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Involvement of *Gugenheimella bovis* in digital dermatitis lesions of dairy cows

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23 **Abstract**

24 Digital dermatitis (DD) of cattle leads to lameness and a decrease of milk production and is
25 responsible for major economic losses worldwide. Although a bacterial aetiology is generally
26 accepted, it still is unclear which microorganisms cause and/or maintain the disease. Recently, a
27 previously undiscovered bacterial species, *Guggenheimella bovis*, has been isolated from the
28 front of two DD lesions in Swiss cattle and suggested as a potential pathogen.

29 The aims of the present study were to determine the prevalence of *G. bovis* in 58 German cows
30 suffering from DD via dot blot hybridization, and to analyse the spatial distribution of *G. bovis*
31 within the affected tissue by fluorescence in situ hybridization (FISH). A species-specific probe,
32 GUBO1, was designed and evaluated. In none of the 58 samples *Guggenheimella* could be
33 detected, while cultured *G. bovis* was reliably identified by GUBO1. Further FISH experiments
34 were carried out on two additional biopsies of Swiss cattle tested positive for *G. bovis* by
35 quantitative PCR and permitted visualization of the newly discovered bacteria in situ. In these
36 biopsies *G. bovis* proved to be tissue invasive forming characteristic spherical microcolonies not
37 only within the bacterial biofilm but also in seemingly unaffected parts of the tissue not yet
38 reached by the advancing bacterial front. Although the presence of *G. bovis* does not constitute an
39 essential premise for DD, it seems likely that the bacterial species involved in DD vary, and that
40 in some cases *G. bovis* is crucial for the development of DD lesions.

41

42 Keywords: *Guggenheimella*, digital dermatitis, in situ hybridization, biofilm

43

44

45 **1. Introduction**

46 Digital dermatitis (DD) was first described by Cheli & Mortellaro in 1974 (Cheli and Mortellaro,
47 1974) and is an ulcerative acute or chronic inflammatory disease affecting the bovine foot. DD
48 lesions most frequently involve the plantar skin areas proximal to the coronet of the hind limbs of
49 dairy cattle (Blowey and Sharp, 1988; Read and Walker, 1998) and constitute an intensely
50 painful condition, which may persist for weeks and even months impairing the general condition
51 of the affected cattle. Episodes of lameness, weight loss and decrease of milk yield are
52 consequences frequently described (Blowey, 1990; Hernandez et al., 2002; Laven, 2001; Laven
53 and Logue, 2006; Murray et al., 1996). DD has been observed in various parts of the world
54 (Brown et al., 2000; el-Ghoul and Shaheed, 2001; Enevoldsen et al., 1991; Holzhauer et al.,
55 2006; Milinovich et al., 2004; Rodriguez-Lainz et al., 1998; Somers et al., 2003; van Amstel et
56 al., 1995; Weaver and Court, 1993; Wells et al., 1999), its incidence increasing constantly over
57 the past decades (Read and Walker, 1998; Somers et al., 2003; Wells et al., 1999). Up to 90% of
58 the dairy cattle herds have been found to be affected (Laven and Logue, 2006; Read and Walker,
59 1998; Rodriguez-Lainz et al., 1996; Rodriguez-Lainz et al., 1998; Somers et al., 2003; Wells et
60 al., 1999). Thus it constitutes an important economic factor and warrants intensive research.
61 However, although a bacterial involvement is evident, the aetiology of DD is still under
62 discussion. Treponemes but also various other eubacterial organisms have been isolated from DD
63 lesions and have been supposed as potential pathogens (Blowey et al., 1994; Choi et al., 1997;
64 Collighan and Woodward, 1997; Demirkan et al., 1998; Grund et al., 1995; McLennan and
65 McKenzie, 1996; Moter et al., 1998; Walker et al., 1995).

66 Recently, high numbers of a previously undiscovered bacterial species, *Guggenheimella bovis*,
67 have been found in two independent cases of DD (Simmental x Red Holstein heifers) in

68 Switzerland. The obligate anaerobic short to coccoid Gram-positive rods have been isolated from
69 the very front of both lesions and display a chymotrypsin-like proteolytic activity (Wyss et al.,
70 2005). Both findings suggest an important role of *G. bovis* in the aetiology of DD. In the present
71 study dot blot hybridization experiments were performed to determine the prevalence of *G. bovis*
72 in German dairy cows suffering from DD. Furthermore, fluorescence in situ hybridization (FISH)
73 was used to analyse the distribution of *G. bovis* within DD lesions and its topographical relation
74 to other potential pathogens.

75

76

77 **2. Materials and methods**

78 **2.1. Processing of tissue specimens for dot blot hybridization.** Biopsies (0,7cm in diameter)
79 were taken from typical DD lesions of 58 affected dairy cows (Holstein Friesian breed (n = 49),
80 Red Holstein breed (n = 5), Fleckvieh (n = 4)) from different farms in Germany and transported
81 to the laboratory immediately. DNA isolation, subsequent PCR amplification and preparation of
82 dot blot membranes were performed as described previously (Choi et al., 1997; Moter et al.,
83 2006). Briefly, part of the 16S rRNA gene out of the bulk DNA was amplified using the broad
84 range bacterial primers TPU1 5'-AGA GTT TGA TCM TGG CTC AG-3' (corresponding to
85 positions 8 to 27 in the *E. coli* 16S rRNA gene) and RTU3 5'-GWA TTA CCG CGG CKG CTG-
86 3' (corresponding to complementary positions 519 to 536 in *E. coli* 16S rRNA). Successful
87 amplification was verified by agarose gel electrophoresis.

88

89 **2.2. Processing of tissue specimens for FISH.** Parts of the tissue of each of the 58 biopsies were
90 fixed and embedded in cold polymerizing resin (Technovit 8100, Kulzer, Wehrheim, Germany)
91 as previously reported (Moter et al., 1998). The blocks were sectioned on a rotary microtome
92 (Type DDM 0036, Medim, Baar, Switzerland) using steel knives with hard metal edges. Tissue
93 sections (3-5µm) were straightened on sterile water, placed on silanized slides (Starfrost,
94 Burgdorf, Germany) and stored at 4 °C. Following the evaluation of these samples, two additional
95 biopsies from a study on Swiss cattle (Strub et al., 2007) were included in the experiments and
96 processed in the same way.

97
98 **2.3. Oligonucleotide probes.** Probe EUB 338 (Amann et al., 1990), which is complementary to a
99 region of the 16S rRNA gene conserved in the domain *Bacteria*, was used in dot blot
100 hybridization as positive control to check successful PCR amplification and in FISH to visualize
101 the entire bacterial population in the specimens. The species-specific probe GUBO1 (5'-
102 CCAGTGGCTATCCCTGTGTGAAGG-3'), corresponding to position 135-158 in *Escherichia*
103 *coli* 16S rRNA, was designed after comparative sequence analysis of close phylogenetic
104 neighbours to *G. bovis*. To assess specificity, the probe sequence was compared to all 16S rRNA
105 entries at the EMBL and GenBank databases (as of February 2007), making use of the Husar
106 program package (DKFZ, Heidelberg, Germany), and to the sequences deposited in the
107 Ribosomal Database Project II (Maidak et al., 2001). The probe was checked for its practical use
108 in hybridization experiments by using the program OLIGO (version 4.0).

109
110 **2.4. Bacterial Strains.** To optimize the dot blot hybridization and FISH conditions, *G. bovis*
111 (OMZ 913^T = CIP 108087^T = DSM 15657^T) was used as positive and *Tindallia magadiensis*

112 (DSM 10318^T), being the closest cultured phylogenetic relative, as negative control (Wyss et al.,
113 2005).

114

115 **2.5. Dot blot hybridization.** Dot blot hybridization experiments to detect *G. bovis* were
116 performed as described previously (Choi et al., 1997; Moter et al., 2006). PCR-amplified
117 products gained from fixed cells of *G. bovis* and its closest cultured relative *T. magadiensis* were
118 included in all experiments as positive and negative controls respectively. All hybridizations with
119 the probes EUB 338 (Amann et al., 1990) and GUBO1, both synthesized commercially
120 (biomers.net, Ulm, Germany), were performed at a temperature of 54 °C, while stringency
121 washes were carried out at 60 °C with a washing buffer containing 2x SSC (1x SSC is 0.15 M
122 NaCl plus 0.015 M sodium citrate) - 0.1% SDS for EUB 338 and 5x SSC - 0.2% SDS for
123 GUBO1. After detection of the digoxigenin-labelled probes, X-ray films were exposed to the
124 membranes for 1 to 48 hours. After stripping as reported previously (Moter et al., 2006), identical
125 membranes were re-used for further hybridization experiments.

126

127 **2.6. FISH.** The probe EUB 338 was 5' end-labelled with fluorochrome Cy5 (indodicarbocyanine)
128 and GUBO1 was 5' end-labelled with fluorochrome Cy3 (indocarbocyanine). Both probes were
129 applied simultaneously. FISH experiments were performed as described previously (Sunde et al.,
130 2003), except for mounting the slides with Vectashield containing DAPI (4,6-diamidino-2-
131 phenylindoldihydrochlorid) (Vector Laboratories, Orton Southgate, UK). Hybridizations were
132 carried out at a temperature of 50 °C for 2 to 3 hours. In all experiments fixed cells of *G. bovis*
133 and *T. magadiensis* served as positive and negative controls respectively. To adjust the stringency
134 of GUBO1, FISH experiments were performed incubating fixed cells of *G. bovis* and *T.*

135 *magadiensis* using different hybridization mixes with formamide concentrations rising in steps of
136 5% (v/v) from 0% (v/v) to 75% (v/v). Several pictures with a fixed exposure time were taken of
137 each bacterial species at each level of formamide. The program daime (digital image analysis in
138 microbial ecology) (Daims et al., 2006) was used to measure the signal intensity of the stained
139 bacteria at different concentrations of formamide. While the signal intensity of *T. magadiensis*
140 due to unspecific binding of the probe decreased largely at formamide concentrations of 10% and
141 above, the intensity of *G. bovis* remained stable up to formamide concentrations of 30% (v/v)
142 (data not shown). Thus, FISH of the tissue sections was carried out with hybridization buffer
143 containing 30% (v/v) of formamide.

144
145 **2.7. Epifluorescent microscopy.** To view the bacteria in sections processed for FISH an
146 epifluorescence microscope (AxioPlan II, Zeiss, Jena, Germany) equipped with a 100 W high
147 pressure mercury lamp (HBO 103, Osram, Munich, Germany) and 10x, 40x and 100x objectives
148 was used. Narrow band filter sets HQ F31-000, HQ F41-007 and HQ F41-008 (AHF
149 Analysentechnik, Tübingen, Germany) were used to analyse the DAPI, Cy3 and Cy5 signals
150 respectively. Digital images were generated with an AxioCam HRC (Zeiss) making use of the
151 AxioVision 4.4 software.

152

153

154 **3. Results**

155 **3.1. Dot blot hybridization.** When carried out with the probe EUB 338, dot blot hybridization
156 experiments indicated the presence of bacteria in all of the 58 samples as well as in the positive

157 and negative controls and thus confirmed successful PCR amplification. Using the specific probe
158 GUBO1 under stringent hybridization conditions, *G. bovis* could not be detected in any of the
159 clinical samples while only the positive control yielded a strong signal (Fig. 1).

160
161 **3.2. FISH.** In all FISH experiments performed as determined above cultured *G. bovis* was
162 reliably detected both by the specific probe GUBO1 and by the eubacterial probe EUB 338, while
163 *T. magadiensis* could only be detected by the probe EUB 338 (Fig. 2). All of the examined DD
164 samples from German cattle showed large amounts of various morphological types of bacteria
165 stained by EUB 338 and displayed the characteristic structure of DD ulcers (Fig. 3) with
166 spirochetes or fusiform bacteria being the very outriders invading the tissue at the front-of-lesion
167 (Nordhoff and Wieler, 2005) (Fig. 4). However, *G. bovis* could not be visualized in any of these
168 tissue slides, neither in the superficial parts of the ulcers nor in the centres of the lesions and in
169 particular not at the apical borders (Fig. 3).

170 Subsequently, we submitted two biopsies from a different study (Strub et al., 2007) tested
171 positive for *G. bovis* by quantitative PCR to FISH to determine the role of *Guggenheimella* in the
172 architecture of DD biofilms. We succeeded in visualizing *G. bovis* in these tissue sections in high
173 numbers. Only few of these bacteria appeared as single cells, while most of them formed
174 characteristic spherical microcolonies. Some of these colonies were observed among the other
175 bacteria in clearly affected areas of the biopsy, but the majority of them could be found in deeper,
176 seemingly unaffected parts of the tissue (Fig. 5). The biofilm structure of the *Guggenheimella*-
177 positive ulcers and the bacterial morphotypes involved differed considerably from the
178 characteristic, spirochete-dominated lesions we observed in the 58 biopsies of German cows (Fig.
179 4, Fig. 5).

180

181

182 **4. Discussion**

183 While the findings of Wyss et al. (2005) strongly suggest an aetiological role of *G. bovis* in the
184 two examined cases of DD, the bacteria being isolated from the very front of the lesions and
185 displaying a proteolytic activity, it is unlikely that their involvement is constitutional for the
186 formation of DD ulcers in cattle. In none of the 58 examined tissue samples from German cows
187 *G. bovis* could be found. It is thus not present in the lesions at all or else only present in numbers
188 below the detection limits of FISH and dot blot hybridization. Even in the latter case it remains
189 questionable if such minute amounts of a bacterial species are likely to influence the pathogenetic
190 process of DD in a significant way.

191 While this work was in progress, another study on the prevalence of *G. bovis* in DD lesions was
192 conducted (Strub et al., 2007). Strub et al. examined tissue samples of 20 affected cows from
193 Swiss farms by quantitative PCR and detected *G. bovis* in four out of 20 animals, concluding that
194 an involvement of this organism in the aetiology of DD is improbable considering the low
195 prevalence – a conclusion which is consistent with the results of our epidemiology on German
196 cattle.

197 Nonetheless, the FISH experiments on two of these biopsies tested positive for *Guggenheimella*
198 turned out to be of the utmost importance. The results obtained underline that FISH is a valid tool
199 offering detailed information about the tissue distribution of one or more bacterial species in DD
200 biofilms. They prove that previous detection of *Guggenheimella* (Strub et al., 2007; Wyss et al.,
201 2005) has not been due to contamination by environmental bacteria. *G. bovis* can be part of the
202 bacterial population in DD lesions and it is tissue invasive. As the organism could even be

203 visualized in unaffected parts of the biopsy way ahead of the advancing bacterial front, one can
204 conjecture that its role for the development of the DD biofilm might be an important one, that, in
205 certain cases, it might prepare the ground for the following bacterial invasion. Considering the
206 striking morphological differences between these Swiss lesions on the one hand and the 58
207 German lesions on the other, it is tempting to speculate whether there is more than just one entity
208 of DD, that the process of mixed bacterial infection and inflammation leading to the ulcers is not
209 always alike, and that in one of at least two entities the participation of *G. bovis* might be
210 decisive. However, further and more comprehensive epidemiological data about the various
211 potential DD pathogens, *Guggenheimella bovis* among them, need to be gained. One cannot
212 overestimate the importance of in situ techniques for this purpose.

213

214

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218

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- 306

307 **Figure captions**

308 **Fig. 1.** Dot blot hybridizations of the identical membrane using the eubacterial probe EUB 338
309 (a) and the species-specific probe GUBO1 (b). In lanes A to E and fields F1 to F3 PCR-amplified
310 products from DD lesions of 58 affected German dairy cows were applied. In fields F10 and F11
311 PCR products from *G. bovis* (F10) and its closest cultured relative, *T. magadiensis* (F11) were
312 applied as positive and negative controls respectively. Fields F4 to F9 were left empty. (a) Strong
313 signals in all fields prove successful PCR amplification. (b) *G. bovis* was not detected in any of
314 the clinical samples, while the positive control yielded a strong signal.

315 **Fig. 2.** Simultaneous hybridization of fixed cells of *G. bovis* (a and c) and *T. magadiensis* (b and
316 d) with the probes EUB 338-Cy5 (magenta) and GUBO1-Cy3 (bright orange). (a and c) Identical
317 microscopic fields show detection of *G. bovis* by both EUB 338 (a) and GUBO1 (b) whereas
318 detection of *T. magadiensis* by EUB 338 only (b) and not GUBO1 (d) proves specificity of the
319 FISH experiment.

320 **Fig. 3.** FISH on a tissue section of a typical DD lesion using probes EUB 338-Cy5 and GUBO1-
321 Cy3 and unspecific nucleic acid stain DAPI. (a and b) Overview. (a) Overlay of the Cy5- and
322 FITC-filter sets shows the bacterial biofilm (magenta) while background fluorescence (green)
323 allows orientation within the tissue. (b) Identical microscopic field using the Cy3-filter set. (c to
324 f) Higher magnifications of the inserts. (c) Overlay of the Cy5-, FITC- and DAPI-filter sets
325 shows massive bacterial invasion (magenta), autofluorescent erythrocytes (green) and host cell
326 nuclei (blue) in the superficial part of the ulcer. (d) No *G. bovis* is seen in the same microscopic
327 field using the Cy3- and DAPI-filter sets. (e and f) Likewise, *G. bovis* was not detected in the
328 central part of the biofilm.

329 **Fig. 4.** FISH of the apical border of the ulcer (as indicated in Fig. 3a) using EUB 338-Cy3 and
330 DAPI. Overlay of Cy3-, FITC- and DAPI-filter sets shows bacteria (orange) and cell nuclei
331 (blue) in the deepest part of the biofilm. Single spirochetes (arrowheads) invade the tissue at the
332 front of lesion.

333 **Fig. 5.** FISH on a tissue section of a DD biopsy tested positive for *G. bovis* by PCR.
334 Simultaneous hybridization with the probes EUB 338-Cy5 and GUBO1-Cy3 combined with
335 DAPI stain. (a-c) Overlay of the Cy3-, Cy5-, FITC- and DAPI-filter sets. (a) The overview shows
336 a massive bacterial biofilm (magenta) and also distinct round colonies of *G. bovis* (orange). (b)
337 High resolution of the insert shows two microcolonies of *G. bovis* visualized by GUBO1 (orange)
338 close to the bacterial biofilm (magenta). Note that the bacterial morphotypes involved in this
339 lesion differ considerably from those in Fig. 3 and Fig. 4. (c) High magnification of the insert
340 shows a solitary microcolony of *G. bovis* detected by GUBO1 in seemingly intact tissue distant
341 from the bacterial front.

342

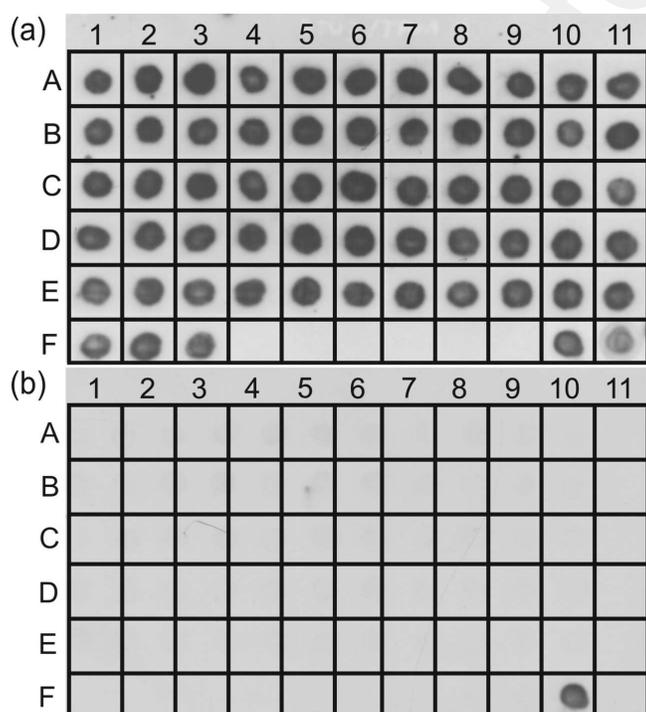
343 The online version of this paper contains two supplementary movie files.

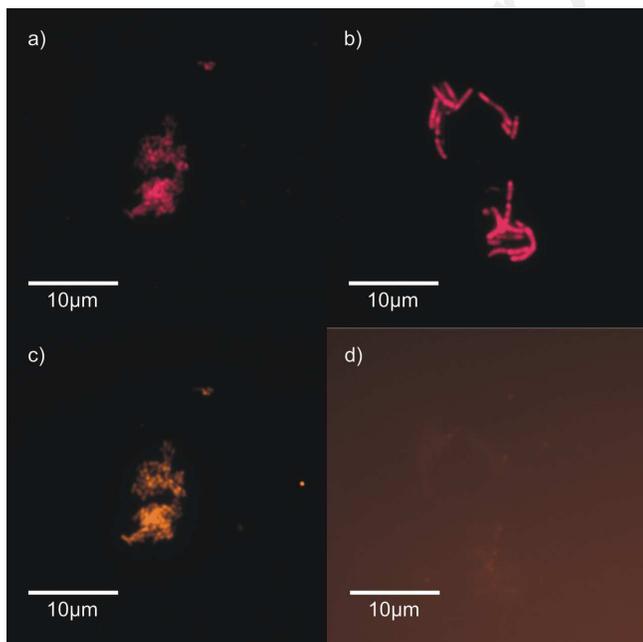
344

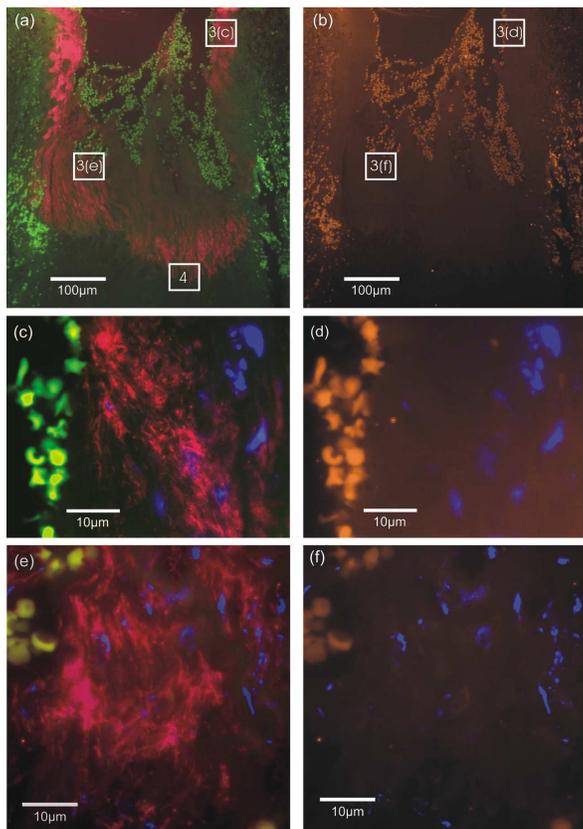
345 **Movie 1.** Typical DD lesion with spirochetes and fusiform bacteria invading the tissue.

346 Deconvolution of a Z-stack reveals the spiral morphotype of the bacterial outriders (orange)
347 detected by the eubacterial probe EUB 338-Cy3.

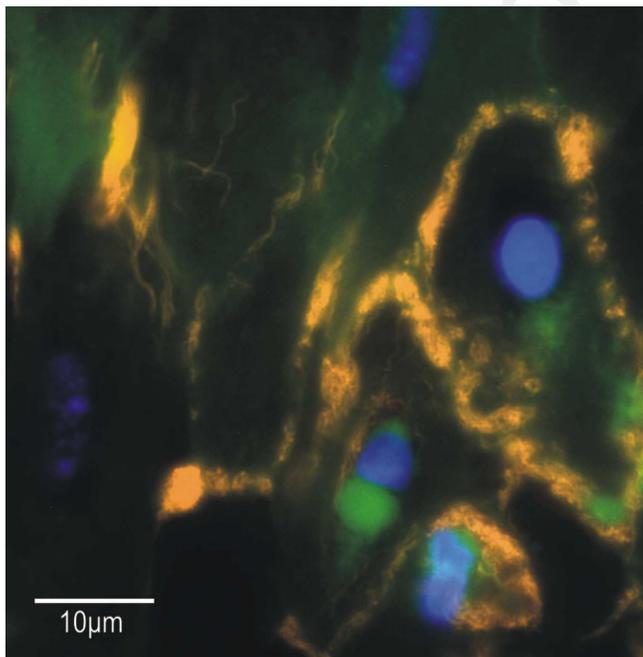
348 **Movie 2.** FISH of a *Guggenheimella*-positive DD ulcer. Z-stacking through the section shows the
349 spherical shape of the *G. bovis* microcolony (orange) detected by GUBO1-Cy3. Note the
350 considerable morphological differences between the bacteria visualized by EUB 338-Cy5
351 (magenta) and those in *Guggenheimella*-negative lesions (movie 1). Note the absence of
352 spirochetes.

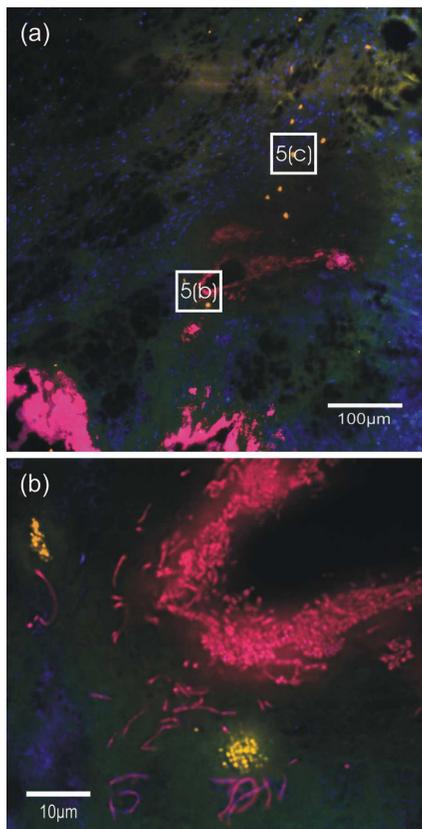






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