Spirochetes from Digital Dermatitis Lesions in Cattle Are Closely Related to Treponemes Associated with Human Periodontitis

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Digital dermatitis (DD), first described in 1974 by Cheli and Mortellaro (R. Cheli and C. Mortellaro, p. 208–213, in *Proceedings of the 8th International Conference on Diseases of Cattle*, 1974), is a major problem in dairy cows and beef cattle causing significant economic losses worldwide. Lesions are typically found at the volar skin proximal to the heel bulbs. Microscopic examination of biopsies or touch preparations of these lesions revealed a variety of different bacterial morphotypes including significant numbers of spirochetes which often represent the predominant morphotype. We used comparative 16S rRNA sequence analysis to determine the diversity and phylogeny of these hitherto unclassified DD spirochetes. Results indicate that those lesions looked at so far contained at least five spirochetal phylotypes, all clustering within the genus *Treponema*. Phylotype DDKL-4 was nearly identical (99.4% similarity) to that of a nonpathogenic human treponeme, *T. phagedenis*. Two phylotypes DDKL-3 and DDKL-13 were closely related to those from treponemes commonly found in human periodontitis lesions, i.e., *T. denticola* and *T. vincentii*, exhibiting 95 and 98% similarity, respectively. The other two phylotypes, DDKL-12 and DDKL-20, had no close relatives to any cultivable treponemal species but clustered to previously described group IV oral treponemes. Preliminary analysis using in situ hybridization with fluorescently labeled oligonucleotide probes against smears from DD biopsies revealed that from all lesions analyzed so far, *T. denticola*-like spirochetes were detected in the highest proportion of all spirochetal morphotypes.

Digital dermatitis (DD), first described by Cheli and Mortellaro (6), is an acute or chronic ulcerative epidermitis in cattle that most commonly affects the skin immediately above the coronet between the heel bulbs (4). It is characterized clinically by an erosion of the superficial layers of the epidermis due to the loss of keratin, epithelial hyperplasia and hypertrophy, pain and swelling at the diseased sites, and a typical foul odor. Early lesions often show granulomatous strawberry-like ulcerations, whereas older lesions exhibit a grayish-brown color. DD usually leads to lameness and to a significant decrease in body weight and milk production (4). The disease is found with an incidence varying from 5 to 60% and a prevalence of 2 to 30%, rising to 80% in some herds and causing substantial economic loss in cattle dairies and the meat industry worldwide (38).

Microscopic analysis of specimens taken from DD lesions revealed a variety of different bacterial morphotypes, including gram-negative rods and spirochetes. Spirochetes are often found in great numbers not only in superficial lesions but also in deeper layers of the dermis (5, 11, 25). The etiology of DD is not yet established. However, the presence of high numbers of bacteria, including spirochetes, which apparently invade deeper tissues as well as successful antibiotic therapy strongly suggests a role of bacteria in the etiology of the disease (25). So far only a few organisms belonging to the genera *Prevotella*, *Porphyromonas*, *Fusobacterium*, and *Bacteroides* have been isolated (23). A recent paper reported the isolation of two groups of spirochetes with characteristics most consistent with the genus *Treponema* (36). However, the exact chemotaxonomic or

phylogenetic classification of these organisms has not yet been accomplished.

To facilitate further studies on the etiology of DD and to establish the role of spirochetes, we decided to identify DD spirochetes by comparative 16S rRNA sequence analysis. Phylogenetic analysis based on 16S rRNA sequence comparison is well established and has been used successfully to identify yet uncultured pathogenic organisms (26) and endosymbionts (1, 24) or to describe the microbial diversity of complex microbial ecosystems such as mixed infections (8) and environmental microbiota (2, 33, 37). The advantages of using this approach are obvious. It reliably allows a phylogenetically relevant classification, the design of rRNA-targeted oligonucleotide probes or primers for amplification or hybridization assays, and hence a thorough population analysis by fluorescence in situ hybridization (FISH). We therefore established a representative 16S rRNA gene library by cloning in vitro-amplified almost full-length 16S rRNA genes of DD spirochetes into *Escherichia coli*. Comparative sequence analysis of recombinant clones enabled us to describe five treponemal phylotypes, some of which were closely related to culturable oral treponemes commonly found in human periodontitis. Preliminary in situ hybridization analysis using fluorescently labeled phylotype-specific oligonucleotide probes revealed a differential distribution of the appropriate organisms in biopsies taken from several DD lesions.

MATERIALS AND METHODS

Light microscopy. Biopsies (1 by 1 cm) from eight infected dairy cows (Land Brandenburg, Germany) were taken from the plantar sites of bulbs that showed the typical clinical appearance of DD. Biopsies from four cows were pooled and processed for DNA preparation while biopsies from another four cows were used for FISH (see below). Touch preparations of all biopsies were examined by dark-field microscopy for the presence of motile spirochetes or were analyzed by light microscopy after Gram staining. For histologic examination, part of the tissue was fixed in buffered 5% formaldehyde solution, dehydrated, and embedded in paraffin wax. Sections were stained with hematoxylin-eosin (H&E).

Electron microscopy. For scanning electron microscopy, sections of the biopsy material were fixed according to the OTOTO method (17), dried with Peldri II

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(Ted Pella, Redding, Calif.), spattered with 5 nm of gold-palladium, and examined on a Philips EM 400 scanning electron microscope.

For transmission electron microscopy, tissue blocks (1 by 1 mm) were fixed in a solution consisting of 2.5% glutaraldehyde diluted in 1.65% $K_2Cr_2O_7$ at 4°C for 1 h. After postfixation in 1.5% osmium tetroxide, specimens were negatively stained with 1% uranyl acetate, dehydrated, and embedded in Epon 812 (Serva, Heidelberg, Germany). Ultrathin sections (60 nm) were placed onto collodion-coated copper grids, stained with lead citrate, and examined with a Philips EM 400 transmission electron microscope.

DNA preparation. For DNA isolation, DD biopsies from four dairy cows were pooled, minced with a scalpel, suspended in an appropriate volume of lysis buffer (500 mM Tris-HCl, pH 9.0, 20 mM EDTA, 10 mM NaCl, 1% sodium dodecyl sulfate [SDS]), and incubated at room temperature for 2 h. Proteinase K (final concentration of 200 µg/ml; Boehringer, Mannheim, Germany) was added, and incubation was continued at 37°C overnight. All further steps were performed as previously described (8).

PCR amplification and cloning. Amplification of spirochete 16S rRNA genes (16S rDNA) was performed by using the spirochete-specific forward primer RE-TREP (5'-ccg aat tgg tgg aca ac GT[T/C] TTA AGC ATG CAA GTC-3'; corresponding base positions 46 to 63 in *E. coli* 16S rRNA) and the universal primer RE-RTU1500 (5'-ccc ggg atc caa gct TG[T/C] TAC CTT GTT ACG ACT T-3'; corresponding base positions 1492 to 1509 in *E. coli* 16S rRNA). Underlined lowercase sequences at the 5' end represent the *EcoRI*, *SalI* or *XmaI*, *BamHI*, and *HindIII* restriction sites for RE-TREP or RE-RTU1500, respectively. Amplification was done in final volume of 100 µl by adding 1 µg of DNA, 200 µM deoxynucleoside triphosphates, buffer (50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 9.0), 2.5 U of *Taq* polymerase (Gibco BRL, Eggenstein, Germany), and 0.3 µM each primer. After the addition of mineral oil, PCR was performed in an OmniGene thermal cycler (Hybaid, Teddington, England); DNA was first denatured at 93°C for 2 min followed by 30 cycles of 93, 55, and 72°C for 1 min at each temperature. The PCR products were purified by agarose electrophoresis and eluted by using the QIAquick gel elution kit (Qiagen, Hilden, Germany). The cloning of amplified 16S rDNA was performed as previously described (8).

Plasmid DNA preparation and sequence analysis. Plasmid DNAs were isolated from 3-ml overnight cultures of *E. coli* recombinants as previously described (32). The final volume of plasmid DNA was 50 µl in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). The sequences of inserts (16S rDNA) were obtained by using the SequiTherm cycle sequencing kit (BioZym, Oldendorf, Germany) and a LI-COR automated sequencer (model 4000; MWG-Biotech, Ebersberg, Germany) according to the manufacturer's protocols. The sequencing primers used were M13 sequencing primers 40 (5'-GTT TTC CCA GTC ACG AC-3') and 24 (5'-AAC AGC TAT GAC CAT G-3') and universal eubacterial primers (19, 39).

Phylogenetic analysis. 16S rDNA sequences were compared with all sequence entries retrievable from current databases (EMBL and GenBank) and unpublished spirochetal sequences (7, 10) by using the sequence analysis program Husar 4.0 (DKFZ, Heidelberg, Germany). For construction of evolutionary trees we used version 3.0 of the TREECON software package (35). A single distance matrix was calculated from about 580 bases (positions 109 to 677 in *E. coli* 16S rRNA). All positions were included. Multiple base changes at single positions were corrected by the method of Jukes and Cantor (16). Dendrograms were constructed by the neighbor-joining method of Saitou and Nei (31). The sequences were also examined for chimeric sequences by using the program CHECK_CHIMERA (20).

Phylo-type-specific rRNA-based oligonucleotide probes. Specific oligonucleotide probes DDK3 (5'-CCC TTA TTC ACA TGA TTA CCG T-3', corresponding to position 481 to 502 in *E. coli* 16S rRNA), DDK4 (5'-ACA GTC TCG CTT CAC TTT G TA G-3', corresponding to position 1246 to 1267 in *E. coli* 16S rRNA), and Tre I (5'-ACG CAA GCT CAT CCT CAA G-3', corresponding to position 220 to 238 in *E. coli* 16S rRNA) complementary to 16S rRNA clone sequences (see Results) were designed and used to identify appropriate phylo-types by dot blot or in situ hybridization. The universal primer (RTU3, 5'-G[T/A] TTA CCG CGG C[G/T]G CTG-3', corresponding to position 519 to 536 in *E. coli* 16S rRNA) was used for dot blot hybridization screening of the recombinants.

Dot blot hybridization. Oligonucleotide probes were labeled with digoxigenin by using the DIG oligonucleotide 3'-end labeling kit (Boehringer, Mannheim, Germany) according to the manufacturer's instructions. Heat-denatured (5 min at 100°C) plasmid DNAs (2 µl) with spirochetal rDNA inserts were spotted onto positively charged nylon membranes (Hybond; Amersham, Braunschweig, Germany) and immobilized by UV irradiation at 254 nm for 3 min. Prehybridization was done in hybridization buffer (5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 1% blocking reagent, 0.1% N-laurylsarcosine, 0.1% SDS) at the appropriate hybridization temperatures for 1 h (see below). Hybridization was performed for 1 h in the same buffer after addition of DIG-labeled oligonucleotide (50 pmol). Thereafter, membranes were washed twice in washing buffer (5× SSC, 0.2% SDS) at hybridization temperature for 15 min each. The hybridization temperatures for the probes RTU3, DDK3, DDK4, and Tre I were 54, 55, 59, and 58°C, respectively. Bound DIG-labeled oligonucleotides were visualized by chemiluminescence using lumigen CSPD according to the manufacturer's instructions (Boehringer, Mannheim, Germany).

FISH. For in situ hybridization, bacteria from skin biopsy specimens were released by shaking of the minced tissues in prerduced Hardy broth medium (15) for 1 h. Bacteria were pelleted by centrifugation (45,000 rpm, 10 min at 4°C, Heraeus Labofuge 400) and washed once in cold phosphate-buffered saline (PBS). Fixation and preparation of cell smears were performed as described previously (13), except that the final fixation step in methanol and formaldehyde was omitted. Briefly, bacteria were fixed with 3.7% formaldehyde in PBS and stored at -20°C. For in situ hybridization, 2-µl aliquots were spotted onto glass slides with 12 separate wells coated with 0.01% $CrKSO_4$ and 0.1% gelatin. The slides were air dried and dehydrated in 50, 80, and 99% ethanol for 3 min each.

Oligonucleotides were enzymatically labeled at their 3' ends with Cy3 (Indo-carbocyanin)-dUTP (Amersham, Braunschweig, Germany) or fluorescein-ddUTP (Boehringer, Mannheim, Germany) by using terminal transferase according to the digoxigenin-labeling protocols (Boehringer, Mannheim, Germany).

An aliquot (10 µl) of hybridization solution (0.9 M NaCl, 20 mM Tris-HCl, pH 7.2, 0.01% SDS) containing 50 ng of the appropriate oligonucleotide probe was applied to each well of the slides and incubated in a humid chamber in the dark at 46°C for 2.5 h. The stringency was adjusted by adding formamide in different concentrations to the hybridization solution. The slides were rinsed with distilled water, air dried, and examined under oil immersion with a 100× Neofluar objective on a Zeiss Axioskop (Jena, Germany) equipped with a high pressure mercury bulb (HBO 50) and filter sets 487915 (BP 546/12, FT580, LP590) and 487909 (BP 450-490, FT 510, LP 515). Photomicrographs were taken on a Kodak Ektachrome HC 400 film.

Nucleotide sequence accession numbers. The EMBL accession numbers for the reference spirochetal 16S rRNA sequences used in this study are as follows: *Treponema phagedenis*, M57739; *Treponema denticola*, M71236; *Spirochaeta* sp., M71240; *Treponema pallidum*, M88726; *Treponema bryantii*, M57737; *Treponema pectinovorum*, M71237; *Treponema saccharophilum*, M71238; *Treponema succinifacience*, M57738; *Treponema* sp. M59294; *Spirochaeta isovaleric*, M88720; *Spirochaeta aurantia*, M57740; *Borrelia burgdorferi*, L36160; *Borrelia hermsii*, M60968; *Borrelia anserina*, M72397; *Brachyspira aalborgi*, Z22781; *Serpulina innocens*, M57744; *Serpulina hyodysenteriae*, M57742; *Leptonema illini*, Z21632; *Leptospira interrogans*, X17547; *Leptospira biflexa*, Z12821; *Escherichia coli*, J01859. The 16S ribosomal RNA sequences determined in this study are available from the EMBL, GenBank, and DDBJ nucleotide sequence databases under accession numbers Y08893 for DDKL-3, Y08894 for DDKL-4, Y08895 for DDKL-12, Y08896 for DDKL-13, and Y08897 for DDKL-20.

RESULTS

Morphology and histology of DD lesions. Biopsies were taken from diseased cows showing characteristic wet, exudative lesions as depicted in Fig. 1A. The ulcers were characterized by a dark red proliferative area surrounded by a white epithelial margin extending from the skin to the horn of the heel between two claws. H&E-stained sections revealed histologic features of an acute inflammation characterized by necrosis of the superficial layers, hyper- or parakeratosis, and spongiotic changes. In advanced lesions epidermal layers were completely absent with dermal papillae reaching to the surface thus producing the typical granulomatous appearance. Capillaries were hyperemic with enlarged nuclei of the endothelial cells. Occasionally local hemorrhages occurred. The periphery of the papillary blood vessels was characterized by an infiltrate consisting of mainly polymorphonuclear leukocytes with lower numbers of macrophages or lymphocytes and only a few plasma cells (Fig. 1B). Electron microscopy revealed huge numbers of spirochetes that resided between or inside necrotic cells in epidermal vacuoles that resulted from keratolytic activity (Fig. 1C).

Bacteriological findings. Gram-stained touch preparations of fresh biopsies revealed numerous bacterial morphotypes such as long filaments or short, encapsulated gram-negative bacilli and high numbers of spirochetes (Fig. 2A). Scanning electron microscopy identified at least two spirochetal morphotypes with diameters of 0.27 and 0.17 µm, respectively (Fig. 2B).

Phylogenetic analysis and design of specific hybridization probes. For phylogenetic analysis nearly the complete spirochetal 16S rRNA genes (corresponding to positions 46 to 1509 in *E. coli* 16S rRNA) were amplified from fresh biopsy samples by using a spirochete-specific and a universal primer. To determine the extent of diversity among spirochetes found within

FIG. 1. Clinicopathological appearance of DD lesions. (A) Typical lesions of DD with circumscribed superficial ulcerations of the skin proximal to the bovine coronary band. (B) Histopathology (H&E staining) of DD lesions showing necrotic superficial epidermal layers and inflammatory infiltration of the dermal papillae. (C) Representative transmission electron micrograph showing numerous spirochetes in an advanced stage of a typical DD lesion.

FIG. 2. Microscopic appearance of DD spirochetes. (A) Gram-stained touch preparation of infected tissue. (B) Scanning electron micrograph showing different spirochetal morphotypes (1 and 2) with diameters of 0.17 and 0.27 μm , respectively.

DD biopsies collected from four animals, inserts of 20 randomly chosen recombinant clones were sequenced. Comparative sequence analysis showed that all recombinants except one that exhibited *E. coli* 16S rDNA sequences contained spirochetal rDNA inserts. Phylogenetic analysis revealed five phylotypes: DDKL-3, DDKL-4, DDKL-12, DDKL-13, and DDKL-20. For DDKL-3, DDKL-4, and DDKL-13 nearly complete 16S rDNA sequences (1,313 to 1,428 bases) were determined. A phylogenetic tree (Fig. 3) based upon the comparison of 580 bases (corresponding to positions 109 to 677 in *E. coli* 16S rRNA) shows the relationship of DD spirochetes to known spirochetes. Phylotype DDKL-4, represented by four clones, was nearly identical (99.4% similarity) to the sequence of a non-pathogenic human treponeme, *T. phagedenis* strain K. DDKL-3 was represented by nine clones and showed sequence similarity of approximately 95% to an oral treponeme, *T. denticola*. Phylotype DDKL-13 represented by only one clone revealed 98% similarity to an oral treponeme, *T. vincentii*, and was nearly identical (2-base-pair mismatch) to phylotype sequence NZM3142 previously detected in a subgingival plaque sample of a patient with destructive periodontitis (8). DDKL-12 and DDKL-20, represented by one clone each, had no close relatives among known treponemes, but they clustered to previously described group IV treponemes (8). Two clones that contained chimeric sequences composed of DDKL-3 and DDKL-4 were identified by the program CHECK_CHIMERA (20).

Whole-cell in situ hybridization for direct identification of treponeme phylotypes from DD lesions. To identify and enumerate DD treponemes characterized by comparative sequence analysis directly in material from DD lesions, smears of another four biopsy specimens were processed for in situ hybridization. Oligonucleotide probes DDK4 specific for both *T. phagedenis* and phylotype DDKL-4, DDK3 specific for phylotype DDKL-3 (a close relative of *T. denticola*), and Tre I for phylotype DDKL-13 were used after labeling with Cy3 or fluorescein. The probe Tre I was initially designed for detection of all group I oral treponemes including *T. vincentii* (22). The specificity of each oligonucleotide was tested by dot blot hybridization using recombinant plasmid DNA as a target. As shown in Fig. 4, probe DDK4 and Tre I detected larger treponemes, while DDK3 identified smaller cells with tight spirals. In all samples examined so far the DDKL-3 (*T. denticola*-like) phylotype was detected most frequently, whereas other phylotypes were present in only small numbers. Only a minor pro-

FIG. 3. Phylogeny of DD spirochetes. 16S rRNA-based phylogenetic tree showing the relationships of DD spirochetes (DDKL-) to other spirochetes, using *Escherichia coli* as an outgroup. The tree was based on 100 bootstrap samplings of partial sequences (about 580 bases) by using the Jukes & Cantor (16) correction and the neighbor-joining (31) method. The scale bar represents 10% difference in nucleotide sequences as determined by taking the sum of the length of the horizontal lines connecting two sequences.

FIG. 4. FISH. Different treponemal phylotypes were detected by using fluorescently labeled oligonucleotide probes. Phase-contrast (left) and epifluorescence photomicrographs (right) of the same field are shown for each preparation. (A) Probe Tre 1, labeled with fluorescein-ddUTP, was used for the detection of *T. vincentii*-related organisms. Probes DDK4 and DDK3 labeled with Cy3-dCTP were used for the detection of *T. phagedenis* (panel B) or *T. denticola*-like (panel C) phylotypes, respectively.

portion of spirochetal morphotypes were not detected by the three probes used.

DISCUSSION

As shown by light and electron microscopy, spirochetes were present in large numbers in touch preparations or tissue sections of lesions typical for DD in cattle. They far outnumber other bacterial morphotypes, and their presence is always associated with necrotic changes of the infected tissue, suggesting a possible etiologic role of these organisms. Different spirochetal morphotypes varying in length, width, and number of endoflagella have been described before (11, 14, 25, 38). These observations correspond well with a recent report on the successful isolation of two distinct spirochetal phenotypes from papillomatous digital dermatitis and interdigital dermatitis, suggesting that these organisms belonged to the genus *Treponema* (36). However, despite the description of some phenotypic traits the exact taxonomic classification for these isolates has not been determined.

In contrast, the phylogenetic approach using comparative 16S rRNA sequence analysis not only provided a reliable taxonomic classification but also allowed the design of diagnostic hybridization probes useful for direct visualization of novel organisms and further epidemiologic studies. Although complete 16S rRNA sequences will be necessary for determinative phylogenetic description, the comparison of partial sequences comprising all relevant spirochetal signature sequences has been shown to be sufficient for the description of treponemal diversity within a complex microbial population of a subgingival plaque sample (8). The validity of this approach and the accuracy of the sequence analysis have recently been documented by successful isolation and subsequent analysis of the entire 16S rRNA gene sequence from a number of novel hitherto uncultured human oral treponemes that were previously identified by molecular genetic techniques (40). These data indicated that a critical analysis of partial 16S rRNA sequences provides appropriate phylogenetic information. However, it is crucial to carefully check all sequence entries for the presence of chimeric sequences (18, 30). By using the CHECK_CHIMERA program, we identified two chimeras composed of two parental treponeme sequences that were cross-amplified at positions corresponding to bases 310 to 360 and 660 to 760 in *E. coli* 16S rRNA. Our data clearly indicate that spirochetes associated with DD in cattle belong to the genus *Treponema*, thus corroborating prior observations that suggested this taxonomic affiliation on the basis of phenotypic traits, e.g., size, insertion or number of periplasmic flagella, antigenic characteristics and enzymatic activity (14, 36). A report on a positive *B. burgdorferi* serology in DD cattle, suggesting that this organism might be implicated in DD, is explained either by the presence of cross-reacting antibodies or by the fact that animals included in these studies were coinfecting with *B. burgdorferi* (3). Our study, however, clearly shows that spirochetes present in DD lesions are not borreliae but belong without any doubt to the genus *Treponema*.

To our surprise, two phylotypes, DDKL-3 and DDKL-13, were closely related to oral treponemes, *T. denticola* (95% similarity) and *T. vincentii* (98% similarity), commonly associated with human periodontal infections. This striking relationship to human oral treponemes is underscored by the observation that monoclonal antibody H9-2 directed against a 37-kDa endoflagellar sheath protein of *T. pallidum* (21) and used in the past to identify yet uncultured invasive oral treponemes (pathogen-related oral spirochetes [PROS]) in patients with severe periodontitis or acute necrotizing ulcerative gingivitis

(27, 28) cross-reacted with treponemes found within DD lesions (data not shown). Recently we showed that PROS constitute a heterogeneous group of spirochetes that belong to phylogenetic group I of oral treponemes, a group that includes *T. vincentii* and related organisms (9). Phylotypes DDKL-12 and DDKL-20 do not bear close resemblance to any known treponeme. They cluster, however, to group IV of oral treponemes, a taxon that has been defined by comparative 16S rRNA analysis (8).

Mere numeric association suggested that phylotype DDKL-3, a close relative of the well-established oral pathogen *T. denticola* (12), might play a role in the pathogenesis of DD lesions. In contrast to other DD treponemes, *T. denticola*-like organisms occur in high proportions. However, the number of DD specimens analyzed so far is too small. A detailed epidemiologic study is needed. Another criterion for assessing their pathogenic role would be to identify tissue-invasive spirochetes commonly found in chronic DD lesions. We are currently approaching this problem by using fluorescence-labeled oligonucleotide probes in situ hybridization experiments. However, to finally determine the etiologic relevance of single species in mixed infections it will be necessary to study bacterial interactions. Such an interaction has been shown for heel abscesses in sheep, where the causative organisms *Fusobacterium necrophorum* and *Actinomyces* (*Corynebacterium*) *pyogenes* occur in different proportions but complement each other's ability to stimulate growth and to attack phagocytes (29). The virulence determinants of bacteria present in DD lesions have yet to be investigated. Molecular epidemiologic studies using FISH may guide us to successfully design such experiments aimed at elucidating microbial interactions in mixed infections, an area of increasing importance for both veterinary and human medicine (34). As compared to periodontal infections, the analysis of these interactions in DD might be easier due to the relatively small number of bacterial species associated with this disease. Hence, it seems possible to use DD as a model system for studying the pathogenesis and prevention of human periodontitis.

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