Serpulina alvinipulli sp. nov., a new *Serpulina* species that is enteropathogenic for chickens

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Strain C1^T is an anaerobic spirochaete that causes intestinal disease in chickens. Multilocus enzyme electrophoresis analysis and 16S rRNA sequence comparisons have indicated that this spirochaete is a *Serpulina* strain. In these investigations, various phenotypic and genomic properties useful for establishing a taxonomic identity for strain C1^T were studied. As determined by electron microscopy, cells of the spirochaete measured 8–11 × 0·22–0·34 μm and had a typical spirochaete ultrastructure. Each cell had 22–30 flagella. C1^T cells formed weakly β-haemolytic colonies on trypticase soy agar plates containing 5% bovine blood. The spirochaete reached maximum population densities of 10^9 cells ml^{-1} with a 2–4 h population doubling time in brain heart infusion broth containing 10% calf serum (BHIS broth). C1^T cultures in BHIS broth were positive in tests for hippurate hydrolysis and negative for indole production. Glucosamine, *N*-acetylglucosamine, glucose, fructose, maltose and mannose were growth substrates for the spirochaete in heart infusion broth containing 7% calf serum (HS broth). During growth in HS broth beneath an O_2/N_2 (1:99) atmosphere, cells of the spirochaete consumed O_2 and glucose and produced H_2, CO_2, acetate, butyrate and ethanol. Strain C1^T DNA had a G+C content of 24·6 mol%. Based on DNA–DNA hybridization analyses, the DNA of strain C1^T exhibited 24–39% relative reassociation with DNA of *Serpulina hyodysenteriae, Serpulina innocens, Serpulina pilosicoli, Serpulina murdochii* and *Serpulina intermedia*. These results indicate that chicken spirochaete strain C1^T has many phenotypic properties common to *Serpulina* species and, based on DNA hybridization analysis, represents a unique *Serpulina* species. For this new species the name *Serpulina alvinipulli* is proposed, for which the type strain is C1^T (= ATCC 51933^T).

Keywords: *Serpulina alvinipulli* sp. nov., enteropathogenic bacteria, spirochaetes, chickens

INTRODUCTION

Unidentified spirochaetes have been implicated as transmissible, aetiological agents of intestinal disease in commercially raised chickens. Spirochaetes at high population densities have been observed colonizing the caecal mucosae of egg-laying hens with diarrhoea (Swayne *et al*., 1992; Trampel *et al*., 1994). Spirochaetes have been detected more frequently in faecal samples from chickens with intestinal disorders than in samples from healthy birds (Dwars *et al*., 1989).

Evidence for the enteropathogenic nature of a specific chicken spirochaete was provided by Davelaar *et al*. (1986), who demonstrated that the cells of a spirochaete isolated from a diarrhoeic hen both colonized the caeca and induced diarrhoea in chickens fed pure cultures of the spirochaete. The bacterium was weakly haemolytic, did not produce indole and had 26–30 periplasmic flagella per cell (13–15 inserted at each cell end). To our knowledge, this spirochaete has not been characterized further.

Swayne *et al*. (1995) described a weakly haemolytic spirochaete, strain C1^T (91-1207/C1^T), that biochemically and morphologically resembled the bacterium isolated by Davelaar *et al*. (1986). The spiro-

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Abbreviation: MEE, multilocus enzyme electrophoresis.

chaete was isolated from a chicken with diarrhoea and was enteropathogenic for both 1-d-old chicks and laying hens. Strain C1^T has a similar cell ultrastructure and shares certain phenotypical traits with the swine intestinal spirochaetes *Serpulina hyodysenteriae* and *Serpulina innocens*. Nevertheless, this chicken spirochaete could be distinguished from the swine spirochaetes based on 16S rDNA restriction patterns and multilocus enzyme electrophoretic (MEE) profiles (Swayne *et al.*, 1995). A 1416 bp region of the chicken spirochaete 16S rRNA gene had 98–99 % sequence similarity with corresponding regions of *S. hyodysenteriae* strain B78^T, *S. innocens* strain B256^T and *Serpulina pilosicoli* strain P43/6/78^T 16S rRNA genes, indicating that C1^T is properly considered as a *Serpulina* species (Stanton *et al.*, 1996).

The goal of the research described in this article was to determine physiological and genomic characteristics useful for the taxonomic identification of chicken spirochaete strain C1^T. The results of these studies indicate that strain C1^T represents a new *Serpulina* species. For this species, we propose the name *Serpulina alvinipulli* sp. nov. ('*Serpulina* of a diarrhoeic chicken') and use this species designation throughout this paper. A culture of the proposed type strain *S. alvinipulli* C1^T has been deposited with the American Type Culture Collection under the accession number ATCC 51933^T.

METHODS

Bacterial strain and culture conditions. *S. hyodysenteriae* strains B78^T and B204 were used as control strains in these studies. *S. alvinipulli* strain C1^T was originally provided by David Swayne, Ohio State University, and has been preserved in the NADC stock culture collection. Cells of these spirochaetes were cultured routinely in brain heart infusion broth containing 10 % calf serum (BHIS) beneath an initial culture atmosphere of O_2/N_2 (1:99). Culture media and incubation conditions followed those used for *Serpulina* (*Treponema*) *hyodysenteriae* (Stanton & Lebo, 1988). All investigations used cultures in the exponential phase of growth, that is when the culture OD_{620} was 0·9–1·0 (anaerobic culture tubes, 18 mm path length), equivalent to $2–5 \times 10^8$ cells ml^{-1} culture (direct cell counts, Petroff–Hausser counting chamber). Calf serum was heat-treated (56 °C for 30 min) prior to addition to culture media.

DNA analysis. DNA was extracted and purified from various *Serpulina* species by using a modified Marmur technique (Marmur, 1961; Stanton *et al.*, 1991). DNA relatedness was estimated by the S1 nuclease method (Crosa *et al.*, 1973) as described previously (Grimont *et al.*, 1980). The mol% G+C content of *S. alvinipulli* DNA was determined by thermal denaturation using DNA at a final concentration of 50 μg ml^{-1} in $0{\cdot}1 \times$ SSC (Stanton *et al.*, 1997). A Beckman DU 650 spectrophotometer equipped with a Peltier temperature-controlled cuvette holder was used to determine T_m values (Beckman Instruments). *S. hyodysenteriae* B78^T DNA (25 ± 1 mol%) was used as a control.

Phase-contrast and electron microscopy. *S. alvinipulli* C1^T cell suspensions for phase-contrast micrography were prepared and photographed as described for *S. pilosicoli* (Trott *et al.*, 1996a). For electron microscopy of negatively stained bacteria, cells of strain C1^T were harvested from 1 ml BHIS culture (approximately 2×10^8 bacteria, direct cell counts) by centrifugation (microfuge, 14000 ***g*** for 5 min). The pelleted bacteria were washed once by suspending them in 0·5 ml sterile distilled water and, after a second centrifugation step, the final cell pellet was resuspended in 0·1 ml water. A sample of the cell suspension was mixed with an equal volume of 2 % phosphotungstic acid (pH 7) and viewed by electron microscopy as described previously (Trott *et al.*, 1996a). Twenty cells were examined to determine their dimensions and the number of flagella attached at each end of the cell.

Biochemical tests. Biochemical characteristics were determined for three different BHIS cultures of strain C1^T. A standard test for hippurate hydrolysis (Smibert & Krieg, 1981) was used with the following modifications. *S. alvinipulli* C1^T cells were harvested from 0·1 ml BHIS culture by centrifugation in a microfuge (16000 ***g*** for 10 min), washed once in 0·5 ml TBS (Tris-buffered saline: 0·01 M Tris, 0·85 % NaCl, pH 7·4) and resuspended in 0·02 ml TBS. The final cell suspension was added to 0·4 ml 1 % sodium hippurate solution and incubated at 37 °C. After 30 min, 0·2 ml ninhydrin reagent was layered on top and the reaction mix incubated at 37 °C for 6–8 h. The aqueous phase became purplish-blue if cells gave a positive reaction (hydrolysed hippurate). Cultures of *S. pilosicoli* P43/6/78^T (hydrolysing hippurate) and *S. hyodysenteriae* B78^T (non-hydrolysing hippurate) were used as controls. Indole production was assayed by the Erlich indole test (Finegold & Martin, 1982) using *S. alvinipulli* C1^T cultures in BHIS broth ($3–5 \times 10^8$ cells ml^{-1}). *S. hyodysenteriae* B78^T and *S. innocens* B256^T cultures were used as positive and negative controls, respectively, for the indole reaction. The API AN-IDENT test system for various enzymic activities was used according to the manufacturer's instructions (bioMérieux Vitek).

Growth substrates. Methods for identifying growth substrates of *S. alvinipulli* strain C1^T followed those used for *S. hyodysenteriae* (Stanton & Lebo, 1988). A chemical compound was considered to support growth if *S. alvinipulli* final cell densities in medium containing the compound were more than twofold greater than cell densities in medium without added substrate. Final population densities were determined by direct cell counts (Petroff–Hausser counting chamber) after cultures reached maximum optical density readings (OD_{620}). HS (heart infusion plus serum) broth used in these studies contained 7 % (v/v) heat-treated calf serum. For testing starch as a growth substrate, the calf serum was heat treated a second time at 65 °C for 30 min to inactivate endogenous starch-hydrolysing activity.

Analysis of metabolic substrates and products. Metabolic products were determined for *S. alvinipulli* cells growing in 7 ml HS broth (heart infusion broth, 10 % serum, 0·2 % glucose) beneath an initial O_2/N_2 (1:99, by vol.) atmosphere. Calf serum used in the media for metabolic studies was heat treated as described above and was additionally heated at 65 °C for 30 min to inactivate endogenous, O_2-scavenging activities that interfered with assays of O_2 consumption by bacterial cultures. Methods and equipment for analysing volatile fatty acids and alcohols by GLC have been reported previously (Stanton & Lebo, 1988; Trott *et al.*, 1996a). Gases produced (H_2 and CO_2) and consumed (O_2) by the bacteria were identified and quantified by GC using samples of the culture atmosphere as described previously (Trott *et al.*, 1996a), except that argon was used as a carrier gas to detect H_2. To avoid introducing air (O_2), samples of culture atmospheres were taken through the stoppers of culture tubes placed within a Coy anaerobic chamber and by positive displacement (after injecting an equal volume of O_2-free water into the culture tube). Uninoculated culture tubes containing sterile media were incubated and used as controls for all assays.

RFLP-PCR analysis of 16S rDNA. A portion (approximately 558 bp) of the *rrs* gene of *S. alvinipulli* was amplified and analysed for restriction fragment length polymorphisms (RFLPs; Stanton *et al.*, 1997). The PCR amplification (forward primer, 5′-GGAAACGCCTCGGATACTGT-3′; reverse primer, 5′-CCTTCCTCCTACTTGAACGTA-3′) was specific for *Serpulina* species and *Brachyspira aalborgi* (Stanton *et al.*, 1997). The restriction enzymes *Sau*3A, *Taq*I, *Hin*fI, *Mbo*II and *Sph*I were used according to the manufacturer's directions. Restriction fragments were separated by electrophoresis (65 V for 2 h) on a 4% NuSieve (3:1) gel (FMC Bioproducts) in 1 × TAE buffer and visualized by ethidium bromide staining (Sambrook *et al.*, 1989; Stanton *et al.*, 1997). Fragment sizes were estimated by comparison with DNA fragments of known size (size range 36–2645 bp; D-15 DNA marker, Novex Technology).

RESULTS

DNA relatedness and G + C content

The estimated DNA relatedness between *S. alvinipulli* $C1^T$ DNA and the DNA of other *Serpulina* species ranged between 24 and 39% (Table 1), indicating that $C1^T$ is a distinct *Serpulina* species. In two determinations using the thermal denaturation method, the G + C content of *S. alvinipulli* $C1^T$ DNA was 24·6 mol%. In parallel determinations, *S. hyodysenteriae* $B78^T$ DNA and *E. coli* XL-1 DNA had 24·2 and 50·8 mol% G + C, respectively.

Cell morphology

By phase-contrast microscopy (Fig. 1), *S. alvinipulli* $C1^T$ cells had a mean length of 9·5 μm, slightly larger than *Serpulina hyodysenteriae* $B78^T$ cells (mean cell length 7 μm). When examined by electron microscopy (Fig. 2), $C1^T$ cells had a typical spirochaete ultrastructure with equal numbers of flagella attached near each end of the cells. Cells of the spirochaete had an outer sheath, helical protoplasmic cylinder and peri-

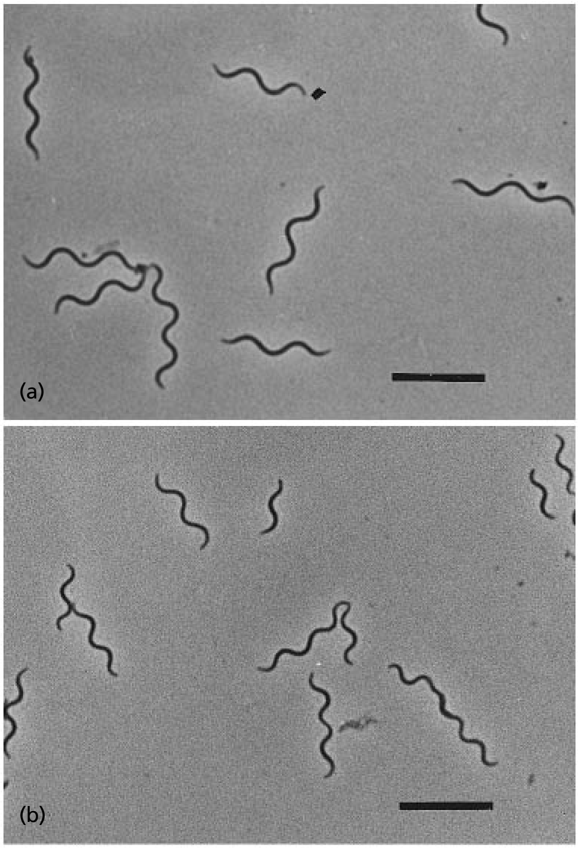

Fig. 1. (a) *S. alvinipulli* $C1^T$ cells. (b) *S. hyodysenteriae* $B78^T$ cells. Phase-contrast photomicrographs of wet-mount preparations. Bars, 10 μm.

Table 1. DNA–DNA relative reassociation between various *Serpulina* species

Relative reassociation values for $C1^T$ were determined in this study. Values for other *Serpulina* species were reported previously (Stanton *et al.*, 1997). Values in parentheses are ΔT_m.

Source of unlabelled DNA	Source of labelled DNA					
	$B78^T$	$B256^T$	WES-B	PWS/A^T	$56–150^T$	$C1^T$
S. hyodysenteriae $B78^T$	100		25	57 (7)	27	33
S. innocens $B256^T$	29	100	22	45 (12)	66 (7)	32
S. pilosicoli $P43/6/78^T$	21	23	78	28	22	
S. pilosicoli WES-B	28	29	100	26	28	24
S. intermedia PWS/A^T	68 (8)	44	30	100	24	39
S. murdochii $56\text{-}150^T$	37	64 (5)		37 (15)	100	35
S. alvinipulli $C1^T$	28	37	28	37	31	100

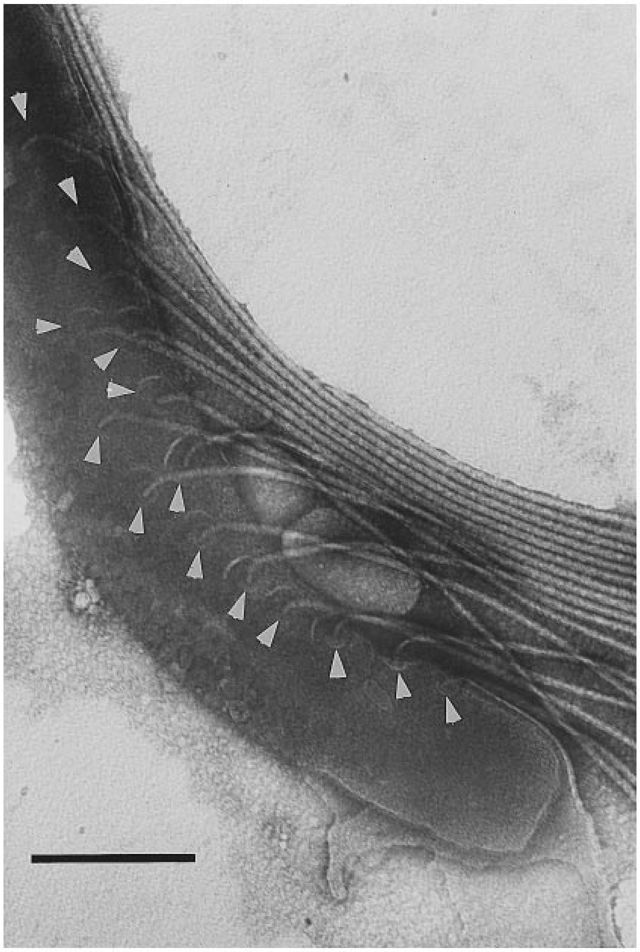

Fig. 2. Electron micrograph of *S. alvinipulli* C1^T cell negatively stained with PTA (2 %, pH 7·0). Disrupted outer sheath enables insertion sites of 15 periplasmic flagella to be seen (white arrowheads). Bar, 0·25 μm.

Table 2. Growth substrates of *S. alvinipulli* C1^T

The following compounds did not support detectable growth of *S. alvinipulli* C1^T: sucrose, D-galactose, D-trehalose, pyruvate, lactose, L-rhamnose, D-melibiose, D-fucose, L-fucose, D-raffinose, L-arabinose, D-ribose, D-xylose, *N*-acetyl-D-galactosamine, D-galactosamine, D-mannitol, glycerol, acetate, lactate, succinate, pebble-milled cellulose, pectin, soluble starch, hog gastric mucin, glycogen, hyaluronic acid, polygalacturonic acid, 50 × MEM amino acids glutathione.

Substrate	**Incubation period (h)**	**Max. population density ($10^{-8}\times$)**
None	36–48	1·3
D-Glucose	24–36	8·0
D-Mannose	36–48	10·0
D-Maltose	24–36	8·0
D-Glucosamine	36–48	9·5
N-Acetyl-D-glucosamine	36–48	9·5
D-Fructose	36–48	3·4

plasmic flagella that appeared to wrap around the protoplasmic cylinder and overlapped in the centre of the cell. The cells measured 8–11 × 0·22–0·34 μm and had 22–30 (mean = 24) flagella per cell with 11–15 attached at each end (Fig. 2). Similar flagellar numbers have been reported for *S. hyodysenteriae* cells (Stanton *et al.*, 1997). Previously, strain C1^T and *S. hyodysenteriae* B78^T cells were reported to have 16 flagella per cell (Swayne *et al.*, 1995). However, in those studies, cross-sections of the spirochaetes were examined by electron microscopy. We believe that more accurate estimates of flagellar numbers can be obtained using negatively stained bacteria and counting both flagella and flagellar attachment sites.

Culture characteristics

S. alvinipulli C1^T cultures were positive for hippurate hydrolysis. Control cultures of *S. hyodysenteriae* B78^T and *S. pilosicoli* P43/6/78^T were negative and positive for hippurate hydrolysis, respectively, as reported previously (Trott *et al.*, 1996a). C1^T cultures did not produce detectable amounts of indole. *S. hyodysenteriae* B78^T gave a positive indole reaction, and *S. innocens* B256^T gave a negative reaction. The population doubling time of C1^T cells in BHIS broth at 37 °C was 2–4 h. Cells of the spirochaete grew more rapidly in BHIS broth at either 39 °C or 42 °C than at 37 °C and did not grow at 32 or 47 °C. Initial culture atmospheres containing 5 % (v/v) or less O_2 did not prevent growth of C1^T. The minimum calf serum concentration supporting optimum growth (highest cell yields in shortest incubation times) in HS broth was 7 %, and this concentration was used in HS broth to determine growth substrates. *S. alvinipulli* C1^T cells used a limited number of tested carbohydrates as growth substrates (Table 2).

The results of the API AN-IDENT test kit for determining the biochemical properties of strain C1^T cultured in BHIS broth are given below in the species description. Results of tests with *S. hyodysenteriae* B204 agreed with those reported previously for strains of this species (Hunter & Wood, 1979; Fellström, 1995; Fellström *et al.*, 1995). Enzymic activities for *S. alvinipulli* C1^T cells were consistent with those reported previously for this strain, except for α-galactosidase (Swayne *et al.*, 1995). Variations in culture conditions may explain this difference.

Interestingly, the biochemical reactivities determined in the AN-IDENT colorimetric tests were not always consistent with growth substrate results of the spirochaete. *S. alvinipulli* C1^T gave a positive reaction for β-galactosidase yet was unable to use lactose for growth (Table 2). *S. alvinipulli* cells were negative in tests for α-

Table 3. Metabolic end products of growing cells of *S. alvinipulli* C1^T

For end product determinations, *S. alvinipulli* and *S. hyodysenteriae* cells were cultured beneath a 1% O_2 atmosphere (balance N_2) in HS broth containing 10% calf serum and 0·2% glucose. During growth, cells of both species consumed O_2 in the culture atmosphere [estimated to be 1–1·2 μmol O_2 (ml culture broth)$^{-1}$].

Product	**Yield (μmol ml^{-1} medium) with:**	
	S. alvinipulli **C1^T**	***S. hyodysenteriae*** **B204**
Acetate	16·8	11·8
Butyrate	0·8	2·9
Ethanol	2·2	TR
CO_2	19·4	22·8
H_2	22·8	32·0

TR, trace [$\leqslant$ 0·2 μmol (ml medium)$^{-1}$].

glucosidase, yet this spirochaete used maltose as a growth substrate (Table 2), suggesting the possible existence of an inducible mechanism for maltose metabolism.

Metabolic products

S. alvinipulli C1^T cells growing in HS broth beneath an atmosphere containing O_2/N_2 (1:99) consumed oxygen and glucose and produced H_2, CO_2, acetate, butyrate and ethanol (Table 3). Unlike *S. hyodysenteriae*, *S. alvinipulli* cells produced significant amounts [2·2 μmol (ml culture broth)$^{-1}$] of ethanol, and the ratio of H_2 to CO_2 produced by *S. alvinipulli* was closer to 1 (Table 3).

RFLP-PCR test

Based on RFLP-PCR analysis of an amplified product of the 16S rRNA gene, *S. alvinipulli* C1^T could be differentiated from *S. innocens* B256^T (*Hin*fI digests) *S. pilosicoli* P43/6/78^T (*Taq*I digest), *S. murdochii* 56–150^T (*Sau*3A and *Hin*fI digests) and from the human intestinal spirochaete *B. aalborgi* (*Mbo*II digest) (Table 4). The chicken spirochaete could not be differentiated from *S. hyodysenteriae* B78^T or *S. intermedia* PWSA^T using these restriction enzymes. Analyses of the predicted sequences of the 16S rDNA amplicons of *B. aalborgii* 513A^T and of the *Serpulina* species for sites of 420 restriction enzymes did not reveal any site unique to the C1^T sequence (Heiman, 1997).

DISCUSSION

Based on 16S rDNA sequence comparisons, MEE analysis in previous studies (Swayne *et al*., 1995; Stanton *et al*., 1996) and phenotypical characteristics determined in these studies, avian intestinal spirochaete strain C1^T is phylogenetically related to *Serpulina* species and should be considered as a *Serpulina* species. The DNA relatedness between strain C1^T and other *Serpulina* species was estimated to be 24–39% (Table 1). As these values are below the cut-off value of 70% DNA relatedness that has been recommended for phylogenetic definition of a species (Wayne *et al*., 1987), strain C1^T is properly considered as a new species of *Serpulina*. We propose the designation *Serpulina alvinipulli* (*Serpulina* of a diarrhoeic chicken) for this new species and

Table 4. Restriction enzyme digestion of 16S rDNA amplicons of *Serpulina* species and *B. aalborgi* 513A^T

A portion of the *rrs* gene was amplified from chromosomal DNA of each strain by PCR and digested using the indicated restriction enzyme (Stanton *et al*., 1997). Values in the table represent fragment sizes (bp) either observed directly after gel electrophoresis (> 75 bp) of the restriction digests or predicted (< 75 bp) based on the 16S rDNA sequences of the strains when the PCR amplification product was cut with a particular restriction enzyme. Fragments below 75 bp could not be detected after gel electrophoresis.

16S rDNA amplicon from	**Restriction fragments (bp)**			
	***Hin*fI**	***Sau*3A**	***Taq*I**	***Mbo*II**
S. hyodysenteriae B78^T	132, 426	558	43, 81, 208, 226	558
S. innocens B256^T	21, 111, 426	558	43, 81, 208, 226	558
S. pilosicoli P43/6/78^T	132, 426	558	43, 43, 183, 208	558
S. intermedia PWS/A^T	132, 426	558	43, 81, 208, 226	558
S. murdochii 56–150^T	21, 111, 426	187, 371	43, 81, 208, 226	558
S. alvinipulli C1^T	132, 426	558	43, 81, 208, 226	558
B. aalborgi 513A^T	132, 422	558	43, 81, 208, 226	85, 473

Table 5. Culture and biochemical characteristics of various *Serpulina* species and *Brachyspira aalborgi* 513A^T

Spirochaete characteristics in the table are based on analyses of *S. alvinipulli* C1^T in these studies and on results from previous studies of the other species (Hunter & Wood, 1979; Hovind-Hougen *et al.*, 1982; Fellström, 1995; Fellström *et al.*, 1995, 1997; McLaren *et al.*, 1997; Stanton *et al.*, 1997). α-gal, α-glu and β-glu: α-galactosidase, α-glucosidase and β-glucosidase, respectively. +/− indicates that some strains possess and other strains lack a particular enzyme activity. A unique 'signature' sequence of nucleotides was identified within the 16S rDNA of *S. pilosicoli* and has been used to design specific PCR tests for that spirochaete (Park *et al.*, 1996; Fellström *et al.*, 1997).

Species	Haemolysis	Indole production	Hippurate hydrolysis	Demonstrated pathogenicity (animal)	Flagella per cell	Enzyme activity:			16S rDNA signature sequence
						α-Gal	α-Glu	β-Glu	
S. hyodysenteriae	Strong	+	−	Yes (swine)	22–28	−	+/−	+	No
S. innocens	Weak	−	−	No	20–26	−	+/−	+	No
S. intermedia	Weak	+	−	No	24–28	−	+	+	No
S. pilosicoli	Weak	−	+	Yes (swine; 1-d-old chicken)	8–12	+/−	+/−	−	Yes
S. murdochii	Weak	−	−	No	22–26	−	−	+	No
S. alvinipulli	Weak	−	+	Yes (chickens)	22–30	−	−	+	No
B. aalborgi	Weak	?	?	No	8	−	−	−	No

recommend that strain C1^T be considered the type strain.

S. alvinipulli C1^T cells have a number of biochemical properties in common with other *Serpulina* species. *S. alvinipulli* is a carbohydrate-fermenting, anaerobic spirochaete that produces ethanol, acetate, butyrate, H_2 and CO_2 from glucose metabolism. Under a N_2/CO_2 (99:1) atmosphere, growing cells consume oxygen, a reaction probably mediated by NADH oxidase, as the *nox* gene has been detected in *S. alvinipulli* C1^T cells (Stanton *et al.*, 1995). Both *S. alvinipulli* and *S. hyodysenteriae* produce more H_2 than CO_2, although the ratio of H_2/CO_2 is lower for *S. alvinipulli* than for *S. hyodysenteriae* (Table 3) and for other *Serpulina* species (Trott *et al.*, 1996a; Stanton *et al.*, 1997).

S. alvinipulli is the first novel *Serpulina* species from chickens to be isolated and characterized. Recent studies have indicated that chickens are also hosts for *S. innocens* and *S. pilosicoli*, for the newly described species *S. intermedia* and *S. murdochii* and for intestinal spirochaetes in an MEE group (group d) that is likely to represent a new *Serpulina* species (Trott *et al.*, 1996b; McLaren *et al.*, 1997; Swayne & McLaren, 1997).

S. alvinipulli cultures have been demonstrated to cause intestinal disease when inoculated into chickens (Swayne *et al.*, 1995). *S. pilosicoli* and *S. intermedia* strains have been implicated as intestinal pathogens of chickens (Swayne & McLaren, 1997). Cells of *S. alvinipulli*, *S. intermedia*, *S. pilosicoli*, *S. innocens* and *S. murdochii* form weakly haemolytic colonies on trypticase soy/blood agar medium. For both taxonomic and clinical diagnostic purposes, there is a growing need for simple, reliable tests to differentiate these weakly haemolytic *Serpulina* species.

An RFLP-PCR test based on an amplified portion of the *rrs* gene has been used to differentiate porcine *Serpulina* species (Stanton *et al.*, 1997). In the present studies, *S. alvinipulli* C1^T could not be distinguished from *S. intermedia* PWS/A^T or *S. hyodysenteriae* B78^T by this test when the restriction enzymes *Hin*fI, *Sau*3A, *Taq*I or *Mbo*II were used. These results indicate that the RFLP-PCR procedure cannot be used by itself. The test may be useful in conjunction with other tests for differentiating *Serpulina* species.

Fellström *et al.* (1997) have proposed a method for organizing porcine intestinal spirochaetes into groups based on type of haemolysis, indole reaction, hippurate hydrolysis and whether or not the spirochaete is pathogenic. These groups roughly correspond to strains of *S. hyodysenteriae* (group I), *S. intermedia* (group II), *S. innocens* (group IIIb and c), *S. murdochii* (group IIIa) and *S. pilosicoli* (group IV) (Stanton *et al.*, 1997). Unfortunately, *S. alvinipulli* C1^T is indistinguishable from *S. pilosicoli* based on these four properties (Table 5), and both species have been isolated from chickens. Nevertheless, *S. alvinipulli* C1^T can be distinguished from *S. pilosicoli* on the basis of flagellar numbers per cell and a 'signature sequence' in the 16S rDNA of *S. pilosicoli* (Table 5). Culture and phenotypical characteristics useful for differentiating *S. alvinipulli* C1^T from the other *Serpulina* species are given in Table 5.

Knowledge of the epidemiology, incidence, economic impact and virulence aspects of intestinal spirochaete diseases is improving as pathogenic species are identified and methods for differentiating pathogenic and non-pathogenic spirochaetes are developed (Dwars *et al.*, 1989; Swayne & McLaren, 1997; van der Zeijst *et al.*, 1997). Methods for differentiating *Serpulina* species and, especially, for identifying weakly haemolytic, enteropathogenic spirochaetes will undoubtedly evolve as additional, diverse strains of each *Serpulina* species are isolated and characterized.

Description of *Serpulina alvinipulli* sp. nov.

Serpulina alvinipulli (al.vi.ni.púl.li. combination of L. gen. adj. *alvini* suffering from diarrhoea and L. gen. n. *pulli* of a chicken; *alvinipulli* of a diarrhoeic chicken, referring to the host animal from which the spirochaete was isolated.

Pathogenic. When inoculated by crop gavage, *S. alvinipulli* cells colonize the caeca of 1-d-old chicks and 14-month-old hens and induce mild lymphocytic typhlitis and diarrhoea. Cells grow by forming weakly haemolytic colonies on trypticase soy agar medium containing 5 % defibrinated bovine blood. *S. alvinipulli* C1 cells have a typical spirochaete ultrastructure, i.e. an outer sheath, helical protoplasmic cylinder and periplasmic flagella wrapped around the protoplasmic cylinder and inside the outer sheath. The cells measure 8–11 × 0·22–0·34 μm and have 22–30 (mean 24) flagella per cell (11–15 inserted at each cell end, with free ends overlapping in the middle of the cell). Growing cells beneath an atmosphere containing O_2/N_2 (1:99) consume oxygen. Cells possess the gene for NADH oxidase. Grows well (3–10 × 10^8 cells ml^{-1}, final population densities) in HS broth containing D-glucose, D-fructose, D-mannose, D-maltose, D-glucosamine or *N*-acetyl-D-glucosamine as growth substrates. Growth not detectable with these potential substrates: sucrose, D-galactose, D-trehalose, pyruvate, lactose, L-rhamnose, D-melibiose, D-fucose, L-fucose, D-raffinose, L-arabinose, D-xylose, D-ribose, D-galactosamine, *N*-acetyl-D-galactosamine, D-mannitol, glycerol, acetate, lactate, succinate, pebble-milled cellulose, soluble starch, pectin, hog gastric mucin, glycogen, hyaluronic acid, polygalacturonic acid, 50 × MEM amino acids or glutathione. *S. alvinipulli* $C1^T$ cells cultured in BHIS (brain heart infusion supplemented with 10 % heat-treated calf serum) broth give positive reactions in tests for hippurate hydrolysis, *β*-glucosidase, *β*-galactosidase, alkaline phosphatase, indoxyl acetate hydrolysis, arginine aminopeptidase, alanine aminopeptidase and glycine aminopeptidase; and negative reactions in tests for indole production, *α*-glucosidase, *α*-galactosidase, *N*-acetyl-glucosaminidase, *α*-arabinosidase, *α*-fucosidase, arginine metabolism, leucine aminopeptidase, proline aminopeptidase, pyroglutamic acid arylamidase, tyrosine aminopeptidase, histidine aminopeptidase, phenylalanine aminopeptidase and catalase. Gram-negative, catalase-negative, aerotolerant anaerobe. Optimum growth temperature, 39–42 °C. No growth at 32 or 47 °C. G + C content of the DNA is 24·6 mol % (T_m). Population doubling time in BHIS is 2–4 h. Requires serum for growth in BHIS broth. Shares high 16S rRNA sequence identity (98–99 %) with other *Serpulina* species (*S. hyodysenteriae*, *S. innocens*, *S. pilosicoli*, *S. intermedia* and *S. murdochii*). Shares 24–39 % DNA relatedness with the other *Serpulina* species based on DNA–DNA relative reassociation studies. Type strain: $C1^T$ (91-1207/$C1^T$) (= ATCC 51933^T).

NOTE ADDED IN PROOF

Ochiai *et al.* recently proposed the re-designation of *Serpulina* species as *Brachyspira* species (*Microbiol Immunol* **41**, 445–452) and published new name combinations in Validation List no. 64 (*Int J Syst Bacteriol* **48**, 327). In accordance with the rules of taxonomy, the bacterium characterized in this paper should be designated *Brachyspira alvinipulli* sp. nov.

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REFERENCES

Crosa, J. H., Brenner, D. J. & Falkow, S. (1973). Use of a single-strand specific nuclease for analysis of bacterial and plasmid deoxyribonucleic acid homo- and heteroduplexes. *J Bacteriol* **115**, 904–911.

Davelaar, F. G., Smit, H. F., Hovind-Hougen, K., Dwars, R. M. & van der Valk, P. C. (1986). Infectious typhlitis in chickens caused by spirochetes. *Avian Pathol* **15**, 247–258.

Dwars, R. M., Smit, H. F., Davelaar, F. G. & Van't Veer, W. (1989). Incidence of spirochaetal infections in cases of intestinal disorder in chickens. *Avian Pathol* **18**, 591–595.

Fellström, C. (1995). Phenotypical characterisation of intestinal spirochaetes isolated from pigs. *Res Vet Sci* **59**, 1–4.

Fellström, C., Pettersson, B., Uhlen, M., Gunnarsson, A. & Johansson, K.-E. (1995). Phylogeny of *Serpulina* based on sequence analyses of the 16S rRNA gene and comparison with a scheme involving biochemical classification. *Res Vet Sci* **59**, 5–9.

Fellström, C., Pettersson, B., Thomson, J., Gunnarsson, A., Persson, M. & Johansson, K.-E. (1997). Identification of *Serpulina* species associated with porcine colitis by biochemical analysis and PCR. *J Clin Microbiol* **35**, 462–467.

Finegold, S. M. & Martin, W. J. (1982). *Bailey and Scott's Diagnostic Microbiology*, p. 668. St Louis, Toronto & London: The C. V. Mosby Company.

Grimont, P. A. D., Popoff, M. Y., Grimont, F., Coynault, C. & Lemelin, M. (1980). Reproducibility and correlation study of three deoxyribonucleic acid hybridization procedures. *Curr Microbiol* **4**, 325–330.

Heiman, M. G. (1997). Webcutter, version 2, alpha release (http://www.firstmarket.com/cutter).

Hovind-Hougen, K., Birch-Andersen, A., Henrik-Nielsen, R., Orholm, M., Pedersen, J. O., Teglbjærg, P. S. & Thaysen, E. H. (1982). Intestinal spirochetosis: morphological characterization and cultivation of the spirochete *Brachyspira aalborgi* gen. nov., sp. nov. *J Clin Microbiol* **16**, 1127–1136.

Hunter, D. & Wood, T. (1979). An evaluation of the API ZYM system as a means of classifying spirochaetes associated with swine dysentery. *Vet Rec* **104**, 383–384.

McLaren, A. J., Trott, D. J., Swayne, D. E., Oxberry, S. L. & Hampson, D. J. (1997). Genetic and phenotypic characterization of intestinal spirochetes colonizing chickens, and allocation of known pathogenic isolates to three distinct genetic groups. *J Clin Microbiol* **35**, 412–417.

Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J Mol Biol* **3**, 208–218.

Park, N. Y., Chung, C. Y., McLaren, A. J., Atyeo, R. F. & Hampson, D. J. (1996). Polymerase chain reaction for identification of human and porcine spirochaetes recovered from cases of intestinal spirochaetosis. *FEMS Microbiol Lett* **125**, 225–229.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Smibert, R. M. & Krieg, N. R. (1981). General characterization. In *Manual of Methods for General Bacteriology*, pp. 409–443. Washington, DC: American Society for Microbiology.

Stanton, T. B. & Lebo, D. F. (1988). *Treponema hyodysenteriae* growth under various culture conditions. *Vet Microbiol* **18**, 177–190.

Stanton, T. B., Jensen, N. S., Casey, T. A., Tordoff, L. A., Dewhirst, F. E. & Paster, B. J. (1991). Reclassification of *Treponema hyodysenteriae* and *Treponema innocen*s in a new genus, *Serpula* gen. nov., as *Serpula hyodysenteriae* comb. nov. and *Serpula innocens* comb. nov. *Int J Syst Bacteriol* **41**, 50–58.

Stanton, T. B., Hanzelka, B. L. & Jensen, N. S. (1995). Survey of intestinal spirochaetes for NADH oxidase by gene probe and by enzyme assay. *Microb Ecol Health Dis* **8**, 93–100.

Stanton, T. B., Trott, D. J., Lee, J. I., McLaren, A. J., Hampson, D. J., Paster, B. J. & Jensen, N. S. (1996). Differentiation of intestinal spirochaetes by multilocus enzyme electrophoresis analysis and 16S rRNA sequence comparisons. *FEMS Microbiol Lett* **136**, 181–186.

Stanton, T. B., Fournié-Amazouz, E., Postic, D., Trott, D. J., Grimont, P. A. D., Baranton, G., Hampson, D. J. & Saint Girons, I. (1997). Recognition of two new species of intestinal spirochetes: *Serpulina intermedia* sp. nov. and *Serpulina murdochii* sp. nov. *Int J Syst Bacteriol* **47**, 1007–1012.

Swayne, D. E. & McLaren, A. J. (1997). Avian intestinal spirochaetes and avian intestinal spirochaetosis. In *Intestinal Spirochaetes in Domestic Animals and Humans*, pp. 267–300. Edited by D. J. Hampson & T. B. Stanton. Wallingford & New York: CAB International.

Swayne, D. E., Bermudez, A. J., Sagartz, J. E., Eaton, K. A., Monfort, J. D., Stoutenburg, J. W. & Hayes, J. R. (1992). Association of cecal spirochetes with pasty vents and dirty eggshells in layers. *Avian Dis* **36**, 776–781.

Swayne, D. E., Eaton, K. A., Stoutenburg, J., Trott, D. J., Hampson, D. J. & Jensen, N. S. (1995). Identification of a new intestinal spirochete with pathogenicity for chickens. *Infect Immun* **63**, 430–436.

Trampel, D. W., Jensen, N. S. & Hoffman, L. J. (1994). Cecal spirochetosis in commercial laying hens. *Avian Dis* **38**, 895–898.

Trott, D. J., Stanton, T. B., Jensen, N. S., Duhamel, G. E., Johnson, J. L. & Hampson, D. J. (1996a). *Serpulina pilosicoli* sp. nov., the agent of porcine intestinal spirochetosis. *Int J Syst Bacteriol* **46**, 206–215.

Trott, D. J., Atyeo, R. F. Lee, J. I., Swayne, D. E., Stoutenburg, J. W. & Hampson, D. J. (1996b). Genetic relatedness amongst intestinal spirochaetes isolated from rats and birds. *Lett Appl Microbiol* **23**, 431–436.

van der Zeijst, B. A. M. & ter Huurne, A. A. H. M. (1997). Molecular biology of intestinal spirochaetes. In *Intestinal Spiochaetes in Domestic Animals and Humans*, pp. 91–108. Edited by D. J. Hampson & T. B. Stanton. Wallingford & New York: CAB International.

Wayne, L. G., Brenner, D. G., Colwell, R. R. & 9 other authors (1987). International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.