



Metabolic and evolutionary patterns in the extremely acidophilic archaeon *Ferroplasma acidiphilum* YT

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4
5 **Metabolic and evolutionary patterns in the extremely acidophilic archaeon *Ferroplasma***
6 ***acidiphilum* Y^T**

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32

33 **Abstract**

34 *Ferroplasmaceae* represent ubiquitous iron-oxidising extreme acidophiles with a number of unique
35 physiological traits. In a genome-based study of *Ferroplasma acidiphilum* Y^T, the only species of the
36 genus *Ferroplasma* with a validly published name, we assessed its central metabolism and genome
37 stability during a long-term cultivation experiment. Consistently with physiology, the genome analysis
38 points to *F. acidiphilum* Y^T having an obligate peptidolytic oligotrophic lifestyle alongside with
39 anaplerotic carbon assimilation. This narrow trophic specialisation abridges the sugar uptake, although
40 all genes for glycolysis and gluconeogenesis, including bifunctional unidirectional fructose 1,6-
41 biphosphate aldolase/phosphatase, have been identified. Pyruvate and 2-oxoglutarate dehydrogenases
42 are substituted by ‘ancient’ CoA-dependent pyruvate and alpha-ketoglutarate ferredoxin
43 oxidoreductases. In the lab culture, after ~550 generations, the strain exhibited the mutation rate of ≥ 1.3
44 $\times 10^{-8}$ single nucleotide substitutions per site per generation, which is among the highest values recorded
45 for unicellular organisms. All but one base substitutions were G:C to A:T, their distribution between
46 coding and non-coding regions and synonymous-to-non-synonymous mutation ratios suggest the neutral
47 drift being a prevalent mode in genome evolution in the lab culture. Mutations in nature seem to occur
48 with lower frequencies, as suggested by a remarkable genomic conservation in *F. acidiphilum* Y^T
49 variants from geographically distant populations.

50

51 **Introduction**

52 *Ferroplasma acidiphilum* Y^T (DSM 12658^T) from the family *Ferroplasmaceae*, order
53 *Thermoplasmatales*, phylum *Euryarchaeota* are iron-oxidising extreme acidophiles that require small
54 amounts (0.02 % w/vol) of yeast extract for growth and populate environments with low pH values and
55 rich in sulfur compounds and metals in the form of sulfides^{1,2}. Various ecological aspects related to this
56 widely distributed archaeal group were reviewed earlier³. Deep metagenomic and metaproteomic
57 investigations of microbial communities of acid mine drainage (AMD) biofilms in Iron Mountain (CA,
58 USA) inhabited *inter alia* by the members of the family *Ferroplasmaceae*, have been conducted to
59 provide some insights into, and hypotheses on, their metabolism and physiology^{4,5,6}. A number of
60 uncommon biochemical features have also earlier been revealed for *F. acidiphilum* Y^T, such as an
61 unusually high proportion of iron-containing proteins in the proteome and low pH optima for the
62 enzyme activities *in vitro*^{7,8,9}. Despite aforementioned research milestones on *Ferroplasmaceae*, there is
63 a further need in investigation of metabolism of *F. acidiphilum* Y^T, important in the relation to the
64 practical applications and for filling the void in our understanding of fundamental mechanisms of its
65 lifestyle. In particular, there is still no consensus on the major mechanisms of carbon assimilation and
66 hence on the major role of *Ferroplasma* spp. play in the environment (apart from the ferrous oxidation,
67 which is well established and characterised in detail). Suggested patchiness of the genomic pools of, and
68 frequent recombinations in genomic variants in *Ferroplasma* spp. and “*Ferroplasma acidarmanus*” fer1
69 in their natural environment¹⁰ that could also be linked with a certain mosaicism of assemblies resulting
70 from metagenomic data from a multitude of clonal variants, could also be verified by the analysis of a
71 genome from geographically distant, yet closely related sibling with 100% SSU rRNA gene sequence
72 identity. For this, the high-quality, ungapped genome from a characterised reference isolate from a
73 similar environment represents a good opportunity.

74 Here, we present the genome-based and wet-lab analysis of *F. acidiphilum* Y^T in the context of its niche
75 adaptation, nutrients acquisition, energy and carbon metabolic pathways and its relatedness with

76 phylogenetic neighbours. Furthermore, we provide an overview of the *in vitro* genome evolution
77 patterns during the long-term maintenance of the strain in the laboratory culture.

78

79 **RESULTS AND DISCUSSION**

80 **Genome stability and evolution**

81 *General genome features.*

82 The size of the genome of *F. acidiphilum* Y^T is 1.826.943 bp, G+C content 36.49 %, the total gene
83 number was predicted to be 1773 (excluding 19 CDS with pseudogene qualifiers) with a coding density
84 of 86.4 %; 508 genes were revealed to code for hypothetical proteins. Loci for 5S, 16S and 23S rRNA
85 are not arranged in a single operon, but scattered in the chromosome; 46 tRNAs were predicted.

86 *Genome sequence comparison of *F. acidiphilum* Y^T with “*F. acidarmanus*” strain *fer1*.*

87 Strains *F. acidiphilum* Y^T and “*F. acidarmanus*” *fer1* have zero mismatches in their 16S rRNA gene
88 sequences, which, nevertheless, does not prove by itself that both belong to the same species. It was
89 therefore worth to assess their relatedness by using the Average Nucleotide Identity (ANI) analysis
90 (<http://enve-omics.ce.gatech.edu/ani/>¹¹). The analysis suggested the median ANI value of 98.7 % (Fig.
91 S1), which is well above the commonly accepted cut-off (95 %) for separation of two species based on
92 the whole-genome comparisons. In addition to that, the application of the online Genome-to-Genome
93 Distance Calculator (GGDC 2.0 tool, <http://ggdc.dsmz.de/distcalc2.php>¹²) using all three default
94 calculation formulae suggested DNA-DNA hybridization (DDH) values 73.1, 85.5 and 85.80% and
95 DDH values $\geq 70\%$ with the probabilities 83.97, 97.3 and 98.38, correspondingly. To sum up, both
96 analyses suggested that based on their genomic sequences, *F. acidiphilum* Y^T and “*F. acidarmanus*”
97 belong to the same species, despite showing some physiological differences reported earlier⁵.
98 Interestingly, the geographical separation of these two organisms (and many others, as one can judge
99 from metagenomic assemblies in public sequence databases) has not lead to a great deal of speciation.
100 This may also suggest that their geographical separation occurred relatively recently and that despite the

101 affiliation of these archaea to a very special niche, they must be rather robust to, and persistent in the,
102 non-acidic environments, which allows them to disseminate and colonise the sulfidic, low-pH niches
103 across the planet. Seemingly under natural conditions the evolution of such small genome-sized (and
104 hence having a narrow metabolic repertoire), slowly metabolising organisms is on-going at lower rates,
105 which restricts the genome evolution and therefore prevents the divergence and speciation. This is also
106 in line with the suggestion that small and compact genomes, as well as single-copy rRNA genes are the
107 signs for minimising metabolic costs in habitats where neither a broad metabolic repertoire, nor high
108 numbers of paralogous proteins are needed to accommodate growth under very constant and stagnant
109 environmental conditions.

110 ***Horizontally transferred genomic islands in *F. acidiphilum* Y^T.***

111 Horizontally transferred genomic islands (GIs) were identified in the complete genome sequence of *F.*
112 *acidiphilum* Y^T by the Seqword Gene Island Sniffer (SWGIS) program¹³, IslandViewer program
113 package comprising three different GI prediction algorithms¹⁴ and by GOHTAM¹⁵. Joint results of GI
114 identification by different methods are shown in Fig. 1. Nine putative GIs characterized by alternative
115 oligonucleotide usage (OU) patterns were detected by SWGIS and IslandViewer programs predicted
116 three shorter GIs. GOHTAM returned many short regions with atypical tetranucleotide and/or codon
117 usage; however, not all of them necessarily were of a lateral origin. Predicted GIs mostly harboured
118 genes with unknown functions, a few transposases and several enzyme-coding genes including a gene
119 cluster of archaeal sulfocyanin-containing respiratory system and a beta-lactamase in GI [126,000-
120 156,681] and a cluster of genes encoding CRISPR-associated proteins in seventh GI [905,732- 938,099]
121 (see below for more details). Our findings indicate that the horizontal gene transfer might play an
122 important role in the evolution of metabolic pathways of *F. acidiphilum* Y^T and in the acquisition of a
123 resistance against viruses.

124 GIs identified were searched for tetranucleotide pattern similarity through the database of 17,984 GIs
125 detected in 1,639 bacterial and chromosomal sequences (see the database at

126 www.bi.up.ac.za/SeqWord/sniffer/gidb/index.php)¹⁶. Significant compositional similarity of GIs from
127 *F. acidiphilum* Y^T was found with GIs of many other archaea and bacteria belonging to distant
128 taxonomic units. However, the highest similarity was observed between GIs of *F. acidiphilum* and
129 another acidophilic archaeon *Thermoplasma volcanium* GSS1. Remarkably, among recipients of GIs
130 from other extremophiles, there were several *Bacteroides* species.

131 The factor playing an important role in the genome evolution and lateral gene transfer are transposases.
132 In total, 80 transposases have been predicted, among them 28 belonged to IS4 family proteins and 10
133 were affiliated with MutS transposase mutator family proteins (COG3328). As it was suggested earlier¹⁷
134 the MutS homologs are abundant in *Euryarchaeota* and could be indicative to the gene transfer from
135 bacteria to archaea. Other genes encode IS605 OrfB family transposases and ISA0963 transposases,
136 IS2000 family protein, MULE, OrfA of protein families, consistently with the previous reports of
137 *Thermoplasmatales* to commonly carry numerous ISs of the families IS4 IS5, IS256, IS481, ISA1214
138 and IS2000/605/607¹⁸.

139 ***Mismatch repair and recombination.***

140 Recombination and mismatch repair proteins were represented by the DNA resolvase (FAD_0665)
141 exhibiting a relatively low similarity to its counterparts from methanogens and bacteria. DNA-repair
142 helicase FAD_1466 was similar to archaeal Rad25 proteins, FAD_1503 exhibited 30% identity with
143 *Sulfolobales* XPD/Rad3-related DNA helicases and with another DNA repair protein FAD_1564. Genes
144 FAD_0550 and FAD_0559 encode DNA repair and recombination proteins RadA and RadB, archaeal
145 homologs of RecA and Rad51, respectively; the latter is considered of being *Euryarchaeota*-specific¹⁹.
146 Mismatch repair proteins, MutS-like ATPases (FAD_0765-0766), were most similar to MutS proteins
147 from bacteria and *Thermoplasmatales*. The genome encodes a number of endonucleases, namely of the
148 type II restriction endonuclease FAD_0313 exhibiting a high similarity only with bacterial proteins, two
149 gene copies for endonucleases of types III (FAD_1157, 1370), IV (FAD_0129, 1301) and of type V
150 (FAD_0403) as well as Fen1 (FAD_0558) and PolX (FAD_1333) endonucleases.

151 **Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR).** The *F. acidiphilum* Y^T
152 genome revealed the presence of two clusters of Clustered Regularly Interspaced Short Palindromic
153 Repeats (CRISPR) separated by one operon encoding the CRISPR-associated (Cas) proteins and ten
154 genes, which are not related to CRISPR (Fig. 2). CRISPRs and Cas proteins represent a microbial small
155 RNA-based interference system found in most archaea and many bacteria; the CRISPR-Cas system
156 functions as the adaptive microbial immune system against invading viruses and plasmids, and it also
157 has a role in microbial pathogenesis, DNA repair, and biofilms²⁰. The cluster CRISPR1 of *F.*
158 *acidiphilum* Y^T is quite large and contains 133 identical and 3 degenerated direct repeats (30 bp long)
159 separated by 135 different spacers of similar size (34-39 bp) (Fig. 2). The cluster CRISPR2 is smaller
160 with 31 direct repeats (31 bp each) separated by 30 different spacers (35-38 bp, with spacer 5 being 62
161 bp). Neither spacers, nor repeats from these clusters share any sequence similarity to each other. The
162 rather large size of both CRISPR1 and CRISPR2 arrays might be indicative of high activity of the *F.*
163 *acidiphilum* Y^T CRISPR system^{21,22}. The NCBI Blast analysis of the *F. acidiphilum* Y^T CRISPR spacers
164 revealed no homologous sequences present in the available viral genomes or plasmids suggesting that its
165 CRISPR targets have yet to be discovered. Only the spacer 2 from cluster CRISPR2 was found to be
166 identical to a region in a gene encoding the hypothetical protein FACI_IFERC00001G0010 in the “*F.*
167 *acidarmanus*” genome, a large uncharacterized protein with the predicted UvrD-like helicase and
168 restriction endonuclease type II-like domains. Although the “*F. acidarmanus*” genome also encodes two
169 CRISPR clusters and eight *cas* genes, their repeat sequences showed no similarity one to another
170 suggesting that their CRISPR systems are not related. The eight *cas* genes of *F. acidiphilum* Y^T are
171 associated with the cluster CRISPR1 and are expected to be co-transcribed (*cas6*, *cas10*, *cas7*, *cas5*,
172 *cas3*, *cas4*, *cas1*, and *cas2*) (Fig. 2). Based on the *cas* gene arrangement and the presence of *cas10*, the
173 *F. acidiphilum* Y^T CRISPR-Cas system can be classified as a CRISPR subtype I-D, which is similar to
174 the type III system²³. This is consistent with the fact that most archaea contain the CRISPR subtypes A,
175 B, or D²⁴. In most of the type I and III CRISPR systems, Cas6 proteins cleave long pre-CRISPR RNA

176 (crRNA) transcripts generating mature crRNAs containing a single spacer with flanking repeat
177 fragments²⁵. Based on sequence, the type I-D CRISPR repeats have been predicted to form hairpin
178 structures, which are recognized by Cas6 proteins and cleaved at the 3'-base of the stem-loop. Analysis
179 of the *F. acidiphilum* Y^T CRISPR1 and CRISPR2 repeats revealed that they can form similar hairpin
180 structures suggesting that both CRISPR1 and CRISPR2 pre-crRNAs can be processed by the single *F.*
181 *acidiphilum* Y^T Cas6 protein. Comparison of amino acid sequences of the *F. acidiphilum* Y^T Cas
182 proteins with GenBank identified the Cas1 and Cas2 proteins from *Picrophilus torridus* (an acidophilic
183 archaeon and the closest phylogenetic neighbour of *Ferroplasmaceae*) as the top BLAST hits (50% and
184 46% sequence identity, respectively). However, other Cas proteins from *F. acidiphilum* Y^T were more
185 similar to the corresponding Cas proteins from the metagenomic assembly dubbed “*Ferroplasma* sp.
186 Type II” (58% to 75% sequence identity).

187 ***Analysis of mutations over the long-term cultivation in vitro.***

188 Comparison of two variants of genomes of *F. acidiphilum* Y^T (i.e. the original culture deposited in the
189 DSMZ in 1998 (DSM 12658^T) and the culture continuously grown in laboratory with re-inoculation
190 intervals of 24.5 days for 11 years) revealed 116 single-nucleotide substitutions (see Supplementary
191 Table S2 for details on substitutions and single-nucleotide polymorphism) randomly scattered across the
192 chromosome (Fig. 1), green arrowheads on the outer circle). 115 out of 116 were GC to AT
193 substitutions; such nucleotide shift is a common tendency for spontaneous single-base substitutional
194 mutations²⁶ and indicates that *F. acidiphilum* Y^T genome with already low GC content is prone to
195 further AT enrichment. Among substitutions, 12 (about 11 %) were detected in non-coding sequences
196 that is consistent with the overall coding percentage (86.4 %) in the genome. From bases' substitutions
197 in coding sequences, 34 of 103 (i.e. 33%) were synonymous. Majority of 69 non-synonymous base
198 substitutions resulted in non-conservative amino acid changes and only in 7 cases resulted in conserved
199 ones. 11 genes had two substitution sites (Table S2). Substitutions in coding regions mostly occurred in
200 genes with known functions but also in 17 genes encoding hypothetical proteins (almost all these

201 proteins contain one or more conserved domains). Some base substitutions occurred in genomic islands
202 1, 2 and 4, specifically in the GI 1, which contains gene clusters coding for ribosomal proteins (Fig. 1)
203 and Table S2). Distribution of substitutions in other GIs showed evidence of those in functional genes,
204 only one mutation occurred in a hypothetical gene.

205 Base-substitutional mutation rate per nucleotide position per generation calculated for *F. acidiphilum* Y^T
206 was within the highest range of that in other unicellular organisms, i.e. was similar or higher than that in
207 *Mesoplasma florum*²⁷, which until now had the highest record of mutation rates per base per generation.
208 According to the data²⁷, in prokaryotic organisms, viruses and most (except four) unicellular eukaryotes
209 base substitution rates per site per cell division fit the regression plot $\log_{10}u = -8.663 - 1.096\log_{10}G$ (u and
210 G are mutation numbers and genome sizes, respectively, and $r^2 = 0.872$) (Fig. 3). *F. acidiphilum* Y^T,
211 however, occupies an outstanding position in this respect with remarkable 0.02 (conservative estimates)
212 mutations per generation per genome (as a comparison, this figure for *Escherichia coli* is of approx.
213 0.001). We hypothesize that one of the possible reasons of these outstanding mutation rates may be the
214 earlier observed abnormal abundance of intracellular iron in the cells of *F. acidiphilum* Y^T⁸, which may
215 under oxidative stress conditions be linked with excessive DNA damage by Fenton reaction. Another
216 factor, which may contribute to the high mutation rates, is the error-prone DNA polymerase IV
217 (FAD_1298), which is capable of inducing mutations at sevenfold higher rate than under its
218 deficiency²⁸. The experimental validation of above hypotheses though is yet to be conducted.

219

220 **Energy and carbon metabolism**

221 ***Oxygen respiration and iron oxidation***

222 The detailed biochemical study of the respiratory chain of *F. acidiphilum* strain Y^T has recently been
223 reported²⁹. Interestingly, the genes coding for electron flow chain involved in iron oxidation in *F.*
224 *acidiphilum* Y^T were located in the identified Genomic Island/GI 1 (126,000-156,681), similarly to that

225 in *P. torridus* and *Cuniculiplasma divulgatum*, where the origin of the respiratory complexes has also
226 been attributed to the lateral gene transfer^{30,31}.

227 Related to the synthesis of the Fe-S systems we have detected the cysteine desulfurase gene
228 (FAD_0633), co-clustered genes *sufC* and *sufB* genes (probably related to the above) and the
229 hypothetical protein with a low similarity level to bacterial SufD like protein (FAD_1089-1087). There
230 were 6 ORFs in the genome related to ferredoxin synthesis (FAD_0146 [COG0348]; FAD_0257
231 [COG1146]; FAD_1078 [COG1145]; FAD_1661 [COG2440]; FAD_1852 [COG1146] and FAD_1160
232 [COG2440]). Most of them contain 4Fe-4S clusters, providing low potential electron donors for redox
233 processes in *F. acidiphilum* Y^T.

234 ***Amino acids metabolism.***

235 Genome inspection of *F. acidiphilum* Y^T revealed incomplete synthesis pathways for histidine,
236 isoleucine, leucine and valine (Fig. S2) pointing at the dependence on external sources and hence
237 supporting the role of *Ferroplasma* in the environment as iron-oxidising proteolytic ‘scavengers’. The
238 well-developed capacity for degrading amino acids is encoded by the *F. acidiphilum* Y^T genome. For
239 example, we found the genes for the degradation of histidine via urocanate (FAD_1379) and
240 tryptophane via kynurenine to anthranilate (FAD_0101-0104) and 2-oxoacid dehydrogenase complex
241 (FAD_1290-1291). Transamination of aspartate and glutamate via aspartate aminotransferases
242 (FAD_0393, 0538 and 1098) and glutamate dehydrogenase (FAD_0434) generates corresponding
243 branched-chain 2-oxoacids, oxaloacetate and 2-oxoglutarate, which are citric acid cycle intermediates.

244 Bioleaching pilot plant, from where *F. acidiphilum* Y^T was isolated, contained ore particles of various
245 sizes, where this archaeon may encounter anoxic microenvironments. Physiological studies performed
246 on *F. acidiphilum* Y^T denoted this strain as a facultative anaerobe, coupling chemoorganotrophic growth
247 on yeast extract to the reduction of ferric iron⁵. However, the detected reduction cannot be recognised as
248 respiratory reactions since the obtained biomass was very low and close to no substrate-control.
249 Nevertheless, we looked for corresponding genes relevant to a certain metabolic activity of *F.*

250 *acidiphilum* Y^T strain under anaerobic conditions. Pyruvate can be converted to acetyl-CoA by a
251 ferredoxin-dependent pyruvate oxidoreductase (POR, FAD_0567-0568). Obtained product may be
252 converted to acetate by an ADP-forming acetyl-CoA synthetase thus providing substrate level
253 phosphorylation step of pyruvate fermentation. Additionally, the *F. acidiphilum* Y^T genome possesses
254 all genes necessary for complete arginine fermentation, i.e. arginine deiminase pathway. This ‘ancient’
255 catabolic route, converting arginine to ornithine, carbon dioxide, ATP and ammonium constitutes a
256 major source of energy for some obligate anaerobic bacteria and fermenting archaea^{32,33}. Produced
257 ammonium increases the intracellular pH and has been shown to be important for survival of various
258 prokaryotes in acidic environment³⁴. The arginine deiminase pathway was probably present in the last
259 universal common ancestor (LUCA) to all the domains of life and its genes evolved independently,
260 undergoing complex evolutionary changes leading to a later assemblage into a single cluster with
261 functional interdependence³³. It must be noted that all three genes of the arginine deiminase pathway,
262 namely arginine deiminase (FAD_0428), ornithine transcarbamoylase (FAD_1523) and carbamate
263 kinase (FAD_0067) are not in a single operon, but are located distantly one from another in the *F.*
264 *acidiphilum* Y^T genome; the above has so far not been detected in any other but very closely related
265 extremely acidophilic archaea.

266 Arginine fermentation route is not the only signature of ancient anaerobic LUCA metabolism, which
267 occurs in the *F. acidiphilum* Y^T genome. Following the method described elsewhere^{35,36}, we identified
268 several other genes of the ancient metabolic core including 6 methyltransferases (FAD_0113, 0367,
269 1012, 1218, 1562 and 1651), 5 SAM-dependent methyltransferases (FAD_0758, 0931, 1052, 1315 and
270 1729) and ferredoxin (FAD_0146) in addition to several subunits of the H⁺/Na⁺-antiporter
271 Mrp/hydrogenases and related complexes (FAD_0579-0584). The acquisition of this antiporter
272 comparable to [NiFe] hydrogenases was proposed as a crucial step at the early stages of bio-energetic
273 evolution, which allowed conversion of geochemical pH gradient into the biologically more useful Na⁺
274 gradient³⁷. Noteworthy, all these protein families are typical for strict anaerobes and rarely occur in

275 genomes of aerotolerant or facultatively anaerobic prokaryotes, harbouring heme-copper oxygen
276 reductases³⁶. *F. acidiphilium* Y^T can be an example of such rare organisms that possess both LUCA
277 candidate gene protein families alongside the cytochrome oxidases.

278 ***TCA cycle in F. acidiphilium Y^T***

279 As observed in a multitude of studies, for a successful isolation of many prokaryotes, and especially
280 archaea, the yeast extract should be added into the cultivation media as an essential component and a
281 source of numerous cofactors and nutrients but also oligopeptides and amino acids. These nutrients are
282 fundamental substrates feeding many metabolic pathways, including tricarboxylic (citric) acid cycle
283 (TCA). This cycle is likely the central metabolic hub of *F. acidiphilium* Y^T, while most proteins
284 involved in the canonical TCA cycle were identified in genome, except for 2-oxoglutarate (OG)
285 dehydrogenase complex (Fig. 4). In common with some other archaea^{38,39}, the conversions of pyruvate
286 to acetyl-CoA and of 2-OG to succinyl-CoA are catalysed by the respective pyruvate:ferredoxin
287 oxidoreductase (POR, FAD_0567-0568) and alpha-ketoglutarate:ferredoxin oxidoreductase (KOR,
288 FAD_0712-0713). Although both enzymes were initially characterised as extremely oxygen-sensitive,
289 POR and KOR activities have been demonstrated also in a number of obligately aerobic organisms^{40,41}.
290 Compared to their anaerobic counterparts, these enzymes are oxygen-tolerant, exhibit lower rates and
291 have an unusual subunit structure^{42,43}. Noteworthy, it has been suggested⁴⁴ that to support biosynthetic
292 reactions some aerobic prokaryotes might utilise KOR for the reductive carboxylation of succinyl-CoA
293 to 2-OG. Given that succinyl-CoA synthetase, succinate dehydrogenase, fumarate hydratase and malate
294 dehydrogenase are the enzymes that catalyse reversible reactions, the formation of 2-OG from
295 oxaloacetate via malate, fumarate, succinate and succinyl-CoA is apparently plausible for *F.*
296 *acidiphilium* Y^T (Fig. 4). This finding suggests that, while relying primarily on amino acids catabolism
297 for carbon, *F. acidiphilium* Y^T can recruit the partially reverse, or reductive, TCA cycle as the additional
298 anabolic strategy to produce important precursors for biosynthesis. This strategy was demonstrated in a
299 number of archaea and acidophilic bacteria⁴⁵.

300 Although we did not quantify the expression of all genes involved in TCA cycle, the transcriptomic
301 analysis of succinate dehydrogenase and malate dehydrogenase revealed that both enzymes were
302 expressed to a similar extent (Fig. 4). Recently, these enzymes were identified in *Ferroplasma* proteome
303 as proteins induced during anaerobic growth coupled with ferric iron reduction^{46,47}. It is therefore most
304 likely that under these conditions, in order to provide the terminal electron acceptor (Fe^{3+}) with reducing
305 power, the catabolic function of TCA cycle prevails over the anabolic.

306 In connection with the inability to use acetate as the sole carbon source, the key enzymes of the
307 glyoxylate cycle, isocitrate lyase, and malate synthase, could not be identified in the *F. acidiphilum* Y^T
308 genome. Concluding the description for the oxidative, partially “anaerobic” TCA cycle, it becomes
309 apparent that due to the capability of KOR for the reductive carboxylation, *F. acidiphilum* Y^T cells
310 possess an enzymatic machinery permitting to convert succinyl-CoA into 2-OG while fixing inorganic
311 carbon. 2-OG can be directly converted into amino acids by glutamate dehydrogenase (FAD_0434),
312 which assimilates ammonium and besides biosynthetic function can be regarded as a part of nitrogen
313 metabolism. Additionally, glutamate can be also formed from 2-OG by an aspartate aminotransferase
314 (FAD_1098) yielding oxaloacetate (Fig. 4).

315 ***Glycolysis/ Gluconeogenesis.***

316 Growth on amino acids requires a gluconeogenic pathway for carbohydrate synthesis⁴⁸ and in line with
317 that all genes for a reverse glycolytic pathway have been identified. Interestingly, *Ferroplasma*
318 possesses a gene encoding a bifunctional gluconeogenetic fructose 1,6-bisphosphate
319 aldolase/phosphatase, a strictly anabolic enzyme, which is discussed as being an ancestral enzyme
320 type⁴⁹. Consistently, homologues for classical (glycolytic) fructose 1,6-bisphosphate aldolases are
321 missing. Although *F. acidiphilum* Y^T was reported to be unable to use sugars as the sole carbon source,
322 genes coding for some essentially irreversible reactions of glycolysis, besides aldolase, appear to be
323 present in the genome. These are glucokinase (FAD_0277), phosphofructokinase (FAD_0353). Thus, it
324 is likely that the absence of corresponding transporters preclude the uptake of external glucose, which,

325 nevertheless, can be metabolised in phosphosugars and pentoses if synthesised *de novo* by *F.*
326 *acidiphilum* Y^T cells. In consistency with findings in other archaea⁵⁰, the oxidative pentose phosphate
327 pathway is lacking in *F. acidiphilum* Y^T, but its reductive part is fully present and likely operative (Fig.
328 4).

329 ***Putative CO₂ assimilation mechanisms through gene expression analysis.***

330 Earlier it was reported that *F. acidiphilum* Y^T was able to incorporate into its biomass the inorganic
331 carbon in the form of ¹⁴CO₂^{1,51}. The genome analysis however did not suggest a clear assimilatory
332 pathway whereas a number of carboxylation reactions may have led to the incorporation of CO₂ into the
333 biomass. Besides mentioned above reductive carboxylation of succinyl-CoA to 2-OG by KOR, it is
334 possible that also POR enzyme is used in the reverse direction for anabolic purposes to support
335 biosynthetic reactions. Additionally, the *F. acidiphilum* Y^T genome harbours two enzymes whose
336 activity in the carboxylation direction might be involved in CO₂ fixation: phosphoenol pyruvate
337 carboxylase (PEPC) (FAD_1044) and NAD-binding malate oxidoreductase (malic enzyme FAD_0703)
338 (Fig. 4).

339 Expression of genes for these four enzymes along with succinate and malate dehydrogenases was
340 detected and quantified by real-time PCR. Prior to perform the RT-PCR assays we estimated the nucleic
341 acids ratio in *F. acidiphilum* Y^T culture harvested after 4 days, which corresponded to the late
342 exponential/early stationary growth phase. This value provides an indication of cellular RNA levels, i.e.
343 metabolic state, and is independent of the number of cells examined. The estimated RNA/DNA ratio of
344 7.81 indicated that *F. acidiphilum* Y^T cells were actively metabolising at this state. Two housekeeping
345 genes, *gyrB* and *rpl2* exhibiting constitutive levels of expression, were selected as standards to quantify
346 the relative abundance of *F. acidiphilum* Y^T gene transcripts involved in both, TCA cycle and in
347 anaplerotic CO₂ assimilation (Table S1). Compared to *gyrB* transcripts, we detected a slightly higher
348 transcription level of *rpl2* (the structural component of the large 50S ribosomal subunit), which reflected
349 the active metabolic state of *F. acidiphilum* Y^T. Noteworthy, while comparable with expression levels of

350 the references, relative amounts of *sdhA*, *sdhD* and *mdhI* transcripts were significantly reduced (40-200-
351 fold) as compared to those of POR, KOR and malic enzyme. The PEPC transcripts were detected in
352 quantities similar to those of *gyrB* (Fig. 4). As far as only PEPC catalyses irreversible carboxylation, the
353 RT-PCR data confirm that direct carboxylation reactions do contribute to the inorganic carbon uptake by
354 *F. acidiphilum* Y^T cells. We are aware that to confirm unambiguously the contribution of POR, KOR
355 and malic enzyme to the total cellular carbon formation, more in-depth biochemical studies of
356 anaplerosis are needed.

357 ***Transport mechanisms of F. acidiphilum* Y^T are habitat-specific**

358 To thrive in environmental settings with high concentrations of metals and metalloids (iron, copper,
359 cadmium, zinc and arsenic) *F. acidiphilum* Y^T must possess the corresponding set of important transport
360 mechanisms. Various genes coding for cation diffusion facilitator family, manganese/divalent cation and
361 tellurium resistance ABC transporters were detected in the *F. acidiphilum* Y^T genome (Table S3). These
362 transporters increase tolerance to divalent metal ions such as cadmium, cobalt, tellurium and zinc.
363 Besides, they may provide essential cofactors like molybdate and tungsten for diverse enzymes.

364 *F. acidiphilum* Y^T is native to arsenic-rich environments, and to withstand the arsenite stress the genome
365 encodes the ATP-dependent arsenite efflux pump. Genes for homologues of arsenite-sensitive regulator
366 (FAD_1795) and arsenite efflux pump permease (FAD_1796) were found located in a single operon. A
367 gene encoding for an arsenite efflux pump ATPase located distantly from the *ars* operon on the
368 chromosome was also identified (FAD_1514). With regard to the phosphorus, the *F. acidiphilum* Y^T
369 genome possesses one sodium-dependent phosphate transporter FAD_1510 and three inorganic
370 phosphate:H⁺ symporters (FAD_1260, 1738, 1753). Previously we described the narrow specialisation
371 of *F. acidiphilum* Y^T in uptake of organic substrates, highlighting that this strain was not capable of
372 growth on any of tested compounds, including organic acids, alcohols and single amino acids, common
373 sugars and related compounds¹. The addition of yeast extract was observed to be essential for growth
374 with the optimum concentration at 200 mg l⁻¹. In concordance with these observations, *F. acidiphilum*

375 Y^T genome is lacking genes for the transport and assimilation of common organic compounds other than
376 amino acids, and has only one identifiable integral carbohydrate ABC transporter (FAD_1026-1028).
377 Herewith, at least 7 oligopeptide/peptide ABC transporters and 17 transporters for amino acids were
378 found. Additionally to this cluster of transporters, the *F. acidiphilum* Y^T genome harbours 48 genes for
379 transporters belonging to the Major Facilitator Superfamily (MFS). Although poorly characterised, this
380 large and diverse group of secondary transporters was found to participate in the export of structurally
381 and functionally unrelated compounds and in the uptake of a variety of substrates including ions, amino
382 acids and peptides^{52,53}. These MFS-affiliated genes were found to be located nearby genes for
383 membrane and transposase IS4 family proteins, amino acids transporters or vitamins biosynthesis.
384 Certain speculation on various possible functionalities might be done in this relation. *F. acidiphilum* Y^T
385 MFS-related proteins exhibited the most significant similarity mostly to the counterparts from
386 *Thermoplasmatales* known to possess highest number of MFS proteins among other *Euryarchaeota* (13
387 in average) according to <http://supfam.org/SUPERFAMILY>⁵⁴. In this context, the number of MFS-
388 related genes found in *F. acidiphilum* Y^T genome (48) is within the range (in average, 40 per genome)
389 for *Thermoplasmatales* that occupy the same or similar environments.

390 Consistently with the abundance of oligopeptide/peptide transporters, the genome of *F. acidiphilum* Y^T
391 encodes 16 cytoplasmic and membrane-associated proteases and aminopeptidases, including tricorn
392 protease FAD_0691 and its integrating factors F2 (FAD_0645) and F3 (FAD_0317) both possessing the
393 aminopeptidase activity. In conjunction with these factors, tricorn protease can degrade oligopeptides in
394 a sequential manner, yielding free amino acids⁵⁵. Besides this sophisticated cell-associated proteolytic
395 machinery, the genome of *F. acidiphilum* Y^T encodes three secreted acid proteases thermopsins
396 (FAD_0679, 0833 and 1292). Thus, in concordance with physiology, the genome analysis indicates that
397 *F. acidiphilum* Y^T has a metabolism specialised in efficiently converting proteins and peptides into
398 amino acids. Noteworthy, the growth of the strain *F. acidiphilum* Y^T is strongly affected by the presence
399 of yeast extract in amounts greater than 200 mg l⁻¹ and is completely inhibited at concentrations greater

400 than 2 g l⁻¹. As we realised from the genome analysis, the membrane of *F. acidiphilum* Y^T is likely to be
401 well supplied with numerous protein- and amino acid-transporting complexes determining exceptional
402 nutrient-scavenging capabilities. If this is true, the sudden entry into the cytoplasm of an abundance of
403 nutrients could overwhelm the respiratory metabolism with reducing power that would generate
404 damaging level of toxic oxygen species, such as hydroxyl radicals and peroxides. Additionally, the *F.*
405 *acidiphilum* Y^T cytoplasm would become overloaded by organic compounds, which could provoke the
406 cell death by dehydration.

407 Additionally to the oligotrophic adaptation, the growth was not detected on the yeast extract alone
408 without ferrous iron, which serves as the electron donor^{1,5}. Taken together, these data point to *F.*
409 *acidiphilum* Y^T as an obligate peptidolytic chemomixotrophic oligotroph.

410 *F. acidiphilum* Y^T genome does not harbour any of known pathways of CO₂ fixation, thus suggesting
411 that the capability of *F. acidiphilum* to assimilate inorganic carbon^{1,51} is probably a result of anaplerotic
412 CO₂ assimilation. An intriguing point to mention is the ubiquity of *F. acidiphilum* with their remarkable
413 conservation of genomes. The ability to iron oxidation is solely characteristic to *Ferroplasmaceae*
414 family members among all up to date cultivated and studied *Thermoplasmatales* archaea, which
415 represents a certain advantageous/niche speciation trait and might contribute to the broad distribution of
416 these archaea. This is in a strong contrast with *Picrophilus* or *Thermogymnomonas* spp. that have so far
417 been detected exclusively on Japanese Isles.

418 One could speculate on another argument for the possible ancient origin of these archaea reflected in
419 amino acid/peptides dependence, which was suggested to exist in first heterotrophs and which seems to
420 be linked to sulfur-containing environments⁵⁶. In concordance with this hypothesis, the genes for several
421 protein families from an apparent ancient anaerobic core of the LUCA, e.g. for ferredoxin, several
422 subunits of the Mrp-antiporter/hydrogenase family, numerous S-adenosyl methionine (SAM) dependent
423 methyltransferases that rarely occur in aerobic prokaryotes^{35,36}, were found in the *F. acidiphilum* Y^T
424 genome.

425 One of the interesting observations was a relatively high number of single nucleotide substitutions in the
426 genome of *F. acidiphilum* Y^T after ~550 generations *in vitro*. We hypothesize that such a high mutation
427 rate could be caused by faster growth rates under optimal conditions in the culture, which is untypical
428 for these archaea in their real life in natural habitats where they tend to exhibit a remarkable genomic
429 conservation even in geographically distant populations. Analysis of nucleotide substitutions suggests
430 that the genome is prone to the further decrease in GC content. The ratios of synonymous to non-
431 synonymous amino acid substitutions and the distribution of single nucleotide substitutions between
432 coding and non-coding regions suggest that at least under optimal cultivation conditions, the neutral
433 drift is a prevalent mode of the genome evolution *in vitro*. This hypothesis certainly requires a deeper
434 experimental analysis with parallel cell lines run in continuous bioreactors and for a greater number of
435 generations.

436

437 **Methods**

438 ***Reference strain and growth conditions***

439 *F. acidiphilum* Y^T (DSM 12658^T) was deposited to the DSMZ collection in 1998, and since then
440 maintained in the laboratory, in 2008 the original isolate was retrieved from DSMZ for genome
441 sequencing. *F. acidiphilum* Y^T was routinely grown on the Medium 9K containing 25 g/l of
442 FeSO₄·7H₂O, supplemented with 0.02 % (w/vol) of yeast extract until the mid-exponential phase at as
443 described previously¹. For calculation of single substitution mutation rates, the 100-ml cultures were
444 grown in Erlenmeyer flasks under optimal conditions¹ since deposition of the strain to the DSMZ Strain
445 Culture collection in 1998. As an inoculum, 10 ml of culture were used each time, with 164 repeated
446 growth experiments. The final culture (2008) was subjected to the DNA extraction and sequencing.
447 Isolation of DNA from both variants was conducted using Genomic DNA isolation kit (QIAGEN,
448 Hilden, Germany).

449 ***Sequencing and assembly***

450 *De novo* sequencing data production of *F. acidiphilum* Y^T was conducted at the Liverpool University
451 Genome Centre on a 454 FLX Ti (454 Life Sciences, Branford, CT, USA) using a standard library (34
452 x) coverage. In addition, a library sequencing using Illumina 2000 was done at Fidelity Systems (short
453 paired-end 400 bp, av. read 100, coverage x 639) and at the Sequencing Facility of the Helmholtz Centre
454 for Infection Research (Braunschweig, Germany) (single end, 36 nt in average, x 233 coverage).
455 Genome assembly and gap closure were performed by Fidelity Systems Ltd. (Gaithersburg, MD, USA)
456 using Phred/Phrap and Consed^{57,58,59} have been operated for the final sequence assembly. DupFinisher⁶⁰
457 was used for the correction of repeat mis-assemblies and 384 Sanger end-sequenced fosmids for the
458 generation of a single scaffold (0.98 x coverage). For the full closure, a number of direct sequencing
459 reactions has been conducted⁶¹. The genome was automatically annotated at Fidelity Systems (USA)
460 using Fgenesb:2.0 and manually curated using GenDB v. 2.2.1 annotation system Ribosomal RNA
461 genes were identified via BLAST searches⁶² against public nucleotide databases and transfer RNA
462 genes using tRNAScan-SE v. 1.21⁶³. The CRISPRFinder web service was used for the identification of
463 CRISPRs⁶⁴. The genome of *F. acidiphilum* Y^T variant grown in the lab for ~550 generations was
464 sequenced using Illumina (average coverage: 233) and was further mapped on the assembled type strain
465 genome. The genome sequence of *F. acidiphilum* Y^T has been deposited to the GenBank/EMBL/DDBJ
466 with the accession number CP015363.

467 ***RNA isolation and quantitative reverse transcription PCR analysis (Q-RT-PCR).***

468 Q-RT-PCR was used to estimate the abundance of ten target genes transcripts (Table S1). *F.*
469 *acidiphilum* Y^T cells were collected after 4 days (corresponding to onset of stationary phase) by
470 centrifugation at 9000 rpm for 15 min of 15 - 25 ml culture and total RNA was immediately purified
471 using miRVANA kit (Ambion). RNA samples were treated with Turbo DNA-free kit (Ambion Austin,
472 TX, USA). To eliminate the residual DNA contamination present in the RNA preparations, a second
473 DNase treatment (DNase I, Invitrogen) was included prior to complementary DNA (cDNA) production.
474 cDNA synthesis was performed with SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA,

475 USA) with 100 ng of total RNA and 2 pmol of Random Hexamer Primer (Thermo Fisher Scientific)
476 according to the manufacturer's instruction. All RT-PCR experiments were performed using an ABI
477 7500 Fast Real-Time PCR System thermocycler. Gene-specific primers and TaqMan® probes (Table
478 S1) were designed using Primer Express® software v.2.0 (Applied Biosystems, USA). 5'-6-FAM and 3'-
479 BHQ1 labelled TaqMan® probes were obtained from Biomers (Germany). RNA samples were tested in
480 triplicates along with "No Template Control" (NTC). The reaction mixtures for Taqman® Q-RT-PCR
481 were as follows: 0.3 µM final concentration of each primer, 0.2 µM TaqMan probe, cDNA template
482 equivalent to 1 ng of RNA starting material, 12.5 µl of 2X TaqMan® 5 Universal PCR Master Mix (PE
483 Applied Biosystems) and ultrapure water added to the final volume of 25 µl. The reactions were
484 performed under the following conditions: 2 min at 50 °C followed by 10 min at 95 °C, followed by 40
485 cycles of 15 s at 95 °C and 1 min at 60 °C. PCR specificity and product detection was checked by
486 examining the temperature-dependent melting curves of the PCR products and by sequencing of cloned
487 amplicons.

488 Generation of quantitative data by RT-PCR is based on the number of cycles needed for amplification-
489 generated fluorescence to reach a specific threshold of detection (the Ct value). RT-PCR amplification
490 was analysed using an automatic setting for the baseline and threshold values and using the relative
491 standard curve method. Standards for all amplifications were prepared using known amounts of cloned
492 target templates. Amplicons were generated by PCR amplification of the target genes from genomic
493 DNA. The resulting amplicons were then purified using the Wizard SV Gel and PCR Clean-up System
494 kit (Promega, Madison, WI, USA), and cloned in pGEM®-T Easy Vector System I (Promega). After
495 cloning, plasmids were extracted using the QIAprep Spin Miniprep kit (Qiagen, Hilden, Germany) and
496 DNA concentrations were measured using a Nanodrop® ND-1000 spectrophotometer. Standard curves
497 were based on serial dilution ranging between 10^7 and 10^1 gene copies. Ct values were then
498 automatically generated by software and exported for calculation of average Ct and standard deviation
499 (SD) values of triplicates. The comparative method using *gyrB* mRNA as the normalizer was performed

500 as described elsewhere⁶⁵. For normalization based on multiple, most stably expressed housekeeping
501 genes, we used a ribosomal *pL2* gene, which has equal to *gyrB* reaction efficiency (*E*) value of 1.90^{66,67}.

502

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511

512 **Conflict of interest**

513 The authors declare no conflict of interest.

514

515 **Authors' contributions**

516 O.V.G., M.M.Y. and P.N.G conceived the research. O.V.G, H.T., O.N.R, S.L., A.F.Y, A.G., A.S., D.R.,
517 C.B, M.F, T.Y.N., M.M.Y, and P.N.G did the genome analysis. V.L., F.S. and M.M.Y. did the qPCR
518 experiments. O.V.G, M.M.Y and P.N.G wrote the manuscript.

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520

521 Supplementary Information is available at the Journal website.

522

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685

686 **Figure legends**

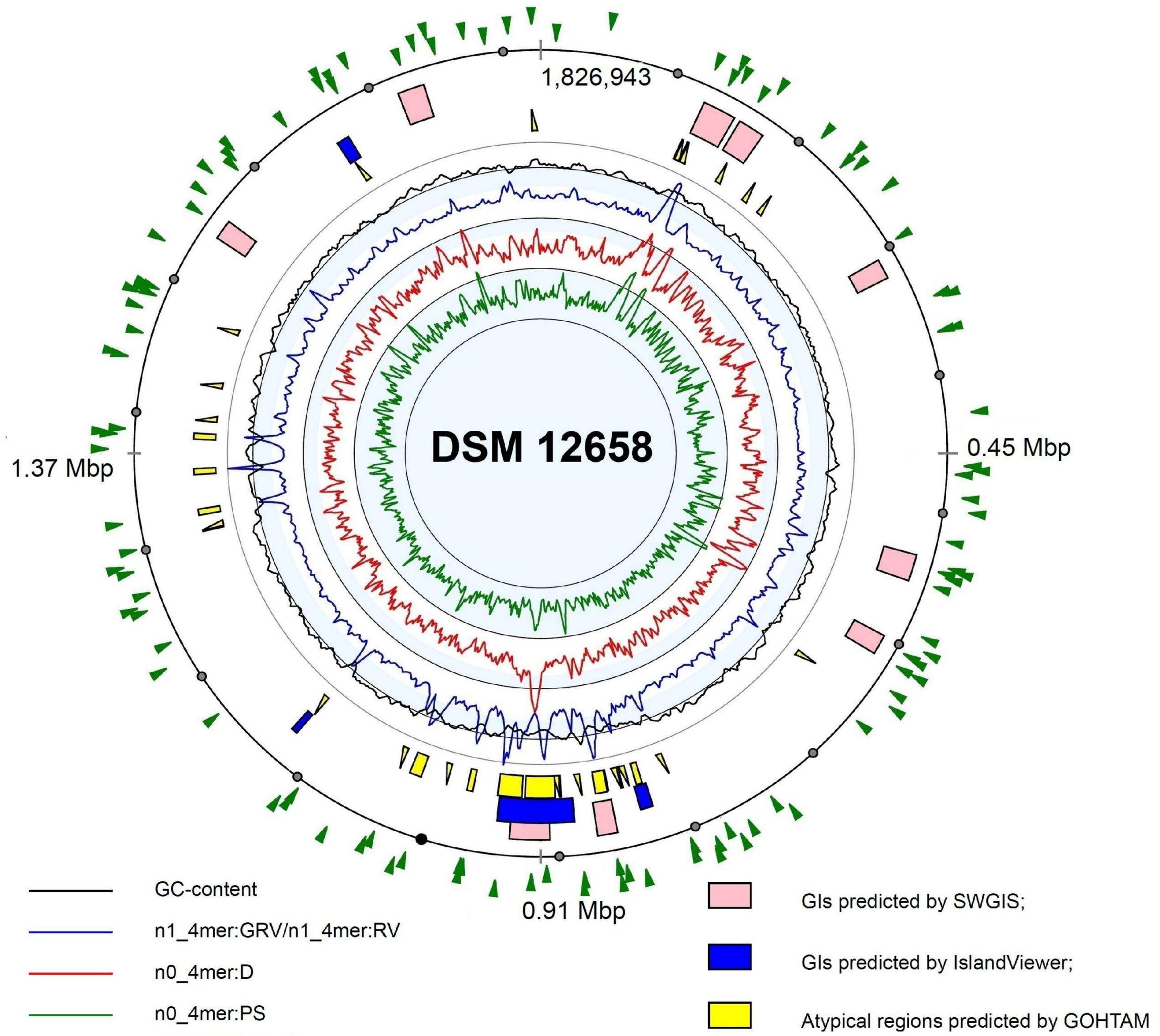
687 **Figure 1. The genome genomic islands (GI) of *F. acidiphilum* Y^T.** Localization of GIs on the chromosome of *F.*
 688 *acidiphilum* Y^T, as predicted by SWGIS (pink boxes), IslandViewer (blue boxes) and GOTHAM (yellow boxes). Histograms
 689 in the inner cycles of the atlas depict variations of the following oligonucleotide usage parameters: GC-content (black curve);
 690 ratio of generalized to local relative variances calculated for tetranucleotide usage patterns normalized by the GC-content
 691 (blue curve, n1_4mer:GRV/n1_4mer:RV); distances between not-normalized local tetranucleotide usage pattern and the
 692 global one calculated for the complete chromosome (red curve, n0_4mer:D); asymmetry between not-normalized

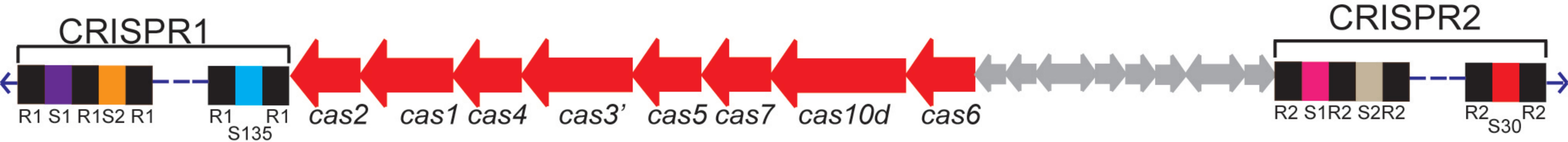
693 tetranucleotide usage patterns calculated for the direct and complement DNA strands (green curve, n0_4mer:PS). Use of
 694 these parameters for GI identification and their standard abbreviations were explained in more detail⁶⁸. Green arrowheads
 695 (outer circle) indicate single-nucleotide substitutions in the genome of *F. acidiphilum* Y^T after ~550 generations in the
 696 laboratory culture (s. Supplementary Table S2 for more details).
 697

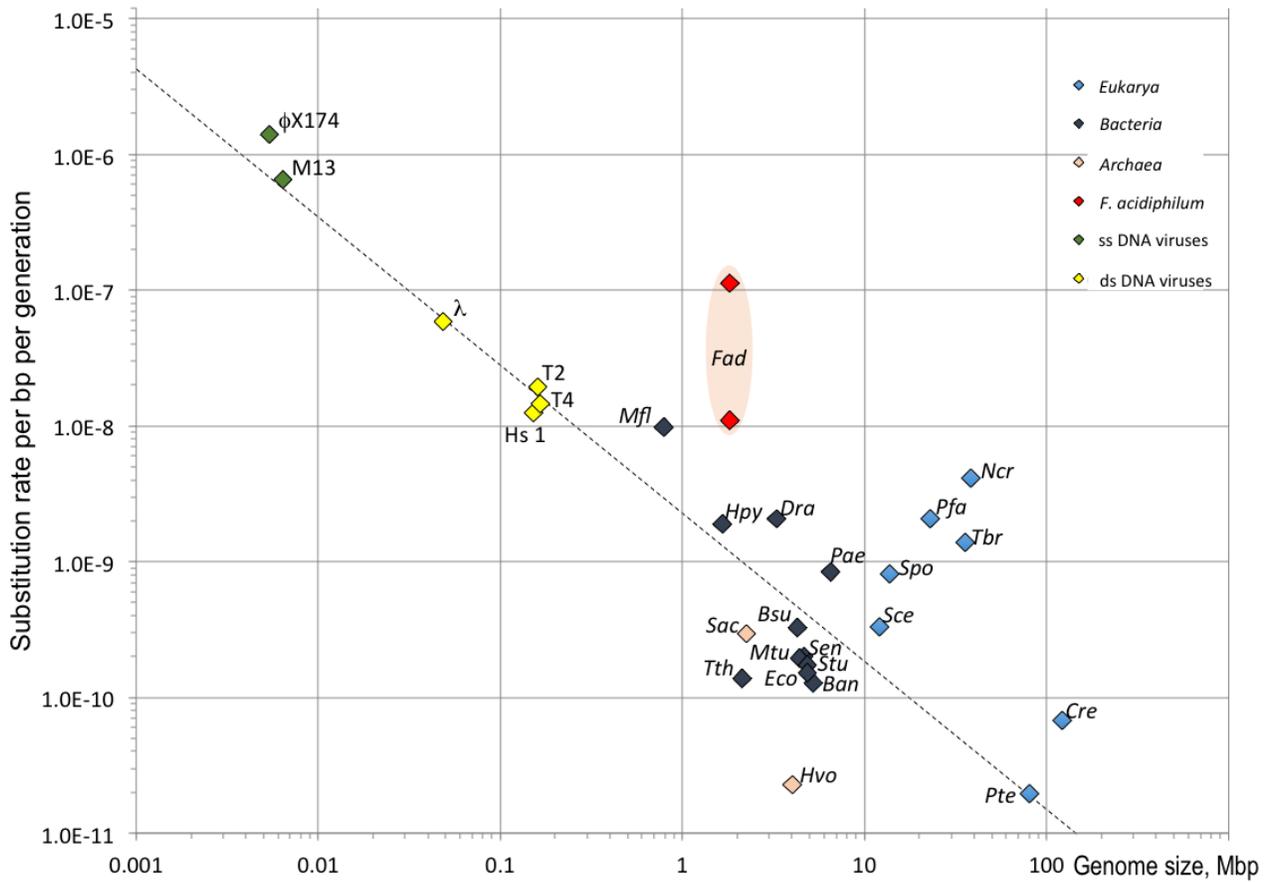
698 **Figure 2. Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) locus in *F. acidiphilum* Y^T** with one
 699 operon encoding the CRISPR-associated (Cas) proteins (red arrows). CRISPR system belongs to the Subtype I-D. Ten genes
 700 not related to CRISPR are shown in grey. The cluster CRISPR1 contains 137 identical direct repeats of 30 bp separated by
 701 136 different spacers of 34-39 bases. The Cluster CRISPR2 is shorter and has 31 direct repeats (31 bp each) with 30 different
 702 spacers (35-62 nt). Spacers and repeats in Clusters 1 and 2 show no sequence similarity one to another.
 703

704 **Figure 3. Single-nucleotide mutations accumulated during cultivation of *F. acidiphilum* Y^T.** Base-substitutional
 705 mutation rates per site per generation plotted vs genome sizes. The data on mutation rates of unicellular organisms and
 706 viruses and the regression plot ($\log_{10}u = -8.66 - 1.096\log_{10}G$, where u and G are mutation numbers and genome size,
 707 respectively) are taken from²⁹. Single-stranded DNA viruses: ϕ X174, phage *phi*174; M13, phage M13. Double-stranded
 708 DNA viruses: λ , phage lambda; T2, phage T2; T4, bacteriophage T4, Hs 1, Herpes simplex virus. Bacteria: *Bsu*, *Bacillus*
 709 *subtilis*; *Ban*, *Bacillus anthracis*; *Dra*, *Deinococcus radiodurans*; *Hpy*, *Helicobacter pylori*; *Mfl*, *Mesoplasma florum*; *Mtu*,
 710 *Mycobacterium tuberculosis*; *Pae*, *Pseudomonas aeruginosa*; *Sen*, *Salmonella enterica*; *Stu*, *Salmonella typhimurium*; *Tth*,
 711 *Thermus thermophilus*. Archaea: *Fad*, *Ferroplasma acidiphilum*; *Hvo*, *Haloferax volcanii*; *Sac*, *Sulfolobus acidocaldarius*.
 712 Eukarya: *Cre*, *Chlamydomonas reinhardtii*; *Ncr*, *Neurospora crassa*; *Pfa*, *Plasmodium falciparum*; *Sce*, *Saccharomyces*
 713 *cerevisiae*; *Spo*, *Schizosaccharomyces pombe*; *Tbr*, *Trypanosoma brucei*; *Pte*, *Paramecium tetraurelia*. Pink area reflects the
 714 distribution of mutation rates in *Ferroplasma* with the higher point value corresponding to all detected base substitutions in
 715 the strain cultured for ~550 generations as compared with the original genome, and lower value representing the most
 716 conservative mutation rate prediction (all mutations with frequency values <5% and SNPs in the original genome were
 717 excluded).
 718

719 **Figure 4. Proposed citric acid cycle and related enzyme reactions in *F. acidiphilum* Y^T.** The enzymes are as follows: 1,
 720 pyruvate kinase (FAD_1603); 2, PEP carboxykinase (FAD_1050); 3, PEP carboxylase (FAD_1044); 4, NAD-binding malic
 721 enzyme / malate dehydrogenase (FAD_0703); 5, pyruvate : ferredoxin oxidoreductase (FAD_0567-0568); 6, citrate synthase
 722 (FAD_1100); 7, aconitate hydratase (FAD_0701); 8, isocitrate dehydrogenase (FAD_1632); 9, 2-oxoglutarate:ferredoxin
 723 oxidoreductase (FAD_0712-0713); 10, succinyl-CoA synthetase (FAD_0709-710); 11, succinate dehydrogenase
 724 (FAD_0714-0717); 12, fumarate hydratase (FAD_1630); 13, malate dehydrogenase (FAD_0718); 14, glutamate
 725 dehydrogenase (FAD_0434); 15, aspartate aminotransferase (FAD_1098); 16, phosphoenolpyruvate synthase (FAD_1233);
 726 17, phosphoglycerate mutase (FAD_0440, FAD_1169, FAD_1350); 18, 2-phosphoglycerate kinase (FAD_1810); 19,
 727 glyceraldehyde-3-phosphate dehydrogenase (FAD_0549); 20, triosephosphate isomerase (FAD_0107); 21, fructose-2,6-
 728 bisphosphatase (FAD_0332); 22, 6-phosphofructokinase (FAD_0353); 23, bifunctional phosphoglucose/phosphomannose
 729 isomerase (FAD_0562); 24, phosphoglucomutase/phosphomannomutase (FAD_0602); 25, transketolase (FAD_1477-1476);
 730 26, transaldolase (FAD_1201; FAD_1475); 27, ribulose-phosphate 3-epimerase (FAD_0295). Abbreviations used: Fd,
 731 electron carrier ferredoxin; NAD, nicotinamide adenine dinucleotide; CoA, Coenzyme-A; PEP, phosphoenolpyruvate; UQ,
 732 ubiquinone. Enzymes labeled in blue are potentially involved in anaplerotic assimilation of CO₂. Their relative expression
 733 levels, analysed by RT-qPCR and indicated by the numbers in the central box, were obtained by normalization of the total
 734 RNA added and using transcripts of DNA gyrase subunit B (*gyrB*) as the internal reference (value 1.0). Normalization using
 735 *gyrB* was additionally validated vs transcripts of gene for ribosomal L2 protein. Average normalisation data derived from
 736 triplicates with standard deviation below 5%.







Pentose phosphate pathway

