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1 **Three unique groups of spirochetes isolated from digital dermatitis lesions in UK**  
2 **cattle.**

3

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5

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23 **Abstract**

24 Bovine digital dermatitis (BDD) is a severe infectious cause of lameness which  
25 has spread through dairy cattle populations worldwide, causing serious welfare and  
26 agricultural problems. Spirochetes are the main organisms implicated and have  
27 previously proven difficult to isolate. This study aimed to isolate and characterise the  
28 range of spirochetes associated with BDD in the UK. Twenty three spirochete isolates  
29 were obtained from 30 BDD lesions, which by 16S rRNA gene and *flaB2* gene analysis  
30 clustered within the genus *Treponema* as three phylogroups; groups 1 (*Treponema*  
31 *medium*/*Treponema vincentii*-like), 2 (*Treponema phagedenis*-like) and 3 (*Treponema*  
32 *denticola*/*Treponema putidum*-like). The treponemes displayed large genotypic and  
33 phenotypic diversity between phylogroups and differed from named treponeme species.  
34 A previously isolated contagious ovine digital dermatitis spirochete was located within  
35 one of the three phylogroups, group 3, and could also be identified within this group on  
36 the basis of phenotype testing, suggesting BDD and contagious ovine digital dermatitis  
37 may share the same aetiological agent. A strain isolated from a bovine interdigital  
38 dermatitis lesion, could be identified as part of BDD isolate group 2, suggesting bovine  
39 interdigital dermatitis and BDD may have the same causative agent. Two common  
40 enzyme activities, C4 esterase and C8 esterase lipase, were identified in all BDD  
41 associated treponemes suggesting common metabolic pathways for sharing this novel  
42 niche or even common virulence traits. Further studies are required to determine whether  
43 the three groups of novel treponemes are representative of new treponeme taxa and to  
44 delineate how they interact with bovine tissues to cause disease.

45 **Keywords:** Bovine digital dermatitis, *Treponema*, 16S rRNA gene, phenotyping

## 46 1. Introduction

47 Bovine digital dermatitis (BDD) is an ulcerative foot disease found in dairy cattle,  
48 initially reported in Italy in 1974 (Cheli and Mortellaro, 1974) and which has  
49 subsequently been identified worldwide. The main clinical feature of BDD is lameness  
50 resulting from a lesion immediately above the coronet between the heel bulbs (Blowey  
51 and Sharp, 1988). BDD results in large welfare and economic problems; hence  
52 prevention and treatment of this disease is of great importance (Demirkan et al., 2000;  
53 Read et al., 1992). The etiology of BDD has not yet been completely determined;  
54 however, the majority of evidence suggests involvement of spirochetes. Spirochetes have  
55 frequently been found in large numbers, deep inside BDD lesions (Blowey et al., 1992;  
56 Demirkan et al., 1998; Read et al., 1992) and molecular methods have further implicated  
57 and identified them as belonging to the genus *Treponema* (Choi et al., 1997; Demirkan et  
58 al., 1998).

59 The *Treponema* species are very difficult to maintain in culture, although some  
60 progress has been made in isolation from BDD lesions. Eight spirochetes were isolated  
61 from BDD lesions in the USA, with seven of these isolates forming a distinct phenotypic  
62 group (Walker et al., 1995). A further four USA BDD spirochetes were identified as  
63 similar to *Treponema phagedenis* (Trott et al., 2003). A spirochete isolated from a BDD  
64 lesion in the UK was identified as similar to the USA isolates (Demirkan et al., 2006),  
65 whilst a German BDD spirochete (*Treponema brennaborensis*) has been identified as  
66 quite different (Schrank et al., 1999). We deduced that there was a need for further  
67 isolation of BDD treponemes given the small number of isolations and that a previous  
68 molecular survey (Choi et al., 1997) suggested a more diverse treponemal community

69 than the above isolation data suggests. In the present study, we attempted to determine  
70 the range of spirochetes present in BDD lesions from a number of farms in the UK. We  
71 isolated and characterised 23 spirochete strains and compared against other relevant  
72 treponemes.

73

## 74 **2. Materials and Methods**

### 75 *2.1 Isolation and cultivation.*

76 Single biopsies were taken from 30 Holstein-Friesian cows with BDD from  
77 Merseyside (n=12), Cheshire (n=9), Shropshire (n=4) and Gloucestershire (n=5), UK,  
78 with a total of 9 farms included in the study (Tables 1 and 2). The BDD biopsies were  
79 collected during the housing period when BDD cases are reported to peak (Blowey and  
80 Sharp, 1988; Somers et al., 2005); in this case from October 2003 through to July 2004.  
81 All farms sampled were medium-sized commercial dairy operations (~200-300 cows)  
82 using cubicle type housing and had suffered recent outbreaks of BDD. All biopsies were  
83 from typical bovine digital dermatitis (BDD) lesions, except for one sample taken from a  
84 case of bovine interdigital dermatitis (IDD) (see Table 1). After cleaning the foot surface  
85 by brushing and washing with sterile PBS, a 3 mm punch biopsy was taken from the  
86 centre of the lesion, washed in sterile phosphate buffered saline (pH 7.4), placed in oral  
87 treponeme enrichment broth (OTEB: Anaerobe Systems, Morgan Hill, CA, USA)  
88 containing rifampicin (5 µg/ml) and enrofloxacin (5 µg/ml) and transferred to the  
89 laboratory. The biopsy was transferred into an anaerobic cabinet (85% N<sub>2</sub>, 10% H<sub>2</sub> and  
90 5% CO<sub>2</sub>, 36°C) and diced into small fragments (~1 mm<sup>3</sup>). The fragments were inoculated  
91 into fresh OTEB supplemented with 10% fetal calf serum (FCS), antibiotics as above and

92 incubated for 24 hours. Bacteria were then subcultured on fastidious anaerobe agar  
93 (FAA) plates (LabM, Bury, UK) supplemented with 5% defibrinated sheep blood, 10%  
94 FCS and antibiotics as above, for ~2 weeks. Single colonies were inoculated into growth  
95 media without antibiotics and subculture only repeated if cultures were not deemed pure  
96 by phase contrast microscopy and 16S rRNA gene sequencing. Isolates were stored at -  
97 80°C in growth medium containing 10% glycerol.

### 98 2.2 Reference strains.

99 Reference strains *Treponema vincentii* ATCC 35580, *T. vincentii* D2A2 and  
100 *Treponema medium* ATCC 700293<sup>T</sup> were a kind donation from Dr. H. E. Allison, School  
101 of Biological Sciences, University of Liverpool, UK. *Treponema phagedenis* CIP62.29  
102 was obtained from the Institut Pasteur, France.

### 103 2.3 Gene sequencing and phylogenetic analyses.

104 For isolation of genomic DNA, 7 ml of exponential phase spirochete culture was  
105 centrifuged (5000 X g, 10 min, 4°C in a bench-top centrifuge). DNA was then extracted  
106 from the cell pellet using Chelex-100 as previously described (Chua et al., 2005) and  
107 stored at -20°C.

108 *Treponeme* 16S rRNA gene and *flaB2* gene PCR were carried out as described  
109 previously (Demirkan et al., 2001). Amplified PCR products were sequenced  
110 commercially and complete genes assembled using the Staden sequence analysis package  
111 (Staden, 1996). Gene sequences were aligned using CLUSTALW (Thompson et al., 1994)  
112 and phylogenetic trees were calculated with the neighbor joining method (bootstrap  
113 values based on 1000 iterations) using nucleotide substitution rates calculated according  
114 to the Kimura two-parameter model implemented in MEGA2 (Kumar et al., 2001).

115 *2.4 Enzyme activities.*

116 Enzyme profiles for each strain were determined using the APIZYM system  
117 (bioMérieux, Lyon, France) according to the manufacturer's instructions, with each test  
118 in triplicate. Validation of APIZYM testing used *Treponema vincentii* ATCC 35580 (LA-  
119 1) as a test microorganism with identical enzyme activities identified to those previously  
120 reported (Schrack et al., 1999).

121 *2.5 Nucleotide sequence accession numbers.*

122 The complete list of Genbank accession numbers for 16S rRNA and *flaB2* gene  
123 sequences determined as part of this study are shown in Table 1 except: *T. phagedenis*  
124 CIP62.29 16S rRNA gene accession EF645248, *T. medium* ATCC 700293, *T. vincentii*  
125 ATCC 35580 and *T. vincentii* D2A2 *flaB2* gene sequences accession numbers EF061285,  
126 EF061286 and EF061287 respectively.

127

128 **3. Results**

129 *3.1 Spirochete isolation.*

130 Twenty three spirochete isolates were obtained from BDD lesions (Table 1). Most  
131 cultures required 2-3 repeated passages onto supplemented FAA plates before non-  
132 contaminated, single spirochete strain cultures were obtained. To identify optimal serum  
133 for growth in liquid medium, isolated strains were inoculated into OTEB containing 10%  
134 FCS or rabbit serum (RS). From the 23 spirochetes isolated, several strains exhibited  
135 better growth (substantial increase in total cell number) with RS (than FCS) as an OTEB  
136 growth supplement (Table 1). We failed to isolate spirochetes from every biopsy (Table



137 2), as might be expected with fastidious anaerobe isolations. However, two different  
138 strains were isolated from single lesion biopsies from two cows.

### 139 *3.2 Growth characteristics.*

140 Isolated strains inoculated onto FAA plates only demonstrated growth with blood and/or  
141 serum present suggesting that growth of the spirochetes was serum dependent. Optimal  
142 growth (substantial increase in colony size and number) was achieved with both serum  
143 and sheep blood FAA supplements present. The spirochetes could be divided into three  
144 groups (groups 1, 2 and 3) on the basis of growth characteristics in OTEB (Table 1) and  
145 on sheep blood FAA plates. When group 1 isolates were inoculated onto FAA plates  
146 supplemented with 10 % RS; nine days incubation produced translucent, circular, convex,  
147 colonies between 0.1 and 3 mm in size. After a further week, colonies were larger (2-3  
148 mm) with  $\beta$ -hemolysis underneath and a lawn had swarmed between colonies. A second  
149 group of isolates (group 2) demonstrated swarms of individual, circular colonies when  
150 inoculated onto FCS-supplemented FAA. After 14 days, colonies were typically a final  
151 size of 0.2-2 mm in size, convex, circular and translucent with no local hemolysis. When  
152 group 3 isolates were inoculated onto FCS-supplemented FAA, the agar surface was  
153 covered in translucent, circular, convex single colonies of ~0.2 mm after just 4 days,  
154 which after a further week grew to a final size of ~0.5-2 mm. After 3 weeks, distinct,  $\beta$ -  
155 hemolysis appeared underneath colonies which was more penetrative (visible from the  
156 under side of the plate) than that exhibited by group 1 isolates. Furthermore, irregular  
157 shaped projections appeared to have grown from many colonies. The projections had a  
158 characteristic metallic sheen and after a further week the protrusions had swarmed across  
159 the entire plate. An ovine spirochete strain G179 isolated in this laboratory previously

160 (Demirkan et al., 2001) demonstrated identical growth characteristics to the group 3  
161 isolates.

### 162 3.3 16S rRNA gene analysis.

163        Approximately 1420 bp of the 16S rRNA gene were sequenced for each of the 23  
164 isolates and sequences aligned against a large number of relevant 16S rRNA gene  
165 sequences (Fig. 1). On phylogenetic tree construction, the 23 isolates separated into three  
166 distinct phylogroups corresponding exactly to the three groups identified using growth  
167 characteristics. 16S rRNA gene sequence identity shared within each phylogroup was  
168 high with individual members of groups 1, 2 and 3 sharing a minimum sequence identity  
169 of 100%, 99.9% and 99.7% respectively. In contrast, phylogroups displayed a large  
170 amount of 16S rRNA gene sequence diversity between them, with group 1 and group 2  
171 being least similar with 90.1% identity whilst group 2 and group 3 were most similar  
172 although with only 92.3% sequence identity. Group 1 strains were most closely related to  
173 DDKL-13 (an uncultivated German BDD spirochete sequence) with 99.84% sequence  
174 identity, then *T. medium* ATCC 700293<sup>T</sup> (99.6%) and to a lesser extent *T. vincentii*  
175 ATCC 35580 (99.0%). All group 2 isolate 16S rRNA gene sequences were identical,  
176 except T167, and were identical to an uncultivated German BDD spirochete sequence  
177 DDKL-4, several previously reported American BDD isolates *Treponema* sp. 2-1498,  
178 *Treponema* sp. 3A, *Treponema* sp. 4A (Trott et al., 2003; Walker et al., 1995) and G356  
179 (*Treponema* sp. HW-2003) isolated from a BDD lesion in the UK (Demirkan et al.,  
180 2006). T167 contained a single nucleotide substitution difference from the identical  
181 sequences. The group 2 isolate 16S rRNA sequences were also identical to the closest  
182 designated species *T. phagedenis* CIP62.29 except for a single nucleotide substitution and

183 were related to a lesser extent to *Treponema pallidum* spp.. Group 3 isolates were closely  
184 related to a spirochete isolated in this laboratory from a case of contagious ovine digital  
185 dermatitis (CODD) (Demirkan et al., 2001) with ~99.8% sequence identity, then to  
186 bovine isolate *Treponema* sp. 1-9185MED (~99.0%) from America (Walker et al., 1995),  
187 then to *Treponema putidum* ATCC 700334<sup>T</sup> (~96.6%), *Treponema denticola* ATCC  
188 35405<sup>T</sup> (~95.7%) and then an uncultivated German BDD spirochete sequence DDKL-3  
189 (~94.05%).

#### 190 3.4 *flaB2* gene analysis.

191 Further phylogenetic analysis was undertaken by comparing *flaB2* across a  
192 number of isolates. Approximately 510 bp of the *flaB2* gene were sequenced in 14 BDD  
193 isolates and reference strains *T. medium* ATCC 700293<sup>T</sup>, *T. vincentii* ATCC 35580 and *T.*  
194 *vincentii* D2A2. Initially, the *flaB2* gene sequences were aligned against a number of  
195 reported treponeme *flaB2* sequences and trimmed to the shortest length. After  
196 phylogenetic tree construction (Fig. 2), the 14 isolates could be divided into the same  
197 three distinct groups already identified by growth characteristics and 16S rRNA gene  
198 analysis. Amongst group 1 isolates there was 100% nucleotide sequence identity in the  
199 *flaB2* gene. The strains were placed closest to *T. medium* ATCC 700293<sup>T</sup> and grouped to  
200 a lesser extent with *T. vincentii* strains (corresponding to 97.6% and 96.8% *flaB2*  
201 nucleotide sequence identity respectively). The group 2 isolates were located together in  
202 the *flaB2* phylogenetic tree as a group and two distinct subgroups could be identified  
203 within the phylogroup. Interestingly *T. phagedenis* Kazan 5 was situated in one subgroup  
204 with group 2 BDD isolates, as it had 100% nucleotide sequence identity with T119A and  
205 T320A. The second subgroup consisted of 4 isolates which shared between 97.0% and

206 99.4% nucleotide sequence identity. In agreement with 16S rRNA gene phylogenetic  
207 analysis, using *flaB2*, identified group 3 isolates as most closely related to a spirochete  
208 isolated from a CODD lesion. The CODD isolate actually clustered within the group 3  
209 isolates with the ovine isolate *flaB2* and strain T3552B *flaB2* sharing 99.4% sequence  
210 identity whilst T354A and T3552B *flaB2* only shared 97.8% sequence identity. The  
211 group 3 isolates are then shown to be most closely related to a *T. denticola* isolate (ATCC  
212 33521) sharing only ~80.9% sequence identity.

### 213 3.5 Enzyme activities.

214 The enzyme activities of the BDD associated spirochetes, compared with other  
215 relevant treponemes, showed (Table 3) that the three groups had specific enzyme patterns  
216 and were different to patterns for previously designated *Treponema* species. The enzyme  
217 profiles for each of the previously categorised three groups were identical within each  
218 group and different between groups, in good correlation with the genetic analyses and  
219 growth characteristics. Only C4 esterase and C8 esterase lipase enzyme activity was  
220 present in all three BDD treponeme groups. Interestingly, the previously reported ovine  
221 isolate which shared identical 16S rRNA gene sequence with the group 3 isolates also  
222 shared an identical enzyme profile with the group 3 isolates.

## 223 4. Discussion

224 This study has isolated and characterised a large panel of spirochetes associated  
225 with BDD in dairy cattle. This has been a significant advance as studies of this emerging  
226 and spreading disease have been previously severely hampered by the difficulties in  
227 isolating and maintaining these organisms *in vitro*. Although previous reports have  
228 implicated BDD as a polyspirochetal infection (Choi et al., 1997; Moter et al., 1998) only

229 on two (of 21) occasions were we able to isolate more than one spirochete phylotype  
230 from a single BDD lesion; although this might be expected given the fastidious nature of  
231 these anaerobes. The BDD spirochetes' 16S rRNA gene sequences identified the isolates  
232 as all belonging to the genus *Treponema*, in agreement with previously reported  
233 phylogenetic analyses (Choi et al., 1997; Demirkan et al., 1998). From five different  
234 spirochete 16S rRNA gene fragment sequences previously identified in BDD lesions  
235 (Choi et al., 1997), three of the gene fragments clustered with the 16S rRNA gene  
236 sequences of the strains isolated here. DDKL-3, DDKL-4 and DDKL-13, were reported  
237 by the authors to be most similar to *T. denticola*, *T. phagedenis* and *T. vincentii*  
238 respectively on the basis of 16S rRNA gene sequence similarity. In this study, the isolates  
239 showed similar relationships; with the group 1, 2 and 3 isolates sharing high 16S rRNA  
240 gene sequence similarities to *T. medium/T. vincentii*, *T. phagedenis* and *T. denticola/T.*  
241 *putidum* respectively, all isolated from human tissues (Chan et al., 1993; Smirbert, 1984;  
242 Umemoto et al., 1997; Wallace et al., 1967; Wyss et al., 2004). The reason only three of  
243 five potential phylogroups identified as populating BDD lesions are isolated here may be  
244 the result of culture bias given the fastidious nature of treponemes.

245         The results presented here clearly indicate that there is heterogeneity between  
246 these isolated BDD associated treponemes and that they generally fall into one of three  
247 well defined groups. It is intriguing that, by each of the means of characterisation used in  
248 this study, they divided into these groups and that each assay provided the same  
249 clustering, be it growth characteristics, gene sequences or enzyme activity patterns. Given  
250 the large number of similarities within groups and the differences to currently designated  
251 taxa, we believe further studies are required to delineate whether these BDD associated

252 treponemes represent new taxa. Whilst the group 3 isolates could be proposed as a new  
253 species as they are within the 97% 16S rRNA sequence identity limit to closest relative  
254 (Stackebrandt and Goebel, 1994), the group 1 and 2 isolates have nearly identical 16S  
255 rRNA gene sequences to closest relatives suggesting that DNA:DNA hybridisations and  
256 further phenotypic studies are required before it can be determined whether they  
257 represent novel taxa. Certainly the taxonomic status of the group 2, *T. phagedenis*-like  
258 treponemes have been discussed before without final taxonomic proposition (Trott et al.,  
259 2003) and such taxonomy is deterred as *T. phagedenis* itself is not currently in J.  
260 Euzéby's list of valid bacterial names (Euzéby, 1997).

261       The ovine spirochete isolate clustered with the group 3 isolates in 16S rRNA gene  
262 and *flaB2* phylogenetic trees, and also lay within this group of isolates on the basis of  
263 growth characteristics and enzyme activities. This is in agreement with previous work  
264 from this laboratory proposing that BDD and CODD may share a common etiological  
265 agent (Dhawi et al., 2005). Whilst the majority of strains were isolated from typical BDD  
266 lesions, strain G187 was isolated from an IDD lesion, a different clinical manifestation of  
267 the bovine foot. Strain G187 was identical to other group 2 BDD isolates on the basis of  
268 genotypic and phenotypic analysis suggesting that BDD and IDD share such treponemes  
269 as common etiological agents, as reported previously (Walker et al., 1995). All group 1  
270 isolates were from a single farm (Table 1) suggesting this group may not be present in all  
271 BDD lesions or that culture bias has prevented subsequent isolation. All treponemes  
272 isolated from BDD lesions to date, including the three groups isolated here and the quite  
273 different *T. brennaborensis*, have C4 esterase and C8 esterase lipase enzyme activity.  
274 Further investigation is required to determine if these enzyme activities represent

275 common metabolic pathways required for sharing this novel niche or maybe even  
276 common virulence traits.

277         The three phylogroups are related to quite different treponeme species (Fig. 1),  
278 with group 1 and 3 isolates related to treponemes involved in human periodontal disease  
279 (Asai et al., 2002; Wyss et al., 2004) and group 2 isolates related to a human commensal  
280 treponeme from the human urogenital tract (Wallace et al., 1967). A fluorescence *in situ*  
281 hybridization (FISH) study has suggested that the *T. phagedenis*-like and *T. medium*-like  
282 spirochetes were found deep inside lesions whilst *T. denticola*-like spirochetes were only  
283 found in the superficial layers (Moter et al., 1998). As the group 2, *T. phagedenis*-like  
284 isolates have been identified in BDD lesions from UK, USA and Germany (Choi et al.,  
285 1997; Demirkan et al., 2006; Walker et al., 1995) and are most commonly isolated; the  
286 largest proportion of recent BDD research has been centred on them with some  
287 evidence of pathogenic potential obtained (Elliott et al., 2007; Zuerner et al., 2007).  
288 However, as the group 1 and 3 isolates are most closely related to human periodontal  
289 disease treponemes and in this study are reported to produce  $\beta$ -hemolysis, a virulence  
290 characteristic of pathogenic spirochetes (Hyatt et al., 1994; Lee et al., 2002); the  
291 importance of these 2 groups of treponemes may be currently underestimated. Hence,  
292 given the fastidious nature of treponemes, a large molecular investigation (e.g. using  
293 PCR) is needed to further delineate the relationship of the three phylogroups and further  
294 treponemes with BDD more effectively. Also, whilst disease has been experimentally  
295 transmitted from foot to foot using lesion material (Read and Walker, 1996), experiments  
296 using single isolates/mixes of the BDD treponemes should be attempted to try and fulfil

297 Koch's postulates. Hopefully, a combination of the aforementioned studies would finally  
298 identify the role that each of these treponeme phylotypes plays in BDD lesions.

299

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303 career of the late Tony Hart, a microbiologist of considerable international stature who  
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403 **Tables:**

404 TABLE 1. Spirochetes isolated from BDD lesions in this study.

Strain <sup>a</sup>	Isolation date	Location	Serum <sup>b</sup>	Group <sup>c</sup>	16S rRNA gene accession No.	<i>flaB2</i> gene accession No. <sup>d</sup>
T184	6/10/03	Merseyside, Farm 1, cow 1	RS (9)	1	AY387410	-
T19	7/10/03	Merseyside, Farm 1, cow 2	RS (9)	1	EF061249	EF061271
T54	7/10/03	Merseyside, Farm 1, cow 3	RS (9)	1	EF061250	EF061272
T56	7/10/03	Merseyside, Farm 1, cow 4	RS (9)	1	EF061251	EF061274
T18A	7/10/03	Merseyside, Farm 1, cow 5	RS (9)	1	EF061252	EF061273
T18B	7/10/03	Merseyside, Farm 1, cow 5	FCS (4)	3	EF061270	-
T167	8/12/03	Cheshire, Farm 1, cow 1	FCS (7)	2	EF061253	EF061279
T257	12/1/04	Cheshire, Farm 2, cow 1	FCS (7)	2	EF061257	EF061280
T296A	12/1/04	Cheshire, Farm 2, cow 2	FCS (7)	2	EF061258	-
T354A	12/1/04	Cheshire, Farm 2, cow 3	FCS (4)	3	EF061267	EF061283
T354B	12/1/04	Cheshire, Farm 2, cow 3	FCS (7)	2	EF061259	-
T136	4/2/04	Shropshire, Farm 1, cow 1	FCS (7)	2	EF061255	EF061282
T119A	4/2/04	Shropshire, Farm 1, cow 2	FCS (7)	2	EF061256	EF061275
T52A	4/2/04	Shropshire, Farm 1, cow 3	FCS (7)	2	EF061254	EF061281
T320A	24/2/04	Merseyside, Farm 2, cow 1	FCS (7)	2	EF061261	EF061276
T380A	24/2/04	Merseyside, Farm 2, cow 2	FCS (7)	2	EF061262	-
T2721A	24/2/04	Merseyside, Farm 2, cow 3	FCS (7)	2	EF061260	EF061277
T3552B	24/2/04	Merseyside, Farm 2, cow 4	FCS (4)	3	EF061268	EF061284
W35	17/3/04	Cheshire, Farm 3, cow 1	FCS (7)	2	EF061264	-
G819CB	7/5/04	Gloucestershire, Farm 1, cow 1	FCS (4)	3	EF061269	-
G187	12/5/04	Gloucestershire, Farm 2, cow 1	FCS (7)	2	EF061266	-
G169A	13/5/04	Gloucestershire, Farm 3, cow 1	FCS (7)	2	EF061265	EF061278
T323C	20/7/04	Merseyside, Farm 2, cow 5	FCS (7)	2	EF061263	-

405 <sup>a</sup> All strains were isolated from typical bovine digital dermatitis (BDD) lesions, except  
 406 G187 which was isolated from a case of bovine interdigital dermatitis (IDD).

407 <sup>b</sup> Preferred OTEB serum supplement for optimal growth is described (as determined by  
 408 total cell numbers). The number of days for optimal growth is shown in brackets.

409 <sup>c</sup> Group designation according to growth characteristics.

410 <sup>d</sup> A dash indicates that the sequence was not determined.

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412 TABLE 2. Spirochete isolations compared with the number of biopsies sampled.

Location	Cows sampled <sup>a</sup>	Spirochetes isolated	Cows yielding more than one isolate <sup>b</sup>
Merseyside, Farm 1	6	6	cow 5 (2)
Merseyside, Farm 2	6	5	-
Cheshire, Farm 1	2	1	-
Cheshire, Farm 2	5	4	cow 3 (2)
Cheshire, Farm 3	2	1	-
Shropshire, Farm 1	4	3	-
Gloucestershire, Farm 1	2	1	-
Gloucestershire, Farm 2	1	1	-
Gloucestershire, Farm 3	2	1	-

413 <sup>a</sup> A single biopsy was taken from each cow.

414 <sup>b</sup> The total number of spirochetes obtained is indicated in brackets.

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421 TABLE 3. Enzyme activities of the 23 bovine spirochetes compared with other relevant  
422 treponemes.

Species/Group <sup>a</sup>	Strain	Enzyme Activity <sup>b</sup>																		
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
<i>T. brennaborensis</i> <sup>c</sup>	DSM 12168 <sup>T</sup>	+	+	+	-	-	-	-	-	-	+	+	-	+	-	+	-	+	-	-
<i>T. medium</i> <sup>d</sup>	ATCC 700293 <sup>T</sup>	+	+	+	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-
<i>T. vincentii</i> <sup>c</sup>	ATCC 35580	-	-	-	-	+	-	-	-	-	+	+	-	+	-	-	-	+	-	-
<i>T. phagedenis</i> <sup>e</sup>	Reiter	-	-	-	-	-	-	-	-	-	+	-	-	+	+	-	-	+	-	-
<i>T. putidum</i> <sup>f</sup>	ATCC 700334 <sup>T</sup>	+	+	+	-	+	-	-	+	+	+	+	+	+	-	+	+	-	-	-
<i>T. denticola</i> <sup>f</sup>	ATCC 35405 <sup>T</sup>	-	+	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-
<b>Group 1</b>	<b>5 isolates</b>	+	+	+	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-
<b>Group 2</b>	<b>14 isolates</b>	+	+	+	-	-	-	-	-	-	+	+	-	+	+	-	-	+	-	+
<b>Group 3</b>	<b>4 isolates</b>	-	+	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-
Ovine spirochete <sup>d</sup>	G179	-	+	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-
Bovine isolates <sup>g</sup>	7 isolates	+	+	+	-	-	-	-	-	-	+	+	-	+	+	-	-	+	-	-
Bovine isolate <sup>g</sup>	1-9185 MED	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-

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424 <sup>a</sup> Nearest species designated relatives (according to 16S rRNA gene sequence identity)  
425 are shown above the bovine treponemes and previously reported bovine and ovine  
426 isolates are shown below.

427 <sup>b</sup>As determined by the APIZYM system. Enzymes tested: 1, alkaline phosphatase; 2, C4  
428 esterase; 3, C8 esterase lipase; 4, C14 lipase; 5, leucine arylamidase; 6, valine  
429 arylamidase; 7, cystine arylamidase; 8, trypsin; 9, chymotrypsin; 10, acid phosphatase;  
430 11, naphtholphosphohydrolase; 12,  $\alpha$ -galactosidase; 13,  $\beta$ -galactosidase; 14,  $\beta$ -  
431 glucuronidase; 15,  $\alpha$ -glucosidase; 16,  $\beta$ -glucosidase; 17, N-acetyl-  $\beta$ -glucosaminidase;  
432 18,  $\alpha$ -mannosidase; 19,  $\alpha$ -fucosidase.

433 <sup>c</sup> APIZYM results have been previously reported (Schrank et al., 1999).

434 <sup>d</sup> determined in this study.

435 <sup>e</sup> APIZYM results have been previously reported (Dettori et al., 1995).



436 <sup>f</sup> APIZYM results have been previously reported (Wyss et al., 2004).

437 <sup>g</sup> APIZYM results have been previously reported (Walker et al., 1995).

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438 **Figure Captions:**

439 FIG. 1. Phylogenetic tree based on 16S rRNA gene sequence comparison over ~1420  
440 aligned bases showing relationship between strains isolated here (bold type) and related  
441 16S rRNA gene sequences. Bootstrap confidence levels are shown as percentages of  
442 nodes and only values above 40% are shown. Accession numbers are shown next to each  
443 strain/16S rRNA gene fragment clone in brackets.

444 \* Previously reported 16S rRNA gene sequences from Bovine Digital Dermatitis lesions.

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461 FIG. 2. Phylogenetic tree based on *flaB2* gene sequence comparison over 510 aligned  
462 bases showing relationship of bovine spirochetes isolated in this study (bold type) with  
463 other reported treponeme sequences. Accession numbers are shown next to each strain in  
464 brackets. Bootstrap confidence levels are shown as percentages of nodes and only values  
465 above 40% are shown.

466 \*Oral treponeme *flaB2* genes sequenced as part of this study.

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