Guggenheimella bovis gen. nov., sp. nov., isolated from lesions of bovine dermatitis digitalis

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Dermatitis digitalis is an economically important ulcerative disease of undetermined aetiology affecting the hooves of cattle. Material was examined from two independent cases of this disease in Switzerland. Cultures from the advancing front of both lesions yielded large numbers of closely related short, mesophilic, non-motile, non-spore-forming, anaerobic, proteolytic, Gram-positive rods. The 16S rRNA gene sequences of strains OMZ 913^T and OMZ 915 were identical and indicate *Tindallia magadiensis* and *Eubacterium saphenum* as their closest relatives.

Phenotypically, the novel isolates are clearly distinguished from related bacteria by protein and antigen patterns, by cellular fatty acids and by API ZYM activities. The diamino acid of the Gram-positive cell wall is ornithine and the G + C content of OMZ 913^T DNA is 44.4 mol%. The phylogenetic distance from recognized taxa in the phylum *Firmicutes* is sufficient to place these bovine isolates into a novel genus and species, for which the name *Guggenheimella bovis* gen. nov., sp. nov. is proposed, with OMZ 913^T (= CIP 108087^T = DSM 15657^T) as the type strain.

Dermatitis digitalis (DD), also known as Mortellaro disease or strawberry foot, is an economically important ulcerative disease affecting the bovine foot in an increasing number of countries including Germany, Italy, Japan, the Netherlands, Switzerland, the UK and the USA and may be the dominant cause of lameness in dairy cows (Blowey & Sharp, 1988; Choi *et al.*, 1997; Collighan & Woodward, 1997; Collighan *et al.*, 2000; Demirkan *et al.*, 1998; Luginbühl & Kollbrunner, 2000; Moter *et al.*, 1998; Schrank *et al.*, 1999; Shibahara *et al.*, 2002; Walker *et al.*, 1995). The reported spread between herds by veterinarians, foot trimmers and purchased animals as well as curing of the condition by antibiotic therapy are characteristics of an infectious disease (Laven, 2001). However, despite extensive research, a specific infectious agent has not been identified, and it has been suggested that the disease may be a polymicrobial infection (Döpfer *et al.*, 1997). The range of bacteria cultured from DD lesions includes *Campylobacter sputorum* (Shibahara *et al.*, 2002),

Porphyromonas levii, *Fusobacterium necrophorum*, *Fusobacterium nucleatum*, *Prevotella oralis*, *Prevotella denticola*, *Prevotella bivia*, *Treponema brennaborense* (Schrank *et al.*, 1999) and as-yet unnamed treponemes (Demirkan *et al.*, 1999; Stamm *et al.*, 2002; Walker *et al.*, 1995). By using culture-independent methods, such as immunocytochemistry and 16S rRNA gene sequence analysis, additional organisms were detected in lesions and were related to *Bacteroides levii*, *Borrelia burgdorferi*, *Mycoplasma hyopharyngis* and several *Treponema* species (Collighan & Woodward, 1997; Demirkan *et al.*, 1998; Moter *et al.*, 1998). Treponemes have been detected deep in the affected tissue by *in situ* hybridization using fluorescent 16S rRNA-targeted oligonucleotide probes; however, the eubacterial organisms seen at the front of the lesion appeared not to be treponemes (Moter *et al.*, 1998) and remain unidentified.

In the present study, we used a rich, liquid medium, OMIZ-Pat, and a limit-dilution culture technique developed for fastidious oral anaerobes (Wyss *et al.*, 1996) to isolate bacterial strains from the advancing front of the lesions of two independent cases of DD in Switzerland. Two 2-year-old Simmental × Red Holstein heifers from small farms (each with about 20 dairy cows and their calves) developed lameness and typical DD about 2 months after returning from their (separate) summering alps. In one of the farms, it was the first incidence of DD, while, on the second farm, several cases had had to be treated in the previous year. After

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Abbreviation: DD, dermatitis digitalis.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain OMZ 913^T is AY272039.

A transmission electron micrograph, SDS-PAGE profiles of whole-cell proteins, API ZYM activities and cellular fatty acid profiles are available as supplementary material in IJSEM Online.

local anaesthesia, the affected foot was scrubbed and washed and a punch sample was taken from the dried surface; the diseased tissue was then surgically removed by scalpel and the macroscopically healthy tissue at the base of the wound (i.e. the front of the lesion) was sampled by punch biopsy. At the same time, a blood sample was taken for serological analysis. Lesion samples were placed in reducing transport fluid (Loesche *et al.*, 1972) and sent in a cool box to the laboratory, where they were processed within 14 h after surgery. Surgery combined with locally and intravenously administered oxytetracycline resulted in rapid healing (Luginbühl & Kollbrunner, 2000).

Samples were mechanically dispersed and plated to obtain cultures by limit dilution in 96-well flat-bottom microtitre plates, using medium OMIZ-Pat (containing 1% heat-inactivated human serum) supplemented with 1 mg rifampicin, 100 mg fosfomycin, 5 mg polymyxin and 30 mg nalidixic acid l^{-1} as described by Wyss *et al.* (1996). Novel isolates and reference strains *Eubacterium limosum* DSM 20543^T, *Eubacterium brachy* VPI D6B-23^T (Sundquist), *Eubacterium nodatum* ATCC 33099^T, *Eubacterium sulci* ATCC 35585^T and *Mogibacterium timidum* ATCC 33094 were cultured in medium OMIZ-Pat. For growth of *Eubacterium saphenum* ATCC 49989^T, OMIZ-Pat was enriched with 5% fetal bovine serum and 10 mM L-lysine and, for *Tindallia magadiensis* DSM 10318^T, the pH of complete OMIZ-Pat was raised by the addition of 2 g $NaHCO_3$ l^{-1} and 35 mM NaOH. Cellular fatty acid methyl esters were prepared and analysed using gas chromatography according to the instructions of the Microbial Identification System (MIDI). The G + C content of strain OMZ 913^T DNA was determined by HPLC according to Mesbah *et al.* (1989). The peptidoglycan of OMZ 913^T cells was analysed by GC/MS after total hydrolysis and derivatization according to Groth *et al.* (1996). API ZYM tests, growth assays and protein, antigen and glycan analyses on blots after SDS-PAGE were performed as described previously (Bickar & Reid, 1992; Wyss, 1998; Wyss *et al.*, 1996). For immunoblots, sera of heifers JE and RI and the alkaline phosphatase-conjugated anti-bovine IgG mAb BG 18 (Sigma) were used at 1:50 and 1:1000 dilutions, respectively.

Cell lysis, amplification of 16S rRNA genes and 16S rRNA gene sequencing procedures are described in detail elsewhere (Dewhirst *et al.*, 1999). For identification of closest relatives, the sequence of strain OMZ 913^T was compared to the 16S rRNA gene sequences of over 9000 microorganisms in our database and 79 000 sequences in the Ribosomal Database Project (Cole *et al.*, 2003) and GenBank. Phylogenetic trees were constructed on 1479 base comparisons using the neighbour-joining method (Saitou & Nei, 1987). TREECON was used for the construction and drawing of evolutionary trees (Van De Peer & De Wachter, 1993).

In view of the constant exposure of cattle hooves to soil and dung, it is not surprising that DD lesions present as

polymicrobial infections, where it is hardly possible to ascribe definitively a pathogenic role to any specific member of the consortium. As in other natural habitats, it appears that only a fraction of its inhabitants have been recognized, let alone isolated. Recently, methods to isolate and characterize fastidious anaerobes have been improved considerably, as exemplified by the routine isolation of treponemes from the consortia in periodontal lesions (Wyss *et al.*, 2001).

Clinically, the two animals appeared indistinguishable. Yet, phase-contrast microscopy revealed a striking difference in the bacterial populations from the two lesion-surface suspensions: both contained high concentrations of bacteria; however, spirochaetes were detected in only one sample. While the suspensions obtained from the front of the two lesions microscopically showed only few bacteria, both yielded cultures of small Gram-positive rods as well as mycoplasmas (identified as *Mycoplasma fermentans*; not shown). Such observations may prove helpful in the search for an aetiological agent in these complex consortia.

From each sample of the advancing front of the two independent lesions, a representative strain was isolated and characterized: OMZ 913^T from heifer JE (Supplementary Fig. A in IJSEM Online) and OMZ 915 from heifer RI presented as short to coccoid, Gram-positive rods with no evidence for motility. No evidence for spore formation was seen by phase-contrast or transmission electron microscopy or by resistance to heat shock (15 min at 65 °C). Phenotypically, the two independent isolates were indistinguishable, as shown for enzyme activities (Supplementary Table A), cellular proteins (Supplementary Fig. B) and cellular fatty acids (Supplementary Table B). Analysis of the bacterial protein patterns after SDS-PAGE shown in Supplementary Fig. B reveals important differences in all interstrain comparisons except the two novel isolates. The only differences between these two strains were observed when their protein blots were immunolabelled with sera obtained from the two animals (not shown). End-product analysis according to Holdeman & Moore (1975) showed that both strains produce butyrate and minor amounts of isovalerate, isobutyrate, propionate and acetate; this in contrast to *Tindallia magadiensis*, which, in our media, produced mainly acetate, propionate and isovalerate (not shown), consistent with the original report by Kevbrin *et al.* (1998).

The peptidoglycan of OMZ 913^T contains ornithine as its diamino acid and could be classified as murein type A4β L-Orn-D-Asp according to Schleifer & Kandler (1972) or type A21.4 in the listing at the DSMZ (<http://www.dsmz.de/species/murein.htm>).

The sequences of the 16S rRNA genes determined for these two strains were identical and placed them within the phylum *Firmicutes*, which contains many low-G + C Gram-positive species. The closest relative was *Tindallia magadiensis*, at about 90% similarity (Fig. 1). This difference in 16S rRNA gene sequence suggests a phylogenetic

Fig. 1. Phylogenetic position of *Guggenheimella bovis* gen. nov., sp. nov. within the class 'Clostridia' of the phylum Firmicutes. The closest cultured relative of *G. bovis* is *Tindallia magadiensis*. Bar, 5% difference in nucleotide sequences.

difference sufficient to assign the presented DD isolates to a novel genus. The G + C content of 44.4 mol% determined for the DNA of OMZ 913^T is also in accord with that of its closest relatives (Cato *et al.*, 1985; Kevbrin *et al.*, 1998; Nakazawa *et al.*, 2000). In terms of 16S rRNA gene sequences (Fig. 1), enzyme activities (Supplementary Table A), cellular proteins (Supplementary Fig. B) and cellular fatty acids (Supplementary Table B), the novel isolates were found to be completely concordant, yet clearly distinct from the low-G + C species *E. brachy*, *E. limosum*, *E. nodatum*, *E. saphenum*, *E. sulci*, *Mogibacterium timidum* and *Tindallia magadiensis*.

The novel isolates OMZ 913^T and OMZ 915 required the complex components yeast extract, peptone and serum for growth in OMIZ-Pat but were neither dependent on nor stimulated by carbohydrates tested each at 2 g l⁻¹ (summarized in the species description). Growth was strictly anaerobic, as indicated by a failure to grow to the surface in OMIZ-Pat deep-agar tubes or on Columbia blood agar plates, when incubated in 10% CO₂ in air. The organisms can be considered mesophilic, since comparable growth was observed at 33 and 37 °C, but no growth was detectable at 25 or 45 °C. pH dependence of growth was tested at 37 °C in OMIZ-Pat medium adjusted in steps of 0.5 pH units between pH 5.5 and 10.0. Growth of OMZ 913^T was obtained in a pH range of 6.5–9.0 with an optimum between 7.5 and 8.0, distinctly lower than the pH 9.5 for *Tindallia magadiensis* tested in the same medium (not shown). After 7–10 days anaerobic incubation, the novel isolates formed shiny, white opaque colonies, approx. 0.5 mm in diameter, on OMIZ-Pat solidified with 1% agarose. Growth was also seen on Columbia agar plates with 5% laked human blood and in brain heart infusion broth supplemented with 5% fetal calf serum and 5 mM oxaloacetate, but the minimal inoculum required to initiate growth was three orders of magnitude greater than in OMIZ-Pat (data not shown).

In contrast to the fastidious novel isolates, their closest cultured relative, the environmental *Tindallia magadiensis*, has no requirement for yeast extract, peptone or serum.

These complex media components did, however, influence the cellular fatty acid composition of *Tindallia magadiensis* (Supplementary Table B).

Even more fastidious than the bovine isolates is another close relative, *E. saphenum* ATCC 49989^T, associated with periodontitis (Uematsu *et al.*, 1993), which showed only poor growth in medium OMIZ-Pat unless supplemented with 5% fetal bovine serum and 10 mM L-lysine (not arginine). Furthermore, *E. saphenum* decolorized the phenol red indicator concomitant with proliferation in lysine/FCS-supplemented OMIZ-Pat, in marked contrast to the novel isolates, whose growth resulted in a colour change to violet. The observed fastidiousness of *E. saphenum* including a requirement for lysine is consistent with the literature (Uematsu *et al.*, 1993).

The two novel DD isolates OMZ 913^T and OMZ 915 are also distinct from the other reference strains under test in displaying a chymotrypsin-like activity (Supplementary Table A). While it is tempting to associate proteolytic activity with pathogenic potential, it should be cautioned that this activity was detected so far only with an artificial chromogenic substrate. Nevertheless, the presence of these potentially proteolytic organisms at the advancing front of the lesion of two independent cases of DD is suggestive of their aetiological role in this disease. Further investigation of this possibility will require both larger-scale epidemiological studies and histological localization experiments with fluorescently labelled 16S rRNA-targeted DNA probes.

Description of *Guggenheimella* gen. nov.

Guggenheimella (Gug.gen.heim.el'la. L. dim. suff. -ella; N.L. fem. n. *Guggenheimella* after the Swiss microbiologist Bernhard Guggenheim, for his contributions to health research).

Obligately anaerobic, mesophilic, asaccharolytic, non-motile, non-spore-forming, Gram-positive, short to coccoid rods. Belongs to the class 'Clostridia' within the phylum Firmicutes. The cell-wall diamino acid is ornithine. The type species is *Guggenheimella bovis*.

Description of *Guggenheimella bovis* sp. nov.

Guggenheimella bovis (bo'vis. L. gen. n. *bovis* of the cow, referring to the source of isolation).

Displays the following properties in addition to those given in the genus description. Cells are 0.4 × 0.5–1.5 µm. The type strain has murein type A4β L-Orn–D-Asp and its DNA has a G + C content of 44.4 mol%. Growth on OMIZ-Pat agar or on Columbia blood agar in GasPak jars yields shiny, white-opaque colonies, approx. 0.5 mm in diameter, within 7–10 days at 37 °C. Liquid cultures in OMIZ-Pat become turbid within 3 days, with a marked change of the phenol red indicator to violet. Cellular fatty acids are linear, non-hydroxylated saturated C16 and C18 as well as unsaturated C18. End products are butyrate and

minor amounts of isovalerate, isobutyrate, propionate and acetate. Mesophilic; no growth at 25 or 45 °C. The pH compatible with growth is 6.5–9.0, with an optimum between pH 7.5 and 8.0. Enzymic activities detected by API ZYM are acid and alkaline phosphatases, C4 and C8 esterases, leucine arylamidase, chymotrypsin and naphthol phosphohydrolase. Activities not detected by API ZYM test are trypsin, α - and β -galactosidases, α - and β -glucosidases, lipase C14, cystyl arylamidase, valine arylamidase, β -glucuronidase, *N*-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase. The following carbohydrates do not affect growth: D-arabinose, L-arabinose, D-cellobiose, D-fructose, D-fucose, L-fucose, D-galactose, D-galacturonic acid, D-glucose, D-glucuronic acid, glycogen, D-lactose, D-maltose, D-mannitol, D-mannose, D-melibiose, L-rhamnose, D-ribose, L-sorbose, starch, D-sucrose, D-trehalose, D-xylose and L-xylose. Growth in OMIZ-Pat is resistant to fosfomycin (100 mg l⁻¹), rifampicin (1 mg l⁻¹), polymyxin (5 mg l⁻¹) and nalidixic acid (30 mg l⁻¹) alone or in combination.

The type strain is OMZ 913^T (=CIP 108087^T=DSM 15657^T). Two independent strains were isolated from heifers with dermatitis digitalis in Switzerland; other habitats are not known. The 16S rRNA gene sequence of the type strain has been deposited under accession number AY272039.

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