

2018-05-31

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<http://hdl.handle.net/10026.1/11401>

10.1099/ijsem.0.002816

International Journal of Systematic and Evolutionary Microbiology
Microbiology Society

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Evaluation of the genus *Thiothrix* Winogradsky 1888 (Approved Lists 1980) emend. Aruga et al. 2002: reclassification of *Thiothrix* disciformis to *Thiolinea* disciformis gen. nov., comb. nov., and of *Thiothrix* flexilis to *Thiofilum* flexile gen. nov., comb. nov., with emended description of *Thiothrix*.

--Manuscript Draft--

Manuscript Number:	IJSEM-D-18-00034R3
Full Title:	Evaluation of the genus <i>Thiothrix</i> Winogradsky 1888 (Approved Lists 1980) emend. Aruga et al. 2002: reclassification of <i>Thiothrix</i> disciformis to <i>Thiolinea</i> disciformis gen. nov., comb. nov., and of <i>Thiothrix</i> flexilis to <i>Thiofilum</i> flexile gen. nov., comb. nov., with emended description of <i>Thiothrix</i> .
Article Type:	Taxonomic Description
Section/Category:	New taxa - Proteobacteria
Keywords:	<i>Thiothrix</i> ; Thiotrichales; <i>Thiolinea</i> ; <i>Thiofilum</i> ; filamentous Bacteria; Gammaproteobacteria
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Manuscript Region of Origin:	UNITED KINGDOM
Abstract:	<p><i>Thiothrix</i> is the type genus of the Thiotrichaceae in the Thiotrichales of the Gammaproteobacteria, comprising nine species of sulfur-oxidising filamentous Bacteria, which are variously autotrophic, heterotrophic or have mixed metabolic modes. Within the genus, 4 species show 16S rRNA gene identities lower the Yarza threshold for the rank of genus (94.5 %) - <i>Thiothrix</i> disciformis, <i>Thiothrix</i> flexilis, <i>Thiothrix</i> defluvii and <i>Thiothrix</i> eikelboomii - as they show no affiliation to extant genera, a polyphasic study was undertaken including biochemical, physiological and genomic properties and phylogeny based on the 16S rRNA gene (<i>rrs</i>), recombination protein A (<i>RecA</i>), polynucleotide nucleotidyltransferase (<i>Pnp</i>), translation initiation factor IF-2 (<i>InfB</i>), glyceraldehyde-3-phosphate dehydrogenase (<i>GapA</i>), glutaminyl-tRNA synthetase (<i>GlnS</i>), elongation factor EF-G (<i>FusA</i>) and concatamers of 53 ribosomal proteins encoded by <i>rps</i>, <i>rpl</i> and <i>rpm</i> operons, all of which support the reclassification of these species. We thus propose <i>Thiolinea</i> gen. nov. and <i>Thiofilum</i> gen. nov. for which the type species are <i>Thiolinea</i> disciformis gen. nov., comb. nov. and <i>Thiofilum</i> flexile gen. nov., comb. nov. We also propose that these genera are each circumscribed into novel families Thiolinaceae fam. nov. and Thiofilaceae fam. nov., and that <i>Leucothrix</i> and <i>Cocleimonas</i> are circumscribed into Leucotrichaceae fam. nov. and provide emended descriptions of <i>Thiothrix</i> and Thiotrichaceae.</p>

1 **Evaluation of the genus *Thiothrix* Winogradsky 1888 (Approved Lists**
2 **1980) emend. Aruga *et al.* 2002: reclassification of *Thiothrix disciformis* to**
3 ***Thiolinea disciformis* gen. nov., comb. nov., and of *Thiothrix flexilis* to**
4 ***Thiofilum flexile* gen. nov., comb nov., with emended description of**
5 ***Thiothrix*.**

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12 Running title: Reclassification of *Thiothrix* spp. to *Thiolinea* gen. nov. and *Thiofilum* gen.
13 nov.

14 **KEYWORDS:** *Thiothrix*, *Thiotrichales*, *Thiolinea*, *Thiofilum*, filamentous *Bacteria*,
15 *Gammaproteobacteria*

16 **NON-STANDARD ABBREVIATIONS:** *Tla.*, *Thiolinea*; *Tfm.*, *Thiofilum*; Δp , proton motive
17 force; rMLST, ribosomal mutli-locus sequence typing; RuBisCO, ribulose 1,5-bisphosphate
18 carboxylase/oxygenase.

19 **Abstract**

20 *Thiothrix* is the type genus of the *Thiotrichaceae* in the *Thiotrichales* of the
21 *Gammaproteobacteria*, comprising nine species of sulfur-oxidising filamentous *Bacteria*,
22 which are variously autotrophic, heterotrophic or have mixed metabolic modes. Within the
23 genus, 4 species show 16S rRNA gene identities lower the Yarza threshold for the rank of
24 genus (94.5 %) – *Thiothrix disciformis*, *Thiothrix flexilis*, *Thiothrix defluvii* and *Thiothrix*
25 *eikelboomii* – as they show no affiliation to extant genera, a polyphasic study was undertaken
26 including biochemical, physiological and genomic properties and phylogeny based on the
27 16S rRNA gene (*rrs*), recombination protein A (RecA), polynucleotide nucleotidyltransferase
28 (Pnp), translation initiation factor IF-2 (InfB), glyceraldehyde-3-phosphate dehydrogenase
29 (GapA), glutaminyl-tRNA synthetase (GlnS), elongation factor EF-G (FusA) and
30 concatamers of 53 ribosomal proteins encoded by *rps*, *rpl* and *rpm* operons, all of which
31 support the reclassification of these species. We thus propose *Thiolinea* gen. nov. and
32 *Thiofilum* gen. nov. for which the type species are *Thiolinea disciformis* gen. nov., comb.
33 nov. and *Thiofilum flexile* gen. nov., comb. nov. We also propose that these genera are each
34 circumscribed into novel families *Thiolinaceae* fam. nov. and *Thiofilaceae* fam. nov., and
35 that *Leucothrix* and *Cocleimonas* are circumscribed into *Leucotrichaceae* fam. nov. and
36 provide emended descriptions of *Thiothrix* and *Thiotrichaceae*.

37

38 The genus *Thiothrix* Winogradsky 1888 (Approved Lists 1980) emend. Aruga *et al.* 2002 [1-
39 3]) is the type genus of the family *Thiotrichaceae*, in turn the type family of the order
40 *Thiothrichales* of the *Gammaproteobacteria*. It comprises filamentous sulfur-oxidising
41 *Bacteria* that form numerous structures including rosettes and holdfasts and which grow
42 chemolithoautotrophically, heterotrophically and/or mixotrophically or possibly
43 chemolithoheterotrophically, with considerable debate regarding their exact metabolic mode
44 dating back to Winogradsky's 1888 study. In addition to the 9 species with validly published
45 names, a large number of names have been mentioned without valid or effective publication.
46 For example, a further 10 species names appear in the *Index Bergeyana* [4] and a further
47 name appears in the *Supplement to the Index Bergeyana* [5]; however, the strains of all 11 of
48 these 'species' have been lost, and as they do not appear on the *Approved Lists* in any case,
49 their names have no standing. Whilst some common features are shared across the 9 species
50 with validly published names, there are a number of considerable differences, which has led
51 to previous studies investigating the true nature of the genus, often concluding that in spite of
52 an enormous phylogenetic and physiological diversity, that the genus should remain as-is [6].
53 Here we present a polyphasic analysis including phylogenetic and genomic evidence, and
54 thus the case for the reclassification of members of *Thiothrix* into two novel genera,
55 *Thiolinea* gen. nov. and *Thiofilum* gen. nov.

56 For the sake of clarity and to avoid confusion, we must define some metabolic modes used
57 herein that are sometimes misinterpreted or misused in the literature. We herein use
58 "mixotrophy" to refer to autotrophy and heterotrophy simultaneously occurring in an
59 organism, typified by assimilation of carbon dioxide into biomass at the same time as the
60 assimilation of *e.g.* a sugar or carboxylate, and is accompanied by the oxidation of an
61 inorganic electron donor in order to generate proton motive force (Δp) which is used to yield
62 ATP and NAD(P)H which are used to fuel the assimilation of carbon dioxide. We use

63 “chemolithoheterotrophy” to refer to the oxidation of an inorganic electron donor as a source
64 of auxiliary energy (ATP and/or NAD(P)H) during heterotrophic growth, which is typified by
65 a complete lack of carbon dioxide assimilation (*pace* anapleurotic reactions). The umbrella
66 term for general mixed metabolisms (*i.e.* chemolithoheterotrophy, mixotrophy *etc*) that we
67 use herein is “mixed metabolic modes” – some studies refer to general mixed metabolism as
68 “mixotrophy”, though this is really incorrect and breeds significant confusion.

69 The 9 species with validly published names are (type species first) *Thiothrix nivea*
70 (Rabenhorst 1865) Winogradsky 1888 (Approved Lists 1980), emend. Larkin and
71 Shinabarger 1983 [1-2, 7]); *Thiothrix caldifontis* (Chernousova *et al.*, 2009 [8]), *Thiothrix*
72 *defluvii* (Howarth *et al.*, 1999 [9]), *Thiothrix disciformis* (Aruga *et al.*, 2002 [3]), *Thiothrix*
73 *eikelboomii* (Howarth *et al.*, 1999 [9]), *Thiothrix flexilis* (Aruga *et al.*, 2002 [3]), *Thiothrix*
74 *fructosivorans* (Howarth *et al.*, 1999 [9]), *Thiothrix lacustris* (Chernousova *et al.*, 2009 [8])
75 and *Thiothrix unzii* (Howarth *et al.*, 1999 [9]). They have been isolated principally from
76 activated sludge and sulfidic lakes or groundwaters (Table 1), and other strains have been
77 isolated from diverse locations including as symbionts of the cave amphipod *Niphargus ictus*
78 G. Karaman [10] that inhabits sulfidic waters, and the marine amphipod *Urothoe poseidonis*
79 Reibish [11]. It is worth noting that *Thiothrix* spp. have also been observed attached to other
80 members of the *Eukarya* such as *Cladophora* spp. Kütz, *Vaucheria* spp. A. P. de Candolle
81 [12], and *Drunella grandis* Eaton [13], and to members of the “*Cyanobacteria*” [12], without
82 determination of their status as symbionts or commensals. It is also worth noting that
83 intracellular parasitic *Bacteria* have been observed within *Thiothrix* spp. [13] grown *in situ*,
84 and which could give rise to apparent phenotypic or chemotaxonomic variation of cultures *in*
85 *vitro*.

86 Of the 9 species with validly published names, many are not possible to reclassify owing to
87 their absence from public culture collections, the current deposit *status* being: *T. caldifontis*

88 (DSMZ; lost by VKM); *T. defluvii* (lost, as far as we can tell – never deposited); *T.*
89 *disciformis* (DSMZ, JCM); *T. eikelboomii* (ATCC); *T. flexilis* (DSMZ, JCM); *T.*
90 *fructosivorans* (ATCC); *T. lacustris* (DSMZ; lost by VKM); *T. nivea* (ATCC, DSMZ), and *T.*
91 *unzii* (ATCC). Thus, this study can only focus on *T. nivea*, *T. flexilis* and *T. disciformis* by
92 way of making any formal reclassification, since type strains of each new combination must
93 be available in two public service collections per Rules 27 and 30 of the *International Code*
94 *of Nomenclature of Prokaryotes* (hereafter “the Code”). As such, our focus is on these taxa
95 but we will note the positions of those species than cannot be formally reclassified at this
96 time to facilitate further work, whilst not formally declaring new combinations, merely as
97 “incidental mentions” per Rule 28*b* of the *Code*.

98 Of the other members of the *Thiotrichaceae*, the genera *Thiomargarita*, *Achromatium*,
99 *Thiobacterium*, *Thioploca* and *Thiospira* do not have cultures available, thus are not included
100 in our analyses. Although the genus *Beggiatoa* was included in this family in recent editions
101 of *Bergey’s Manual*, there is very low 16S rRNA (*rrs*) gene identity of members of this genus
102 to the rest of the *Thiotrichaceae* – it was recently found to cluster entirely separately from the
103 rest of this family [49] and the 16S rRNA gene identity of *Beggiatoa alba* B18LD^T (type
104 species) to that of *Thiothrix nivea* JP2^T (type species of type genus of *Thiotrichaceae*) is
105 85.4 %, which falls below the Yarza median for members of the same class (86.35 %, the
106 Yarza parameters being explained in the next two paragraphs), let alone the same family. As
107 such, we have not considered *Beggiatoa* spp. herein.

108 Historically, many taxonomic studies have divided *Thiothrix* spp. into the ‘*T. nivea* group’
109 and the ‘Eikelboom type 021N group’ and so on - we have not followed this convention as it
110 somewhat clouds the phylogenetic picture. Phylogenetic trees on the basis of the 16S rRNA
111 (*rrs*) gene from *Thiothrix* spp. and the only other canonical *Thiotrichaceae* that have 16S
112 rRNA gene data available - *Leucothrix* spp. and *Cocleimonas flava* - are given in Figure 1,

113 with full details of construction given in the figure legend. It can be seen from Figure 1 that
114 *Thiothrix* spp. cluster into 3 distinct lineages on the basis of gross tree topology, which we
115 have termed Clade X (*T. nivea*, *T. fructosivorans*, *T. caldifontis*, *T. lacustris* and *T. unzii*),
116 Clade Y (*T. disciformis* and *T. eikelboomii*) and Clade Z (*T. defluvii* and *T. flexilis*). Within
117 Clade X, the 16S rRNA gene identities to *T. nivea* are 94.4 – 95.2 %. A threshold 16S gene
118 identity value of 94.5 % for genus level relationships was documented by Yarza *et al.* [14],
119 which we have termed ‘the Yarza threshold’ [15] and have applied to clarifying the
120 systematics of other *Thiotrichales* [16-17] and other *Gammaproteobacteria* [18], as well as
121 the *Betaproteobacteria* and the *Hydrogenophilalia* [15]. On the basis of the Yarza threshold,
122 the species in Clade X mostly constitute a single genus, within which *T. nivea* takes priority
123 as the type species, however *T. fructosivorans* stands at 94.4 % 16S rRNA gene identity to *T.*
124 *nivea* and thus *could* constitute a separate genus on the basis of the Yarza threshold alone.
125 Since it does not form a separate line of descent from the rest of Clade X, we have retained it
126 as a member of the genus *Thiothrix*, though it should be noted that it exhibits growth on
127 fructose and catalase enzyme activity, both of which are not found in any other Clade X
128 organisms. Clade Y members have 16S rRNA gene identities of 90.9 to 92.3 % to *T. nivea*,
129 indicating that they, on the basis of the Yarza threshold, are not in the same genus as *T. nivea*,
130 but since they have 16S rRNA gene identities to one another of 94.8 %, Clade Y species are
131 both within one genus. Clade Z organisms have identities to *T. nivea* of 89.2 – 89.5 %,
132 indicating that they are not members of the same genus either, but since they have identities
133 to one another of 96.9 %, both Clade Z species would form one genus. All clades are
134 sufficiently distant from *L. mucor* and *C. flava* to not belong to those genera (shown in Table
135 1) – though it is worth noting that all Clade X to Z organisms have the helix 18 deletion of
136 the 16S rRNA gene, but *L. mucor* and *C. flava* do not, confirming common ancestry of these
137 Clades as distinct from *Leucothrix* spp. and *Cocleimonas* spp.

138 In terms of higher taxa systematics and 16S rRNA gene identities, we have previously
139 defined a series of ‘Yarza medians’ as a system of cut-off values for family (92.25%), order
140 (89.20%), class (86.35%) and phylum (83.68%) based on values determined by Yarza *et al.*
141 [14], and which we have, again, applied across the *Gammaproteobacteria* [15-16, 18],
142 *Betaproteobacteria* and *Hydrogenophilalia* [15]. Consideration of these parameters with gene
143 identities given in the previous paragraph and in Table 1 would indicate that Clade X and
144 Clade Y all belong to the same order, but not to the same family, with Clade Y forming a
145 distinct family from that in which Clade X falls (since the latter is *Thiothrix sensu stricto*,
146 *Thiotrichaceae* is retained as the name for this family); as such, Clade Y will form the type
147 genus of a novel family within the *Thiotrichales*. Clade Z also constitutes a novel family, but
148 falls into a separate order of the *Gammaproteobacteria*, distinct from the *Thiotrichales* but
149 we will not consider this further within this study since a much larger issue must be
150 considered at the same time, *viz.* the systematics of the higher taxa of the
151 *Gammaproteobacteria*. Since both *Leucothrix* and *Cocleimonas* are sufficiently distant from
152 *T. nivea* to potentially constitute a separate class, and further work must be undertaken to
153 delineate this. We will, however, define a new family for Clade Z, with position *incertae*
154 *sedis* pending further work – given the size of the *Gammaproteobacteria* and the number of
155 species therein and the necessity for rMLST-type approaches with long concatamers, it may
156 not be possible to ascertain this accurately based on the current algorithm, software and
157 hardware limitations, even within high-performance computing. We can, however, determine
158 that *Leucothrix* and *Colceimonas* do not fall within the *Thiothrichaceae* or our two novel
159 families, and so we circumscribe them on the basis of their 16S rRNA gene identities as a
160 novel family, *Leucotrichaceae* fam. nov., but we place this *incertae sedis* pending further
161 work, whilst rejecting it from the *Thiothrichales sensu stricto*.

162 For those taxa with sequenced genomes, phylogenetic studies were performed on the basis of
163 a range of ‘housekeeping’ genes other than the 16S rRNA gene (above) commonly used in
164 taxonomic studies (Figure 2), selected on the basis of their involvement in a diversity of
165 pathways and systems, namely those encoding polyribonucleotide nucleotidyltransferase (EC
166 2.7.7.8, *pnp*); translation initiation factor IF-2 (EC 3.6.5.3, *infB*); glyceraldehyde-3-phosphate
167 dehydrogenase (NAD⁺, EC 1.2.1.12, *gapA*); glutaminyl-tRNA synthetase (EC 6.3.5.7, *glnS*)
168 and elongation factor EF-G (EC 3.6.5.3, *fusA*) and recombination protein A (*recA*). Gene
169 sequences were obtained from public databases of genes from *Thiothrix* and *Leucothrix* spp.,
170 and *Thiomicrospira pelophila* DSM 1534^T (also from the *Thiotrichales* of the
171 *Gammaproteobacteria*), genes from which were used as the outgroup. We employed
172 *Thiothrix* sp. AAV1 as a proxy for *T. fructosivorans* (16S rRNA gene identity 99.9% and 16S
173 rRNA gene tree position shown in Supplementary Figure S1). We have provided full details
174 of tree construction in the figure legends for these trees. A further analysis was performed
175 using amino acyl sequences derived from genes encoding ribosomal proteins in the form of
176 53-gene concatamers, obtained from databases of the ribosomal multilocus sequence typing
177 (rMLST) online platform [19], again, with full details provided in the legend (Figure 3).

178 Across almost all phylogenetic analyses of amino acyl sequences derived from housekeeping
179 genes performed, *Thiothrix* spp. fell into the same three clades per the 16S rRNA gene
180 analyses, branching from highly supported nodes (Figure 2), *pace* RecA, the tree for which
181 did not have well-supported nodes. The GapA and GlnS trees did not show full resolution of
182 Clade Y and Clade Z, but this was clear across all of the other trees. In spite of these minor
183 variations in single gene trees, the concatamer analysis of 53 ribosomal protein-coding genes
184 gave the same overall topography as the 16S rRNA gene tree (Figure 3), with three well-
185 divided clades originating from well-supported nodes with bootstrap values of 99-100 % over

186 5,000 replicate reconstructions. These data support the division of *Thiothrix* into 3 genera, in
187 line with the 16S rRNA gene identities across the genus.

188 Clade X (*Thiothrix* spp. *sensu stricto*) organisms are united by chemolithoautotrophic or
189 seemingly obligately mixotrophic growth and have cells 0.7-2.5 μm wide and 0.7-6.5 μm
190 long. They are catalase negative (*pace* a weak reaction in *T. fructosivorans*) but oxidase
191 positive. They form filaments which do not contain knots or branches and which almost all
192 (*pace T. unzii*) have polysaccharide sheaths. Salt is not required for growth and temperature
193 optima are 24-30 $^{\circ}\text{C}$, with pH optima of 7.0-8.0 and maxima of 8.2-8.6. Ribulose-1,5-
194 bisphosphate carboxylase/oxygenase (RuBisCO, EC 4.1.1.39) from the Calvin-Benson-
195 Bassham cycle is found in two key forms in the '*Proteobacteria*' – form I (composed of 8
196 CbbL and 8 CbbS subunits) and form II (a dimer of CbbM, which is evolutionarily related to
197 CbbL, [20-21]). The form I RuBisCOs present in the organisms considered here can be
198 further divided into forms IAq (cytoplasmic) and IAc (carboxysomal). In Clade X, genes
199 encoding form IAq and form II RuBisCO were found in all strains for which a genome
200 sequence exists, with *T. nivea* and *T. caldifontis* also having form IAc. The form IAc genes in
201 *T. nivea* and *T. caldifontis* are collocated with genes encoding carboxysome shell proteins,
202 carboxysomal carbonic anhydrase and the RuBisCO assembly factor [22]. For both species,
203 carboxysome genes are followed by genes homologous to those encoding inorganic carbon
204 transporters, similar to observations in other chemolithoautotrophs (*e.g.* [16] and [47]). Based
205 on the presence of genes encoding carboxysome components, and the (likely) inorganic
206 carbon transporters, these two species could express mechanisms for the concentration of
207 carbon dioxide, allowing growth under low carbon dioxide partial pressures.

208 All *Thiothrix sensu lato* oxidise thiosulfate to elementary sulfur and [then] sulfate, possessing
209 genes encoding elements of the canonical Kelly-Friedrich pathway ('sox pathway') of sulfur
210 oxidation (found complete in *Paracoccus* spp. from the *Alphaproteobacteria* and in both

211 complete and partial forms across the *Gammaproteobacteria*, *Betaproteobacteria* and
212 *Acidithiobacillia*, [23]), but in different degrees of fragmentation around the genome (Table
213 1), which we have also observed in other members of the *Thiotrichales* (Scott *et al.*, under
214 review). Sulfide dehydrogenase (flavocytochrome *c*, EC 1.8.2.3, Fcc – used in the oxidation
215 of sulfide to elementary sulfur) genes and sulfide:quinone reductase (EC 1.8.5.4, Sqr – used
216 in the oxidation of sulfide to polysulfide, in the formation of elementary sulfur granules)
217 genes were also present in all *Thiothrix sensu lato*, correlating with these species
218 accumulating elementary sulfur granules during growth. Additionally, all Clade X organisms
219 have adenosine 5'-phosphosulfate reductase (EC 1.8.4.8, AprAB), which is found in sulfate-
220 reducing anaerobes but also in aerobic sulfur oxidisers, operating to oxidise sulfite to sulfate.
221 Organisms across all three clades also have the reverse form of dissimilatory (bi)sulfite
222 reductase (EC 1.8.99.5, DsrAB), which oxidises sulfide to sulfite in aerobes. The Clade X
223 enzyme data are similar to the findings in “*Thiothrix ramosa*”, which affiliates to this clade
224 [40].

225 All members of all clades have a full complement of genes encoding enzymes of Krebs’
226 cycle, including the E3 subunit of the 2-oxoglutarate dehydrogenase complex –
227 dihydrolipoamide dehydrogenase (*lpdA*, EC 1.8.1.4) – which is either missing at gene level or
228 present but does not result in *in vivo* enzyme activity of the complex in most obligate
229 autotrophs, cutting Krebs’ cycle into Smith’s biosynthetic horseshoe [16, 24-27] and
230 preventing heterotrophic growth since the cycle cannot be for energetic purposes, only for
231 biosynthesis. It is not known if this complex is expressed in *Thiothrix* spp., but the potential
232 for heterotrophic growth is at least present. Genes encoding the enzymes of the glyoxylate
233 shunt were found in all *Thiothrix sensu lato*, permitting growth on acetate and other C₂
234 compounds, as sole carbon sources, in principle [28], but genes encoding enzymes of C₁
235 compound metabolism (*viz.* methanol dehydrogenases (*mxhF* and *xoxF* EC 1.1.2.7 [29]),

236 Quayle pathway (ribulose monophosphate pathway [30]) and serine cycle [31]) were not
237 found. In terms of glycolytic (or gluconeogenic) pathways, the full complement of genes
238 encoding enzymes of the Entner-Doudoroff [32], Embden-Meyerhoff-Parnas and pentose
239 phosphate [33] was found in all clades examined.

240 The complete transaldolase variant Calvin-Benson-Bassham cycle enzymes could be
241 accounted for in all three clades with the exception of ribulose-1,5-bisphosphate
242 carboxylase/oxygenase (RuBisCO, EC 4.1.1.39), which was not found in Clade Z (or in *L.*
243 *mucor*); all Clade Y species have RuBisCO form IAq (a cytoplasmic form, [20]) and some
244 also have form IAc (carboxysomal), whereas Clade X have either form IAq or form II (also
245 cytoplasmic), and no carboxysomal forms. These data would imply that Clade Z are obligate
246 heterotrophs and whilst they may oxidise thiosulfate to sulfate to provide Δp and thus
247 synthesise ATP to permit chemolithoheterotrophic growth (per some other members of the
248 *Thiotrichales*, e.g. *Methylophaga thiooxydans* [34-35]). They thus do not fix carbon dioxide
249 into biomass *sensu stricto*, but will no doubt assimilate it to a minor degree *via* anaplerotic
250 fixation (e.g. pyruvate carboxylase, EC 6.4.1.1) and cannot be considered autotrophic. These
251 data also imply that Clade Y species have diverse means for carbon dioxide fixation, both
252 evolved for life at higher carbon dioxide partial pressures in which oxygen is present, without
253 having any means to concentrate carbon dioxide or live at low carbon dioxide partial
254 pressures. Clade X have the further ability to live at both much higher carbon dioxide partial
255 pressures using form II RuBisCO or much lower partial pressures using the carboxysomal
256 form, IAc, where oxygen is at lower partial pressures [20] – Clade X has been found in
257 sulfidic wells and springs (karstic and thus high in dissolved bicarbonate), and on
258 amphiphods living in karstic ecosystems [10], which would be in keeping with the presence
259 of form II RuBisCO, which operates more effectively in high carbon dioxide partial pressures
260 than form IAc, which is optimised for lower partial pressures. There is a paucity of ecological

261 data on Clade Y since they have only been isolated from activated sludge thus far, but given
262 that organic matter decomposes with rapidity in activated sludge, we anticipate that the
263 carbon dioxide partial pressures therein to be high, thus precluding the necessity of carbon
264 dioxide concentrating mechanisms in the form of carboxysomes.

265 Substrate profiles for heterotrophic (or mixotrophic) growth are quite restricted in Clade X -
266 with hexoses and disaccharides not being used (*pace* fructose and sucrose in *T.*
267 *fructosivorans*), which is unusual given Krebs' cycle is complete at genome level and there
268 are no lesions of glycolysis *pace* the pentose phosphate pathway in two species. Acetate is the
269 only substrate used universally in Clade X. Whilst Clades Y and Z can grow on sugar
270 alcohols (*viz.* mannitol and glycerol), Clade X organisms cannot – a distinguishing and useful
271 diagnostic feature. It is worth noting that the growth of *T. fructosivorans* on fructose can
272 probably be ascribed to the presence of the fructose transport system genes in the genome
273 sequence, which are absent from all other species examined. *T. nivea* and *T. unzii* both
274 hydrolyse gelatine and starch but the rest of the clade cannot. All species of all clades can use
275 ammonium or nitrate as their sole nitrogen source and some species can use nitrite. *T.*
276 *caldifontis* is the only *Thiothrix* sp. for which diazotrophy has been reported, which is
277 supported by the presence of *nif* genes encoding the molybdenum-iron nitrogenase (EC
278 1.19.6.1), which were also found in *T. nivea*, but not in any other species examined. GC
279 fractions in Clade X are 49.3 – 52.0 mol% *in vitro*, or 50.5 – 54.9 mol% *in silico*.

280 All genomes examined for Clades X, Y and Z contain genes for the *cbb₃* cytochrome *c*
281 oxidase and *bd-I* quinol oxidase, both of which are also common to *L. mucor*, which
282 additionally has the *aa₃* cytochrome *c* oxidase, but this is not found in Clades X, Y or Z. The
283 *cbb₃* oxidase has a high oxygen affinity in general and this would be in keeping with the
284 microaerophilic nature of many organisms in these clades, growing best at low oxygen partial
285 pressures, and the absence of the *aa₃*-type oxidase would be in keeping with very weak

286 growth under high oxygen partial pressures. As the *bd-I* ubiquinol oxidase has been
287 implicated in promoting survival to oxidative stress, this is also in keeping with the lifestyle
288 of these organisms [36].

289 For the sake of completeness and to benefit those reading ecological studies or literature on
290 *Thiothrix* species without validly published names, consideration was given to the various
291 “*Thiothrix*” 16S rRNA gene sequences curated into the GenBank™ and IMG databases, a
292 phylogenetic analysis of which is given in Supplementary Figure S1. Of these, two groups of
293 sequence are worthy of consideration – the first is “*Thiothrix ramosa*”, a well-characterised
294 isolate from a sulfidic spring in what is now Latvia, subjected to extensive physiological and
295 phylogenetic characterisation in the 1990s [37-41] – probably the best studied *Thiothrix*
296 isolate in terms of sulfur physiology. It was identified as related to *T. nivea*, though it is now
297 lost from culture (Elena Odintsova, *personal communication*) and the name was never validly
298 or effectively published. Our analysis confirms that “*T. ramosa*” would still fall within the
299 genus *Thiothrix sensu stricto*, with the closest relative being *T. lacustris* with a 16S rRNA
300 gene identity of 97.7 % - *T. lacustris* also being obtained from a sulfidic freshwater
301 ecosystem of similar latitude, but lacking the characteristic branching observed in “*T.*
302 *ramosa*”, which is absent from other *Thiothrix* spp. “*T. ramosa*” was shown to grow on a
303 range of energy sources, including substituted thiophenes (the metabolism of which is very
304 poorly understood), thus *T. lacustris* DSM 21227^T (genome sequence available) may prove a
305 valuable model organism for these metabolic pathways if it indeed shares this trait. The
306 second group of note are the large number of *Thiothrix*-affiliated cloned 16S rRNA and
307 rRNA gene sequences obtained from *Niphargus ictus* specimens from the Frassassi Caves in
308 Italy, which have sequences deposited in the GenBank™ database and most of these probably
309 represent close relatives of *T. fructosivorans*, the type strain of which was isolated from
310 activated sludge in the USA.

311 We conclude that extant *Thiothrix* spp. in culture actually represent three distinct genera, on
312 the basis of the clades defined in this study, the properties of which are curated at genus level
313 in Table 2, with comparison to other genera of the *Thiotrichales*. Since Clade X contains the
314 type species (*T. nivea*), the genus that it circumscribes must retain the name *Thiothrix*, in
315 accordance with Rule 39a of the *Code*. As such, *T. nivea*, *T. unzii*, *T. caldifontis*, *T.*
316 *fructosivorans*, *T. lacustris* are considered *bona fide* members of the genus *Thiothrix*. We
317 propose that Clade Y circumscribes a novel genus for which we propose the name *Thiofilum*
318 gen. nov., for which the type species is *Thiofilum flexile* gen. nov., comb. nov. *T. defluvii*
319 would be circumscribed into *Thiofilum* too, but the lack of available strains means we cannot
320 make this formal reclassification and “*Thiofilum defluvii*” will have to be subject of future
321 work, if ever *T. defluvii* cultures are found and deposited accordingly. Clade Z circumscribes
322 another new genus, for which we propose the name *Thiolinea* gen. nov., for which the type
323 species is *Thiolinea disciformis* gen. nov., comb. nov. Again, *T. eikelboomii* undoubtedly
324 would be circumscribed within *Thiolinea*, the lack of strains deposited in international
325 collections makes it impossible to formally reclassify it at this time, thus “*Thiolinea*
326 *eikelboomii*” will be the subject of future work if and when the strain is made available to the
327 public in 2 or more international service collections. Obviously our mention of “*Tfs. defluvii*”
328 and “*Tla. eikelboomii*” are merely ‘incidental mentions’ under Rule 28b of the *Code* and do
329 not constitute or attempt to constitute any formal prioritisation or laying any claim to future
330 publication of these two names.

331

332 **Description of *Leucotrichaceae* fam. nov.**

333 *Leucotrichaceae* (Leu.co.tri.cha.ce'ae. N.L. fem. n. *Leucothrix*, type genus; -aceae suffix to
334 denote family; N.L. fem. pl. n. *Leucotrichaceae*, the *Leucothrix* family).

335 This family is circumscribed on the basis of 16S rRNA gene sequences and comprises the
336 genera *Leucothrix* (type genus) and *Cocleimonas*. Ubiquinone-8 (UQ-8) is the dominant
337 respiratory quinone. Dominant fatty acids are palmitoleic (C_{16:1}), palmitic (C_{16:0}) and
338 vaccenic (C_{18:1}) acids. G+C fractions are 43.4 to 49.5 mol%. Contain *aa*₃ and *cbb*₃ type
339 cytochrome *c* oxidases and the *bd*-I ubiquinol oxidase. Found in seawater and on/in marine
340 green algae and marine gastropods. Falls within the *Gammaproteobacteria*, with the position
341 at order level *incertae sedis* at present.

342 Type genus: *Leucothrix*.

343 **Description of *Thiolinea* gen. nov.**

344 *Thiolinea* (Thi.o.li'ne.a. Gr. neut. n. *theion* sulfur, brimstone, L. transliteration *thium*, sulfur;
345 L. fem. n. *linea*, a string, a cord; N.L. fem. n. *Thiolinea*, sulfur string)

346 Heterotrophic. Gram-stain-negative cells, forming filaments with holdfasts and that may
347 exhibit branching. Rosettes are formed and filaments may contain knots, but do not have a
348 polysaccharide sheath. Do not form endospores or exospores. Thiosulfate is oxidised during
349 heterotrophic growth but the energetic effects have not been examined. Have RuBisCO
350 genes. Nitrate is not reduced. The 16S rRNA genes have about 92.5 – 93.5 % sequence
351 identity to that of *Thiothrix nivea* JP2^T. Ubiquinone-8 (UQ-8) is the dominant respiratory
352 quinone. Major fatty acids are palmitoleic (C_{16:1}), palmitic (C_{16:0}) and vaccenic (C_{18:1}) acids -
353 the dominant unsaturated fatty acid is vaccenic acid. Members of the *Thiothrichaceae* in the
354 *Thiotrichales* of the *Gammaproteobacteria*.

355 Type species is *Thiolinea disciformis*, isolated from activated sludge suffering from bulking
356 in Japan).

357 **Description of *Thiolinea disciformis* gen. nov. comb. nov.**

358 *Thiolinea disciformis* (dis.ci.for'mis. L. masc. n. *discus*, a disc (from Gr. masc. n. *diskos*); L.
359 fem. adj. suffix *-formis* (from L. fem. n. *forma*, figure, shape or appearance) in the shape of;
360 N.L. fem. adj. *disciformis*, disc-shaped, after the main cell morphology).

361 Type species of *Thiolinea*. Basonym: *Thiothrix disciformis* Aruga *et al.* 2002.

362 Gram-stain negative. Sugar-grown cells are oxidase-positive and violently catalase-positive.
363 Heterotrophic, and oxidises thiosulfate or sulfide during heterotrophic growth. Forms
364 fingerprint-like colonies on glucose-acetate agar. Cells are mostly rod shaped but morphology
365 is variable, particularly with regard to length. Most cells are discoid or ovoid and 1.2 – 3.0
366 μm diameter and 0.5 – 3.0 μm in length, forming slightly bent, sheath-free filaments greater
367 than 0.5 mm in length that can reach several millimetres. Some cells in filaments are elongate
368 or swollen. Septa between cells of filaments are clearly defined. Volutin (polyphosphate)
369 granules are absent. Sudanophilic (lipid) granules are present. Temperature range of growth is
370 14-32 °C, with optimum growth 25-30 °C and no growth at 4 °C or at 37 °C. Growth is fully
371 inhibited by 85 mM (0.5 % w/v) sodium chloride. Elementary sulfur globules are deposited
372 within the invaginated inner membrane when grown in the presence of thiosulfate or sulfide,
373 which are oxidised to sulfate. Carbon sources for heterotrophic growth include hexose sugars
374 (glucose, fructose, mannose), disaccharides (sucrose, maltose, trehalose), intermediates of
375 Krebs' cycle (succinate, malate, citrate), carboxylates (pyruvate, acetate, butyrate,
376 hydroxybutyrate), amino acids (glutamate, aspartate, alanine), alcohols (mannitol, glycerol),
377 but not benzoate, xylose, erythritol, galactose, lactose, melibiose, raffinose, arabinose,
378 lactate, ethanol, propan-1-ol, sorbitol, formate, gelatine or starch. Does not require vitamins
379 for growth. Does not reduce nitrate. Has genes encoding *cbb₃* cytochrome *c* and *bd-I*
380 ubiquinol type terminal oxidases, and complete Krebs' cycle plus the glyoxylate shunt. Has
381 genes encoding form II RuBisCO and does not have carboxysomal genes. G+C fraction is
382 45.1 mol% from the genome sequence. Dominant respiratory quinone is ubiquinone-8.

383 Dominant fatty acids in glucose-and-acetate grown cells are palmitoleic (C_{16:1}), palmitic
384 (C_{16:0}) and vaccenic (C_{18:1}) acids.

385 Type strain is B3-1^T (= DSM 14473^T = JCM 11364^T), isolated from activated sludge
386 suffering from bulking (Japan).

387 **Description of *Thiolineaceae* fam. nov.**

388 *Thiolineaceae* (Thi.o.li.ne.a.ce'ae. N.L. fem. n. *Thiolinea*, type genus; *-aceae* suffix to denote
389 family; N.L. fem. pl. n. *Thiolineaceae*, the *Thiolinea* family)

390 This family is circumscribed on the basis of 16S rRNA gene sequences and comprises the
391 genus *Thiolinea* (type genus). Ubiquinone-8 (UQ-8) is the dominant respiratory quinone.

392 Dominant fatty acids are palmitoleic (C_{16:1}), palmitic (C_{16:0}) and vaccenic (C_{18:1}) acids. G+C
393 fractions are 45.1 to 46.3 mol% on the basis of genome sequence data. Falls within the order
394 *Thiotrichales* of the *Gammaproteobacteria*.

395 Type genus: *Thiolinea*

396 **Description of *Thiofilum* gen. nov.**

397 *Thiofilum* (Thi.o.fi'lum. Gr. neut. n. *theion* sulfur, brimstone, L. transliteration *thium*, sulfur;
398 L. neut. n. *filum*, filament, thread; N.L. neut. n. *Thiofilum*, sulfur filament).

399 Members of the *Thiothrichaceae* in the *Thiotrichales* of the *Gammaproteobacteria*.

400 Obligately heterotrophic. Gram-stain-negative filamentous bacteria. Filaments do not have
401 polysaccharide sheaths but form holdfasts and rosettes and may become knotted, but are not
402 branched. Do not form endospores or exospores. Thiosulfate can be oxidised to provide Δp,
403 which can be used to generate ATP for chemolithoheterotrophic growth. Nitrate is reduced to
404 nitrite. The 16S rRNA genes have about 91 – 92 % sequence identity to that of *Thiothrix*

405 *nivea* JP2^T. Ubiquinone-8 (UQ-8) is the dominant respiratory quinone. Major fatty acids are
406 palmitoleic (C_{16:1}), palmitic (C_{16:0}) and vaccenic (C_{18:1}) acids – the dominant unsaturated fatty
407 acid is vaccenic acid.

408 Type species is *Thiofilum flexile*, isolated from activated sludge suffering from bulking in
409 Japan).

410 **Description of *Thiofilum flexile* gen. nov. comb. nov.**

411 *Thiofilum flexile* (fle'xi.le. L. neut. adj. *flexile*, flexile, pliable).

412 Type species of *Thiofilum*. Basonym: *Thiothrix flexilis* Aruga *et al.* 2002.

413 Gram-stain-negative. Sugar-grown cells are oxidase- and catalase-positive. Obligately
414 heterotrophic. Forms fingerprint-like colonies on glucose-acetate agar. Cells are rod shaped
415 but morphology is variable, particularly with regard to length. Most cells are discoid or ovoid
416 and 1.0 – 4.0 µm diameter and 0.5 – 5.5 µm in length, forming slightly bent, sheath-free
417 filaments greater than 0.5 mm in length that can reach several millimetres. Some cells in
418 filaments are elongate or swollen. Septa between cells of filaments are clearly defined.
419 Rosettes and holdfasts are observed in some strains. Volutin (polyphosphate) granules are
420 usually absent. Sudanophilic (lipid) granules are present. Temperature range of growth is 14-
421 37 °C, with optimum growth 20-30 °C and no growth at 4 °C or at 42 °C. Growth is good in
422 0 to 170 mM (0 – 1.0 % w/v) sodium chloride but is inhibited slightly at 340 mM (2.0 %
423 w/v). A small number of elementary sulfur globules are deposited within the invaginated
424 inner membrane when grown in the presence of thiosulfate or sulfide, which are either not
425 oxidised or only weakly oxidised to sulfate. Trichomes collected *in situ* do not contain sulfur
426 deposits. Carbon sources for heterotrophic growth include hexose sugars (glucose, fructose,
427 mannose), disaccharides (sucrose, maltose, trehalose), intermediates of Krebs' cycle

428 (succinate, malate, citrate), carboxylates (lactate, propionate, pyruvate, acetate,
429 hydroxybutyrate), amino acids (glutamate, aspartate, alanine), alcohols (mannitol), but not
430 benzoate, glycerol, butanol, xylose, galactose, melibiose, erythritol, raffinose, arabinose,
431 rhamnose, ethanol, propan-1-ol, sorbitol, formate, gelatine or starch. Does not require
432 vitamins for growth. Reduces nitrate to nitrite. Has genes encoding *cbb*₃ cytochrome *c* and
433 *bd*-I ubiquinol type terminal oxidases, and complete Krebs' cycle plus the glyoxylate shunt.
434 Does not have RuBisCO genes. G+C fraction is 44.3 mol% from the genome sequence.
435 Dominant respiratory quinone is ubiquinone-8. Dominant fatty acids in glucose-and-acetate
436 grown cells are palmitoleic (C_{16:1}), palmitic (C_{16:0}) and vaccenic (C_{18:1}) acids.
437 Type strain is EJ2M-B^T (= DSM 14609^T = JCM 11135^T), isolated from activated sludge
438 suffering from bulking (Japan).

439 **Description of *Thiofilaceae* fam. nov.**

440 *Thiofilaceae* (Thi.o.fi.la.ce'ae. N.L. neut. n. *Thiofilum*, type genus; *-aceae* suffix to denote
441 family; N.L. pl. n. *Thiofilaceae*, the *Thiofilum* family).

442 This family is circumscribed on the basis of 16S rRNA gene sequences and comprises the
443 genus *Thiofilum* (type genus). Ubiquinone-8 (UQ-8) is the dominant respiratory quinone.

444 Dominant fatty acids are palmitoleic (C_{16:1}), palmitic (C_{16:0}) and vaccenic (C_{18:1}) acids. G+C
445 fractions are around 44.3 mol% on the basis of genome sequence data. Falls within the
446 *Gammaproteobacteria*, with the position at order level *incertae sedis* at present.

447 Type genus: *Thiofilum*

448 **Emended description of *Thiotrichaceae* Garrity *et al.* 2005**

449 *Thiotrichaceae* (Thi.o.tri.cha.ce'ae. N.L. fem. n. *Thiothrix*, type genus; *-aceae*, suffix
450 denoting family; N.L. fem. pl. n. *Thiotrichaceae*, the *Thiothrix* family).

451 This family is circumscribed on the basis of 16S rRNA gene sequences and includes the
452 genus *Thiothrix* on this basis, and historically has contained *Achromatium*, *Thiobacterium*,
453 *Thiomargarita*, *Thioploca* and *Thiospira*, but their position is impossible to determine given
454 the paucity of sequence data. They should, however, not be regarded as rejected from this
455 family. Type genus are filamentous organisms that deposit elementary sulfur during growth.

456 Type genus: *Thiothrix*.

457 **Emended description of *Thiothrix* Winogradsky 1888 (Approved Lists 1980) emend.**

458 **Aruga *et al.* 2002**

459 *Thiothrix* (Thi'o.thrix. Gr. masc. n. *theion* sulfur, brimstone, L. transliteration *thium*, sulfur;
460 Gr. fem. n. *thrix* hair; N.L. fem. n. *Thiothrix*, sulfur hair).

461 Members of the *Thiothrichaceae* in the *Thiotrichales* of the *Gammaproteobacteria*. Gram-
462 stain-negative filamentous organisms. Filaments have sheaths in most species and do not
463 form knots. Autotrophs using form IAq and form II RuBisCO. Cells are rods with rounded
464 ends 0.7 – 2.5 µm in diameter and 0.7 – 6.5 µm in length. Form white colonies with fibrous
465 margins. Cells have polar tufts of fimbriae. Use thiosulfate or sulfide to support
466 chemolithoautotrophic growth, with concomitant production of sulfate. All species can
467 assimilate carbon from acetate and most can use succinate. None grow on mannitol or
468 glycerol. Nitrate is reduced to nitrite. Major fatty acids are palmitoleic (C_{16:1}), palmitic (C_{16:0})
469 and vaccenic (C_{18:1}) acids - the dominant unsaturated fatty acid is palmitoleic acid. Genes
470 encoding *cbb₃* and *bd-I* terminal oxidases are found in all species. G+C fractions are 51.1-
471 54.9 mol% (from genome sequences). Can be isolated from sulfidic wells, springs and lakes,
472 from activated sludge, and from the bodies of amphipods living in sulfidic environments.

473 Type species is *Thiothrix nivea*.

474 **Acknowledgements**

475 We thank Dr Elena V. Odintsova of the University of Birmingham (Birmingham, UK) and Dr
476 Ann P. Wood, previously of King's College London (London, UK) for discussions on the
477 properties of "*Thiothrix ramosa*" and confirming its loss from culture. We also acknowledge
478 Dr Keith Jolley (University of Oxford, UK) and his team, for the rapid curation of IMG/ER
479 sequences into the rMLST database, and Dr Jolley for providing excellent support with the
480 platform.

481 **Financial Information**

482 The authors received no specific funding for this work.

483 **Conflict of interest**

484 The authors declare they have no competing interests.

485 **Ethical statement**

486 No human, animal or other work that would require ethical approval was undertaken in this
487 study.

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618

619

621 **Table 1.** Comparative properties of *Thiothrix* spp., *Leucothrix* spp. and *Cocleimonas flava*.
622 Data refer to type strains unless otherwise indicated.
623 Data are curated from [1-3] and [6-9] or obtained from genome sequence data held in the
624 Integrated Microbial Genomes (IMG) database.
625 *N.D.*, not determined/no data available; +, positive or present; -, negative or absent; ±, weakly
626 positive.
627 * Values in square brackets are *in silico* values derived from genome sequence data in the
628 IMG public database, other values are from *in vitro* determinations.
629 † Genome parameters are from *Thiothrix* sp. AAV1, which has 99.9% 16S rRNA gene
630 identity to *T. fructosivorans* Q^T.

631

632 **Table 2.** Curated properties of the genera *Thiothrix*, *Thiolinea* gen. nov. and *Thiofilum* gen.
633 nov. and their respective families. Data are from [1-3] and [6-9].

634

635

636 **Figure 1.** Phylogenetic trees on the basis of the 16S rRNA (*rrs*) gene, showing the positions
637 of *Thiothrix* species, divided into Clade X, Clade Y and Clade Z, versus *Leucothrix* spp. and
638 *Cocleimonas flava*. Type species of genera are shown in bold type. Nucleotide sequences
639 were aligned using MUSCLE [42] without use of any pre-sets for speed that reduce accuracy.
640 Aligned sequences were tested for best fit to models on the basis of the Bayesian information
641 coefficient (BIC) in MEGA 7.0.26 [43], and the trees reconstructed accordingly, using the
642 Tamura-Nei model [44] with a discreet gamma distribution to model rate differences across
643 sites (maximum likelihood parameter = 0.1664; neighbour joining/minimum evolution shape
644 parameter = 5). Trees shown are the optimal trees, with numbers at nodes indicating the
645 percentage of 5,000 bootstrap replications in which the topology was preserved (values
646 <70 % omitted for clarity). All positions at which there was less than 95 % coverage were
647 omitted from the final analysis, in which 1,551 nt were used. Branch lengths are to scale and
648 indicate the number of substitutions per site – bars represent 0.02 substitutions per site for all
649 trees shown. The outgroup of each tree is *Thiomicrospira pelophila* DSM 1534^T from the
650 *Piscirickettsiaceae*, also in the *Thiothrichales* per all members of the ingroup. Maximum
651 likelihood tree shown had the highest log-likelihood after 5,000 replications (-4806.27).
652 Neighbour joining and minimum evolution trees shown had the optimal sum of branch length
653 0.523 and 0.525, respectively. Accession numbers are given in parentheses and refer to the
654 GenBank or Integrated Microbial Genomes (IMG) databases (the latter contain an underscore
655 “_”).

656

657 **Figure 2.** Maximum likelihood trees for the genera *Thiothrix* and *Leucothrix*, on the basis of
658 amino acyl sequences derived from a range of housekeeping genes encoding
659 polyribonucleotide nucleotidyltransferase (EC 2.7.7.8, *pnp*); translation initiation factor IF-2
660 (EC 3.6.5.3, *infB*); glyceraldehyde-3-phosphate dehydrogenase (NAD⁺, EC 1.2.1.12, *gapA*);
661 glutaminyl-tRNA synthetase (EC 6.3.5.7, *glnS*); elongation factor EF-G (EC 3.6.5.3, *fusA*),
662 and recombination protein A (*recA*). *Thiothrix* clades X, Y and Z are indicated and type
663 species of genera are shown in bold type. Nucleotide sequences were converted into amino
664 acyl sequences using the bacteriological code, in frame, and were aligned using MUSCLE
665 [42] without use of any pre-sets for speed that reduce accuracy. Aligned amino acyl
666 sequences were tested for best fit to models on the basis of the Bayesian information
667 coefficient (BIC) in MEGA 7.0.26 [43], and the trees reconstructed accordingly, using the Le
668 and Gascuel model [45] with a discreet gamma distribution to model rate differences across
669 sites, and invariant sites. Trees shown are the optimal trees, with numbers at nodes indicating
670 the percentage of 5,000 bootstrap replications in which the topology was preserved (values
671 <70 % omitted for clarity). All positions at which there was less than 95 % coverage were
672 omitted from the final analysis, in which the number of amino acyl residues used was: Pnp
673 348, InfB 789, GapA 332, GlnS 549, FusA 689, RecA 341. Branch lengths are to scale and
674 indicate the number of substitutions per site – bars represent 0.05 or 0.10 substitutions per
675 site. The outgroups are sequences from *Thiomicrospira pelophila* DSM 1534^T from the
676 *Piscirickettsiaceae*, also in the *Thiothrichales* per all members of the ingroups. Maximum
677 likelihood trees shown had the highest log-likelihoods after 5,000 replications. Accession
678 numbers in parenthesis relate to the IMG database.

679

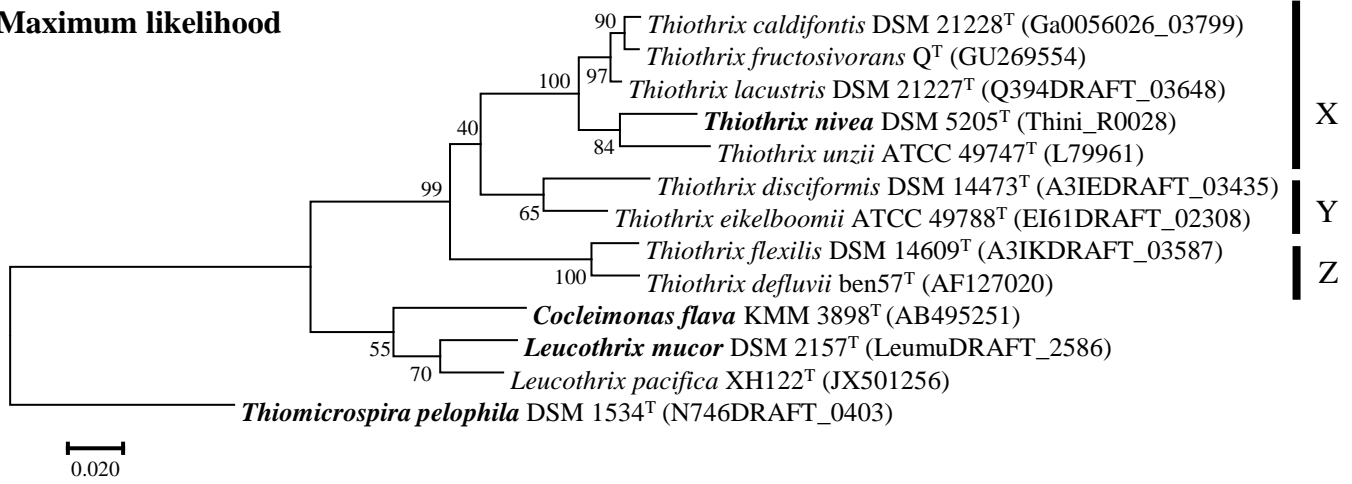
680 **Figure 3.** Maximum likelihood tree of *Thiothrix* and *Leucothrix* spp. using amino acyl
681 concatamer sequences derived from 53 ribosomal protein genes, namely *rpsA* to *rpsU*, *rplA*
682 to *rplF*, *rplL* to *rplX* and *rpmA* to *rpmJ*. Sequences were extracted at gene level and
683 concatenated using the ribosomal multilocus sequence typing (rMLST) online platform [19]
684 from genome sequences publically available in the IMG database. Gene concatamers were
685 then translated in frame using the bacterial code in MEGA 7.0.26 [43] and the derived amino
686 acyl concatamers were then aligned using MUSCLE [42] without any pre-sets, and the
687 aligned dataset tested for the best model fit on the basis of the lowest BIC in MEGA. The tree
688 was thus reconstructed using the Le and Gascuel model [45] with amino acid frequencies
689 estimated from the data and with a discreet gamma distribution to model rate differences
690 across sites, and invariant sites (parameter = 0.5754). Tree shown is the optimal tree, with
691 numbers at nodes indicating the percentage of 5,000 bootstrap replications in which the
692 topology was preserved (values <70 % omitted for clarity). All positions at which there was
693 less than 95 % coverage were omitted from the final analysis, in which 6,668 aa were used.
694 Branch lengths are to scale and indicate the number of substitutions per site – bar represents
695 0.1 substitutions per site for all trees shown. The outgroup is *Thiomicrospira pelophila* DSM
696 1534^T from the *Piscirickettsiaceae*, also in the *Thiothrichales* per all members of the ingroup.
697 Maximum likelihood tree shown had the highest log-likelihoods after 5,000 replications (-
698 49,806.6). Values in parentheses are Genome ID numbers relating to the rMLST database.
699
700

Clade	N/A			Clade Z		Clade Y		Clade X				
Order	Incertae sedis, pending further study					Thiotrichales						
Family	Leucotrichaceae fam. nov.			Thiofilaceae fam. nov.		Thiolineaceae fam. nov.		Thiotrichaceae				
Genus	Leucothrix		Cocleimonas	Thiofilum gen. nov.		Thiolinea gen. nov.		Thiothrix sensu stricto				
Characteristic	<i>Leucothrix mucor</i> DSM 2157 ^T	<i>Leucothrix pacifica</i> XH122 ^T	<i>Cocleimonas flava</i> KMM 3898 ^T	<i>Thiothrix defluvii</i> Ben57 ^T	<i>Thiothrix flexilis</i> EJ2M-B ^T	<i>Thiothrix disciformis</i> B3-1 ^T	<i>Thiothrix eikelboomii</i> AP3 ^T	<i>Thiothrix nivea</i> JP2 ^T	<i>Thiothrix unzii</i> A1 ^T	<i>Thiothrix lacustris</i> DSM 21227 ^T	<i>Thiothrix caldifontis</i> DSM 21228 ^T	<i>Thiothrix fructosivorans</i> ATCC 49748 ^T
Source of type strain	<i>Monostroma</i> sp., USA	Surface seawater, South Pacific Gyre	<i>Umbonium</i> sp., Russia	Activated sludge, Australia	Activated sludge, Japan	Activated sludge, Japan	Activated sludge, USA	Sulfidic well water, USA	Activated sludge, USA	Sulfidic lake water, Russia	Sulfidic spring water, USA	Activated sludge, USA
% 16S rRNA (rrs) gene sequence identity to:												
<i>Thiothrix nivea</i> JP2 ^T	85.6	88.8	87.9	89.5	89.2	90.9	92.3	100.0	94.8	95.0	95.2	94.4
<i>Thiothrix flexilis</i> Ben57 ^T	88.8	88.0	87.4	96.9	100.0	91.3	90.5	89.1	88.0	90.2	89.9	90.1
<i>Thiothrix disciformis</i> B3-1 ^T	89.2	87.6	86.4	92.2	91.3	100.0	94.8	90.8	90.5	91.5	90.9	90.9
Helix 18 deletion of 16S rRNA gene	-	-	-	+	+	+	+	+	+	+	+	+
Colonial properties:												
Colour (reflected light)	White	White	Yellow	N.D.	White	White	White	White	N.D.	White	White	N.D.
Margin	Entire	Entire	Entire	N.D.	Fingerprint-like	Fingerprint-like	Fingerprint-like	Fibrous margin	N.D.	Fibrous margin	Fibrous margin	N.D.
Cell properties:												
Morphology	Rod	Rod	Rod	Rods, cylinders, barrels	Rods, discs, ovoid	Rods, discs, ovoid	Rods, discs, cubes, barrels,	Rods	Rods	Rods	Rods	Rods
Length (µm)	1.8-2.8	2.5-3.0	1.6-1.8	5.0-10.0	0.5-5.0	0.5-3.0	1.0-8.0	3.0	0.7-3.0	4.4-6.3	3.2-6.5	1.2-2.5
Diameter (µm)	0.5-1.0	0.4-0.5	0.3-0.4	1.0-2.0	1.0-4.0	1.2-3.0	0.6-8.0	1.0-1.5	0.70-1.5	0.9-2.3	0.9-2.2	1.2-2.5
Fimbriae	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	+	+	+	N.D.	N.D.	+
Filament properties:												
Filaments formed	+	+	-	+	+	+	+	+	+	+	+	+
Sheathed	-	-	-	-	-	-	-	+	-	+	+	+
Knots found	+	-	-	+	-	N.D.	+	-	-	-	-	-
pH optimum	N.D.	8.0	8.5-9.5	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	7.0	8.0	N.D.
Oxidation of thiosulfate	-	-	+	N.D.	N.D.	N.D.	+	+	+	+	+	+
Carbon sources for heterotrophic growth:												
Sucrose	+	+	N.D.	N.D.	+	+	+	-	-	-	-	+
D-fructose	+	+	N.D.	N.D.	+	+	+	-	-	-	-	+
D-glucose	+	+	+	N.D.	+	+	+	-	-	-	-	-
Acetate	N.D.	N.D.	-	N.D.	+	+	+	+	+	+	+	+
Glycerol	+	+	-	N.D.	-	+	+	-	-	-	-	-
Mannitol	+	+	+	N.D.	+	+	+	-	-	-	-	-
Genes present encoding:												
<i>cbb₃</i> cytochrome <i>c</i> oxidase	+	N.D.	N.D.	N.D.	+	+	+	+	N.D.	+	+	+†
<i>aa₃</i> cytochrome <i>c</i> oxidase	+	N.D.	N.D.	N.D.	-	-	-	-	N.D.	-	-	-†
<i>bd-I</i> ubiquinol oxidase	+	N.D.	N.D.	N.D.	+	+	+	+	N.D.	+	+	+†
RuBisCO	-	N.D.	N.D.	N.D.	-	Form II	Form IAq	Form IAq Form IAc Form II	N.D.	Form IAq Form II	Form IAq Form IAc Form II	Form IAq† Form II
Kelly-Friedrich pathway gene cluster fragmentation	-	N.D.	N.D.	N.D.	<i>soxYZAXB</i>	<i>soxZY</i> <i>soxY</i> <i>soxXA</i> <i>soxY</i>	<i>soxBXAZY</i> <i>soxY</i> <i>soxY</i>	<i>soxZY</i> <i>soxXYZA_Y</i> <i>soxZ</i> <i>soxB</i> <i>soxY</i> <i>soxXA</i>	N.D.	<i>soxYZA</i> <i>soxZ</i> <i>soxB</i> <i>soxXA</i> <i>soxY</i>	<i>soxB</i> <i>soxZYAZYZ</i> <i>soxXA</i>	<i>soxY</i> † <i>soxB</i> <i>soxYZ</i> <i>soxY</i> <i>soxZAY</i>

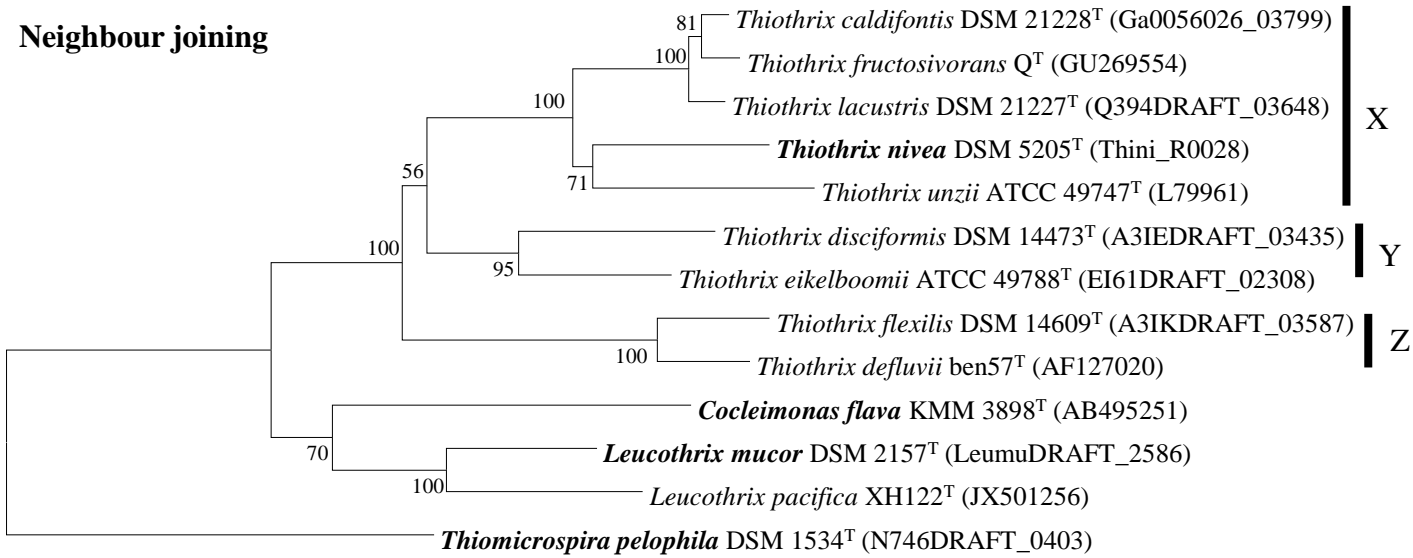
Enzyme activities:												
Oxidase	+	+	+	<i>N.D.</i>	+	+	+	+	+	N.D.	+	+
Catalase	+	+	+	<i>N.D.</i>	+	+	+	-	-	-	-	+
G+C fraction (mol%) *	49.5 [47.8]	46.2	43.4	<i>N.D.</i>	44.0 [44.3]	44.0-45.0 [45.1]	44.0-45.0 [46.3]	52.0 [54.9]	49.3	51.4 [51.3]	52.0 [50.5]	N.D. [51.1]†

	<i>Leucotrichaceae</i> fam. nov.	<i>Thiofilaceae</i> fam. nov.	<i>Thiolineaceae</i> fam. nov.	<i>Thiotrichaceae</i>
Genera	<i>Leucothrix</i> <i>Cocleimonas</i>	<i>Thiofilum</i>	<i>Thiolinea</i>	<i>Thiothrix</i>
Colony colour	White, Yellow	White	White	White
G+C fraction (mol%)	43.4 – 47.8	44.0	44.1-46.3	49.3-54.9
Cell diameter × length (µm)	0.3-1.0 × 1.6-3.0	1.0-4.0 × 0.5-10.0	0.6-8.0 × 1.0-8.0	0.7-2.5 × 0.7-6.5
Catalase activity	+	+	+	-
Pigments	Carotenoids in some species	-	-	-
Gliding motility	-	+	+	+
Carbon sources				
Glycerol	±	-	+	-
Mannitol	+	+	+	-
Acetate	-	+	+	+
Butyrate	-	-	+	-
Glucose	+	+	+	-
Mannose	-	+	+	-
Trehalose	+	+	+	-
Reduction of nitrate	-	+	±	+
Temperature minima (°C)	0-4	10-14	14	4-7
pH minima	5.5	7.0	6.5-7.0	6.2-7.0
pH maxima	11.0	7.9	7.9-8.5	8.2-8.6
Metabolic modes	Heterotrophic	Heterotrophic Chemolithoheterotrophic	Autotrophic Heterotrophic Mixotrophic	Autotrophic Heterotrophic Mixotrophic
Dominant non-substituted fatty acid	Palmitoleic or vaccenic	Vaccenic	Vaccenic	Palmitoleic or palmitic

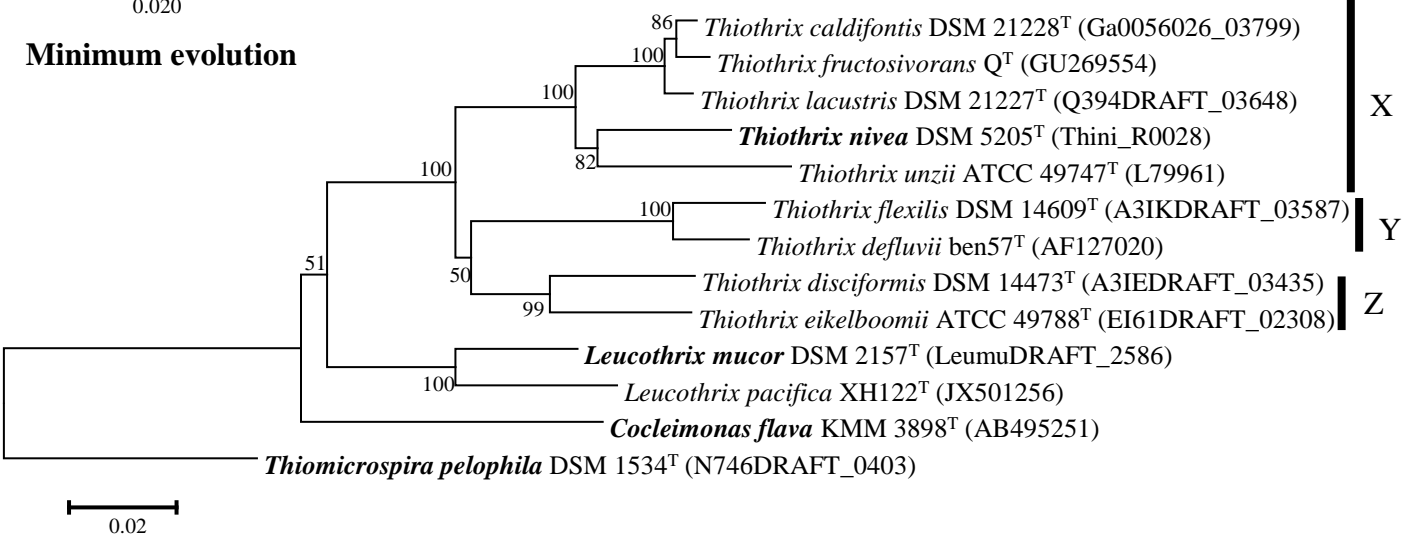
Maximum likelihood

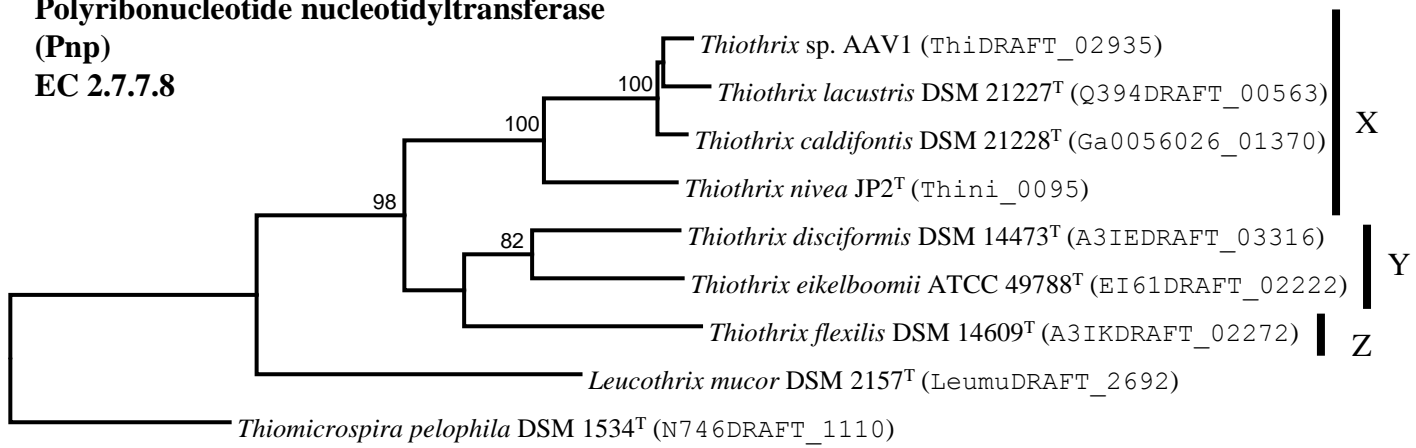
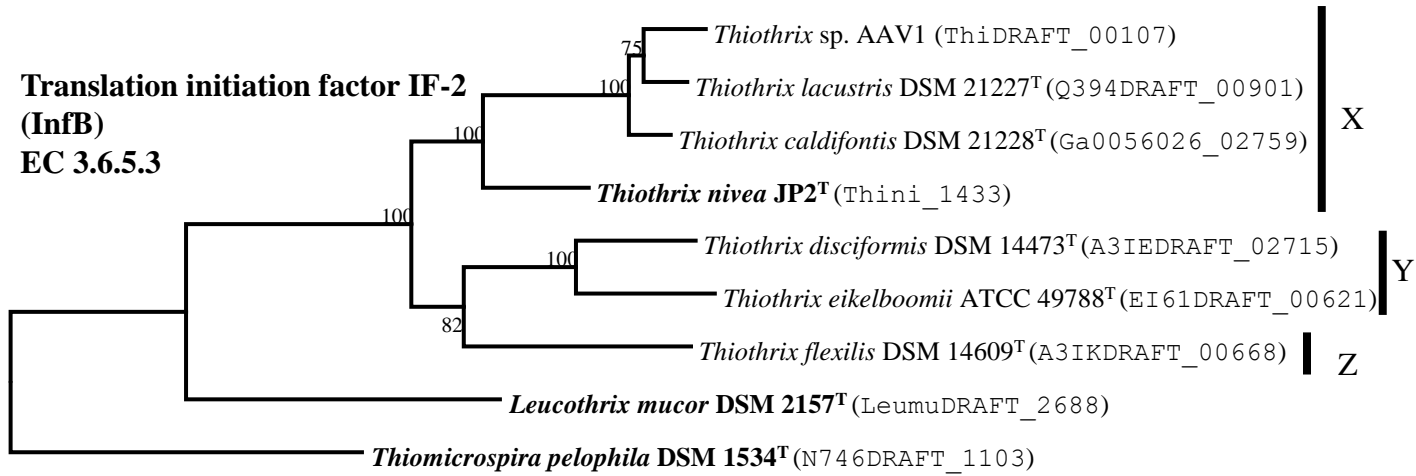
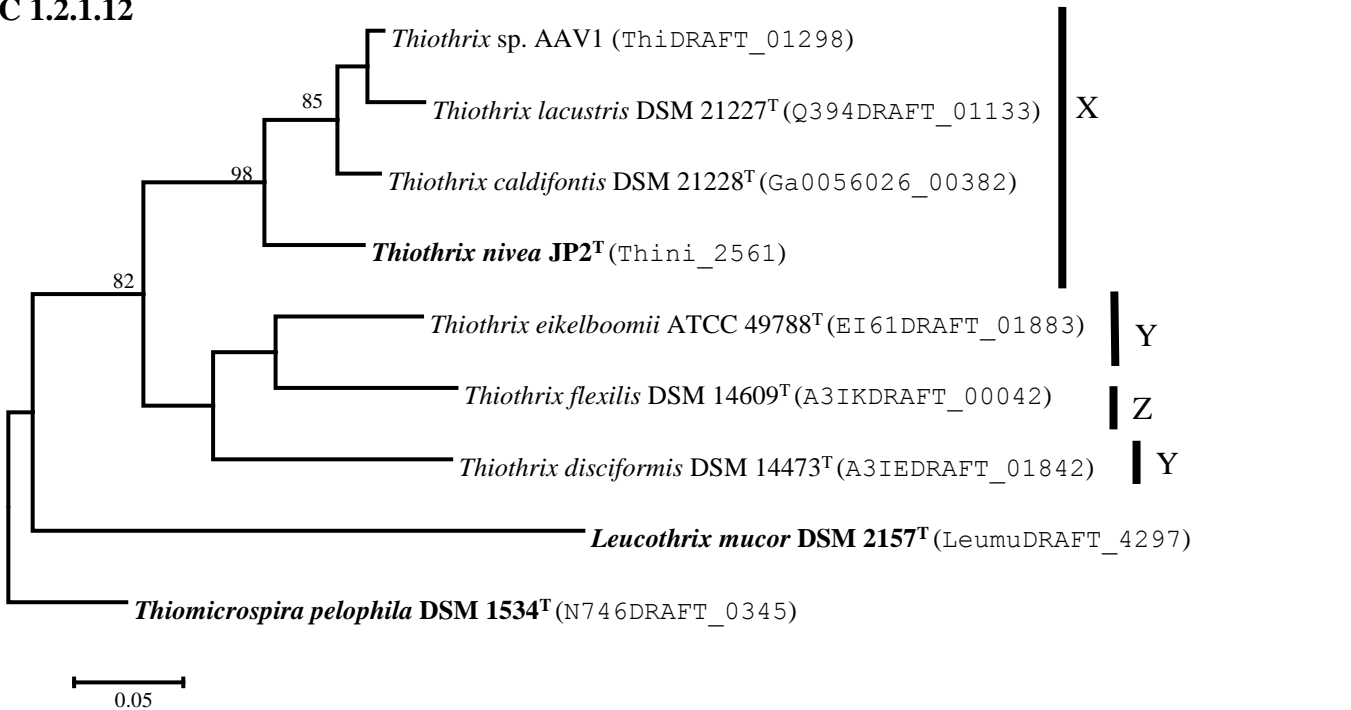


Neighbour joining



Minimum evolution

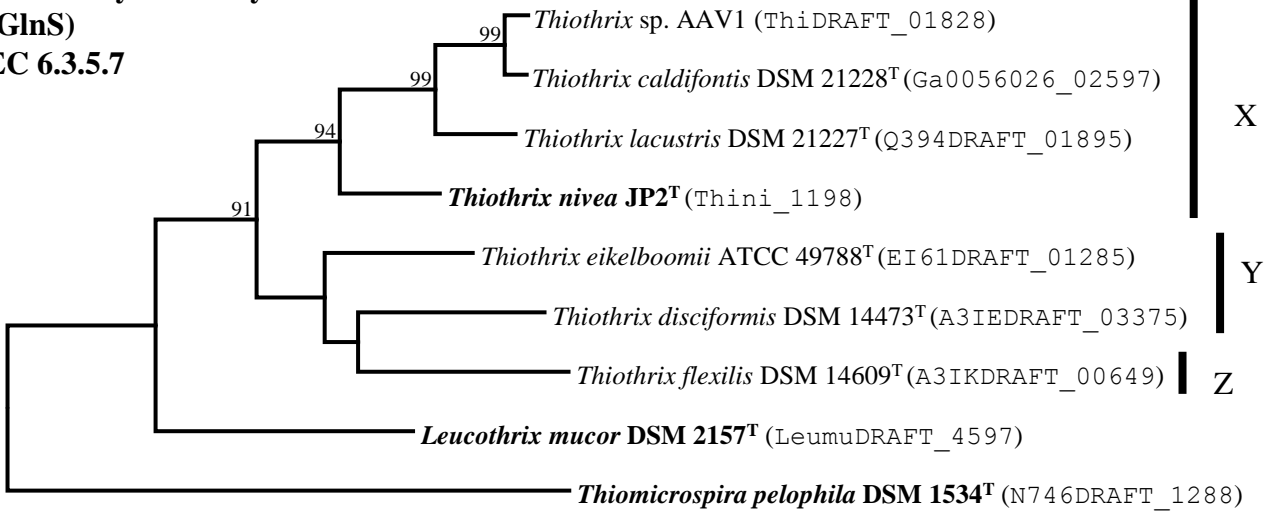


Polyribonucleotide nucleotidyltransferase**(Pnp)****EC 2.7.7.8****Translation initiation factor IF-2****(InfB)****EC 3.6.5.3****Glyceraldehyde-3-phosphate dehydrogenase****(GapA)****EC 1.2.1.12**

Glutaminyl-tRNA synthetase

(GlnS)

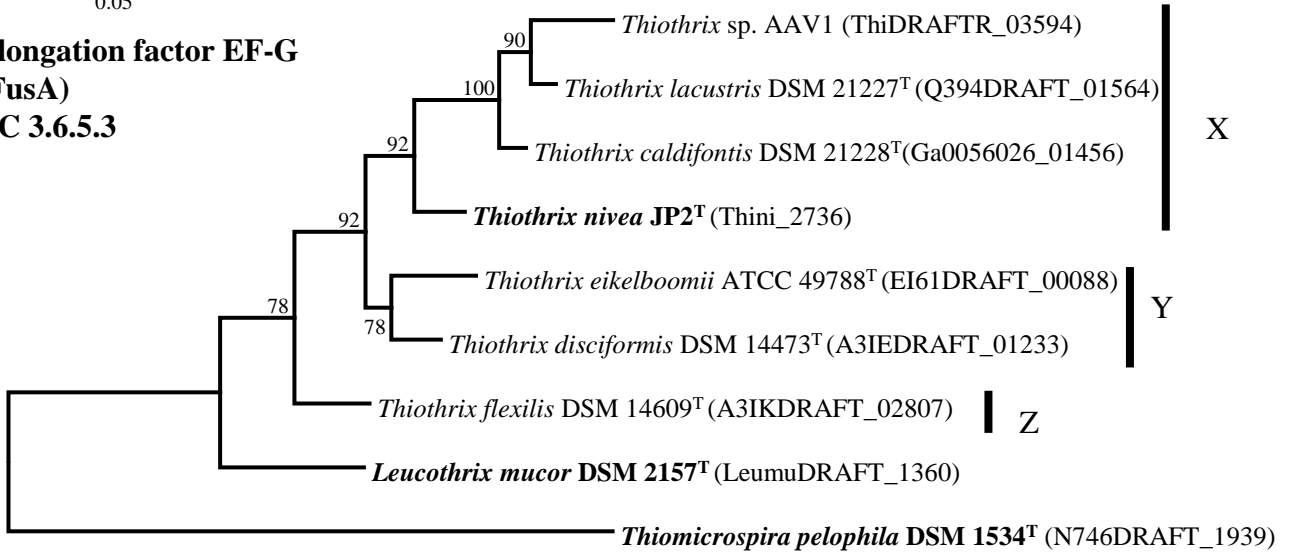
EC 6.3.5.7



Elongation factor EF-G

(FusA)

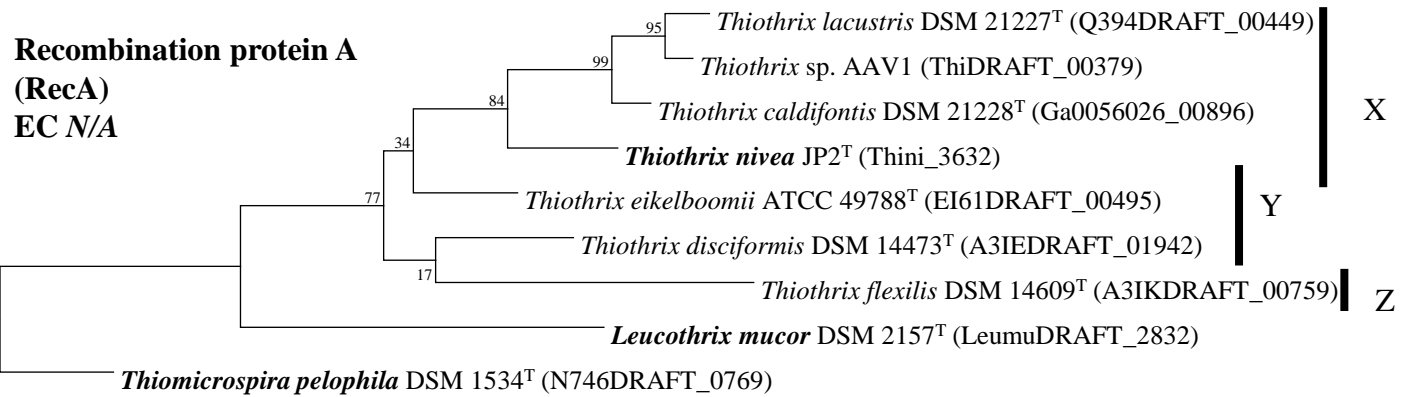
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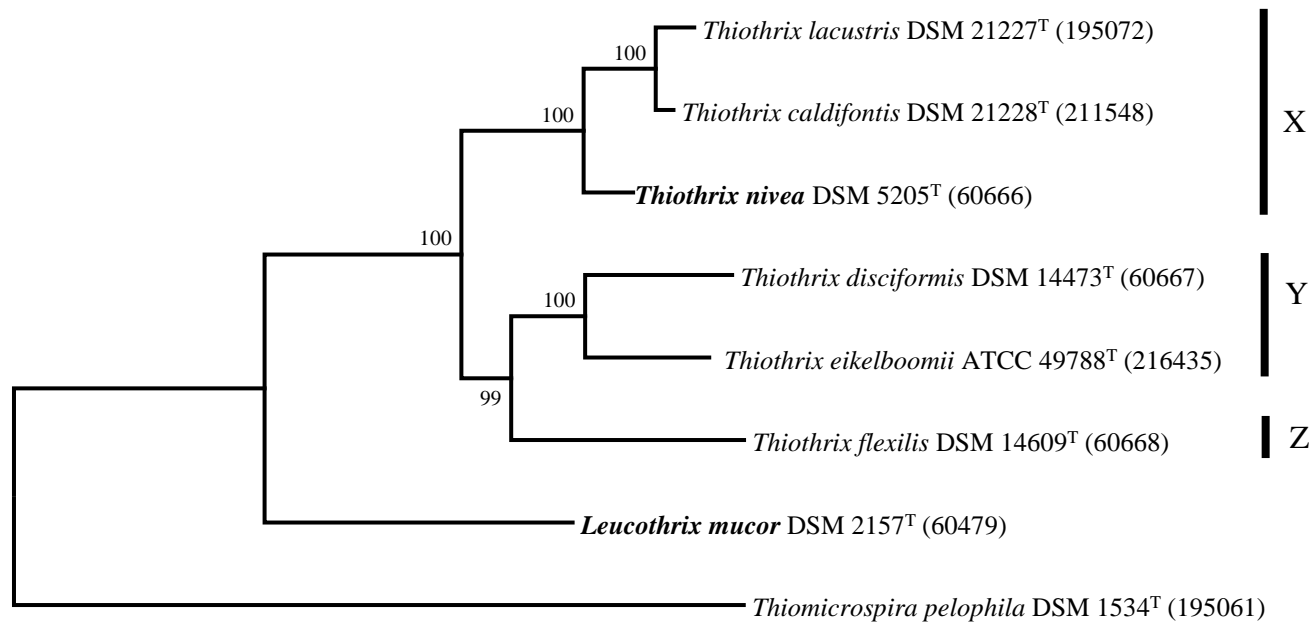


Recombination protein A

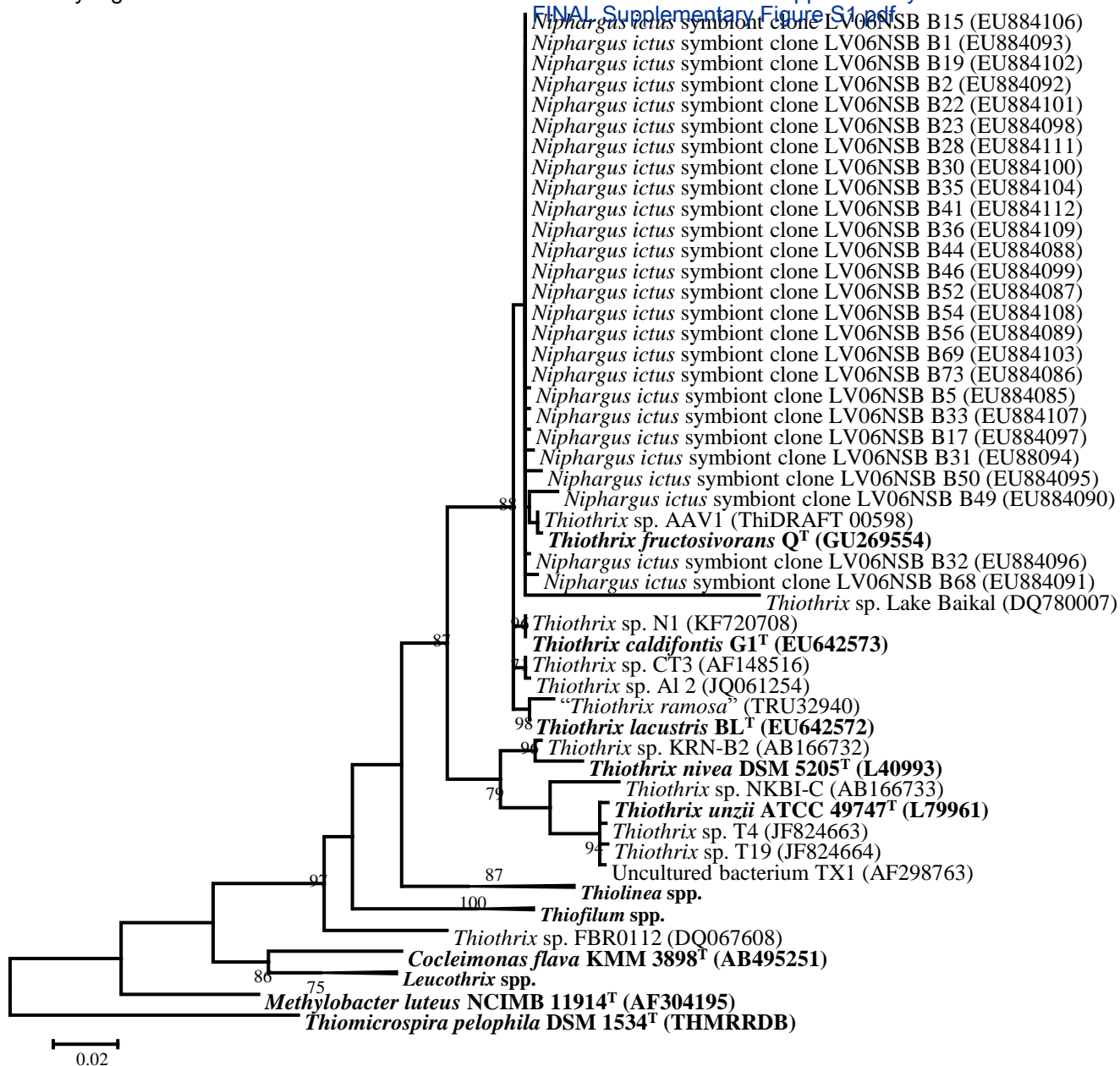
(RecA)

EC N/A





0.1



Supplementary Figure S1. Maximum likelihood tree on the basis of the 16S rRNA (*rrs*) gene, showing *Thiothrix*, *Thiolinea* gen. nov. and *Thiofilum* gen. nov., with relation to sequences in the GenBank and IMG databases that purport to be “*Thiothrix*”, including *Thiothrix* sp. AAV1, which we have used the genome sequence of as a proxy for *T. fructosivorans* (16S rRNA gene identity of AAV1 to Q^T is 99.9%) and “*T. ramosa*”, a well-studied organism that has since been lost. Sequences were extracted at gene level from the GenBank and IMG databases and aligned using MUSCLE without any pre-sets, and the aligned dataset tested for the best model fit on the basis of the lowest BIC in MEGA. The tree was thus reconstructed using the two parameter method of Kimura [46] with a discreet gamma distribution to model rate differences across sites, and invariant sites (parameter = 0.3695). Tree shown is the optimal tree, with numbers at nodes indicating the percentage of 5,000 bootstrap replications in which the topology was preserved (values <70 % omitted for clarity). All positions at which there was less than 95 % coverage were omitted from the final analysis, in which 809 nt were used. Branch lengths are to scale and indicate the number of substitutions per site – bar represents 0.02 substitutions per site for all trees shown. The outgroup is *Thiomicrospira pelophila* DSM 1534^T from the *Piscirickettsiaceae*, also in the *Thiothrichales*. Maximum likelihood tree shown had the highest log-likelihoods after 5,000 replications (-3989.24). Values in parentheses are GenBank and IMG accession numbers – the latter have an underscore (“_”).