

Microbial Ecology

A new species of the γ -proteobacterium *Francisella*, *F. adeliensis* sp. nov., endocytobiont in an Antarctic marine ciliate and potential evolutionary forerunner of pathogenic species --Manuscript Draft--

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Abstract:	<p>The study of the draft genome of an Antarctic marine ciliate, <i>Euplotes petzi</i>, revealed foreign sequences of bacterial origin belonging to, and implying symbiotic relationships with the γ-proteobacterium <i>Francisella</i> that includes pathogenic and environmental species. TEM and FISH analyses confirmed the presence of a <i>Francisella</i> endocytobiont in <i>E. petzi</i>, which we then isolated and found to be a new species, named <i>F. adeliensis</i> sp. nov.. <i>F. adeliensis</i> grows well at wide ranges of temperature, salinity, and carbon dioxide concentrations implying that it may colonize new organisms living in deeply diversified habitats. The <i>F. adeliensis</i> genome includes the <i>igl</i> and <i>pdp</i> gene sets (<i>pdpC</i> and <i>pdpE</i> excepted) of the <i>Francisella</i> pathogenicity island needed for intracellular growth. Consistently with an <i>F. adeliensis</i> ancient symbiotic lifestyle, it also contains a single insertion-sequence element. Instead, it lacks genes for the biosynthesis of essential amino acids such as cysteine, lysine, methionine and tyrosine. In a genome-based phylogenetic tree, <i>F. adeliensis</i> forms a</p>

	<p>new early branching clade, basal to the evolution of pathogenic species. The correlations of this clade with the other clades raise doubts about a genuine free-living nature of the environmental <i>Francisella</i> species isolated from natural and man-made environments, and suggest that <i>F. adeliensis</i> should be considered a pioneer in the <i>Francisella</i> colonization of eukaryotic organisms.</p>
<p>Response to Reviewers:</p>	<p>We would like to gratefully acknowledge the Reviewer #1 for the appreciation of our work and for the suggestion directed to improve the original version of the manuscript. According to his/her suggestion, the <i>F. adeliensis</i> genome was screened for the <i>Francisella</i> Pathogenicity Island. Two genes (<i>pdpC</i> and <i>pdpE</i>) were found lacking from the gene sets encoding the Type VI secretion system, and the implication of this loss was discussed.</p> <p>With regard to the comment of Reviewer #2, we have carried out a TEM analysis to better visualize the <i>F. adeliensis</i> localization inside the host, and dedicated a new multi-panel figure to this localization. However, in relation to the Reviewer criticism that “The authors are using a word of symbiotic against this bacterium [without] confirming whether this <i>F. adeliensis</i> has some mutual functions as the symbiotic bacteria for the host cell”, we need to point out that our manuscript does not deal with <i>Francisella/Euplotes</i> symbiotic relationships. We did not claim at all about species-specificity relationships. In fact, in the Discussion section of the manuscript we have written that “Growing well at temperatures ranging from 4 to 30 °C and promptly adapting to 0-35 ‰ variations in the ambient salinity, <i>F. adeliensis</i> appears capable of colonizing other organisms independently of their adaptation to live in marine, brackish or lacustrine habitats of either cold, or temperate areas”. We understand and use the term “symbiosis” (cytobiont) according to the original definition of Heinrich Anton de Bary (1879): “The living together of unlike organisms”.</p>

23 **Abstract**

24 The study of the draft genome of an Antarctic marine ciliate, *Euplotes petzi*, revealed foreign sequences
25 of bacterial origin belonging to, and implying symbiotic relationships with the γ -proteobacterium
26 *Francisella* that includes pathogenic and environmental species. TEM and FISH analyses confirmed the
27 presence of a *Francisella* endocytobiont in *E. petzi*, which we then isolated and found to be a new species,
28 named *F. adeliensis* sp. nov.. *F. adeliensis* grows well at wide ranges of temperature, salinity, and carbon
29 dioxide concentrations implying that it may colonize new organisms living in deeply diversified
30 habitats. The *F. adeliensis* genome includes the *igl* and *pdp* gene sets (*pdpC* and *pdpE* excepted) of the
31 *Francisella* pathogenicity island needed for intracellular growth. Consistently with an *F. adeliensis* ancient
32 symbiotic lifestyle, it also contains a single insertion-sequence element. Instead, it lacks genes for the
33 biosynthesis of essential amino acids such as cysteine, lysine, methionine and tyrosine. In a genome-based
34 phylogenetic tree, *F. adeliensis* forms a new early branching clade, basal to the evolution of pathogenic
35 species. The correlations of this clade with the other clades raise doubts about a genuine free-living nature of
36 the environmental *Francisella* species isolated from natural and man-made environments, and suggest that *F.*
37 *adeliensis* should be considered a pioneer in the *Francisella* colonization of eukaryotic organisms.

38

39 Keywords: endosymbiosis, microbial associations, polar microbiology, environmental *Francisella*,
40 *Francisella* phylogeny, *Euplotes*

41

42 **Introduction**

43 Like their multicellular descendants, also single-celled eukaryotes host a huge variety of bacteria. Ciliates in
44 particular are a preferential and stable home to bacteria, which may be carried either attached as epibionts to
45 the cell body surface, as is the case of the association between a group (designated as ‘epixenosomes’) of
46 Verrucomicrobia and *Euplotidium itoi* [1], or enclosed as endocytobionts inside the cell body. Being a
47 principal component of the diet of ciliates, that are mostly phagotrophic and filter-feeding, bacteria can easily
48 escape digestion and adopt a new intracellular lifestyle [2]. Roughly 250 ciliate species, among the nearly
49 10,000 that are in total known, have been detected to be hosts of endocytobiont bacteria. Large-size species
50 of *Paramecium*, *Euplotes* and *Spirostomum* may be home also of mixed populations of unrelated species of
51 bacteria [3, 4].

52 The knowledge of the biology and life cycle of endocytobiont bacteria in ciliates is essentially limited to
53 species of *Holospora* and *Caedibacter*, that are colonizers of the nuclear apparatus of freshwater species of
54 *Paramecium* [5, 6]. These symbionts have been successfully isolated from host-cell homogenates, but any
55 attempt of cultivation outside their hosts has failed as in the case of any other bacterial symbiont of aerobic
56 ciliates [7].

57 A substantial contribution to improve this knowledge may now be provided by the isolation and
58 cultivation of *Francisella* bacteria living as endocytobionts in marine species of *Euplotes*, a genus which is
59 quite rich also in freshwater species extensively studied for their symbiotic associations with polymorphic
60 populations of *Polynucleobacter* [8]. *F. endociliophora*, earlier described as a novel subspecies of *F.*
61 *noatunensis* [9], is the first *Francisella* that has been isolated and genome-sequenced from a marine species
62 of *Euplotes*, *E. raikovi*, dwelling in temperate waters [10]. Here we report the isolation and genome
63 sequencing of another new species of *Francisella*, *F. adeliensis* sp. nov., living as endocytobiont in a bipolar
64 (Antarctic and Arctic) species of *Euplotes*, *E. petzi*.

65 The genus *Francisella* comprises species classified as facultative intracellular γ -proteobacteria
66 potentially noxious to their hosts [11, 12]. *F. tularensis*, with its three subspecies, is a specialized
67 intracellular pathogen of both invertebrate and vertebrate hosts, human beings included [13, 14]. *F.*
68 *noatunensis*, with its two subspecies adapted to different hosts' temperatures, is the etiological agent of the
69 fish disease known as francisellosis [15, 16]. The endosymbiotic *F. persica* (ex *Wolbachia persica*) [17],
70 together with the generalists *F. philomiragia* and *F. novicida*, may harm human beings with a compromised
71 immune system [18-20].

72 The position that *F. adeliensis* takes in the genome-based phylogenetic tree provides new insights on
73 *Francisella* diversity and helps to decipher the emergence of symbiosis and the evolution of pathogenicity in
74 this genus.

75

76 **Materials and Methods**

77 ***E. petzi* cultures**

78 The *E. petzi* cells were isolated from a sample of seawater and sandy bottom collected by means of a
79 sediment trap from Adelie Cove in Antarctica, at a depth of 27 m, a temperature of -1.2 °C and a salinity of
80 34 ‰. Cultures were maintained in the laboratory in cold rooms, at 4 °C, under a cycle of 12 h of very low

81 light and 12 h of dark, as previously described [21]. The green alga *Dunaliella tertiolecta* was used as food
82 source.

83

84 **Fluorescent in situ hybridization (FISH)**

85 *E. petzi* cells were collected from severely starved cultures, transferred onto glass slides, fixed with 4 %
86 formaldehyde in phosphate saline buffer (PBS) for 10 min at room temperature, and permeabilized by
87 ethanol gradient (50 %, 80 % and 100 % of ethanol in water, for 10 min each). The fluorescein-labeled probe
88 EUB338 (5'-GCTGCCTCCCGTAGGAT-3') for eubacteria and the Cy3-labeled probe Bwall1448 (5'-
89 CAACCATTGCGCCGGGCCT-3') for *Francisella* were synthesized by Integrated DNA Technologies
90 (Coralville, Iowa, USA). Hybridization was performed following the method described by Hugenholtz et al.
91 [22]. Briefly, 2 µl of each probe solution (50 ng/µl) in 20 µl hybridization buffer (0.9 M NaCl, 20 mM Tris-
92 HCl pH 7.0, 15 % formamide, 0,1 % SDS) were added directly to the cells on slides. Hybridization was
93 performed in a humid chamber at 46 °C for 3 h. Slides were then washed 20 min with washing buffer (318
94 mM NaCl, 20 mM Tris-HCl pH 7.0, 0.1 % SDS) at 48 °C and air dried. Slides were embedded with anti-
95 fading mounting medium and then inspected with a Nikon confocal microscope (Nikon, Amsterdam, The
96 Netherlands).

97

98 **Transmission electron microscopy (TEM)**

99 For TEM analyses, samples were fixed with 2.5% glutaraldehyde and 6% sucrose in 0.1 M cacodylate
100 buffer, pH 7.2, for 2 h at 4 °C. After three washings at 4°C in the same buffer, samples were post-fixed with
101 1% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.2, for 1 h at 4°C, washed in the same buffer, and
102 dehydrated in a gradient ethanol series. Samples were then infiltrated with mixtures of LRWhite
103 resin/ethanol in different percentages, embedded in pure LRWhite resin, and left to polymerize for 2 days at
104 50°C. Resin blocks were cut with a Reichert Ultracut ultramicrotome using a diamond knife. Ultrathin
105 sections (60-80 nm) were collected on copper grids, stained with uranyl acetate and lead citrate, and
106 observed with a JEOL 1200 EXII electron microscope. Micrographs were captured using an Olympus SIS
107 VELETA CCD camera equipped with iTEM software.

108

109 **Isolation, identification and culturing**

110 *F. adeliensis* was isolated from *E. petzi* following the protocol of Sjödin et al. [10]. Briefly, *E. petzi* cell
111 samples were bead beaten and acid treated according to Humrighouse et al. [23], before being diluted in PBS
112 and spread on CHAB (Cysteine Heart Agar Blood) culture plates, supplemented with 10⁵ U/l penicillin and
113 40 mg/l vancomycin as described [24]. The culture plates were incubated at 4 °C for 1 to 2 weeks and
114 monitored for bacterial growth. Colonies were then isolated and maintained in CHAB plates at 4 °C. To
115 identify *F. adeliensis* from other contaminating bacteria, isolated colonies were picked, resuspended in 20 µl
116 of water and immediately lysed by boiling for 3 min. Five µl of each lysed cell suspension were used as
117 template in PCR, run using two sets of primers: fw1 (5'-GCGTTTACCACGGAGTGATT-3') and rv1 (5'-
118 TGGAGCCTAGCGGGATC-3'); fw2 (5'-AGTCAGGGAGGAAGTTTATTTGGTT-3') and rv2 (5'-
119 CACCTTCCTCCGCCTTGT-3'). Positive clones were maintained in CHAB plates for subsequent analysis.

120 For dot-plate analysis, one isolated *Francisella* colony was picked and suspended in 1 ml PBS buffer.
121 Five µl of serial dilution of the *Francisella* suspension were spotted on CHAB plates and incubated at 4, 12,
122 20, 30 and 37 °C. Plates were checked for bacterial growth every 3 days. For growth assays in liquid
123 medium, an overnight culture was used to inoculate aliquots of 50 ml of T medium [25] to reach an OD₆₀₀
124 ranging from 0.01 to 0.05. Flasks were then incubated at different temperatures, salinity and CO₂
125 concentrations. Bacterial growth was monitored by measuring the OD₆₀₀ every day during the first week,
126 then every three days. The number of generation/day were calculated using the Origin 8 software.

127 The presence of the enzymes catalase and oxidase were tested on agar-plates using a 3 % H₂O₂ solution
128 and an oxidase-strip (OXOID- Thermo Fisher Scientific Inc, Monza, Italy), respectively. Motility was
129 determined with the hanging drop technique.

130

131 **Genome sequencing and assembly**

132 Isolated DNA were sequenced using Nextera XT library protocol on an Illumina MiSeq instruments in
133 addition to a Pacific Biosciences RSII system (10-kb library, 2-h movie length), generating a total of 57,926
134 PacBio reads with an average read length of 11,653 bp, using a single-molecular real-time (SMRT) cells.
135 The initial draft of the genome was generated by assembling PacBio reads using the SMRT Analysis system
136 version 2.3.0. Polishing of the draft genome was performed using Illumina reads in berokka and Pilon [26].

137

138 **Phylogenetic analysis**

139 The phylogenetic analysis was inferred using the Neighbor-Joining method [27]. The evolutionary distances
140 among *Francisella* genomes were computed using the number of differences method [28] and are in the units
141 of the number of base differences per sequence. The analysis involved 139 nucleotide sequences, with a total
142 of 213,734 positions in the final dataset. Positions containing gaps and missing data were eliminated.
143 Evolutionary analyses were conducted in MEGA7 [29]. *Fangia hongkongensis* was included as outgroup to
144 generate the genome-based phylogenetic tree.

145

146 **Results**

147 **Identification**

148 Total DNA preparations of *E. petzi* subjected to high throughput sequencing generated 24,800 assembled
149 contigs (Villalobo and Vallesi, unpublished), of which approximately 800 (equivalent to a total of 1.6 Mb)
150 revealed a close similarity to bacterial sequences available from public databases, with the highest matching
151 value of each contig systematically resulting against gene sequences of *Francisella* species.

152 Among the 800 contigs, one of 5091 bp included the 16S and 23S rRNA gene sequences plus the
153 sequences of the tRNA^{Ile} and tRNA^{Ala} genes (Fig. 1A). Therefore, it revealed to be a typical bacterial rDNA
154 operon. Using the SILVA INcremental Aligner bioinformatics tool [30], the 16S rRNA gene sequence of this
155 operon was classified as belonging to *Francisella* with 94.39 % identity and 97 score along 1,480 bp. Given
156 that the 3% cut-off rule [31] for a 16S divergence among species was fulfilled, the new 16S rRNA gene
157 sequence was assumed to belong to a new *Francisella* species for which the proposed name is *Francisella*
158 *adeliensis* nov. sp.. The species name is after that of the Antarctic cove, Adelie, from which *E. petzi*, the *F.*
159 *adeliensis* host, was collected.

160 Analysed in the BLASTN 2.6.1 database [32] for its closest identity, the *F. adeliensis* 16S rRNA gene
161 sequence showed the best alignment (only seven nucleotide variations along 1376 bp) with the 16S gene
162 sequence of an unnamed and uncultured γ -proteobacterium reported to be a chemoautotrophic symbiont on
163 gills of deep-sea clams and mussels collected at a 10-m depth from the fjord of Saanich Inlet, British
164 Columbia [33]. The other two closest counterparts were the 16S sequences of *F. endociliophora* [10] and *F.*
165 *salina* [24], with 96 % of sequence identity along the 1481-bp gene length.

166

167 **Intracellular localization**

168 To verify whether *F. adeliensis* resides as endosymbiont inside *E. petzi*, or it coexists as environmental
169 bacteria with *E. petzi* in culture, *E. petzi* cells were starved for 10 days to avoid any possible bacterial
170 contamination from undigested food, and analyzed by fluorescent in-situ hybridization (FISH) with two
171 distinct probes: one ('EUB338', see Materials and Methods) specific to a 16S rRNA-sequence conserved in
172 most bacterial species, and the second ('Bwall1448') specific to a 23S rRNA region unique to *Francisella*
173 [34]. Both probes generated fluorescent signals within the cytoplasm of *E. petzi* cells (Fig. 1B), and their co-
174 localization provided evidence that *F. adeliensis* was the only guest.

175 Transmission electron microscopy of *E. petzi* cells (deprived of food for not less than one week before
176 being used) confirmed the presence of numerous bacteria (Fig. 2). Only occasionally were they observed to
177 be individually dispersed in the cytoplasm. Each bacterium was confined inside a membranous-bound
178 vesicle (Fig. 2F,G), or it was apparently free in the cytosol (Fig. 2E). Much more often, however, bacteria
179 appeared clustered together in larger fusogenic membrane-bound structures (Fig. C,D), which were quite
180 heterogeneous in size and number of enclosed bacteria, and were usually located in close proximity of the
181 host's somatic and transcriptionally active nucleus (macronucleus).

182

183 Phenotypic traits

184 The isolation of *F. adeliensis* was carried out from *E. petzi* cell lysates following the procedure previously
185 used for *F. endociliophora* [10], taking care to incubate plates at 4 °C. Individual colonies were screened by
186 PCR using two sets of specific primers (Fig. 1A). Primers ('fw1' and 'rv1', see Materials and Methods) of
187 one set were designed to amplify a 360-bp fragment containing a 33-bp sequence lying between the two
188 tRNA coding regions and without counterparts in the rDNA operons of other *Francisella* species. Primers
189 ('fw2' and 'rv2') of the second set were designed to amplify a 660-bp fragment of the 16S rRNA coding
190 region shared among other *Francisella* species. Products sequenced from both amplifications showed to fully
191 match the genomic data, confirming the taxonomic identity of the isolated colonies with *F. adeliensis*.

192 On CHAB plates, *F. adeliensis* colonies look round, white, and slightly mucoidal, formed by rod-shaped
193 and Gram-negative bacteria that are catalase-positive, oxidase-negative, and non-motile. In solid medium,
194 they are visible after 3 days of incubation at temperatures ranging from 20 to 30 °C, and require 6-12 days to
195 grow when incubated at 4 and 10 °C (Fig. 3A). In liquid medium, the highest growth rate was measured at
196 20 and 30 °C, and the lower at 4 °C (Fig. 3B). The mean numbers of generations/day were counted to be

197 0.11, 0.29, 0.53, 0.47 at 4, 10, 20 and 30 °C, respectively, and no growth was observed at 37 °C. Roughly
198 one half of bacteria inoculated on plates at 37 °C died after 16 h of incubation and none survived after 48 h.

199 In the presence of 5 % CO₂, *F. adeliensis* cultures grew with OD₆₀₀ values approximately 60 % lower
200 than those measured in ambient atmosphere (0.04 %). Instead, no significant variation in the growth rate was
201 observed in cultures left to grow in liquid medium containing salt concentrations ranging from 0 to 35 %,
202 implying that *F. adeliensis* is a strongly euryhaline bacterium (data not shown).

203

204 **Genomic features**

205 The *F. adeliensis* genome extends for 2,054,094 bp, a length matching the mean genome size of other
206 *Francisella* species (1.96±0.14 Mbp, Table 1) much more closely than the size of any other bacterial genome
207 (3.82 ±1.8 Mbp) [35]. It contains 1,880 protein coding sequences, 38 tRNA genes, 10 rRNA genes (four 5S
208 rRNA, three 16S rRNA and three 23S rRNA) and one tmRNA gene (Table 1). Its average nucleotide identity
209 (ANI) with the closest *Francisella* genomes is in the range of 77- 78.8 % (Table 2), that is distant from the
210 95-96 % range usually taken as the minimum threshold value to consider two genome sequences as
211 belonging to the same species [36]. Consistently with an intracellular lifestyle, the average 32.6 % G+C
212 content of the *F. adeliensis* genome closely reflects the 32.38 ± 0.24 % content of the other endosymbiotic
213 *Francisella*, and is significantly lower than the average G+C content (49.1 ± 12.4 %) shown by free-living
214 bacteria [35].

215 Based on a search for transposable elements and phages carried out with PHASTER and ISFinder
216 softwares [37, 38], the *F. adeliensis* genome contains prophage sequences like other *Francisella*. However, it
217 includes only one IS*Ftu4* insertion sequence element (E-value 1e⁻¹⁵).

218 Analysis of the *F. adeliensis* genome for the presence of the *igl* (intracellular growth locus)
219 and *pdp* (pathogenicity determinant proteins) genes, components of the so-called ‘*Francisella*
220 pathogenicity island’ responsible for the virulence of *F. tularensis* [39, 40], indicated that all ten *igl* genes
221 were present, but that the five *pdp* gene set lacked the *pdpC* and *pdpE* genes.

222 The observation that *F. adeliensis* requires complex media to grow in culture suggested a loss of genes
223 responsible for the synthesis of essential amino acids. This hypothesis was verified by screening the *F.*
224 *adeliensis* genome for the presence of genes responsible for the synthesis of arginine, cysteine, histidine,
225 lysine, methionine and tyrosine for which the pathogenic *F. tularensis* is known to be auxotrophic [41]. Only
226 the histidine and arginine biosynthesis appeared to be genetically supported, the histidine biosynthesis by the

227 complete set of relevant genes and the arginine biosynthesis by the activity of an *argJ* gene that likely
228 replaces the lack of *argA*, *argD* and *argE* genes [42]. Instead, the biosynthesis of the other four amino acids
229 appeared genetically not supported. The *F. adeliensis* genome lacks the genes *dapD*, *dapC* and *dapE*
230 encoding enzymes responsible for the lysine biosynthesis [43], as well as the gene encoding cystathionine γ -
231 synthase responsible for the methionine and cysteine biosynthesis [44]. With regard to the tyrosine
232 biosynthesis, the genome contains the complete gene set for the shikimate pathway, but it lacks the gene
233 encoding prephenate dehydrogenase which converts prephenic acid to 4-hydroxyphenyl-pyruvic acid [45].
234 In conclusion, *F. adeliensis* shows to be prototrophic for arginine and histidine, and auxotrophic for cysteine,
235 lysine, methionine and tyrosine.

236

237 **Phylogenetic relationships**

238 To assess the *F. adeliensis* interspecific relationships, the *F. adeliensis* genome was compared with the other
239 *Francisella* genomes available from NCBI using 139 gene sequences for a total of 213,734 nucleotide
240 positions. As shown in Fig. 4, *F. adeliensis* forms its own clade with a high statistical support. Together with
241 the clade formed by *F. frigiditurris*, a species recently isolated from the water of a cooling tower [46], it
242 precedes the split of four other major clades in which all the other *Francisella* species are subdivided in full
243 accord with the recently proposed genome-based *Francisella* phylogeny [46, 47]. One of the four clades is
244 specific to species, such as *F. tulariensis* and *F. novicida*, that are pathogenic to terrestrial hosts, and *F.*
245 *persica* (formerly *Wolbachia persica*) isolated from ticks [12, 17]. The second one includes species such as
246 *F. noatuniensis* that are pathogenic to fish, as well as *F. salina* isolated from a seawater sample [46]. The
247 third one includes species such as *F. endociliophora* and *F. halioticida* isolated from marine hosts, together
248 with *F. uliginis* isolated from a seawater sample [9, 12, 46]. And the fourth one is specific to *Francisella*
249 species that have been isolated from waters of cooling systems, and are usually described as ‘environmental’
250 species and regarded as belonging to the genus *Allofrancisella* [48, 49].

251

252 **Discussion**

253 The isolation reported here of *F. adeliensis* from an Antarctic strain of *E. petzi* follows the isolation of *F.*
254 *endociliophora* from *E. raikovi* [10], which is a species distributed in the Caspian and Mediterranean Seas
255 and Eastern Atlantic Ocean [50], and the identification of DNA sequences of a taxonomically undetermined
256 *Francisella* in the genome of *E. focardii* [51], which is a species endemic to Antarctic coastal waters [52].

257 Altogether these findings strongly suggest that *Francisella/Euplotes* associations are relatively common in
258 the marine environment, and two additional considerations reinforce this hypothesis. The first consideration
259 is related to the bipolar biogeographic distribution that characterizes the species structure of the *F.*
260 *adeliensis*'s host, *E. petzi* [21, 53]. Embracing Arctic and White Sea populations in addition to Antarctic and
261 peri-Antarctic ones, this distribution clearly implies that the *F. adelinesis* association with *E. petzi* is likely
262 not restrained to the Antarctic waters where it has been detected. Being extended to the high latitudes of both
263 the hemispheres, it appears to be virtually global and the analysis of other bipolar *Euplotes* species for their
264 symbiotic associations with *F. adeliensis* and/or its close relatives may definitively establish these global
265 dimensions. The second and more significant consideration is related to the psychrophilic and euryhaline
266 behaviour shown by *F. adeliensis*. Growing well at temperatures ranging from 4 to 30 °C and promptly
267 adapting to 0-35 ‰ variations in the ambient salinity, *F. adeliensis* appears capable of colonizing other
268 organisms independently of their adaptation to live in marine, brackish or lacustrine habitats of either cold,
269 or temperate areas.

270 The 16S rRNA gene sequences are the molecules of choice for phylogenetic reconstructions, but their
271 use in devising a *Francisella* phylogenetic tree has frequently been biased by branches supported by low
272 bootstrap values due to the particularly high degree of conservation that these sequences show in
273 *Francisella*. Only the recent availability of genomic data provided more solid grounds to trace the
274 phylogenetic relationships among *Francisella* species, producing phylogenetic trees with more solid statistic
275 support [46, 54]. In the genome-based tree updated with the inclusion of *F. adeliensis* (shown above in Fig.
276 2), *F. adeliensis* branches surprisingly distant from all the intracellular *Francisella*, including *F.*
277 *endociliophora* endocytobiont in *E. raikovi*. It correlates much closer to the two earliest branching clades that
278 are uniquely formed by *Francisella* species, namely *F. frigiditurris*, *Allofrancisella frigididaquae* and *A.*
279 *guangzhouensis*, isolated from cooling towers. As such, they are collectively regarded as environmental
280 species.

281 Granted that these species are really free living—considering the strong acidic conditions used for their
282 isolation, it cannot be excluded that they have actually been isolated from some eukaryotic microorganisms
283 living inside the cooling towers—this correlation implies that *F. adeliensis* foreruns the *Francisella* adaptive
284 evolution in replacing a free-living lifestyle with an intracellular/endosymbiotic style. And the *F. adeliensis*
285 acquisition of the endosymbiotic lifestyle is likely to be quite ancient, considering that a single IS element is
286 present in its genome. In effect, a low number of mobile genetic elements is widely accepted to be a

287 distinctive trait of an ancient stage of intracellular life and an expansion of these elements to be distinctive of
288 initial stages of host restriction [55, 56]. In addition, the finding that *F. adeliensis* is auxotrophic for cysteine,
289 lysine, methionine and threonine, and likely depends on the host for nutrient supply, establishes a close
290 physiological analogy with pathogenic strains of *F. tularensis*, whose virulence depends on the activity of
291 the *Francisella* pathogenicity island cluster of genes [40]. In a mouse model of tularaemia, it has been
292 shown that among these genes *F. tularensis* and *F. novicida* particularly need the expression the *pdpC* gene
293 in order to escape from phagosomes and become free in the cytosol [57]. Neither the *pdpE* gene, which is
294 not directly involved in *F. tularensis* virulence, nor the *pdpC* gene were identified in the *F. adeliensis*
295 genome. In spite of this gene loss, however, evidence from TEM analysis indicates that, in addition to more
296 common fusogenic membrane-bound structures closely recalling the “*Francisella* containing vacuoles”
297 involved in the autophagy-mediated mechanism of *F. tularensis* re-entry into the endocytic compartment
298 [58], *F. adeliensis* may produce cytosolic stages. Although these stages might suggest that *F. adeliensis* is a
299 potential ecological reservoir for the evolution of pathogenic *Francisella*, the observation that it is unable to
300 proliferate at 37 °C should rule out any ability to colonize and be harmful to homothermic, warm-blood
301 organisms.

302

303 **Description of *Francisella adeliensis* sp. nov.**

304 *Francisella adeliensis* (a.de.lien'sis. L. adj. of Adelie) is named after Adelie Cove, the location in Antarctica
305 where the host, the ciliate *Euplotes petzi*, was collected in 2005 [21]. The type strain is deposited at the
306 Swedish Defence Research Institute (FOI), *Francisella* strain collection # FSC1327. Within its host, *F.*
307 *adeliensis* resides in the cytoplasm, as determined by transmission electron microscopy and a FISH analysis
308 carried out with the *Francisella*-specific probe Bwall1448 [34]. Cells are Gram-negative, non-motile,
309 catalase-positive, oxidase-negative and grow at a wide range of temperature (4–30 °C), salinity (0–35 ‰),
310 and carbon dioxide concentrations (0.04–5 %). The *F. adeliensis* complete genome sequence is available at
311 GenBank, with the accession number CP021781, and supporting sequencing data are deposited in Bioproject
312 PRJNA389235.

313

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322

323 **Compliance with Ethical Standards**

324

325 **Conflict of Interest** The authors declare that they have no conflict of interest.

326

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471

472 **Figure legends**

473

474 **Fig 1** *F. adeliensis* identification. **A** Schematic representation of the *F. adeliensis* rDNA operon. The relative
475 positions of fluorescent FISH probes and primers used in colony-PCR are indicated. The 33-bp sequence
476 exclusive of *F. adeliensis* rDNA operon is shown. **B** Fluorescent in situ hybridization of *E. petzi* cells: a,
477 signal from fluorescein-labeled probe EUB338 for all eubacteria; b, signal from Cy3-labeled probe
478 Bwall1448 specific for *Francisella*; c, co-localization of signals of the two labeled probes. Scale bar=20 μ m.

479

480 **Fig 2** Transmission electron microscopy of *E. petzi* cells containing *F. adeliensis*. **A, B** Micrographs of *E.*
481 *petzi* thin sections showing bacteria individually dispersed in the host cytoplasm, or associated together in
482 groups enclosed in membrane-bound compartments. **C–G** Panels showing magnifications of the boxed areas
483 in panels A and B. Abbreviations: MAC, macronucleus; AZM, adoral zone membranelles.

484

485 **Fig 3** *F. adeliensis* growth. **A** Dot-plate analysis of *F. adeliensis* on CHAB agar. Serial dilutions of a *F.*
486 *adeliensis* cell suspension were spotted on plates and the plates incubated at the indicated temperatures for
487 the indicated times (days). **B** Growth curves of *F. adeliensis* in liquid medium incubated at the indicated
488 temperatures. Data from a representative experiment are shown; experiments were repeated three times with
489 equivalent results.

490

491 **Fig 4** Evolutionary relationships of *F. adeliensis*. The optimal tree with the sum of branch length =
492 284909.68814135 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the
493 evolutionary distances used to infer the phylogenetic tree. The scale bar corresponds to 5000 nucleotide
494 differences. Growth style and environment of each species are indicated by colored dots on the right; the six
495 major branches of the tree are enclosed in colored rectangles. The position of *F. adeliensis* is highlighted in
496 bold.

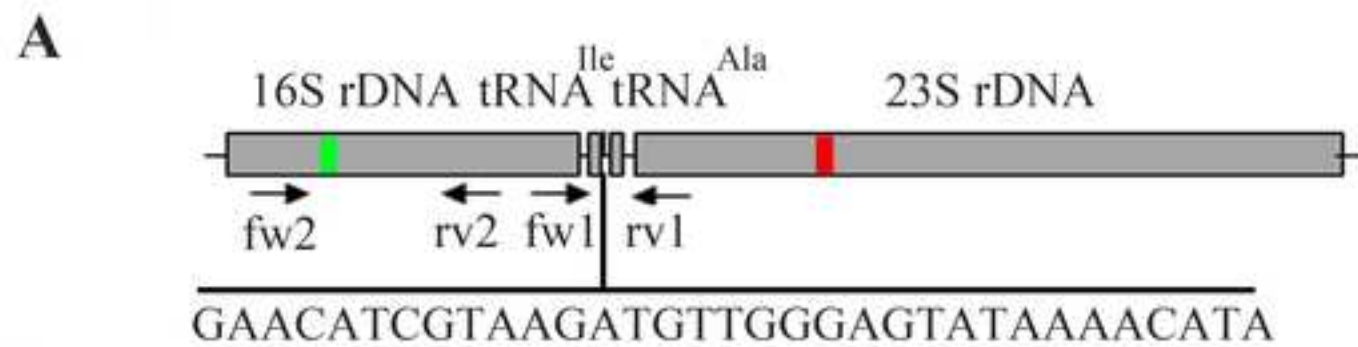
497

Table 1. Genome comparison of ten different *Francisella* species. The accession numbers of the examined genomes are indicated.

Species	Origin	Genome size (bp)	Pred. proteins	tRNAs	rRNA (16S+23S)	% G+C
<i>F. adeliensis</i> CP021781	<i>E. petzi</i>	2,054,094	1,880	38	10 3+3	32.6
<i>F. endociliophora</i> NZ_CP009574	<i>E. raikovi</i>	2,015,987	1,891	38	10 3+3	32.4
<i>Allofrancisella guangzhouensis</i> NZ_CP010427.1	cooling tower water	1,658,482	1,423	38	10 3+3	32.0
<i>F. noatunensis subsp. orientalis</i> FNO24 NZ_CP011922.1	Nile tilapia	1,862,322	1,449	39	10 3+3	32.3
<i>F. philomiragia</i> GA01-2794 NZ_CP009440.1	human	2,148,038	1,999	40	10 3+3	32.4
<i>F. hispaniensis</i> 3523 NC_017449	human	1,945,310	1,798	38	10 3+3	32.3
<i>F. tularensis subsp. holarctica</i> LVS NC_007880	vaccine strain	1,895,994	1,766	38	10 3+3	32.2
<i>F. tularensis subsp. tularensis</i> WY96 NZ_CP012037.1	human	2,005,074	1,871	38	10 3+3	32.4
<i>F. tularensis subsp. mediasiatica</i> FSC147 NC_010677	gerbil	1,893,886	1,659	38	10 3+3	32.3
<i>F. salina</i> TX077308 NC_015696	seawater	2,035,931	1,884	39	10 3+3	32.9

Table 2. ANI in percent between known *Francisella* genomes.

	<i>F. endociliophora</i> NZ_CP009574.1	<i>A. guangzhouensis</i> NZ_CP010427.1	<i>F. noatunensis</i> <i>subsp. orientalis</i> FNO24 NZ_CP011922.1	<i>F. philomiragia</i> GA01-2794 NZ_CP009440.1	<i>F. tularensis subsp.</i> <i>tularensis</i> WY96 NZ_CP012037.1
<i>F. adeliensis</i> CP021781	78.84	77.07	77.58	77.89	77.69
<i>F. endociliophora</i> NZ_CP009574.1	-	78.4	80.44	81.51	80.22
<i>A. guangzhouensis</i> NZ_CP010427.1	-	-	79.30	78.5	78.99
<i>F. noatunensis subsp.</i> <i>orientalis</i> FNO24 NZ_CP011922.1	-	-	-	95.15	82.09
<i>F. philomiragia</i> GA01-2794 NZ_CP009440.1	-	-	-	-	82.39



B

