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1 **First cultivation of health-associated *Tannerella* sp. HOT-286 (BU063)**

2

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30 **Abstract**

31

32 Despite significant advances in recent years in culture-independent molecular microbiology
33 methods, the detailed study of individual bacterial species still relies on having pure cultures
34 in the laboratory. Yet over a third of the approximately 700 different bacterial taxa found in
35 the human oral cavity are as-yet-uncultivated *in vitro*. One such taxon, *Tannerella* sp. HOT-
36 286 (phylotype BU063), is the focus of much interest since it is associated with periodontal
37 health, while *Tannerella forsythia*, its closest phylogenetic neighbour, is strongly associated
38 with periodontal disease. HOT-286, however, has remained uncultivated despite the efforts
39 of several research groups, spanning over a decade. The aim of this study was to cultivate
40 *Tannerella* sp. HOT-286. A heavily-diluted sample of subgingival plaque was inoculated onto
41 culture plates, supplemented with siderophores (pyoverdines-Fe-complex or
42 desferricoprogen) or a neat plaque suspension. After eight days of anaerobic incubation,
43 microcolonies and colonies showing satellitism were passaged onto fresh culture plates
44 cross-streaked with potential helper strains or onto cellulose-acetate membranes placed
45 over lawn cultures of helper strains. Sub-cultured colonies were identified by 16S rRNA-
46 gene-sequencing, and purity confirmed by sequencing 20 clones/library prepared from a
47 single colony. Three of the colonies of interest (derived from pyoverdines- and plaque-
48 supplemented plates) were identified as *Tannerella* sp. HOT-286. The isolates were found to
49 be incapable of independent growth, requiring helpers such as *Propionibacterium acnes* or
50 *Prevotella intermedia* for stimulation, with best growth on membranes over 'helper' lawns. A
51 representative isolate was subjected to phenotypic characterization and found to produce a
52 range of glycosidic and proteolytic enzymes. Further comparison of this novel 'periodontal
53 health-associated' taxon with *T. forsythia* will be valuable in investigating virulence factors of
54 the latter, and possible health benefits of the former.

55

56

57 **Introduction**

58

59 Although the advent of next-generation sequencing has revealed the true diversity of the
60 human oral microbiome, the need for laboratory culture for the comprehensive physiological
61 and pathological characterization of individual bacterial species remains. Approximately 700
62 bacterial taxa/species have been identified in the human oral cavity, based on 16S rRNA
63 gene sequence data (Human Oral Microbiome Database, HOMD, release 13
64 (www.homd.org)). However of these, nearly 250 are as-yet-uncultivated *in vitro* (Chen et al.
65 2010; Dewhirst et al. 2010). Examples of uncultivated oral bacterial taxa include all members
66 of the candidate bacterial Divisions SR1 and GN02 (Camanocha and Dewhirst 2014). Until
67 recently, there were also no cultivated oral phylotypes from the phyla TM7 and *Chloroflexi* –
68 a single oral strain from each phylum has now been successfully cultivated (He et al. 2015;
69 Vartoukian et al. 2016). .

70

71 The recently cultivated TM7 strain, TM7x, has a reduced genome of 705 kb which lacks the
72 genes necessary for essential amino acid biosynthesis; consequently, it is incapable of
73 independent growth and leads an obligately symbiotic relationship with another bacterium,
74 *Actinomyces odontolyticus* (He et al. 2015). Davis et al (2013) have also shown that gene
75 loss in bacteria is associated with auxotrophy for purine, pyrimidine, fatty acid and amino
76 acid synthetic pathways. Bacteria that are metabolically dependent on others may be
77 impossible to grow in pure culture. Conversely, in-vitro cultivation of bacteria in consortia can
78 enable the isolation of previously uncultivated bacteria (Tanaka and Benno 2015; Vartoukian
79 et al. 2010). In particular, species within biofilm communities, such as dental plaque, may
80 depend on each other for metabolic cooperation and intercellular signals (Mihai et al. 2015;
81 Stewart 2012; Vartoukian et al. 2010). Kummerli and co-workers (Kummerli et al. 2009;
82 Kummerli et al. 2014) have reported that the sharing of metabolites such as iron-scavenging
83 siderophores is particularly prevalent in structured bacteria-host environments. It has been

84 suggested that ‘unculturable’ bacteria may have lost the ability to produce siderophores

85 (Lewis et al. 2010), and depend on provision from neighboring bacteria. Indeed, there is

86 evidence that adding siderophores to culture media stimulates the growth of previously

87 uncultivated organisms (D'Onofrio et al. 2010; Guan and Kamino 2001; Vartoukian et al.

88 2016).

89

90 *Tannerella forsythia* is strongly associated with periodontitis (Socransky et al. 1998),

91 possesses several virulence factors (Sharma 2010) and is the only cultivable taxon from the

92 genus *Tannerella*. The as-yet-uncultivated oral phylotype *Tannerella* sp. HOT-286 (clone

93 BU063) is phylogenetically closely-related to *T. forsythia,* but is associated with periodontal

94 health rather than disease (de Lillo et al. 2004; Kumar et al. 2003; Leys et al. 2002). Fodor *et*

95 *al* (2012) included *Tannerella* sp. HOT-286 on their high-priority microorganisms ‘most-

96 wanted’ for genome sequencing list; de Lillo et al (2004) suggested over a decade ago that

97 work to enable culture of *Tannerella* sp. HOT-286 should be urgently prioritised.

98

99 The aim of this study was to cultivate the previously-uncultivated oral phylotype *Tannerella*

100 sp. HOT-286, using several approaches: growth in consortia, addition of siderophores, cross

101 streaking with helper strains, and growth on membranes over helper lawns.

102

103 **Materials and methods**

104

105 Ethical approval for the study was granted by the South West London REC 3 Research

106 Ethics Committee (REF: 10/H0803/161). A 50-year-old female subject with chronic

107 periodontitis, who had not received periodontal or antimicrobial therapy within the previous

108 three months, was recruited for the study with her informed consent. Subgingival plaque was

109 collected with a sterile curette from two deep (7-8 mm) periodontal pockets, pooled and
110 suspended in Reduced Transport Medium (RTM) (Bowden and Hardie 1971).

111

112 The sample was transported within 45 min of collection to an anaerobic workstation (Don
113 Whitley Scientific Ltd.) with an atmosphere of 80% nitrogen, 10% hydrogen and 10% carbon
114 dioxide at 37°C. It was diluted to 10^{-6} in RTM after vortexing for 1 min, and 50 µl of the
115 diluted plaque suspension was used to inoculate multiple pre-reduced Blood Agar Base
116 No.2 (Lab M, UK) / 5% horse blood (BA) plates. A well was made in the center of each agar
117 plate to which was added 150 µl of 0.1 mg/ml solutions of pyoverdines-Fe-complex (Sigma-
118 Aldrich, UK) or desferricoprogen (EMC Microcollections, Germany), 150 µl of neat plaque
119 suspension or 150 µl sterile water.

120

121 After eight days of anaerobic incubation, the mixed cultures were inspected under a plate-
122 dissecting microscope for microcolonies and colonies satelliting around/on larger colonies.
123 The colonies of interest were passaged both onto BA plates, cross-streaked with potential
124 helper strains (*Fusobacterium nucleatum subspecies polymorphum* NCTC 10562, or
125 *Propionibacterium acnes* ATCC 6919), and onto 0.45 µm-pore cellulose acetate membranes
126 (Sartorius, 1110650ACN) overlying fresh *F. nucleatum* or 48-hour *P. acnes* lawn cultures.

127

128 Growth on secondary plates was purified where necessary and DNA extracted using the
129 GenElute Bacterial Genomic DNA kit (Sigma-Aldrich, UK) with the protocol for Gram-positive
130 bacteria, prior to endpoint PCR with ‘universal’ primers 27FYM and 1492R (Lane 1991) as
131 described previously (Vartoukian et al. 2009). For secondary plates showing minimal growth,
132 direct ‘touch’-PCR of single colonies with ‘universal’ primers (Vartoukian et al. 2009) was
133 performed. PCR products were subjected to partial 16S rRNA gene sequencing using primer
134 519R (Vartoukian et al. 2009).

135

136 For cultures identified as *Tannerella* sp. HOT-286 (phylotype BU063), purity was confirmed

137 by sequencing 20 cloned inserts from a library prepared (as described previously

138 (Vartoukian et al. 2009)) using the amplification product of 16S rRNA gene 'touch'-PCR of a

139 single colony with 'universal' primers. Subsequently, the full length of the 16S rRNA gene

140 was sequenced with multiple primers for triple coverage (Vartoukian et al. 2009).

141

142 Colonial and cellular morphology of *Tannerella* sp. HOT-286 strains were determined by:

143 examination under a dissecting microscope, light microscopy after Gram staining and

144 transmission electron microscopy (TEM). For TEM, isolated colonies were gently suspended

145 in 10 mM Tris-HCL buffer (pH 7.4) at a concentration of about 10^8 cells per ml. Samples

146 were negatively stained with 1% (wt/vol) phosphotungstic acid (pH 6.5) for 20 to 30 s. The

147 specimens were examined with a JEOL model JEM-1200EX transmission electron

148 microscope (JEOL USA, Inc., Peabody, MA) operating at 100 kV.

149

150 Enzyme profiles were determined for *Tannerella* sp. HOT-286 isolate SP18_24 and *T.*

151 *forsythia* FDC 338^T using the API ZYM test (BioMerieux, France) and the Rapid ID 32 A

152 anaerobe identification kit (BioMerieux, France) in duplicate.

153

154 Susceptibility to penicillin (1 unit), amoxicillin (10 µg), ampicillin (2 µg), erythromycin (5 µg),

155 tetracycline (10 µg), metronidazole (5 µg), ceftazidine (30 µg), gentamycin (10 µg),

156 chloramphenicol (10 µg) and ciprofloxacin (1 µg) (Oxoid, UK) was determined in duplicate

157 using the disc diffusion method for: a) *Tannerella* sp. HOT-286 SP18_24, using cultures

158 cross-streaked with *P. acnes*, and b) *T. forsythia* FDC 338^T.

159

160 Growth characteristics of *Tannerella* sp. HOT-286 SP18_24 were investigated as follows,

161 with duplicate testing in all cases:

162

163 The ability of pyoverdines-Fe to stimulate growth of this strain on BA under anaerobic
164 conditions was assessed: a) by adding either 150 μl of 0.1mg/ml pyoverdines-Fe or an
165 equivalent volume of sterile water (negative control) to a central well on the plates, or b) by
166 applying a small circular inoculum of live *P. acnes* (positive control) to the center of the
167 plates.

168

169 The effect on growth of SP18_24 of *P. acnes* culture supernatant (CS) or cell-free extract
170 (CFE) was assessed using a method similar to that described above, with addition of test
171 (CS or CFE) or negative control (Nutrient Broth no. 2 (NB; Oxoid, UK) or PBS) agents to a
172 central well, or inoculation with live *P. acnes* as positive control. CS was prepared from a 4-d
173 NB culture of *P. acnes* by centrifuging the culture and passing the supernatant through a 0.2
174 μm-pore filter. CFE was prepared from the same 25 ml broth culture by re-suspending the
175 pellet in 5 ml PBS, sonicating the suspension for three pulses of 2 min, centrifuging and
176 filtering the supernatant.

177

178 The effect of CS/CFE on growth of SP18_24 was also assessed in broth culture. Briefly,
179 SP18_24 was cultured in NB + 1% yeast extract, with or without: CS (50%, v/v), CFE (25%,
180 v/v) or equivalent volumes of plain NB or PBS as controls. Growth was assessed over 16 d
181 using spectrophotometric turbidity measurements at 600 nm.

182

183 Finally, a panel of seven oral bacteria was evaluated alongside *P. acnes* for their stimulatory
184 effect on the growth of SP18_24 as lawn cultures on BA. Small circular inocula of the
185 following bacterial strains were applied to plates: *Streptococcus oralis* (NCTC 7864),
186 *Veillonella dispar* (NCTC 11831), *Actinomyces oris* (ATCC 19246), *Parvimonas micra*
187 (ACTC 33270), *Porphyromonas gingivalis* (ATCC 33277), *Prevotella intermedia* (ATCC
188 25611), *P. acnes* (ATCC 6919) and *F. nucleatum (*NCTC 10562). After 7 d of anaerobic
189 incubation, growth stimulatory effect was graded arbitrarily as 0, +, ++ or +++.

190

191

192 **Results**

193

194 A heavily-diluted subgingival plaque sample was inoculated onto culture plates
195 supplemented either with siderophores or a neat suspension of the plaque sample. Forty-six
196 isolates forming microcolonies or exhibiting satellite growth around other colonies were
197 passaged to fresh plates cross-streaked with helper strains and onto membranes overlying
198 lawn cultures of helpers. Three isolates (two from a pyoverdines-Fe supplemented plate and
199 one from a plaque-supplemented plate) formed several large, cream-coloured colonies on
200 membranes overlying *P. acnes* lawns but showed no, or limited, growth (1-2 tiny colonies)
201 on: (i) secondary plates cross-streaked with *F. nucleatum* and *P. acnes* as helpers, or (ii)
202 membrane cultures over *F. nucleatum* lawns. The isolates were identified as *Tannerella* sp.
203 HOT-286 (phylotype BU063); and the cultures were confirmed pure by sequence analysis of
204 multiple cloned amplicons derived from a single colony of each isolate. The full-length 16S
205 rRNA gene sequences of the three isolates were found to be identical, and 99.2% similar
206 over 1450 bases to *Tannerella* clone BU063 accession number AY008308. The novel
207 sequences were deposited in the GenBank nucleotide sequence database with the following
208 accession numbers: *Tannerella* sp. HOT-286 isolate SP18_4 - KT861600; *Tannerella* sp.
209 HOT-286 SP18_24 - KT861601; and *Tannerella* sp. HOT-286 SP18_26 - KT861602.

210

211 The three isolates showed limited independent growth (Figure 1 A), and were significantly
212 stimulated by *P. acnes*, but not *F. nucleatum* (Figure 1 B). Culture on membranes over *P.*
213 *acnes* lawns resulted consistently in stronger growth than was observed after culture directly
214 on media with *P. acnes* cross-streaks (Figure 1 B and C). The isolates were successfully
215 revived after storage in broth/glycerol at -80°C, although growth was initially sparse,
216 consisting of tiny colonies approximately 0.2 mm in diameter and larger, 1-1.5 mm colonies

217 of the same type (Figure 1 D). After two passages, colonies of *Tannerella* sp. HOT-286 on
218 BA measured, on average, approximately 0.5 mm in diameter after 8 d growth, had a
219 circular or slightly irregular shape, undulate edge, convex profile and convoluted surface.
220 Colonies were grey/off-white in colour, and speckled with opaque cream internal flecks.

221

222 Gram-staining and transmission electron microscopy (TEM) revealed that cells of *Tannerella*
223 sp. HOT-286 were Gram-negative and filamentous (Figure 1 E), measuring 1.2 µm in width,
224 and comprised of segments of varying sizes (Figures 1 F and G). Cells ranged in length from
225 4 to over 50 µm (Figure 1 F). Pili, flagella, or other surface structures were not observed
226 (Figures 1 F and G).

227

228 There was insufficient independent growth of *Tannerella* sp. HOT-286 SP18_24 on BA to
229 perform API ZYM and Rapid ID 32 A tests according to the manufacturer's instructions.
230 Therefore, tests were repeated using SP18_24 biomass harvested from 7-d cultures cross-
231 streaked with *P. acnes*, and compared against results of equivalent tests for *P. acnes*. For
232 some tests, the result was positive for SP18_24 but negative for *P. acnes*, and vice versa,
233 (Table 1), lending credence to the validity of the results. SP18_24 exhibited proteolytic and
234 glycolytic activity and was positive for alkaline phosphatase, acid phosphatase, esterase,
235 esterase lipase and Naphthol-AS-BI-phosphohydrolase.

236

237 *Tannerella* sp. HOT-286 SP18_24 was susceptible to amoxicillin, ampicillin, erythromycin,
238 tetracycline, metronidazole and ceftazidine (with zones of inhibition measuring 30 mm or
239 more in diameter); weakly susceptible to penicillin and chloramphenicol (zones of inhibition
240 of 14-20 mm); and resistant to gentamycin and ciprofloxacin (no zone). The antimicrobial
241 susceptibility profile for *T. forsythia* FDC 338^{T} was the same as that of SP18_24 except that
242 it was strongly susceptible to penicillin, with a 65 mm zone of inhibition.

243

244 Neither pyoverdines-Fe, nor the CS or CFE of *P. acnes* showed any stimulatory effect on the
245 growth of SP18_24 relative to negative controls. Furthermore, SP18_24 did not grow in
246 broth culture, with or without *P. acnes* CS/CFE.

247

248 *P. acnes* and *P. intermedia* showed the strongest growth stimulation (+++) of SP18_24, with
249 dense satelliting growth around *P. acnes* (Figure 4 g), and the development of large colonies
250 of SP18_24 at a distance of up to 25 mm from *P. intermedia* as well as satellite growth
251 (Figure 4 f). *A. oris* and *F. nucleatum* showed moderate growth stimulation (++, Figures 4 d
252 & h). *V. dispar* and *P. gingivalis* were able to weakly stimulate growth of SP18_24 (+,
253 Figures 4 b & e), whereas *S. oralis* and *P. micra* showed no stimulatory capacity (Figures 4
254 a & c).

255

256 The novel *Tannerella* sp. HOT-286 strains have been deposited in culture collections as
257 follows: *Tannerella* sp. HOT-286 SP18_4 – DSMZ XX, JCM 31301; *Tannerella* sp. HOT-286
258 SP18_24 – DSMZ XX, JCM 31302; and *Tannerella* sp. HOT-286 SP18_26 – DSMZ XX,
259 JCM 31303.

260

261 **Discussion**

262

263 Several authors have highlighted the need to cultivate *Tannerella* sp. HOT-286 (de Lillo et
264 al. 2004; Leys et al. 2002; Zuger et al. 2007). Although it was reported that *Tannerella* sp.
265 HOT-286 had been successfully cultivated as part of a consortium (Duran-Pinedo et al.
266 2011), the consortium was lost before a pure culture could be obtained (Frias-Lopez,
267 personal communication). In this study, we used growth in consortia, growth with a cross-
268 streaked helper organism, growth on a membrane over a helper organism lawn, and

269 supplementation with siderophores to allow culture of *Tannerella* sp. HOT-286, leading to its
270 successful isolation in purity. Two of the three novel *Tannerella* isolates were cultured on
271 pyoverdine-Fe-supplemented plates, although growth stimulation by pyoverdine-Fe was not
272 confirmed. Pyoverdine-Fe has been shown to be strongly stimulatory to the difficult-to-
273 culture bacterium *Prevotella* sp. HOT-376 (Vartoukian et al. 2016), demonstrating that
274 growth enhancement by siderophores is a selective phenomenon.

275

276 *Tannerella* sp. HOT-286 was found to be dependent for growth on the proximity of a helper
277 strain, *P. acnes*. It has been observed over the years within the Wade and other labs (Davis
278 et al. 2014) that *P. acnes* stimulates the growth of a number of previously-uncultivated
279 bacteria, although the mechanisms of action are unknown. Interestingly, although co-culture
280 with live *P. acnes* had a strong growth-promoting effect on *Tannerella* sp. HOT-286, this
281 effect was not observed with *P. acnes* CS or CFE, suggesting that the stimulating factor is
282 labile. Furthermore, growth was enhanced more strongly by culturing *Tannerella* sp. HOT-
283 286 on the surface of a membrane over a lawn culture of *P. acnes*, than by culturing the
284 strain directly on agar with *P. acnes* cross-streaks. This would imply either that a greater
285 amount of 'helper' signal, as provided by the larger surface area of a lawn culture than of a
286 narrow cross-streak, is needed for growth; or that separation from the agar surface by a
287 membrane helps protect the recipient from potential growth inhibitors present in the agar
288 medium. It has been shown that hydrogen peroxide, produced during autoclave sterilisation
289 of media which includes both phosphate and agar can inhibit bacterial growth (Tanaka et al.
290 2014).

291

292 Six of eight oral bacterial species, representing four phyla, stimulated the growth of
293 *Tannerella* sp. HOT-286 SP18_24. In general, growth stimulation was observed as
294 satellitism immediately surrounding the helper strain, although *P. intermedia* effected the

295 emergence of several large outlier colonies of SP18_24 at a distance from the helper.
296 Although beyond the scope of this study, a future challenge will be to determine by what
297 mechanism these different helpers stimulate growth of SP18_24, and whether there is a
298 universal or specific mode of action.

299

300 Cells of *Tannerella* sp. HOT-286 were found to be segmented filaments of variable length,
301 confirming the observations of Zuger *et al* (2007) following FISH analysis of BU063 cells.
302 However, their impression that individual segments of cells are of equal length and that
303 consequently, overall cell length is a reflection of the total number of segments present, was
304 not confirmed by our TEM images: whereas the 16 μm cell shown in Figure 1 G had six
305 segments, the longer 50 μm cell in Figure 1 F had only four.

306

307 *Tannerella* sp. HOT-286 is found in high prevalence, but low abundance, in periodontal
308 disease-associated plaques (Zuger et al. 2007), and the relative abundance of this phylotype
309 is estimated to be around 0.05% of subgingival bacteria (HOMD release 13) (Beall et al.
310 2014). Evidence from several studies has indicated that, unlike *T. forsythia*, *Tannerella* sp.
311 HOT-286 is primarily associated with periodontal health (de Lillo et al. 2004; Kumar et al.
312 2003; Leys et al. 2002); Leys et al (2002) reported odds ratios for prevalence in periodontitis
313 of *Tannerella* sp. HOT-286 and *T. forsythia* of 0.1 and 9.9 respectively. The apparent
314 phenotypic dichotomy between these closely-related taxa is clearly of interest, and
315 comparative studies of *Tannerella* sp. HOT-286 and *T. forsythia* could provide some insight
316 into factors involved in the latter's virulence.

317

318 To this end, an enzymatic profile of *Tannerella* sp. HOT-286 was generated and compared
319 to that of *T. forsythia*. The profiles of the two *Tannerella* taxa were similar, despite their
320 different clinical phenotypes. Both taxa produced a range of proteolytic, hydrolytic, lipolytic

321 and saccharolytic enzymes. A comparison of the enzyme activity of the two taxa did not
322 reveal any obvious differences relevant to the virulence of *T. forsythia* although clearly its
323 virulence could be related to factors unconnected to the tests included in the API ZYM and
324 Rapid ID 32 A kits. Antimicrobial susceptibility profiles were also similar for the two taxa.

325

326 Beall and co-workers (2014) isolated individual cells of *Tannerella* sp. HOT-286 by flow
327 cytometry and used multiple displacement amplification to generate a collection of single-
328 cell-amplified genomes with predicted sizes from 3.44 to 4.07 Mb. Putative virulence genes
329 of *T. forsythia* were detected by comparative analysis with the HOT-286 genomes and
330 included genes encoding the PrtH, BspA, NanH and KLIKK proteases (Beall et al. 2014;
331 Ksiazek et al. 2015). Beall *et al* (2014) reported a surprisingly high level of strain
332 polymorphism and substantial nucleotide divergence between the various genomes of
333 *Tannerella* sp. HOT-286. Given that multiple displacement amplification can result in uneven
334 amplification of the genome (Lasken 2012), complete genome sequences are being
335 generated for the three *Tannerella* sp. HOT-286 strains isolated in this study, to enable
336 further comparative genomic analysis with *T. forsythia*.

337

338 Leys and co-workers (2002) showed that subgingival plaque samples were less likely to be
339 dual-colonized with *Tannerella* sp. HOT-286 and *T. forsythia* than would be expected by
340 chance. They suggested a specific exclusionary mechanism, with the possibility that
341 *Tannerella* sp. HOT-286 may provide protection from acquisition of *T. forsythia*. Inverse
342 associations between oral bacteria as a result of antagonistic interactions have been
343 reported for *Streptococcus mutans* and *Streptoccous sanguinis* (Kreth et al. 2005). If
344 confirmed, this could have far-reaching implications in the management of periodontitis. With
345 health-associated *Tannerella* sp. HOT-286 having finally been cultivated, and available for
346 study, such exciting therapeutic possibilities may now be explored.

347

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349

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444

445 **Figure legends**

446

447 Figure 1: Growth characteristics, colony and cellular morphology of *Tannerella* sp. HOT-286.
448 (A-C) Six-day cultures of *Tannerella* sp. HOT-286: (A) No visible growth in absence of helper
449 strain; (B) Satellitism beside *P. acnes* streak on left, no growth beside *F. nucleatum* streak
450 on right; (C) Strong growth on membrane over *P. acnes* lawn. (D) Nine-day culture of
451 *Tannerella* sp. HOT-286 after revival from -80 °C storage showing sparse growth of colonies
452 of variable size; Arrows indicate position of tiny colonies; Bar = 5 mm. (E) Cellular
453 morphology of *Tannerella* sp. HOT-286 by Gram staining; Bar = 10 μm. (F-G) Transmission
454 electron photomicrographs of 2 cells of *Tannerella* sp. HOT-286 (Strain SP18_24): (F) Long
455 cell of about 50 μm in length; Bar = 5 μm. (G) Cell showing segments of variable size; Bar
456 = 2 μm.

457

458 Figure 2: Seven-day cultures of *Tannerella* sp. HOT-286 (SP18_24) showing growth
459 stimulation by several of the eight potential helper strains tested: (a) *Streptococcus oralis*, (b)
460 *Veillonella dispar*, (c) *Parvimonas micra*, (d) *Actinomyces oris*, (e) *Porphyromonas gingivalis*,
461 (f) *Prevotella intermedia*, (g) *P. acnes* and (h) *F. nucleatum*.

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464

	Tannerella sp. HOT-286 (SP18_24) [2]	*Tannerella forsythia* [T] (FDC 338)	*Propionibacterium acnes* (ATCC 6969)
API ZYM			
Alkaline phosphatase	+	+	-
Esterase (C4)	+	+	-
Esterase lipase (C8)	+	+	-
Lipase (C14)	-	-	-
Leucine arylamidase	+	+	-
Valine arylamidase	-	-	-
Cystine arylamidase	-	-	-
Trypsin	+	+	-
α-chymotrypsin	-	-	-
Acid phosphatase	+	+	+
Naphthol-AS-BI-phosphohydrolase	+	+	-
α-galactosidase	-	-	-
β-galactosidase	-	-	+
β-glucuronidase	-	+	-
α-glucosidase	+	-	-
β-glucosidase	-	-	-
N-acetyl-β-glucosaminidase	-	+	+
α-mannosidase	-	-	+
α-fucosidase	-	+	-
Rapid ID 32 A			
Urease	-	-	-
Arginine dihydrolase	-	-	+
α-galactosidase	-	-	-
β-galactosidase	-	+	+
β-galactosidase-6-phosphate	-	+	-
α-glucosidase	+	-	-
β-glucosidase	-	+	-

α-arabinosidase	-	-	-
β-glucuronidase	-	-	-
N-acetyl-β-glucosaminidase	-	+	+
Mannose fermentation	-	-	+
Raffinose fermentation	-	-	-
Glutamic acid decarboxylase	-	-	-
α-fucosidase	+	+	-
Reduction of nitrates	-	-	+
Indole	+	-	+
Alkaline phosphatase	+	+	-
Arginine arylamidase	+	+	+
Proline arylamidase	-	-	+
Leucyl glycine arylamidase	+	+	-
Phenylalanine arylamidase	+	-	-
Leucine arylamidase	+	+	-
Pyroglutamic acid arylamidase	+	-	-
Tyrosine arylamidase	+	+	-
Alanine arylamidase	+	+	+
Glycine arylamidase	-	-	+
Histidine arylamidase	+	+	-
Glutamyl glutamic acid arylamidase	-	-	-
Serine arylamidase	-	-	+

465 **Table 1.** Results of API ZYM and rapid ID 32 A tests [1]

466

467 [1] Reactions graded on a scale of 0-5 with values of 3-5 reported as a positive result (+), as
468 recommended in manufacturer's guidelines

469 [2] Cross-streaks of *P. acnes* present on source plates

470