1   **October 30, 2018:**

2   # Two Chloroflexi classes independently evolved

3   # the ability to persist on atmospheric hydrogen

4   # and carbon monoxide

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Abstract

Bacteria within aerated environments often exist within a variety of dormant forms. In these states, bacteria endure adverse environmental conditions such as organic carbon starvation by decreasing metabolic expenditure and using alternative energy sources. In this study, we investigated the energy sources that facilitate the persistence of the environmentally widespread but understudied bacterial phylum Chloroflexi. A transcriptome study revealed that *Thermomicrobium roseum* (class Chloroflexia) extensively remodels its respiratory chain upon entry into stationary phase due to organic carbon limitation. Whereas primary dehydrogenases associated with heterotrophic respiration were downregulated, putative operons encoding enzymes involved in molecular hydrogen (H_2), carbon monoxide (CO), and sulfur compound oxidation were significantly upregulated. Gas chromatography and microsensor experiments were used to show that *T. roseum* aerobically respires H_2 and CO at a range of environmentally relevant concentrations to sub-atmospheric levels. Phylogenetic analysis suggests that the enzymes mediating atmospheric H_2 and CO oxidation, namely group 1h [NiFe]-hydrogenases and type I carbon monoxide dehydrogenases, are widely distributed in Chloroflexi genomes and have been acquired on at least two occasions through separate horizontal gene transfer events. Consistently, we confirmed that the sporulating isolate *Thermogemmatispora* sp. T81 (class Ktedonobacteria) also oxidises atmospheric H_2 and CO during persistence. This study provides the first axenic culture evidence that atmospheric CO supports bacterial persistence and reports the third phylum to be experimentally shown to mediate the biogeochemically and ecologically important process of atmospheric H_2 oxidation. This adds to the growing body of evidence that atmospheric trace gases serve as dependable energy sources for the survival of dormant microorganisms.

Introduction

Bacteria from the phylum Chloroflexi are widespread and abundant in free-living microbial communities [1–4]. One reason for their success is their metabolic diversity; cultured strains from the phylum include heterotrophs, lithotrophs, and phototrophs adapted to both oxic and anoxic environments [5]. Cultured representatives of the phylum are classified into four classes by the genome taxonomy database [6], the

55　primarily aerobic Chloroflexia and Ktedonobacteria and the anaerobic Anaerolineae
56　and Dehalococcoidia [5]. Numerous studies have provided insight into the metabolic
57　strategies anaerobic classes within Chloroflexi use to adapt to oligotrophic niches [7,
58　8]. Surprisingly little, however, is known about how aerobic heterotrophic bacteria
59　within this phylum colonise oxic environments. Global surveys have reported that
60　Chloroflexi comprise 4.3% of soil bacteria [2] and 3.2% of marine bacteria [3]. However,
61　the most dominant lineages within these ecosystems (notably Ellin6524 and SAR202)
62　have proven recalcitrant to cultivation attempts. Instead, most of our knowledge about
63　the ecophysiological strategies of aerobic heterotrophic Chloroflexi is derived from
64　studies on thermophilic isolates. Various strains from the classes Chloroflexia and
65　Ktedonobacteria have been isolated and characterised from hot springs and
66　geothermal soils [9–13].

67　Within geothermal environments, Chloroflexi strains must endure temporal and spatial
68　fluxes in the availability of organic carbon compounds. It is currently unknown how
69　members of this phylum stay energised in response to these environmental
70　perturbations. Carbon monoxide (CO) and molecular hydrogen (H_2) of both
71　geothermal and atmospheric origin are available in such environments and may be
72　particularly important energy sources for sustaining growth and persistence [14–18].
73　Consistently, genomic and metagenomic studies have revealed that Chloroflexi
74　encode carbon monoxide dehydrogenases [19–21] and hydrogenases [19, 22–24]
75　known to mediate aerobic respiration of these gases. Chloroflexi isolates have been
76　shown to aerobically oxidise CO at a range of environmentally significant
77　concentrations: *Thermomicrobium roseum* can grow chemolithoautotrophically on
78　high concentrations of CO [19] and multiple *Thermogemmatispora* isolates have been
79　shown to oxidise CO, including *T. carboxidovorans* to atmospheric concentrations
80　(0.10 ppmv) [11]. While H_2 oxidation has yet to be reported in aerobic heterotrophic
81　Chloroflexi, strains of the phylum are known to encode the high-affinity group 1h
82　[NiFe]-hydrogenase [22, 25]. This enzyme class has been shown to support bacterial
83　persistence by mediating oxidation of atmospheric H_2 [24, 26–34]. To date,
84　atmospheric H_2 oxidation has only been experimentally confirmed in Actinobacteria
85　[26, 28–30, 32, 35] and two acidobacterial isolates [31, 36].

86　In this study, we investigated the persistence strategies of thermophilic isolates from
87　two classes of Chloroflexi. We focused primarily on *Thermomicrobium roseum* (class

88   Chloroflexia), a strain originally isolated from Toadstool Spring of Yellowstone National

89   Park, USA [9]. This obligately aerobic bacterium is known to grow heterotrophically on

90   a variety of carbohydrates, organic acids, and proteinaceous substrates, and

91   chemolithoautotrophically on carbon monoxide at high concentrations [9, 12, 19].

92   Previous genome analyses have shown *T. roseum* encodes a type I carbon monoxide

93   dehydrogenase and a group 1h [NiFe]-hydrogenase [19, 22]. A combination of

94   transcriptome sequencing and targeted activity assays were used to holistically

95   determine the metabolic basis of persistence in this organism, including demonstration

96   of CO and H_2 oxidation by this strain during carbon-limitation. To generalise these

97   findings, we also investigated *Thermogemmatispora* sp. T81 (class Ktedonobacteria),

98   a cellulolytic thermophilic strain which we previously isolated from geothermal soils in

99   Tikitere, New Zealand [10, 37, 38]. Collectively, our results demonstrate that

100   atmospheric H_2 and CO serve as important energy sources that support the

101   persistence of this phylum.

102

Materials and Methods

Bacterial strains

105   *Thermomicrobium roseum* DSM 5159 [9, 12] and *Thermogemmatispora* sp. T81 [10,

106   37] were imported from the Extremophiles Research Group (GNS Science, Wairakei,

107   New Zealand) culture collection in February 2017. Cultures of both bacterial isolates

108   were routinely maintained in 120 mL serum vials sealed with lab-grade butyl rubber

109   stoppers. Cultures of *T. roseum* cultures contained 30 mL Castenholz media

110   supplemented with 1 g L^{-1} yeast extract and 1 g L^{-1} tryptone, whereas

111   *Thermogemmatispora* sp. T81 cultures were maintained in 30 mL 10% R2A media.

112   For all experiments, both strains were incubated at 60°C at an agitation speed of 150

113   rpm. Gram staining and 16S rRNA gene amplicon sequencing confirmed both cultures

114   were axenic. Sporulation of *Thermogemmatispora* sp. T81 was verified by light

115   microscopy of Gram-stained cultures.

Transcriptomics

117   Transcriptome shotgun sequencing (RNA-Seq) was used to compare gene expression

118   in *T. roseum* cultures under carbon-replete (exponential phase; 10 mL, OD_{600} of 0.3)

119 and carbon-exhausted (stationary phase; 10 mL, OD_{600} of 0.75, 48 hr post OD_{max})

120 conditions. Biological triplicate samples for each condition were harvested by

121 centrifugation (21,000 $\times$ g, 15 min, 4°C), the supernatants were removed, and the cell

122 pellets resuspended in 1 mL of RNAlater Stabilisation Solution (ThermoFisher

123 Scientific) prior to freezing at -20°C. Extraction and sequencing of RNA was performed

124 by Macrogen Inc., Seoul, Korea. Briefly, RNA was extracted using the RNeasy Plant

125 Mini Kit (Qiagen), libraries were constructed using a TruSeq RNA v2 Sample Prep Kit

126 (Illumina), and rRNA was removed using the Ribo-Zero rRNA Removal Kit (Illumina).

127 The resultant complementary DNA was sequenced on an Illumina HiSeq4000 platform

128 using a paired-end, 100 bp high-throughput protocol. Sequence analysis was

129 performed using the automated cloud-based transcriptomics pipeline, AIR (Sequentia

130 Biotech). Briefly, the steps performed were read trimming, read quality analysis using

131 FastQC [39], and read mapping against the *T. roseum* reference genome (NCBI ID:

132 NC_011959.1 [19]) using an intrinsic platform read aligner with default parameters.

133 Aligned reads were checked for quality prior to data normalisation using the trimmed

134 mean of M-values method [40] and the 'normalizaData' command within the R

135 package HTSFilter [41]. A principle component analysis was then performed on the

136 normalised data prior to statistical analysis using edgeR [42] to obtain differential gene

137 expression counts.

Gas chromatography

139 Gas chromatography was used to determine whether the two Chloroflexi strains were

140 capable of oxidising atmospheric levels of CO and H_2. Briefly, sealed serum vials

141 containing stationary-phase cultures of *T. roseum* (72 hr post OD_{max}) and sporulating

142 cultures of *Thermogemmatispora* sp. T81 (294 hr post-inoculation) were opened,

143 aerated, and resealed. They were then amended with H_2 (*via* 1% v/v H_2 in N_2 gas

144 cylinder, 99.999% pure) or CO (*via* 1% v/v CO in N_2 gas cylinder, 99.999% pure) to

145 achieve headspace concentrations of ~14 ppmv. Cultures were agitated (150 rpm) for

146 the duration of the incubation period to enhance H_2 and CO transfer to the cultures

147 and maintain an aerobic environment. Headspace samples (1 mL) were routinely

148 collected using a gas-tight syringe to measure H_2 and CO. Concomitantly, headspace

149 gas concentrations in heat-killed negative controls (30 mL) were measured to confirm

150 that observed rates of gas consumption occurred due to a biotic process. Gas

151 concentrations in samples were measured by gas chromatography using a pulsed

152 discharge helium ionization detector [43, 44]. This customized trace gas analyser

153 (model TGA-6791-W-4U-2, Valco Instruments Company Inc.) is designed to analyse

154 a suite of atmospheric gases across six orders of magnitude in concentrations. Briefly,

155 the system is configured to use two valves as injectors/backflushers, and two valves

156 to front flush or heart cut from the precolumns (Mole Sieve 5A, set at 140°C). Gases

157 are then separated on the main columns (5' X 1/8" HayseSep Db, set at 55°C). The

158 fifth valve is used as a sample loop selector to accommodate a larger range of gas

159 concentrations. Concentrations of H_2 and CO in each sample were regularly calibrated

160 against ultra-pure H_2 and CO gas standards of known concentrations. With the

161 standards used, the limit of detection was 42 ppbv H_2 and 9 ppbv CO.

Kinetic analysis

163 Apparent kinetic parameters of H_2 and CO oxidation were estimated in stationary-

164 phase *T. roseum* cultures. Briefly, cultures were incubated with 100 or 1000 ppmv H_2

165 and 200 or 1500 ppmv CO, and headspace gas samples were measured at various

166 time intervals (0, 2, 4 and 8 hr from substrate addition) by gas chromatography.

167 Reaction velocity relative to the gas concentration was measured at each timepoint

168 and plotted on a Michaelis-Menten curve. Curves of best fit, $V_{\text{max app}}$ values, and $K_{\text{m app}}$

169 values were calculated in GraphPad Prism (version 7.01) using non-linear regression

170 models (enzyme kinetics – substrate vs. velocity, Michaelis-Menten, least squares fit).

Activity staining

172 Hydrogenase and carbon monoxide dehydrogenase activity was stained using whole-

173 cell lysates of stationary-phase cultures of *T. roseum*. Five hundred mL of culture was

174 harvested by centrifugation (10,000 *x g*, 10 min, 4°C), washed in phosphate-buffered

175 saline solution (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4,

176 pH 7.4), and resuspended in 16 mL lysis buffer (50 mM MOPS, pH 7.5, 1 mM PMSF,

177 15 mM $MgCl_2$, 5 mg mL^{-1} lysozyme, 1 mg DNase). The resultant suspension was then

178 lysed by passage through a Constant Systems cell disruptor (40,000 psi, four times),

179 with unbroken cells removed by centrifugation (10,000 *x g*, 20 min, 4°C). Protein

180 concentration was calculated using the bicinchoninic acid assay [45] against bovine

181 serum albumin standards. Next, 20 μg protein was loaded onto native 7.5% (w/v) Bis-

182 Tris polyacrylamide gels prepared as described elsewhere [46] and run alongside a

183 protein standard (NativeMark Unstained Protein Standard, Thermo Fisher Scientific)

184 at 25 mA for 3 hr. For total protein staining, gels were incubated in AcquaStain Protein

185 Gel Stain (Bulldog Bio) at 4°C for 3 hr. For hydrogenase staining [47], gels were

186 incubated in 50 mM potassium phosphate buffer supplemented with 500 µM nitroblue

187 tetrazolium chloride (NBT) in an anaerobic chamber (5% H_2, 10% CO_2, 85% N_2 v/v) at

188 60°C for 4 hr. For carbon monoxide dehydrogenase staining [48], gels were incubated

189 in 50 mM Tris-HCl buffer containing 50 µM NBT and 100 µM phenazine methosulfate

190 in an anaerobic chamber (100% CO v/v atmosphere) maintained at 60°C for 4 hr.

Electrode measurements

192 For *T. roseum* cultures, rates of H_2 oxidation with and without treatment of respiratory

193 chain uncouplers were measured amperometrically, following previously established

194 protocols [49, 50]. Prior to the start of measurement, a Unisense H_2 microsensor

195 electrode was polarised at +800 mV for 1 hr using a Unisense multimeter and

196 calibrated with standards of known H_2 concentration. Gas-saturated PBS was

197 prepared by bubbling the solution with 100% (v/v) of either H_2 or O_2 for 5 minutes. For

198 untreated cells, 1.1 mL microrespiration assay chambers were sequentially amended

199 with stationary-phase *T. roseum* cell suspensions (OD_{600} = 1; 0.9 mL), H_2-saturated

200 PBS (0.1 mL), and O_2-saturated PBS (0.1 mL) stirred at 250 rpm, 37°C. Following

201 measurements of untreated cells, the assay mixtures were treated with 100 µM

202 carbonyl cyanide *m*-chlorophenyl hydrazine (CCCP), 10 µM nigericin, or 10 µM

203 valinomycin. Changes in H_2 concentrations were recorded using Unisense Logger

204 Software (agitation speed of 250 rpm, 37°C). Upon observing a linear change in H_2

205 concentration, initial rates of consumption were calculated over a period of 20 s and

206 normalised against total protein concentration.

207

Phylogenetic analyses

209 Phylogenetic trees were constructed to investigate the evolutionary history and

210 distribution of uptake hydrogenases and carbon monoxide dehydrogenases within the

211 Chloroflexi phylum. Specifically, the catalytic subunits of [NiFe]-hydrogenases (HhyL

212 and homologues) and type I carbon monoxide dehydrogenases (CoxL) were retrieved

213 from Chloroflexi genomes and metagenome-assembled genomes (MAGs) in the NCBI

214 RefSeq database *via* protein BLAST [51] in October 2018. The amino acid sequences

215 were aligned with reference sequences [21, 22] using ClustalW in MEGA7 [52].

216 Evolutionary relationships were visualised by constructing a maximum-likelihood
217 phylogenetic tree in MEGA7 [52]. Specifically, initial trees for the heuristic search were
218 obtained automatically by applying Neighbour-Join and BioNJ algorithms to a matrix
219 of pairwise distances estimated using a JTT model, and then selecting the topology
220 with superior log likelihood value. Gaps were treated with partial deletion and trees
221 were bootstrapped to 100 replicates.

222

Results and Discussion

Thermomicrobium roseum upregulates hydrogenase and carbon monoxide dehydrogenase expression during a coordinated response to organic carbon starvation

227 We compared the transcriptomes of triplicate _T. roseum_ cultures under carbon-replete
228 (exponential growing) and carbon-limited (stationary phase) conditions. 401 genes
229 were significantly upregulated and 539 genes were significantly downregulated by at
230 least two-fold ($p < 10^{-6}$) in response to carbon-limitation **(Figure 1a; Table S1)**. Three
231 major trends were observed with respect to energy acquisition and utilisation. Firstly,
232 genes associated with energetically expensive processes were downregulated,
233 including those encoding ribosomal proteins, cytochrome _c_ and menaquinone
234 biosynthesis enzymes, and the megaplasmid-encoded chemotactic and flagellar
235 apparatus **(Table S1)**. Secondly, there was evidence of mobilisation of internal carbon
236 stores, including an acetoin dehydrogenase complex and an electron transfer
237 flavoprotein complex (ETF). Thirdly, primary respiratory dehydrogenases involved in
238 heterotrophic growth (i.e. type I and II NADH dehydrogenases) were downregulated
239 in favour of those associated with lithotrophic metabolism **(Figure 1a; Table S1)**. In
240 both conditions, the terminal oxidases that mediate aerobic respiration were highly
241 expressed and there was no evidence of the use of other electron acceptors; the
242 cytochrome aa_3 oxidase was expressed in both phases and the alternative cytochrome
243 bo_3 oxidase was upregulated during stationary-phase. In contrast, the F_1F_o-ATPase
244 (ATP synthase) was downregulated, a finding consistent with an expected decrease
245 in the availability of respiratory electron donors during carbon-limitation **(Table S1)**.

Thermomicrobium roseum upregulates genes associated with H_2 and CO metabolism under carbon-limiting conditions. The genes encoding the structural subunits of a group 1h [NiFe]-hydrogenase (*hhySL*; trd_1878-1877) [22, 23, 53], which is known to mediate atmospheric H_2 oxidation [29, 31, 47], were upregulated by an average of 12.6-fold **(Figure 1b)**. Also upregulated were the conserved hypothetical proteins *hhaABC* (trd_1876-1874; 5.5-fold) [25], encoded on the same putative operon as the structural subunits, as well as a separate putative operon of maturation factors (trd_1873-1863; 3.1-fold) **(Figure S1; Table S1)**. The structural (trd_1206-1208) and maturation (trd_1209-1215) subunits encoding a type I carbon monoxide dehydrogenase were upregulated by an average of twofold **(Figure 1c & S1)** in response to carbon limitation. Consistent with previous reports of chemolithoautotrophic growth at high concentrations of CO [19], carbon monoxide dehydrogenase genes were highly expressed in both exponential and stationary phase cultures. **(Figure 1c; Table S1)**. This suggests that *T. roseum* uses CO to supplement available organic carbon during growth (mixotrophy) and persistence.

Overall, the greatest differential in gene expression involved a 19-gene cluster (trd_0160-0142) putatively involved with the oxidation of sulfur compounds. The cluster encodes a soluble heterodisulfide reductase (*hdrABC*), an electron transfer flavoprotein complex (*etfAB*), three sulfur carriers (*dsrE1*, *dsrE2*, *tusA*), three lipoate-binding proteins (*lbpA*), and various hypothetical proteins, which are upregulated by an average of 45-fold during persistence. We predict that this system serves to mobilise sulfur atoms from inorganic or organic sulfur-containing compounds and conjugate them to sulfur carriers. In turn, we hypothesise that the Hdr complex may oxidise the resultant thiol groups to disulfides and transfer the liberated electrons to the aerobic respiratory chain-linked ETF complex. In support of this premise, the upregulated gene cluster encodes components homologous to a system recently shown to mediate the oxidation of diverse sulfur compounds in *Hyphomicrobium denitrificans* [54, 55] and the Hdr complex is most closely related to those of sulfur-oxidising *Sulfobacillus*, *Hyphomicrobium*, and *Acidithiobacillus* strains. It seems plausible that *T. roseum* would benefit from a survival advantage if able to harness reduced sulfur compounds available in geothermal springs. However, further work is needed to verify the activity, substrates, and physiological role of this system.

278   Collectively, these findings show that *T. roseum* is more metabolically flexible than
279   previously thought. **Figure 1d** illustrates the predicted remodelling of the respiratory
280   chain that occurs during the transition from carbon-replete to carbon-limited conditions.
281   The upregulation of enzymes involved in harnessing inorganic compounds, in
282   conjunction with the downregulation of gene clusters involved in organic carbon
283   oxidation, suggests that *T. roseum* has evolved mechanisms to maintain aerobic
284   respiration despite organic carbon fluctuations and deprivation within its environment.

285

286   **_T. roseum_ aerobically oxidises H_2 and CO at a wide range of concentrations,**
287   **including sub-atmospheric levels, during persistence**

288   The high expression levels for genes encoding the group 1h [NiFe]-hydrogenase and
289   type I carbon monoxide dehydrogenase suggested that *T. roseum* may support
290   persistence by oxidising atmospheric H_2 and CO. To test this, we incubated carbon-
291   limited cultures of *T. roseum* in an ambient air headspace supplemented with ~14
292   ppmv of either H_2 or CO and monitored their consumption using gas chromatography.
293   In agreement with our hypothesis, cultures aerobically oxidised both gases in a first-
294   order kinetic process and reached mixing ratios (103 ppbv H_2, 22 ppbv CO) five times
295   below atmospheric levels within 71 hours **(Figure 2a & b)**. This constitutes the first
296   observation of both aerobic H_2 respiration and atmospheric H_2 oxidation within the
297   phylum Chloroflexi.

298   Whole-cell kinetic measurements revealed that *T. roseum* efficiently oxidises H_2 and
299   CO across a wide range of concentrations through hydrogenase and carbon monoxide
300   dehydrogenase activity. The enzymes display a moderate apparent velocity ($V_{\text{max app}}$
301   of 236 nmol H_2 and 166 nmol CO g^{-1} of protein min^{-1}) and moderate apparent affinity
302   ($K_{\text{m app}}$ of 323 nM H_2 and 544 nM CO) for these substrates **(Figure 2c & d)**. With
303   respect to carbon monoxide dehydrogenase, these observations are consistent with
304   the organism being able to utilise CO at elevated concentrations for growth [19] and
305   atmospheric concentrations for persistence. The apparent kinetic parameters of the
306   group 1h [NiFe]-hydrogenase are more similar to those recently described for the
307   verrucomicrobial methanotroph *Methylacidiphilum fumariolicum* (K_m = 600 nM) [56]
308   than to the high-affinity, low-activity hydrogenases of previously described
309   atmospheric H_2 scavengers (K_m < 50 nM) [26, 29, 31]. Altogether, these findings

310   suggest that *T. roseum* can take advantage of the elevated H_2 and CO concentrations

311   when available through geothermal activity and subsist on atmospheric concentrations

312   of these gases otherwise.

313   Consistent with observed whole-cell activities, cell-lysates run on native

314   polyacrylamide gels strongly stained for hydrogenase and carbon monoxide

315   dehydrogenase activity **(Figure 2e)**. The triplet banding pattern observed for the

316   hydrogenase is similar to that shown to be mediated by the group 1h [NiFe]-

317   hydrogenases in *M. smegmatis* [26]. We next verified that the hydrogenase was

318   coupled to the respiratory chain by measuring H_2 oxidation using a H_2 electrode under

319   aerobic conditions. Untreated cells oxidised H_2 at a rapid rate. This activity decreased

320   by 2.5-fold upon addition of the respiratory uncoupler CCCP and ceased upon addition

321   of the ionophore valinomycin **(Figure 2f)**.

322   Findings from the transcriptome analysis and activity studies therefore suggest that *T.*

323   *roseum* persists through oxidation of atmospheric H_2 and CO. We propose that the

324   group 1h [NiFe]-hydrogenase and type I carbon monoxide dehydrogenase directly use

325   electrons derived from atmospheric H_2 and CO to support aerobic respiration **(Figure**

326   **1d).** Due to the genetic intractability of Chloroflexi and the lack of specific hydrogenase

327   or carbon monoxide dehydrogenase inhibitors, we were unable to determine the

328   necessity of either H_2 or CO oxidation for prolonged survival for this organism.

329   However, previous studies have demonstrated that genetic deletion of the group 1h

330   [NiFe]-hydrogenase reduces longevity of *M. smegmatis* cells [27, 28, 57]   and

331   *Streptomyces avermitilis* exospores [29, 30].

332

Scavenging of atmospheric gases is a general persistence strategy within the aerobic heterotrophic Chloroflexi

335   Having demonstrated that *T. roseum* oxidises atmospheric trace gases during

336   persistence, we subsequently investigated whether this is a common strategy in

337   employed by the Chloroflexi. We first analysed the respiratory capabilities of

338   *Thermogemmatispora* sp. T81, a heterotrophic cellulolytic and spolulating thermophile

339   which we previously isolated from geothermal soils from Tikitere, New Zealand [10,

340   38]. Analysis of the organism's genome (Assembly ID: GCA_003268475.1) indicated

341   that it encodes core respiratory chain components similar to *T. roseum*, including

342　primary dehydrogenases (*nuo*, *ndh*, *sdh*), terminal oxidases (*cox*, *cyo*), and ATP

343　synthase (*atp*). The genome also encodes putative operons for the structural subunits

344　of a group 1h [NiFe]-hydrogenase, the maturation factors of this hydrogenase, and

345　structural subunits of a type I carbon monoxide dehydrogenase **(Figure S2)**. However,

346　homologues of the putative heterodisulfide reductase and ETF complexes encoded by

347　*T. roseum* are absent from the *Thermogemmatispora* sp. T81 genome.

348　We verified that sporulating cultures of *Thermogemmatispora* sp. T81 actively

349　consume H_2 and CO. The organism slowly oxidised available H_2 and CO in the

350　headspace to sub-atmospheric levels (120 ppbv H_2, 70 ppbv CO) over ~320 hr **(Figure**

351　**3a & b)**. Although this strain has previously been shown to oxidise carbon monoxide

352　[11], this is the first observation that it can do so to sub-atmospheric concentrations

353　and during persistence. These results suggest that, despite their distinct evolutionary

354　histories and ecological niches, *Thermogemmatispora* sp. T81 and *T. roseum* have

355　both evolved similar metabolic strategies to survive organic carbon limitation.

356　Analysis of the distribution of hydrogenases and carbon monoxide dehydrogenases

357　within publicly available reference genomes showed that genetic capacity for trace gas

358　scavenging is a common trait among aerobic Chloroflexi. Specifically, group 1h [NiFe]-

359　hydrogenases and type I carbon monoxide hydrogenases sequences were encoded

360　in three of the four reference genomes within the Thermomicrobiales (class

361　Chloroflexia) and four of the five reference genomes within the Ktedonobacteriales

362　(class Ktedonobacteria) **(Figure 4a & b)**. The latter includes the genomes of the

363　heterotrophic soil bacterium *Ktedonobacter racemifer* [58] and the nitrite-oxidising

364　bioreactor isolate *Nitrolancea hollandica* [59]. In addition, seven strains within the

365　photosynthetic order Chloroflexales encoded group 1f and/or group 2a [NiFe]-

366　hydrogenases **(Figure S3)**. These hydrogenase classes have been shown to mediate

367　aerobic H_2 oxidation in a range of bacteria, including to sub-atmospheric

368　concentrations in *Acidobacterium ailaaui* and *M. smegmatis* respectively [36, 47].

369　Moreover, a metatranscriptome study showed homologs of the group 1f [NiFe]-

370　hydrogenase of *Roseiflexus* species are highly expressed in geothermal microbial

371　mats at night [60]. Hence, it is likely that the traits of aerobic H_2 respiration and possibly

372　atmospheric H_2 oxidation extends to the photosynthetic strains of this phylum. A range

373　of metagenome-assembled genomes, including from the abundant candidate class

374　Ellin6549 [2, 24], also encoded genes for aerobic H_2 and CO oxidation **(Figure S3 &**

375 **S4)**. Consistent with previous reports, Dehalococcoidia encode group 1a [NiFe]-

376 hydrogenases known to facilitate dehalorespiration [61–63].

377 Our analyses suggest that the capacity for atmospheric H_2 and CO oxidation has

378 evolved on at least two occasions within the Chloroflexi. Phylogenetic trees show that

379 the group 1h [NiFe]-hydrogenases from Chloroflexia and Ktedonobacteria are

380 divergent and fall into two distinct, robustly supported branches **(Figure 4a)**. It is

381 therefore likely that Chloroflexia and Ktedonobacteria independently acquired these

382 enzymes *via* horizontal gene transfer events from other Terrabacteria, rather than

383 vertically inheriting them from a common ancestor. Phylogenetic analysis also

384 suggests that the type I carbon monoxide dehydrogenase has been acquired on two

385 or three occasions in this phylum **(Figure 4b)**. In line with being independently

386 acquired, the putative operons encoding the hydrogenase and carbon monoxide

387 dehydrogenase in *T. roseum* **(Figure S1)** and *Thermogemmatispora* sp. T81 **(Figure**

388 **S2)** are distinctly organized. For example, the structural and accessory factors of

389 carbon monoxide dehydrogenase are encoded in a single putative operon in

390 *Thermogemmatispora* sp. T81 (*coxMSLIG*), but are separated into a structural operon

391 (*coxGSLM*) and accessory operon (including *coxG* and *coxE*) in *T. roseum*. These

392 findings agree with previous inferences of horizontal dissemination of *hhyL* and *coxL*

393 genes [21, 22, 25] and suggest there is strong selective pressure for the acquisition of

394 metabolic enzymes that support persistence.

395

Ecological significance of metabolic flexibility and trace gas oxidation in
Chloroflexi

398 Aerobic heterotrophic bacteria from the phylum Chloroflexi are more metabolically

399 versatile than previously thought. The transcriptome analyses clearly shows that *T.*

400 *roseum* regulates its metabolism in response to organic carbon limitation, enabling

401 persistence on a combination of exogenous inorganic compounds and likely

402 endogenous carbon reserves. In support of this, gas chromatography measurements

403 showed that the bacterium efficiently oxidises H_2 and CO down to sub-atmospheric

404 concentrations during persistence through an aerobic respiratory process. We made

405 similar findings for the ktedonobacterial isolate *Thermogemmatispora* sp. T81,

406 suggesting that trace gas scavenging might be a common persistence strategy

407   employed by aerobic Chloroflexi. Phylogenetic analyses suggest that *T. roseum* and
408   *Thermogemmatispora* sp. T81 horizontally acquired the capacity to oxidise
409   atmospheric H_2 and CO *via* separate events. The apparent convergence in
410   persistence strategies is notable given the distinct evolutionary histories, persistence
411   morphologies (i.e. sporulation in T81), and ecological niches of these bacteria.
412   Resource generalism is therefore likely to be a common ecological strategy for the
413   survival of Chloroflexi in environments where organic carbon may be periodically
414   scarce.

415   More broadly, these findings provides pure culture support for the hypothesis that
416   atmospheric carbon monoxide serves as an energy source for persistence [24]. A
417   range of heterotrophic bacteria have previously been inferred to be capable of
418   oxidising atmospheric CO, including Proteobacteria [64, 65], Actinobacteria [66], and
419   a *Thermogemmatispora* strain [11]; however, the exact physiological role of this
420   process has remained unresolved. We show that the expression and activity of carbon
421   monoxide dehydrogenase is linked to persistence, and provide evidence that
422   atmospheric CO serves as an electron donor for the aerobic respiratory chain in this
423   condition. Indeed, as with atmospheric H_2, atmospheric CO is likely to be a dependable
424   energy source for microbial survival given its ubiquity, diffusibility, and energy density.
425   However, in contrast to atmospheric H_2 [27, 30], it remains to be validated through
426   genetic and biochemical studies that atmospheric CO oxidation can enhance survival
427   of bacteria during persistence. It should also be emphasised that the wide kinetic
428   range of the *T. roseum* carbon monoxide dehydrogenase likely enables this isolate to
429   both persist on atmospheric CO and to grow on CO chemolithoautotrophically [19]
430   (and possibly mixotrophically) within specific microenvironments where carbon
431   monoxide is available at elevated concentrations through geothermal activity.

432   Finally, this study establishes Chloroflexi as the third phylum experimentally shown to
433   scavenge atmospheric H_2, following the Actinobacteria [29, 32, 35, 47, 53] and
434   Acidobacteria [31, 36]. The findings made here are similar to those previously reported
435   for the actinobacterium *Mycobacterium smegmatis* [47, 57] and acidobacterium
436   *Pyrinomonas methylaliphatogenes* [31], both of which also shift from heterotrophic
437   respiration to atmospheric H_2 oxidation in response to energy limitation, including
438   through expressing group 1h [NiFe]-hydrogenases. Given at least four other cultured
439   phyla **(Figure 4a)** and two candidate phyla [24] also encode group 1h [NiFe]-

hydrogenases, it seems increasingly likely that atmospheric H_2 serves as a general energy source for aerobic heterotrophic bacteria. This observation is also potentially biogeochemically significant, given aerobic soil bacteria are known to be the main sink in the global biogeochemical hydrogen cycle [67]. Further work, however, is needed to test these whether these principles extend to the still enigmatic Chloroflexi species inhabiting mesophilic soil environments.

Footnotes

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667

Figures

Figure 1. Differential gene expression of carbon-replete (exponential phase) and carbon-limited (stationary phase) cultures of *Thermomicrobium roseum*. (**a**) Volcano plot showing relative expression change of genes following carbon-limitation. The fold-change shows the ratio of normalised transcript abundance of three stationary phase cultures divided by three exponential phase cultures (biological replicates). Each gene is represented by a grey dot and respiratory genes are highlighted as per the legend. (**b & c**) Heat maps of normalised abundance of the putative operons encoding the structural subunits of the group 1h [NiFe]-hydrogenase (*hhySL*; **a**) and type I carbon monoxide dehydrogenase (*coxLSM*; **b**). The read counts per kilobase million (RPKM) are shown for three exponentially growing and three stationary phase biological replicates. (**d**) Differential regulation of the respiratory complexes mediating aerobic respiration of organic and inorganic compounds. Complexes that are differentially shaded depending on whether they are significantly upregulated (green), downregulated (orange), or unchanged (grey) in carbon-limited compared to carbon-replete cultures. Gene names, loci numbers, and average fold changes in transcriptome abundance are shown for each complex. Note that the physiological role of the highly upregulated *hdr* and *etf* complexes is yet to be experimentally validated.

a

1000

−log₁₀ (*p* value)

100

10

-4 -3 -2 -1 0 1 2 3 4 5 6 7 8
log₂ (fold change)

- ▲ Hydrogenase
- ▼ Carbon monoxide dehydrogenase
- ● Electron transfer flavoprotein
- ■ Heterodisulfide reductase
- ● Succinate dehydrogenase
- + Type I NADH dehydrogenase
- × Type II NADH dehydrogenase
- ○ Cytochrome aa_3 oxidase
- □ Cytochrome bo_3 oxidase
- ◆ ATP synthase

b Hydrogenase structural operon:

RPKM
15,000
10,000
5,000
0

Exponential Cultures
1
2
3

Stationary Cultures
1
2
3

hhyS *hhyL* HP HP HP

c CO dehydrogenase structural operon:

RPKM
100,000
80,000
60,000
40,000
20,000
0

coxG *coxL* *coxS* *coxM*

d

H⁺

Atmospheric H₂   Atmospheric CO

H⁺   H⁺   H⁺

nuoA-E,H-N
trd_1780-1789
0.58 ×

ndh
trd_1911
0.48 ×

sdhABCD
trd_0330-0333
2.8 ×

hhyLH
trd_1877-1878
12.6 ×

coxLMS
trd_1206-1208
2.0 ×

etfAB
trd_0148-0159
22 ×

MQH₂
MQ
H⁺

coxABC
trd_0707-0709
1.1 ×

cyoAB
trd_0557-0558
4.1 ×

atpA-H
trd_1227-1234
0.46 ×

NADH NAD⁺

NADH NAD⁺   Succinate Fumarate   H₂ 2 H⁺   CO CO₂

e⁻ ?   e⁻ ?

O₂ H₂O   O₂ H₂O

ADP + P_i   ATP

Imported organic carbon

2 X-SH

hdrABC
trd_0153-0155
29 ×

X-S-S-X

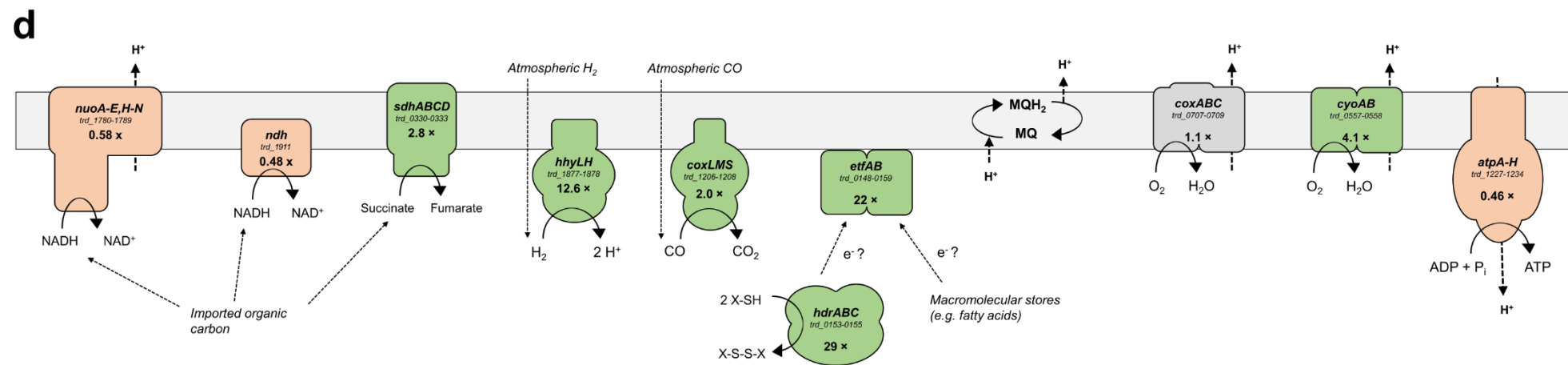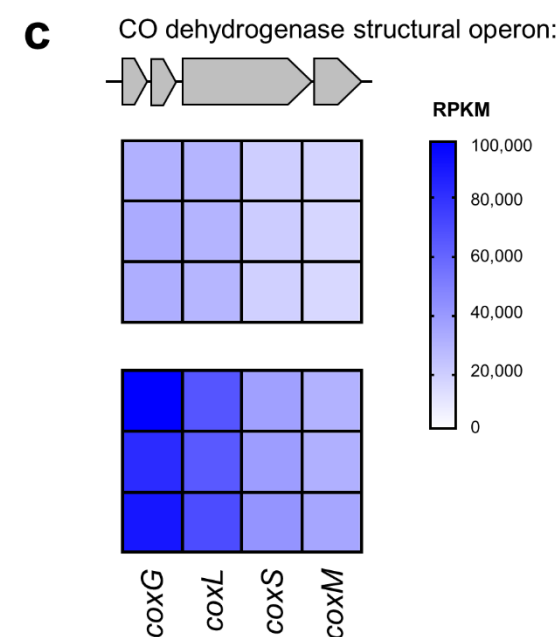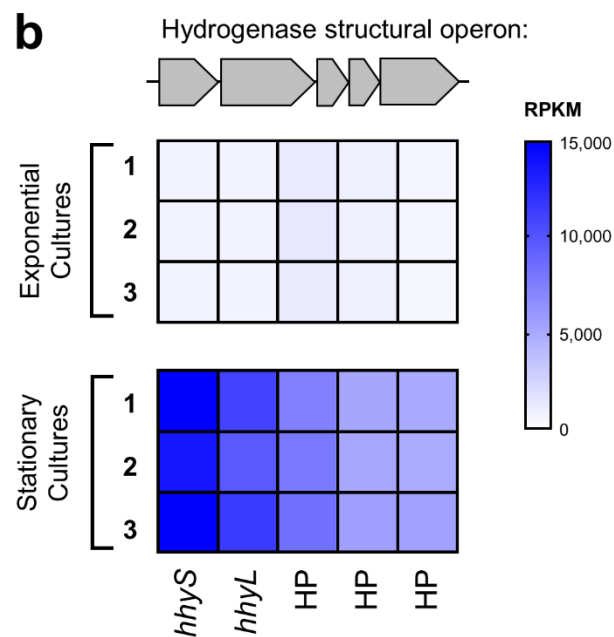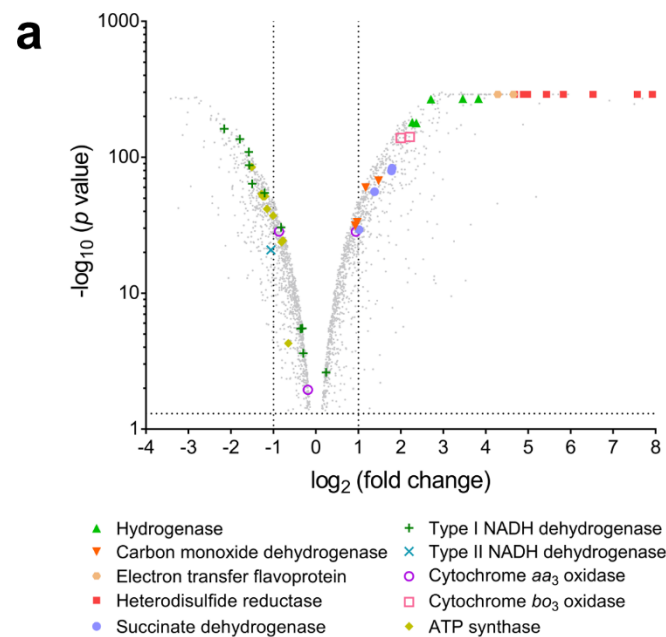*Macromolecular stores (e.g. fatty acids)*

H⁺

687

Figure 2. Hydrogenase and carbon monoxide dehydrogenase activity of *Thermomicrobium roseum* cultures during organic carbon limitation. (**a & b**) Oxidation of molecular hydrogen (H$_2$; **a**) and carbon monoxide (CO; **b**) to sub-atmospheric levels by *T. roseum* cultures. Error bars show standard deviations of three biological replicates, with heat-killed cells monitored as a negative control. Mixing ratios of H$_2$ and CO are displayed on a logarithmic scale and dotted lines show the average atmospheric mixing ratios of H$_2$ (0.53 ppmv) and CO (0.10 ppmv). (**c & d**) Apparent kinetics of H$_2$ (**c**) and CO (**d**) oxidation by *T. roseum* whole cells. Curves of best fit and kinetic parameters were calculated based on a Michaelis-Menten kinetics model. (**e**) Zymographic observation of hydrogenase and carbon monoxide dehydrogenase activity in *T. roseum* whole-cell lysates. The first two lanes show protein ladder and whole protein stained with Coomassie Blue. The other lanes shown hydrogenase and carbon monoxide dehydrogenase activity stained with the artificial electron acceptor nitroblue tetrazolium in a H$_2$-rich and CO-rich atmosphere respectively. (**f**) Amperometric measurements of hydrogenase activity in *T. roseum* whole cells. The rate of H$_2$ oxidation was measured with a hydrogen electrode before and after treatment with the respiratory uncouplers and ionophores carbonyl cyanide *m*-chlorophenyl hydrazine (CCCP), nigericin, and valinomycin.
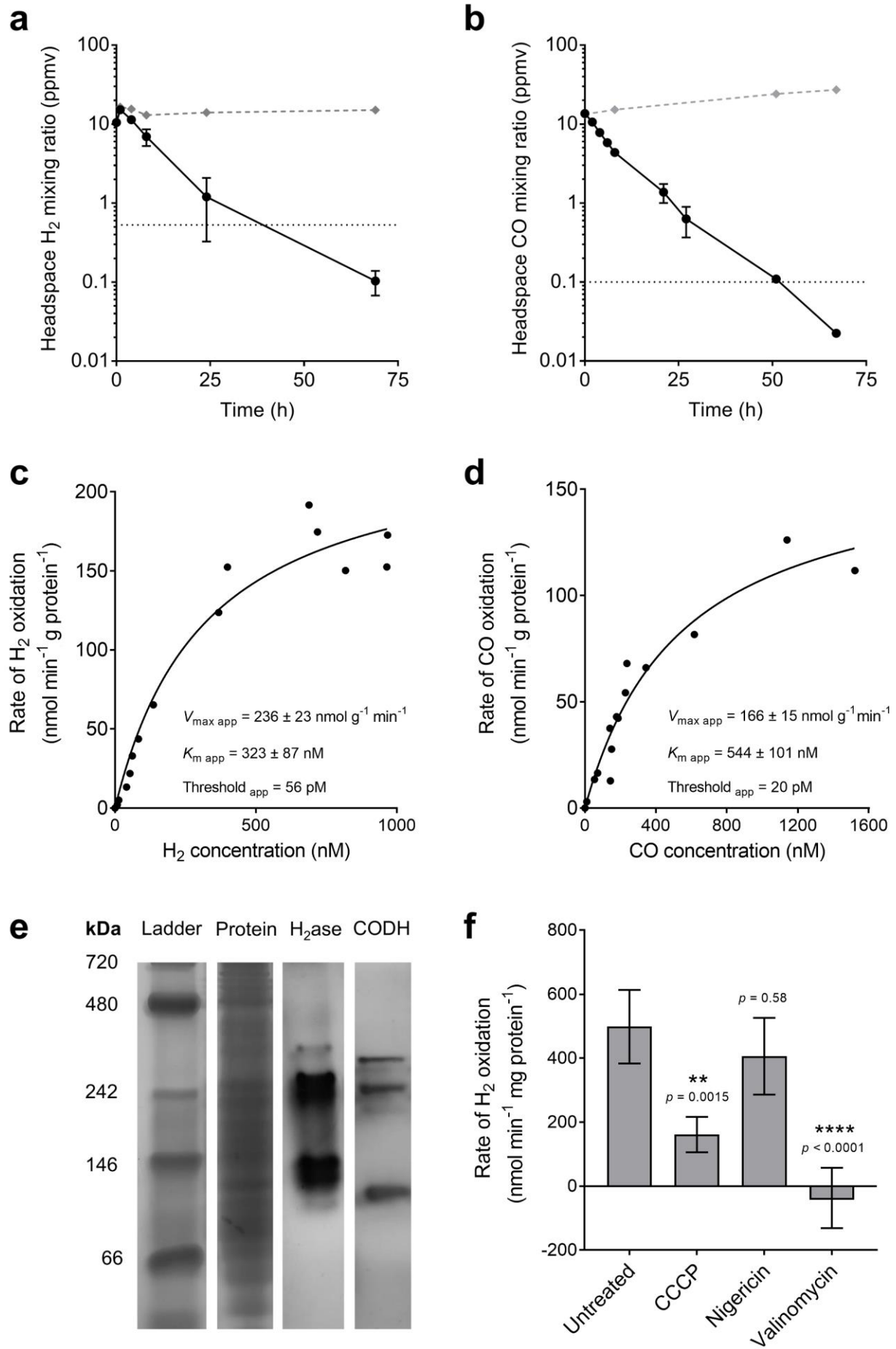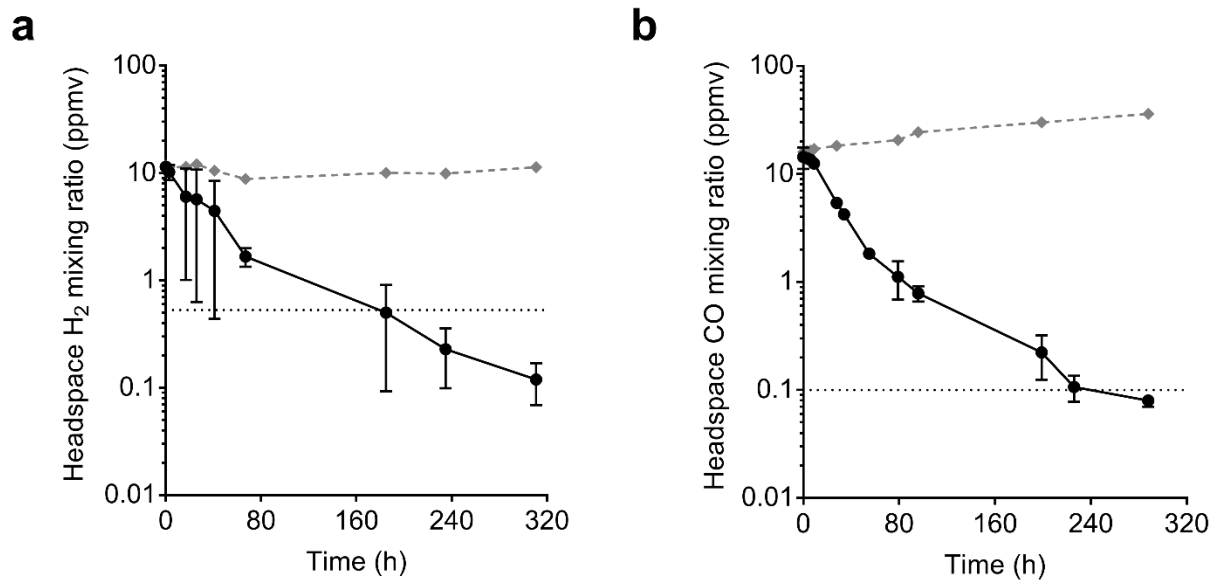
a

b

c

$V_{max\ app} = 236 \pm 23$ nmol g^{-1} min^{-1}

$K_{m\ app} = 323 \pm 87$ nM

Threshold$_{app}$ = 56 pM

d

$V_{max\ app} = 166 \pm 15$ nmol g^{-1} min^{-1}

$K_{m\ app} = 544 \pm 101$ nM

Threshold$_{app}$ = 20 pM

e

f

706

707 **Figure 3. Hydrogenase and carbon monoxide dehydrogenase activity of**
708 ***Thermogemmatispora* sp. T81.** Oxidation of molecular hydrogen (H_2; **a**) and carbon
709 monoxide (CO; **b**) to sub-atmospheric levels by *Thermogemmatispora* sp. T81
710 cultures. Error bars show standard deviations of three biological replicates, with heat-
711 killed cells monitored as a negative control. Mixing ratios of H_2 and CO are displayed
712 on a logarithmic scale and dotted lines show the average atmospheric mixing ratios of
713 H_2 (0.53 ppmv) and CO (0.10 ppmv).

714

715 **Figure 4. Evolutionary history of the group 1h [NiFe]-hydrogenase and type I**

716 **carbon monoxide dehydrogenase.** Phylogenetic trees showing the distribution and

717 evolutionary history of the catalytic (large) subunits of the group 1h [NiFe]-

718 hydrogenase (*hhyL*; **a**) and type I carbon monoxide dehydrogenase (*coxL*; **b**) in the

719 phylum Chloroflexi. Chloroflexi sequences (labelled by class) are shown in bold

720 against reference sequences (labelled by phylum). Trees were constructed using

721 amino acid sequences through the maximum-likelihood method (gaps treated with

722 partial deletion) and were bootstrapped with 100 replicates. The trees were

723 respectively rooted with group 1g [NiFe]-hydrogenase sequences (WP_011761956.1,

724 WP_048100713.1) and type II carbon monoxide dehydrogenase sequences

725 (WP_011388721.1, WP_012893108.1). The distribution of other respiratory uptake

726 hydrogenases within genomes and metagenome-assembled genomes (MAGs) in the

727 phylum Chloroflexi is shown in **Figure S3**. The distribution of type I carbon monoxide

728 dehydrogenases within metagenome-assembled genomes (MAGs) in the phylum

729 Chloroflexi is shown in **Figure S4**.