and represented by the strain C320. An explanation of this finding could be that only one strain of the phenotype IIIb dominates within a herd.

The six strains of the subgroup IIIc all had different 16S rRNA sequences. Interestingly, the strain C555 (identical to C562 and C567) from subgroup IIIb and the strain AN1023:90 of subgroup IIIc shared two unique nucleotide positions. These positions were both found to be cytidine residues, situated at positions 780 and 1139, and explains why these two strains clustered together in all trees. This deviation can be regarded as genetically distinct compared with the other strains in this subcluster. In other terms, the evolution occurred from an ancestor of either phenotype IIIb or IIIc, which had the two cytidine residues at position 780 and 1139. Thereafter, one of the strains changed its phenotypic characteristic from IIIc to IIIb (or vice versa). It is very likely that the evolution of the strain-specific nucleotide positions (Table 4) took place after the events of transition into the two cytidines. At position 225 (Table 4), the strains AN1004:90 and C109, which phenotypically both belong to the group IIIc, were found to have an insertion of a G. To achieve a proper alignment, a gap had to be introduced in this position of the other sequences. As a consequence, this position was omitted from the phylogenetic analysis. Thereby, identical 16S rRNA sequences were obtained for the strains AN1023:90 and C173 of the biochemical group IIIc. Despite this fact, we decided to include both AN1023:90 and C173 in Fig. 3 since they have different 16S rRNA sequences. It was not possible to resolve the two phenotypic variants IIIb and IIIc by 16S rRNA comparison, and IIIb strains should only be regarded as subspecies of *S. innocens*.

S. innocens B256^T (27, 54), with the phenotype of group IIIc (9), was unexpectedly found to form an early branch of the

cluster III instead of being a member of the subcluster IIIb/c. This strain formed a position intermediate between those of the group I/II and III spirochetes in all phylogenetic trees (Fig. 3). The intermediate position was also supported by nucleotide analysis (Tables 4 and 7), which showed that the strain B256^T shared only one (position 1200) out of four unique nucleotides associated with group III. Furthermore, only one out of three residues characteristic for the phenotypes IIIb and IIIc (position 587) was found in the 16S rRNA sequence of *S. innocens* B256^T. The conclusion of these findings is that *S. innocens* B256^T has an ancestor common to those of the strains of the IIIb/c subcluster and probably also those of the IIIa subcluster. This was the only strain in our study for which the biochemical classification did not correlate with the 16S rRNA-based phylogeny, and it is, therefore, not included in Table 7. Our data indicated that *S. innocens* B256^T is not a suitable type strain for biochemical group IIIc.

Cluster IV, including *Serpulina* sp. P43/6/78-like strains. Cluster IV comprised P43-like strains which had biochemical profiles identical to that of the reference strain, *Serpulina* strain P43/6/78. Cluster IV is the most distinct and tightly grouped cluster and comprised WBHIS, which causes spirochetal diarrhea (9, 57). The phylogenetic analysis resulted in a stable branch consistent with bootstrap values of 100%. Fifteen unique nucleotide positions compared with those of the other biochemical groups were found for the strains of this phenotype (Table 7). Three other positions at which only one strain from another phenotype group had a nucleotide in common with group IV were found (positions 178, 221, and 1405 in Table 4). Furthermore, the members of biochemical group IV could hydrolyze hippurate and lacked β-galactosidase activity. These features are not present in any of the other clusters and subclusters. 16S rRNA sequences identical to that of the strain P43/6/78 were obtained from four of the isolates (AN76:92, AN497:93, AN914:90, and C62), which indicates a very homogeneous group of intestinal spirochetes.

WBHIS of cluster IV (P43-like strains) have previously been reported to differ both genetically and phenotypically from *Serpulina* strains belonging to other clusters and subclusters (7, 9, 10, 19, 30, 32, 49, 57). Our results strongly support a significant difference between P43-like strains and serpulinas within the other phylogenetic clusters. The strains were more than 97.8% similar to other *Serpulina* species and strains (Table 5); nevertheless, we agree with previous reports that P43-like strains should be classified into a new species (7, 30, 32).

Secondary-structure modelling. Interestingly, the serpulinas of group IV have six consecutive uridines in a region between positions 175 and 182 (Fig. 1). Moreover, this segment also has a two-base deletion which results in only one of the eight positions (position 181) being shared with any of the other biochemical groups. Therefore, this region is suitable for diagnostic methods which rely on PCR (44). The secondary structure of the 16S rRNA molecule in the segment from base 170 to 183 (198 to 219 in *E. coli*) was reconstructed by comparative analysis (62). Figure 4 presents foldings of this region for *E. coli* (Fig. 4A), *Serpulina* strains of type I, II, and III (Fig. 4B), and two alternative secondary structures for the serpulinas of group IV (Fig. 4C and D). The folding pattern of P43-like intestinal spirochetes in Fig. 4C follows the consensus structure of this region seen for most other bacteria, having a loop region from position 170 to 173 (194 to 198 in *E. coli*). This results in a tetraloop sequence, UUUU, starting in position 176 (208 in *E. coli*), with U · U as the closing base pair. However, U · U is not a dominant or even a main alternative closing pair (63). Therefore, the loop in this region may be a hexamer of uridines. The structure in Fig. 4C also has a very

TABLE 7. Nucleotide positions of the 16S rRNA gene correlated to the biochemical characteristics of porcine intestinal spirochetes[a]

Biochemical group	Nucleotide at position:																				
	99 (139)	100 (140)	163 (—)	175 (199)	176 (200)	177 (208)	179 (210)	180 (211)	182 (218)	188 (224)	229 (264)	530 (590)	569 (629)	587 (647)	937 (999)	942 (—)	974 (—)	975 (1037)	979 (1041)	1060 (1122)	1082 (1143)	1197 (1257)	1200 (1260)	1218 (1278)	1376 (1436)	1396 (1456)
I/II	A	G	A	G	A	G	A	A	C	U	C	C	A	A	G	C	G	U	U	G	A	A	A	U	G
IIIa	A	G	C	G	A	G	A	C	C	U	C	C	G	A	C	G	A	U	U	G	A	U	G	U	G
IIIb/c	A	G	A	G	A	G	A	A	C	U	C	C	G	G	A	C	G	A	U	G	U	G	G	U	G
IV	G	A	A		U	U	U	U		C	U	U	G	A	U	G	C	G	A	C	A	A	A	C	A

[a] Nucleotide positions according to the *Serpulina* consensus sequence shown in Fig. 1. Numbers within parentheses are positions according to the *E. coli* numbering. Dashes within parentheses indicate that no position corresponding to that of *Serpulina* spp. is present in *E. coli*. Dots represent gaps introduced for proper alignment. The strain *S. innocens* B256^T of the biochemical group IIIc is excluded (see text for details).

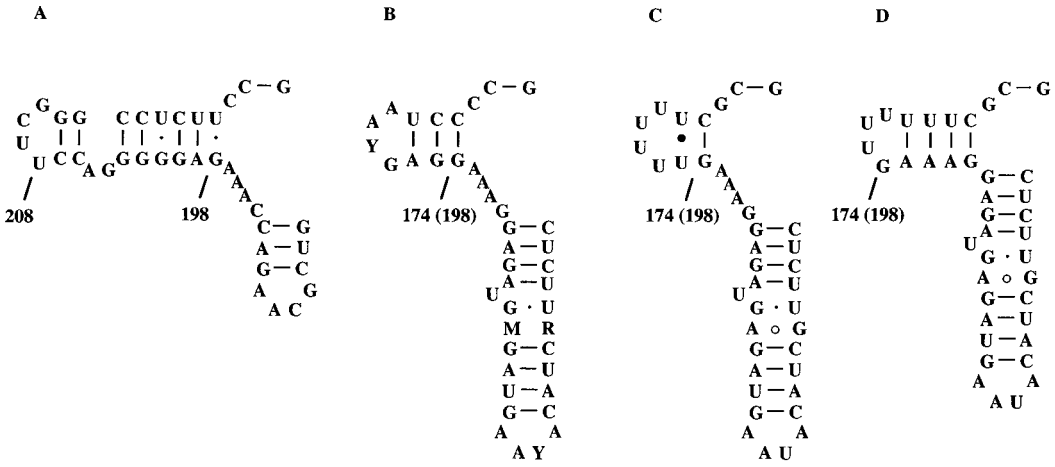

FIG. 4. Secondary structures of the tetraloop region beginning at position 208 (63) according to *E. coli* numbering. The folding of the *E. coli* segment (A) was obtained from the database (14), while the secondary structures of *Serpulina* (B through D) were predicted by the simple empirical approach of comparative sequence analysis. A probable structure of the corresponding region for the *Serpulina* strains belonging to clusters I/II and III is shown in panel B. Two folding patterns (C and D) are presented for the serpulinas of the biochemical group IV. The structure shown in panel C follows a general architecture found for other secondary structures. In panel D, the stretch from nucleotide position 170 to 173 (194 to 197 *E. coli*) has changed from a non-base-paired region into a hypothetical stem structure.

short stem. An alternative folding of this region is visualized in Fig. 4D. Here, the positions 170 to 173 (194 to 198 in *E. coli*) form a stem structure that leads to the tetraloop starting at position 174. The closing base pair, which was found to be of the canonical A · U type normally closing UUUA loops (63), is in this alternative folding of the 16S rRNA molecule closing the uridine-rich, GUUU, tetraloop. The secondary structure in Fig. 4D is considered to be more stable but needs further analysis for verification.

Concluding remarks. The genus *Serpulina* is commonly known as a group of intestinal spirochetes with the predilection to cause gastrointestinal disorders in animals and humans. This is an important group of bacteria in veterinary medicine, and methods for characterization are badly needed. In this work we present the phylogeny of intestinal porcine spirochetes based on sequence analysis of the 16S rRNA gene. We think that the unique nucleotide positions shown in Table 7 confirm the reliability of the defined clusters as a result of truly evolutionary events. Interestingly, the *Serpulina* strains formed clusters and subclusters according to the previously published biochemical classification scheme (9), shown in Table 2. The simple biochemical procedure is not only a valuable diagnostic tool, it also reflects the phylogeny of intestinal porcine spirochetes, namely, cluster I-II, cluster III (with subcluster IIIa and IIIb/c), and cluster IV.

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