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Candidatus Nanopusillus

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1 **1. PHYLUM/CLASS/ORDER/FAMILY:**

2 "Nanoarchaeota"/Nanobdellia/Nanobdellales/Nanobdellaceae

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7 **3. CHAPTER TITLE:**8 *Candidatus Nanopusillus*

9

10 **4. DEFINING PUBLICATION:**

11 Wurch et al. 2016

12

13 **5. AUTHORS NAMES AND INSTITUTIONS:**14 Emily St. John and Anna-Louise Reysenbach, *Biology Department, Portland State University,*15 *Portland, OR, USA*16 Mircea Podar, *Oak Ridge National Laboratory, Oak Ridge, TN, USA*

17

18 **6. ETYMOLOGY:**19 Na.no.pu.sil'lus. Gr. masc. n. *nânos*, a dwarf; L. masc. adj. *pusillus*, very small; N.L. masc. n.20 *Nanopusillus*, a very small member of the *Nanoarchaeota*

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4 **22 7. ABSTRACT:**

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6 The genus *Candidatus Nanopusillus* is comprised of small coccoid cells (~100-400 nm) that live
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8 epibiotically on the surface of archaeal hosts. The first described species, *Candidatus*
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10 *Nanopusillus acidilobi*, is an anaerobic, hyperthermophilic acidophile whose best growth is
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12 observed at 82°C, pH 3.6, cultivated from a hot spring in Yellowstone National Park. *Ca.*
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14 *Nanopusillus acidilobi* cells associate with the *Crenarchaeota* host organism *Acidilobus* sp. 7A.
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16 Archaeal flagella (archaella) have been predicted from the genome sequence and shown to be
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18 expressed in the proteome. A second putative species, *Candidatus Nanopusillus massiliensis*, was
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20 recently reported from human dental plaque and associates with the methanogen
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22 *Methanobrevibacter oralis*. The genome consists of a single scaffold which is highly fragmented
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24 by spans of ambiguous nucleotides, with 16S rRNA gene fragments from *Bacteria*. Both species
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26 have small genomes (~0.6 Mbp) encoding few biosynthetic genes and no apparent ATP synthase
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28 complex genes, suggesting that the nanoarchaeotes rely on their host for production of major
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30 cellular precursors.

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36 *Type species: Candidatus Nanopusillus acidilobi* Wurch et al. 2016

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41 **8. KEYWORDS:**

42 hot spring, hyperthermophile, acidophile, symbiote, *Nanopusillus*

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47 **9. DESCRIPTION:**

48 **42 Ultra-small cocci** (~100-400 nm in diameter) cultivated under **anaerobic** conditions. Species
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50 within *Candidatus Nanopusillus* live as **obligate epibionts** on the surface of specific archaeal
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3 44 hosts, and likely **rely on their hosts for production of the major precursors for cellular**
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5 45 **biosynthesis**. The first described species, *Ca. Nanopusillus acidilobi*, was grown from Cistern
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7 46 Spring, a high-temperature acidic geothermal spring in Yellowstone National Park (YNP). *Ca. N.*
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9 47 *acidilobi* is **hyperthermophilic** and **acidophilic**, with optimal growth observed at 82°C and pH 3.6.
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11 48 Cells are glycosylated, and **archaeal flagella** (archaella) genes are predicted from the genome and
12
13 49 expressed in proteomic data. *Acidilobus* sp. 7A functions as the specific host. A second putative
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15 50 species, *Candidatus Nanopusillus massiliensis*, was recently described from the human oral
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17 51 environment. While the culture is no longer available (Stéphane Alibar 2022, personal
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19 52 communication, 28 November), *Ca. N. massiliensis* cells are **neutrophilic** and **mesophilic**, with
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21 53 best growth observed at 37°C, pH 7, and they grow ectosymbiotically on the surface of
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23 54 *Methanobrevibacter oralis*.

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25 55 *DNA G + C content (mol %): 24 (genome analysis)*

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27 56 *Type species: Candidatus Nanopusillus acidilobi* Wurch et al. 2016

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29 57 Number of *Candidatus* species: 2

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31 58 Family classification: *Nanobdellaceae*

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35 60 **10. NUMBER OF CANDIDATUS SPECIES:**

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41 63 **11. FURTHER DESCRIPTIVE INFORMATION:**

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43 64 **11.1. CELL MORPHOLOGY AND ULTRASTRUCTURE**

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3 65 *Ca. N. acidilobi*, the first described species of the genus *Ca. Nanopusillus*, was cultivated from an
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5 66 acidic, high-temperature hot spring in YNP. Cells are coccoid in shape, approximately 100-300 nm
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7 67 in diameter (Table 1), and are glycosylated (Wurch et al., 2016). Although an S-layer protein was
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9 68 predicted from the sequence of the *Ca. N. acidilobi* genome, the protein was not detected in
10
11 69 proteomic analysis. However, identification of the S-layer protein via trypsin-based proteomics
12
13 70 may be hampered by size and a low quantity of proteolytic sites (Wurch et al., 2016). *Ca. N.*
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15 71 *acidilobi* cells survive on the surface of their crenarchaeotal host, *Acidilobus* sp. 7A (Figure 1), and
16
17 72 electron microscopy has shown distension at the attachment point between host and symbiont
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19 73 (Wurch et al., 2016). This suggests that the YNP *Nanoarchaeota*–host system forms an intimate
20
21 74 association, potentially similar to what has been described for the shallow marine lineage
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23 75 *Candidatus Nanoarchaeum equitans* and its host *Ignicoccus hospitalis*, which appear to form a
24
25 76 bridge-like structure that connects the two organisms and allows for cytoplasmic contact
26
27 77 (Heimerl et al., 2017). A second lineage, *Ca. N. massiliensis* (150-400 nm), was recently reported
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29 78 from the human oral environment and associates with a methanogenic *Euryarchaeota*,
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31 79 *Methanobrevibacter oralis*.

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33 80 <Table 1 near here>

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35 81 <Figure 1 near here>

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39 83 **11.2 NUTRITION AND GROWTH CONDITIONS**

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41 84 Optimal growth of *Ca. N. acidilobi* with its host has been observed at 82°C, pH 3.6. The *Ca. N.*
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43 85 *acidilobi*–*Acidilobus* sp. 7A co-culture can be cultivated anaerobically under a N₂/CO₂ headspace
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45 86 (80:20, v/v) in media containing yeast extract and peptone (Wurch et al., 2016). The newly
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3 87 proposed species *Ca. N. massiliensis* can be grown with its host in modified SAB media under an
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5 88 H₂/CO₂ headspace (80:20, v/v), with optimal growth observed at 37°C, pH 7 (Hassani et al., 2022).
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10 90 **11.3 GENOME FEATURES**
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13 91 A genome sequence has been determined for *Ca. N. acidilobi*, the first cultivated representative
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15 92 of *Ca. Nanopusillus*. The genome is 605,887 bp in length, with 656 predicted protein-coding
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17 93 sequences and a single 5S, 16S and 23S rRNA gene sequence. Genome size is comparable to other
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19 94 terrestrial *Nanoarchaeota* (Table 1), but somewhat larger than the shallow marine taxon *Ca.*
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21 95 *Nanoarchaeum equitans* (see gbm01370). Like other nanoarchaeotes, the *Ca. N. acidilobi*
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23 96 genome points to highly reduced biosynthetic potential, with very minimal genes involved in the
24
25 97 generation of amino acids, nucleotides, lipids or cofactors (Kato et al., 2022; St. John et al., 2019;
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27 98 Waters et al., 2003; Wurch et al., 2016). In contrast to *Ca. Nanoarchaeum equitans*, but like its
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29 99 relatives from New Zealand and Japan, the *Ca. N. acidilobi* genome encodes a suite of genes
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31 100 involved in gluconeogenesis and glycolysis, including several gluconeogenesis genes highly
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33 101 expressed in the proteome (Wurch et al., 2016). Additionally, the *Ca. N. acidilobi* genome does
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35 102 not contain any detectable *trans*-encoded tRNA genes or ATP synthase genes, which have been
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37 103 identified in *Ca. Nanoarchaeum equitans* (Randau, 2012; Waters et al., 2003), and no CRISPR-Cas
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39 104 cassettes have been identified. Like other described *Nanoarchaeota*, the *Ca. Nanopusillus*
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41 105 genome encodes several split protein-coding genes, which have been linked to genome reduction
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43 106 associated with a symbiotic lifestyle (Kato et al., 2022; St. John et al., 2019; Waters et al., 2003;
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45 107 Wurch et al., 2016).
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3 108 By contrast, the *Ca. N. massiliensis* genome quality is poor and consists of a single scaffold
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5 109 which is highly fragmented by spans of ambiguous nucleotides that link the 593 individual
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7 110 contigs. Based on genome quality assessment with CheckM (Parks et al., 2015), 64 to 66 of the
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9 111 unique archaeal marker genes used to estimate genome completeness can be identified in the
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11 112 *Ca. N. massiliensis* genome, depending on whether the scaffold or contig version of the genome
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13 113 sequence is analyzed. In contrast, 117 to 119 archaeal marker genes are detectable in the
14
15 114 complete genomes of *Ca. N. acidilobi*, *Ca. Nanoarchaeum equitans* and *Nanobdella aerobiophila*.
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17 115 Thus, it is likely that ambiguous bases in the *Ca. N. massiliensis* genome hamper gene
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19 116 identification and severely impair genome quality. Nonetheless, analysis of the *Ca. N. massiliensis*
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21 117 genome suggests the presence of genes associated with glycolysis and gluconeogenesis, a single
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23 118 archaeal flagellum gene and the apparent absence of the ATP synthase complex (Hassani et al.,
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25 119 2022).
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35 121 **11.4. ECOLOGY**
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37 122 The first described *Ca. Nanopusillus* species, *Ca. N. acidilobi*, was cultivated with its host from
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39 123 Cistern Spring, a hot spring in Norris Geyser Basin, YNP (pH 4.5, 82°C). A few years prior, a single-
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41 124 cell nanoarchaeote genome "Nst1" was also co-sorted with its putative *Sulfolobales* host "Acd1"
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43 125 from Obsidian Pool, YNP (pH 5.2-5.5, 82°C) (Podar et al., 2013). Although Nst1 was proposed to
44
45 126 represent the unique genus *Candidatus Nanobsidianus stetteri*, reclassification with the Genome
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47 127 Taxonomy DataBase (GTDB) has since placed Nst1 within the *Ca. Nanopusillus* (Parks et al., 2020).
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49 128 Additional single-cell genomics, 16S rRNA gene diversity studies and metagenomic data have
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3 129 suggested that relatives of *Nst1* and *Ca. Nanopusillus* are widely distributed across YNP
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5 130 geothermal springs (Clingenpeel et al., 2013; Jarett et al., 2018; Munson-McGee et al., 2015).
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7 131 The recent description of a putative novel *Ca. Nanopusillus* from human dental plaque
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9 132 (Hassani et al., 2022) raises questions regarding the distribution of the genus. To our knowledge,
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11 133 *Ca.N. massiliensis* represents the first description of a mesophilic, *Euryarchaeota*-dependent,
12
13 134 human-associated nanoarchaeote to date. However, the 16S rRNA gene associated with *Ca. N.*
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15 135 *massiliensis* genome (GenBank/EMBL/DDBJ accession NZ_OV100765.1; locus tag
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17 136 LUA84_RS03675) uses ambiguous bases to link five small contigs, several of which show high
18
19 137 similarity to bacterial sequences, suggesting that the 16S rRNA gene sequence is chimeric. Also,
20
21 138 given the lack of evidence of *Ca. Nanopusillus* in human microbiome datasets, additional
22
23 139 investigation will be crucial to determining the validity of this new species and how it contributes
24
25 140 to our understanding of the distribution and range of host associations found in *Ca. Nanopusillus*.
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35 142 **12. ENRICHMENTS AND ISOLATION PROCEDURES**
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38 143 Enrichment cultures of *Ca. N. acidilobi* and its host *Acidilobus* sp. 7A can be grown in the following
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40 144 medium, containing (per liter of deionized water): NH₄Cl, 0.33 g; KH₂PO₄, 0.33 g; MgSO₄ x 7H₂O,
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42 145 0.33 g; CaCl₂, 0.33 g; KCl, 0.33 g; SL-10 trace metals, 1 ml; Wolfe's vitamin solution, 5 ml of 1000X
43
44 146 solution; yeast extract, 0.3 g; and peptone, 0.5 g. The medium is filter sterilized, and anaerobic
45
46 147 conditions are achieved by three 20-minute rounds of degassing with N₂/CO₂ (80:20, v/v). The
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48 148 final medium is then reduced overnight at 80°C using 100 µM cysteine. Cultivation can be
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50 149 performed at 82°C, pH 3.6. Dilution-to-extinction and optical tweezer selection are effective for
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52 150 isolation of *Ca. N. acidilobi*–*Acidilobus* sp. 7A co-cultures.
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3 151 Cultivation of the *Ca. N. massiliensis*–*M. oralis* co-culture was done using SAB media
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5 152 prepared under an H₂/CO₂ headspace (80/20, v/v), followed by growth at 37°C, pH 7 in an SAB
6
7 153 medium supplemented with D-fructose (0.1 g), vitamins, fatty acids (valeric, isovaleric, 2-
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9 154 methylbutyric and isobutyric acids) and 5% 0.22 µm-filtered bovine rumen.
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156 **13. MAINTENANCE PROCEDURES**

157 For long-term storage, liquid co-cultures of *Ca. N. acidilobi* and *Acidilobus* sp. 7A can be frozen at
158 -80°C with the addition of 10% dimethylsulfoxide.
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14. DIFFERENTIATION OF THE GENUS *CA. NANOPUSILLUS* FROM OTHER GENERA

161 Features differentiating *Ca. Nanopusillus* from *Candidatus Nanoclepta* (see gbm02046), *Ca.*
162 *Nanoarchaeum* (see gbm01370) and *Nanobdella* (Kato et al., 2022) are listed in Table 1. 16S rRNA
163 gene sequence divergence and whole genome sequence identity also distinguish *Ca.*
164 *Nanopusillus* from related lineages in the *Nanoarchaeota*.
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15. TAXONOMIC COMMENTS

167 Based on phylogenetic reconstruction of 16S rRNA genes (Figure 2), *Ca. N. acidilobi* forms a
168 distinct clade with clone and single-cell genome sequences from YNP (YLNA023, OP-9, SCGC AB-
169 777_F03, Nst1). The YNP-specific branch forms part of a larger clade comprised of sequences
170 from other terrestrial hot springs, including locations in Japan (*Nanobdella aerobiophila*),
171 Kamchatka, Russia (CU-1), China (A2, A39) and New Zealand (*Ca. Nanoclepta minutus*), which
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3 172 branches out from marine hydrothermal vent-associated lineages (*Ca. Nanoarchaeum equitans*,
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5 173 MC-1). Due to the high proportion of ambiguous bases (~17%) and similarity to bacterial 16S
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7 174 rRNA gene sequences, the *Ca. N. massiliensis* 16S rRNA gene sequence is not included in the
8
9 175 phylogenetic tree. A concatenated protein tree generated with the GTDB Toolkit (Chaumeil et al.,
10
11 176 2020) based on 53 archaeal marker genes also shows a similar overall topology compared to the
12
13 177 16S rRNA gene tree (Figure 3), with the exception that the deep-sea hydrothermal vent
14
15 178 associated lineage MC-1 clusters with a terrestrial hot spring sequence from Nevada, USA (SpSt-
16
17 179 4), and *Ca. N. massiliensis* forms a small clade with *Ca. N. acidilobi*.
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23 180 Currently, the genus *Ca. Nanopusillus* is classified by GTDB in phylum *Nanoarchaeota*,
24
25 181 class *Nanoarchaeia*, order *Nanoarchaeales* and family *Nanopusillaceae* (Parks et al., 2020).
26
27 182 However, a novel classification system was recently proposed and validly published for the entire
28
29 183 *Nanoarchaeota* phylum, which places *Ca. Nanopusillus* within the class *Nanobdellia*, order
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31 184 *Nanobdellales* and family *Nanobdellaceae* (Kato et al., 2022).
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34 185 <Figure 2 near here>
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36 186 <Figure 3 near here>
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43 188 **16. LIST OF SPECIES OF THE GENUS**
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46 189 **1. *Candidatus Nanopusillus acidilobi* Wurch et al. 2016.**
47
48 190 a.ci.di.lo'bi. N.L. gen. masc. n. *acidilobi*, of acidolobus, growth dependent on the archaeal genus
49
50 191 *Acidilobus*
51
52 192 Distinguishing features are shown in Table 1 and in the genus description. This taxon and its host
53
54 193 were co-cultivated from Cistern Spring, YNP, USA (44.723°N, 110.704°W).
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3 194 *DNA G + C content (mol %): 24 (genome analysis)*

4
5 195 *Type strain: N7A*

6
7 196 *GenBank/EMBL/DDBJ accession (genome): CP010514.1*

8
9 197

10
11 198 **2. *Candidatus Nanopusillus massiliensis* Hassani et al. 2022.**

12
13 199 *mas.si.li.en'sis. L. fem. adj. *massiliensis*, referring to Massilia, the past Roman name of Marseille,*

14
15 200 France where this nano-organism has been discovered

16
17 201 Distinguishing features are listed in the genus description and Table 1. This nanoarchaeote and

18
19 202 its host were co-cultivated from a human dental plaque.

20
21 203 *DNA G + C content (mol %; reported in Hassani et al., 2022): 23.6 (genome analysis)*

22
23 204 *DNA G + C content (mol %; re-calculated with BBmap*

24
25 205 (<https://sourceforge.net/projects/bbmap/>): 24.0 (genome analysis)

26
27 206 *Type strain: Marseille-Q6268*

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29 207 *GenBank/EMBL/DDBJ accession (genome, contigs): CAKLBW000000000.1*

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31 208 *GenBank/EMBL/DDBJ accession (genome, scaffold): OV100765.1*

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35 210 **RELATED ARTICLES**

36
37 211 gbm01370

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39 212 gbm02046

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41 213 fhm00399

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43 214 obm00129

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287 **20. TABLES AND FIGURES**

288 **Table 1.** Major characteristics differentiating members of the *Ca. Nanopusillus* from *Ca.*
 289 *Nanoclepta minutus*, *Nanobdella aerobiophila* and *Ca. Nanoarchaeum equitans*. Data from
 290 Genbank/EMBL/DDBJ records (*Ca. Nanopusillus*); Wurch et al., 2016 (*Ca. Nanopusillus*
 291 *acidilobi*); Hassani et al., 2022 (*Ca. Nanopusillus massiliensis*); St. John et al., 2019 (*Ca.*
 292 *Nanoclepta minutus*); Kato et al., 2022 (*Nanobdella aerobiophila*); Huber et al., 2002; Jahn et
 293 al., 2008; Randau, 2012; Randau et al., 2005; Waters et al., 2003 (*Ca. Nanoarchaeum equitans*).

Characteristic	<i>Ca. Nanopusillus acidilobi</i>	<i>Ca. Nanopusillus massiliensis</i>	<i>Ca. Nanoclepta minutus</i>	<i>Nanobdella aerobiophila</i>	<i>Ca. Nanoarchaeum equitans</i>
Isolation location	YNP	Human mouth	Tikitere, NZ	Oku-shiobara, Tochigi, Japan	Kolbeinsey Ridge
Optimal temperature (°C)	82	37	80–85	65–70	85–90
Cultivation pH	3.6	7.0	6.0	2.5	5.5
Relationship to oxygen	Anaerobe	Anaerobe	Anaerobe	Aerobe	Anaerobe
Cell size, nm	100–300	150–400	~200	200–500	~400
Genome size (bp)	605,887	607,503 ^b	575,637	668,961	490,885
G + C content (mol%)	24	23.6 ^c	32.2	24.9	31.6

1	Partial ATP	-	-	-	-	+
2	synthase					
3	complex genes					
4	CRISPR-Cas	-	n.d.	+	+	+
5	cassette					
6	<i>Trans-spliced</i>	-	n.d.	-	-	+
7	tRNA genes					
8	Host	<i>Acidilobus</i> sp.	<i>Methanobre</i>	<i>Zestosphaer</i>	<i>Metallospha</i>	<i>Ignicoccus</i>
9		7A	<i>vibacter</i>	<i>a tikiterensis</i>	<i>era sedula</i>	<i>hospitalis</i>
10			<i>oralis</i>			

294 ^an.d., no data295 ^bBased on Genbank/ GenBank/EMBL/DDBJ accession OV100765.1296 ^cReported in Hassani et al., 2022

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298 **21. FIGURE CAPTIONS**299 **Figure 1.** Scanning electron micrograph of *Ca. Nanopusillus acidilobi* N7A cells attached to the
300 surface of *Acidilobus* sp. 7A cells. Scale bar, 300 nm.301
302 **Figure 2.** Phylogenetic reconstruction of 16S rRNA genes, showing the position of *Ca.*
303 *Nanopusillus acidilobi* in relation to closely related genera *Ca. Nanoarchaeum*, *Nanobdella* and
304 *Ca. Nanoclepta*, with additional clone, single-cell and metagenome derived sequences. The
305 outgroup (not shown) consists of archaeon GW2011_AR15 (CP010425.1), archaeon
306 GW2011_AR20 (CP010426.1), *Candidatus Tiddalikarchaeum anstoanum* LFW-252_1
307 (CABMEV000000000.1) and *Candidatus Parvarchaeum acidiphilum* ARMAN-4
308 (ADCE00000000.1). 80-100% bootstrap support based on 1000 rapid bootstraps is indicated
309 with filled circles. Scale bar, 0.1 substitutions per nucleotide.

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311 **Figure 3.** Unrooted concatenated protein phylogenetic tree based on 53 marker genes, showing
312 the relative position of *Ca. Nanopusillus* compared to closely related taxa. The tree was
313 constructed using the default GTDB Toolkit release 207_v2 database for archaeal tree building,
314 with the addition of *Ca. Nanopusillus massiliensis*, *Nanobdella aerobiophila* and MC-1. Taxa
315 assigned to *Nanobdellales* (GTDB order *Nanoarchaeales*) are shown. Filled circles indicate 80-
316 100% local support values calculated with the Shimodaira-Hasegawa test (Shimodaira and
317 Hasegawa, 1999). Scale bar, 0.1 substitutions per amino acid.

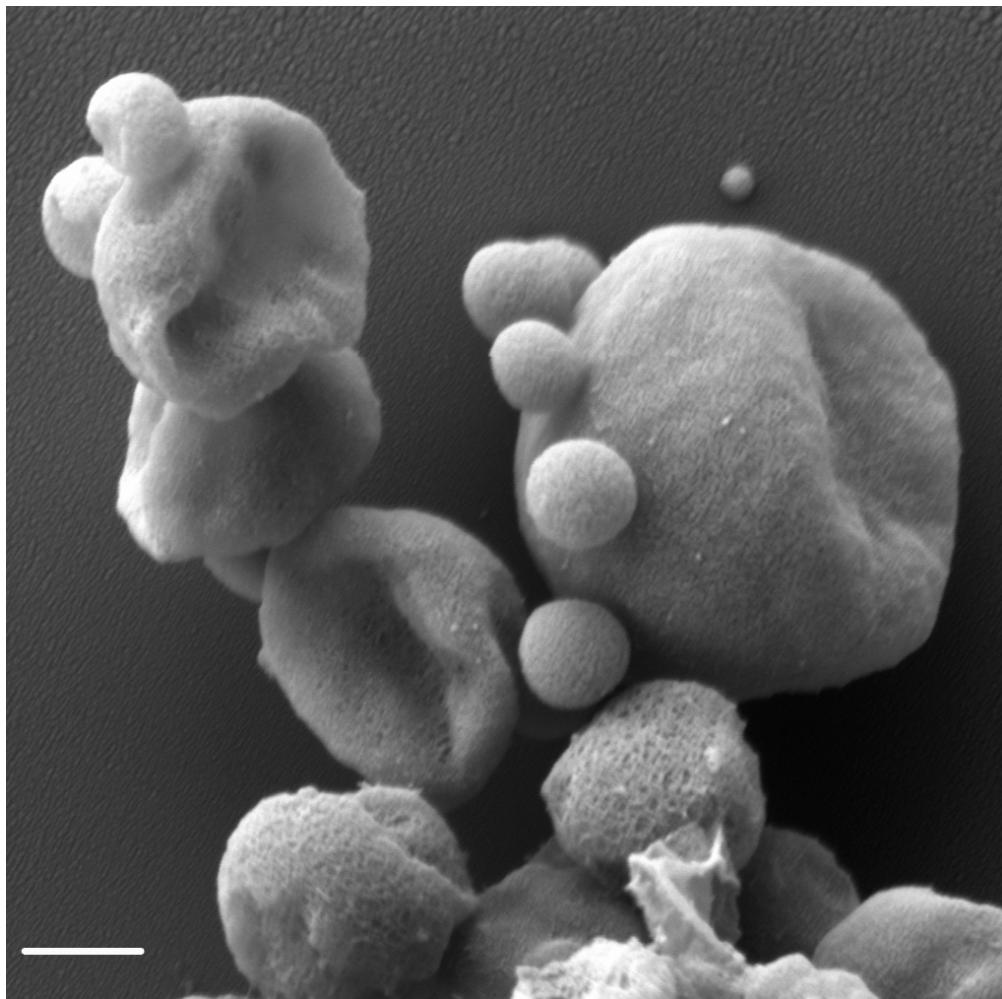


Figure 1. Scanning electron micrograph of *Ca. Nanopusillus acidilobi* N7A cells attached to the surface of *Acidilobus* sp. 7A cells. Scale bar, 300 nm.

86x85mm (300 x 300 DPI)

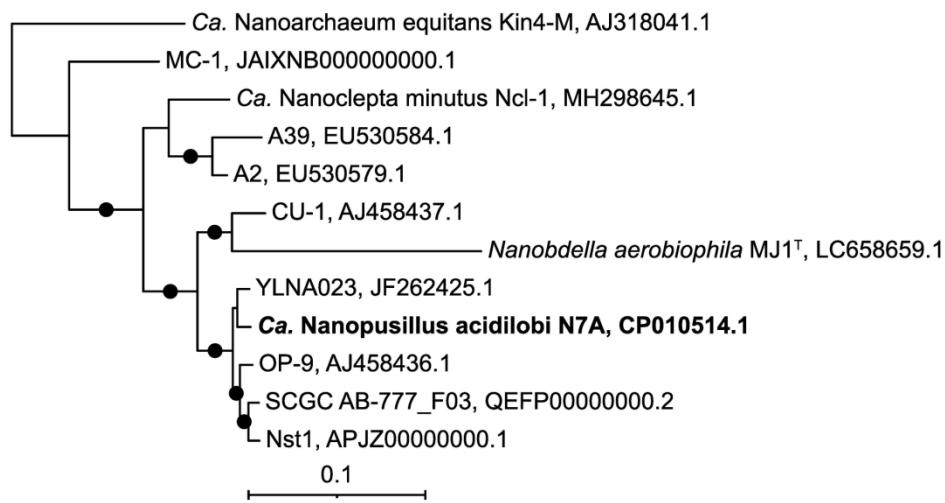


Figure 2. Phylogenetic reconstruction of 16S rRNA genes, showing the position of *Ca. Nanopusillus acidilobi* in relation to closely related genera *Ca. Nanoarchaeum*, *Nanobdella* and *Ca. Nanoclepta*, with additional clone, single-cell and metagenome derived sequences. The outgroup (not shown) consists of archaeon GW2011_AR15 (CP010425.1), archaeon GW2011_AR20 (CP010426.1), *Candidatus Tiddalikarchaeum anstoanum* LFW-252_1 (CABMEV000000000.1) and *Candidatus Parvarchaeum acidiphilum* ARMAN-4 (ADCE00000000.1). 80-100% bootstrap support based on 1000 rapid bootstraps is indicated with filled circles. Scale bar, 0.1 substitutions per nucleotide.

178x88mm (300 x 300 DPI)

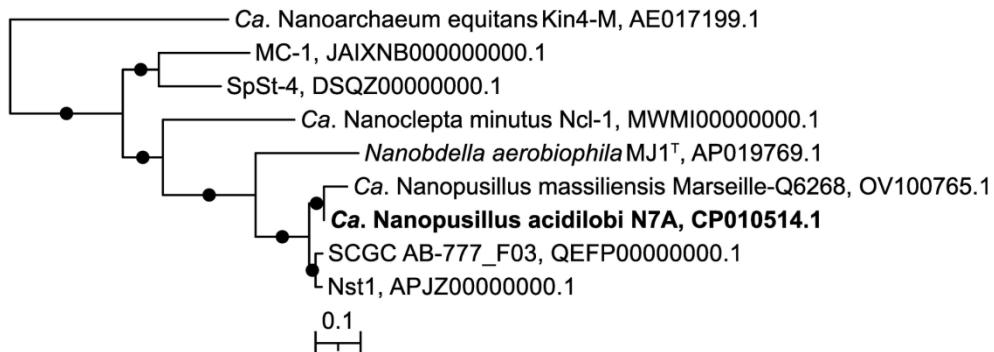


Figure 3. Unrooted concatenated protein phylogenetic tree based on 53 marker genes, showing the relative position of *Ca. Nanopusillus* compared to closely related taxa. The tree was constructed using the default GTDB Toolkit release 207_v2 database for archaeal tree building, with the addition of *Ca. Nanopusillus massiliensis*, *Nanobdella aerobiophila* and MC-1. Taxa assigned to *Nanobdellales* (GTDB order *Nanoarchaeales*) are shown. Filled circles indicate 80-100% local support values calculated with the Shimodaira-Hasegawa test (Shimodaira and Hasegawa, 1999). Scale bar, 0.1 substitutions per amino acid.

178x66mm (300 x 300 DPI)