

Natranaerofaba carboxydovora gen. nov., sp. nov., an extremely haloalkaliphilic CO-utilizing acetogen from a hypersaline soda lake representing a novel deep phylogenetic lineage in the class 'Natranaerobiia'

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***Natranaerofaba carboxydovora* gen. nov., sp. nov., an extremely haloalkaliphilic CO-utilizing acetogen from a hypersaline soda lake representing a novel deep phylogenetic lineage in the class "*Natranaerobiia*"**

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2 **haloalkaliphilic CO-utilizing acetogen from a hypersaline soda lake**
3 **representing a novel deep phylogenetic lineage in the class**
4 **"*Natranaerobiia*"**

5
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18
19
20 **Originality-Significance Statement**

21 This paper describes the first example of an extremely haloalkaliphilic anaerobic bacterium
22 isolated from a hypersaline soda lake capable of CO utilization as electron donor. It represents
23 a member of a novel deep-branching phylogenetic lineage at the level of a new genus and
24 family within the Firmicutes class "*Natranaerobiia*". Based on its basic physiology, the
25 bacterium is an acetogen with a restricted metabolism capable of anaerobic respiration with
26 thiosulfate and nitrate.

1 **Summary**

2 An anaerobic enrichment with CO from sediments of hypersaline soda lakes resulted in a
3 methane-forming binary culture, whereby CO was utilized by a bacterium and not the
4 methanogenic partner. The bacterial isolate ANCO1 forms a deep-branching phylogenetic
5 lineage at the level of a new family within the class "*Natranaerobiiia*". It is an extreme
6 haloalkaliphilic and moderate thermophilic acetogen utilizing CO, formate, pyruvate and
7 lactate as electron donors and thiosulfate, nitrate (reduced to ammonia) and fumarate as
8 electron acceptors. The genome of ANCO1 encodes a full Wood-Ljungdahl pathway allowing
9 for CO oxidation and acetogenic conversion of pyruvate. A locus encoding Nap nitrate
10 reductase/NrfA ammonifying nitrite reductase is also present. Thiosulfate respiration is
11 encoded by a Phs/Psr-like operon. The organism obviously relies on Na-based bioenergetics,
12 since the genome encodes for the Na⁺-Rnf complex, Na⁺-F1F0 ATPase and Na⁺-translocating
13 decarboxylase. Glycine betaine serves as a compatible solute. ANCO1 has an unusual
14 membrane polar lipid composition dominated by diethers, more common among archaea,
15 probably a result of adaptation to multiple extremophilic conditions. Overall, ANCO1
16 represents a unique example of a triple extremophilic CO-oxidizing anaerobe and is classified
17 as a novel genus and species *Natranaerofaba carboxydovora* in a novel family
18 *Natranaerofabacea*.

19

20 Key words: **soda lake, haloalkaliphilic, carboxydophilic, acetogen, "*Natranaerobiiia*"**

1 **Introduction**

2 In view of the possibility of an early soda ocean (created by CO₂ weathering of Na/K-rich
3 basalt crust of volcanic origin) in the geological history of Earth and Mars and a probability of
4 the same in the current ocean on Europa, there is an interest in astrobiology of the existing
5 haloalkaline environments on Earth, such as terrestrial soda lakes and deep sea
6 serpentinization area (Fox-Pawel *et al.*, 2016; Herschy *et al.*, 2014; Kempe and Kazmierczak,
7 2002). One of the potential common substrates for extraterrestrial microbes would be CO,
8 which can form by photolysis of CO₂ under strong UV radiation (King, 2015).

9 Modern saline-alkaline soda lakes represent rare examples of stable highly alkaline
10 natural habitats due to the presence of high concentrations of soluble sodium carbonates,
11 which can reach molar concentrations in hypersaline soda lakes. Such lakes are located in arid
12 areas characterized by a hot climate with long periods of evaporative salt concentration
13 (Schagerl, 2016). Thus, prokaryotes thriving in soda lakes often exhibit triple extremophilic
14 circumstances, i.e. halo-alkalo-thermo-phily (Mesbah *et al.* 2007; 2009; Sorokin *et al.*, 2017).

15 Extensive knowledge has been accumulated in the past three decades on the
16 functionally important microbial communities in soda lakes, both by culture-dependent (Grant
17 and Jones, 2016; Sorokin *et al.*, 2015; Sorokin, 2017) and culture-independent molecular
18 approaches (Vauvorakis *et al.*, 2017; 2019; Zorz *et al.*, 2019). However, several pieces of the
19 puzzle are still missing. In particular, whether anaerobic carbon monoxide oxidation
20 (carboxydrotrophy) is possible under extremely haloalkaline conditions. Aerobic
21 carboxydrotrophy has been shown for a group of highly salt tolerant soda lake alkaliphiles
22 belonging to two closely related genera *Alkalispirillum/Alkalilimnicola* of the
23 *Gammaproteobacteria* (Hoeft *et al.*, 2007; Sorokin *et al.*, 2010). The only anaerobic isolate
24 reported as capable of CO oxidation at high pH is the acetogenic *Alkalibaculum bacchii*, a

1 nonhalophilic, alkalitolerant member of the *Eubacteriaceae* family (*Firmicutes*), isolated
2 from soil (Allin *et al.*, 2010; Liu *et al.*, 2012).

3 Three major types of anaerobic carboxydrotrophy have been established: by acetogens
4 via the Wood-Ljungdahl pathway, resulting in CO₂, acetate and/or ethanol; by methanogens,
5 resulting in methane or acetate; and by hydrogenogens, resulting in H₂ and CO₂ (Diender *et*
6 *al.*, 2015). Since protons are the final electron acceptors of hydrogenogens, the
7 hydrogenogenic metabolism is highly improbable under soda lake conditions, while both
8 acetogenic and methanogenic CO utilization are possible. There is a fourth (marginal)
9 pathway of anaerobic carboxydrotrophy, the direct anaerobic oxidation of CO in presence of a
10 suitable electron acceptor, but it has not yet been well studied, except for sulfate-reducing
11 conditions, exemplified by *Desulfotomaculum* species (Parshina *et al.*, 2010) and
12 *Archaeoglobus fulgidus* (Henstra *et al.*, 2007).

13 Our previous attempts to directly enrich either acetogenic or methanogenic CO-
14 utilizing haloalkaliphiles from soda lakes at mesophilic conditions and low salinity (0.6 M
15 total Na⁺, pH 10, 30°C and 0.2 atm CO in the gas phase) resulted in a positive acetogenic
16 enrichment consisting of five clostridia members distantly related to the genera *Anaerobranca*
17 and *Dethiobacter*. However, efforts to further enrich the consortium failed and the culture was
18 eventually lost. Recently, another trial was undertaken to find out whether CO could replace
19 formate/H₂ as the electron donor for triple extremophilic methyl-reducing methanogens of the
20 class *Methanonatronarchaeia* found in hypersaline soda lakes (Sorokin *et al.*, 2017; 2018).
21 Methane was indeed formed in such enrichments but not directly by methanogens. Instead, a
22 triple extremophilic anaerobic bacterium capable of utilizing CO as the electron donor was
23 responsible for the initial CO conversion. Its phenotypic, phylogenetic and genomic
24 properties are described in this paper.

25

26

1 RESULTS

2

3 **Enrichment and isolation of a haloalkaliphilic carboxydotrophic bacterium**

4 In the primary enrichment, inoculated with anaerobic sediment from a hypersaline soda lake,
5 methane formation at 50 °C in presence of CO/MeOH as substrates was only observed at the
6 minimal CO concentration of 0.05 atm in the gas phase. The onset of methane accumulation
7 occurred after a prolonged lag period (0.3% in the gas phase after 53 d) and reached a
8 maximum of 3.2% at day 85. Interestingly, all CO had already been consumed at day 65,
9 indicating that the CO consumption and methane formation were not synchronized. However,
10 no methane formation occurred in control incubations lacking CO. In the second 1:100
11 transfer of this culture, two major cell morphotypes were observed: bean-shaped motile cells
12 and tiny angular cocci typical for the genus *Methanonatronarchaeum*. This assumption was
13 later confirmed by isolating this methyl-reducing methanogen from the consortium using
14 formate instead of CO as the electron donor. It had 100% 16S-rRNA gene identity to one of
15 the previously isolated strain of *M. thermophilum* (Sorokin *et al.*, 2017; 2018) and it was
16 unable to use CO as the electron donor in pure culture. Therefore, it was concluded that the
17 bean-shaped bacterium was the primary CO-utilizing organism in this consortium, which
18 might have converted part of CO into formate utilized by the methyl-reducing methanogen.
19 The CO-utilizing bacterium was finally separated from the methanogen in serial dilutions
20 using higher CO concentration (10-20% in the gas phase) and omitting MeOH from the
21 medium. Furthermore, we repeated the enrichment at these modified conditions with the
22 inoculum diluted from the start and the same bacterium was selected up to a 10⁻⁸ dilution,
23 indicating that this was the only carboxydotrophic anaerobe capable of growing at the used
24 triple extremophilic conditions. The isolated pure culture strain was designated ANCO1.

25

1 **Phylogeny of strain ANCO1**

2 The 16S rRNA-based phylogenetic analysis placed strain ANCO1 into the *Firmicutes*
3 order *Natranaerobiales* (elevated to the level of a class "*Natranaerobiia*" according to the
4 phylogenomic taxonomy, <https://gtdb.ecogenomic.org>) as a deep-branching lineage (**Fig. 1a**).
5 The closest relative of ANCO1 (90% sequence identity) is the moderately salt-tolerant
6 alkaliphilic peptidolytic anaerobe *Natranaerobaculum magadiense*, isolated from hypersaline
7 soda lake Magadi in Kenya (Zavarzina *et al.*, 2013). A more advanced phylogenomic analysis
8 based on 120 conserved single copy marker genes confirmed the independent position of
9 strain ANCO1 within the class "*Natranaerobiia*" at the level of a new family (**Fig. 1b**): the
10 relative evolutionary divergence (RED) value of the node of *N. carboxydovora* offshoot
11 calculated using GTDBtk was 0.663 (values of 0.773 and 0.629 considered as family and
12 order thresholds, respectively) (Parks *et al.* 2018). AAI values between ANCO1 and members
13 of the type genus *Natranaerobius* did not exceed 48%.

14

15 **Cell morphology**

16 ANCO1 has bean-shaped cells of variable length, from 2 to 5 μm , depending on the
17 growth rate, motile with a few flagella located on the concave side of the cell (**Fig. 2**).
18 Although endospore formation was not observed at the growth conditions used, the genome
19 does contain several extended loci encoding this capacity.

20

21 **Membrane polar lipids**

22 ANCO1 contains a wide range of intact polar lipids (IPLs) (**Table 2**). These all had
23 either a phosphocholine (PC) or phosphoglycerol (PG) polar head group and the PCs were
24 dominant over the PGs. The apolar cores of the IPLs comprised a distinctive range of
25 different structures. The alkyl chains (containing between 14 and 19 carbon atoms) were

1 bound to the glycerol backbone via either ester or ether bonds giving rise to diacyl lipids (two
2 ester bonded chains), diether lipids and mixed acyl/ether lipids (including plasmalogen
3 lipids). Mixed acyl/ether lipids have been reported in anaerobic bacteria, with some
4 exceptions in aerobic bacteria, and are thought to improve cell resistance to extreme external
5 conditions (Grossi *et al.*, 2015). The majority ($\leq 80\%$) of the lipids in ANCO1 were diethers,
6 which are rare in bacteria but are characteristic of archaeal membranes. A number of the
7 bacterial species that have been reported to produce diether lipids belong to thermophiles (e.g.
8 Langworthy *et al.*, 1983; Huber *et al.*, 1992; Hamilton-Brehm *et al.*, 2013). Macrocylic
9 diethers were also detected in ANCO1 as a minor component. These have not previously been
10 described in pure bacterial cultures but have been detected in environments including
11 authigenic carbonates from marine evaporites, probably formed at high temperatures and high
12 salinity (Baudrand *et al.*, 2010; Ziegenbalg *et al.*, 2012), iron sulfide nodules formed during
13 the anaerobic oxidation of methane (van Dongen *et al.*, 2007), geothermal hot springs
14 (Pancost *et al.*, 2006) and iron sulfides from a Mid-Atlantic hydrothermal field (Blumenberg
15 *et al.*, 2007). The archaeal equivalent of this lipid, an isoprenoidal macrocylic diether
16 (known as macrocylic archaeol) has been shown to augment membrane impermeability and
17 stability in thermophilic methanogenic archaea (Koga *et al.*, 1998), which explains its
18 accumulation in extremophilic archaea (Sprott *et al.*, 1991; Bale *et al.*, 2019b).

19 The individual constituents of the core lipids were also analyzed directly in more
20 detail, after hydrolysis of the biomass to cleave off the polar head groups (see Supplementary
21 **Fig. S2** for examples of structures and theoretical hydrolysis schemes). This procedure
22 released fatty acids (FA; formed from ester bound alkyl chains), dimethyl acetals [DA;
23 formed from plasmalogen lipids; van Gelder *et al.* (2014)] and mono and dialkyl glycerol
24 ethers (MGEs and DGEs; formed from ether-bound alkyl chains). Both straight-chain and
25 branched alkyl chains were present. As the DGEs contain two alkyl chains (Supplementary

1 **Fig. S2**), their total number of alkyl carbon atoms range between 29 and 34. Overall
2 conclusion of the lipid analysis is that its unusual composition most probably reflects the
3 triple extremophilic nature of strain ANCO1.

4 The respiratory lipoquinoines were not detectable in ANCO1, either because they were
5 present at a very low level or because they are not produced. The latter is likely since the
6 genome does not contain the genes encoding the enzymes required for menaquinone
7 biosynthesis.

9 **Growth physiology of strain ANCO1**

10 Growth with CO as the electron donor (and other electron donors) was only possible in
11 the presence of at least 100 mg/l of yeast extract. After gradual adaptation, ANCO1 was able
12 to tolerate up to 50% CO in the gas phase. However, the maximum biomass yield reached
13 only 10 mg cell protein l⁻¹ even at highest CO concentration, and the full CO consumption
14 was observed only at 5-10% CO. The only gaseous product detected was CO₂, although most
15 of it was apparently absorbed into the highly alkaline culture medium (**Supplementary Fig.**
16 **S1**). Liquid analysis at the end point in the incubation with 5% CO (11.5 mM) showed
17 formation of two organic products - acetate as a major and formate as a minor component (2.5
18 and 0.8 mM, respectively), indicating that the mode of anaerobic CO conversion in ANCO1 is
19 homoacetogenic (4 mol CO > 1 mol acetate). Both formate and acetate can be utilized by
20 *Methanonatroarchaeum* (as electron donor and C-source, respectively) which might explain
21 why the initial enrichment supported growth of this methyl-reducing methanogen.

22 An enzymatic assay confirmed the presence of anaerobic CODH activity in strain
23 ANCO1 (1.77 ± 0.22 μmol/min of CO conversion per mg protein in cell free extract). The
24 only another compound supporting acetogenic growth from a range of substrates tested (H₂,

1 formate, MeOH, EtOH, PrOH, BuOH, lactate, and sugars) was pyruvate, which was
2 converted into acetate and lactate with a minor formation of H₂ (0.2-0.3 mM).

3 The ability of ANCO1 to use various electron acceptors was first tested with the two
4 positive electron donors, i.e. CO and pyruvate. A definite growth stimulation was observed
5 with fumarate and thiosulfate. The latter was partially reduced to sulfite and sulfide, while
6 succinate formation from fumarate was very weak, which is difficult to explain, especially
7 because fumarate reductase genes are present in the genome (**Table 1**).

8 The genomic data (see below) suggested that ANCO1 has a potential for dissimilatory
9 reduction of nitrate to ammonia (DNRA). However, tests with CO and pyruvate showed
10 neither growth stimulation nor nitrate (5 mM) consumption, and addition of even 2 mM nitrite
11 inhibited growth. Therefore, other potential electron donors (suggested by the genome
12 composition) were tested: formate, H₂ and lactate. The latter two were negative, while
13 anaerobic growth with formate as the electron donor was possible with thiosulfate, fumarate
14 and nitrate/nitrite. The ammonifying growth of ANCO1 was not easy to prove. When nitrate
15 was added at 5 mM from the start, it was converted only to nitrite. At 2 mM initial
16 concentration, however, ammonifying growth with formate as donor was possible up to two
17 further additions of 2 mM portions of nitrate. In total, 6 mM of nitrate were consumed without
18 formation of intermediate nitrite and 4 mM NH₃ was detected in the end of incubation. Nitrite
19 could also support ammonifying growth with formate, but only when it was added at low
20 concentration of 2 mM together with formate (50 mM) to a culture, pre-grown with pyruvate
21 or to a culture started to grow with formate + fumarate. It was possible to add in total up to 6
22 mM nitrite in three portions and the final ammonia production reached 5.3 mM with a
23 prominent biomass increase in comparison to the control without nitrite addition. A direct
24 addition of even 2 mM nitrite to the formate culture from the start revealed the complete
25 absence of growth. Furthermore, formate was the only electron donor for which significant

1 amount of succinate was detected during growth with fumarate as the electron acceptor. On
2 the other hand, sulfidogenic growth on formate with thiosulfate produced the least sulfide
3 from other positive cases (**Table 1**).

4 Lactate as the potential electron donor was also tested with fumarate and thiosulfate as
5 the electron acceptors. In both cases growth was observed, with very active sulfide formation
6 from thiosulfate, but only trace amount of succinate was formed during growth with fumarate.
7 No growth was detected with H₂ at any tested conditions (**Table 1**).

8 Influence of salt and pH on growth of ANCO1 was tested with pyruvate +fumarate.
9 Both profiles showed growth only at a limited range, indicating an extreme specialization of
10 this organism to live in hypersaline soda lake conditions (**Fig. 3**). The salt profile (obtained at
11 pH 9.5) showed that ANCO1 is an extreme halophile, growing within a range of total Na⁺
12 from 2.5 to 4.5 M (optimum at 4 M). It is an obligate alkaliphile, growing within a narrow pH
13 range above 9 and up to 10.5 (optimum at 9.5; the final pH measured at 30 °C). On its
14 temperature dependence, ANCO1 can be qualified as a moderate thermophile with the growth
15 range from 35 to 56 °C and an optimum at 48-50 °C.

16

17 **Functional genomic and proteomic analyses**

18 The genome of ANCO1 (accession number CP054394) was assembled into a single
19 contig of 3.26 MB, which is predicted to be circular and has a GC content of 35.3 mol%.
20 Plasmids were not detected. Annotation produced 3,120 coding sequences. The key functional
21 pathways found in the genome of ANCO1 are summarized in **Figure 4** and the identified
22 genes/proteins are listed in the **Supplementary Table S1**. The key expressed functional
23 proteins are shown on **Supplementary Fig. S3** and listed in **Supplementary Table S2**. The
24 full range of expressed proteins is provided in the **Extended proteomic data file**.

1 The ANCO1 genome contains all the genes of the Wood-Ljungdahl pathway and
2 corresponding enzymes were indeed detected in the proteome of ANCO1 grown on CO and
3 pyruvate. The presence of three CODH-encoding genes in the genome and the detection of
4 CODH activity in cell free extracts confirms the ability of the bacterium to utilize CO as
5 electron donor. ANCO1 encodes for one CODH/ACS cluster, likely involved in CO-oxidation
6 as well as CO₂ fixation. When the organization of this gene cluster is compared to
7 corresponding CODH/ACS clusters of other carboxydophilic genomes, most similarity is
8 observed with the thermophilic acetogens *Moorella thermoacetica* and *Carboxydotherrmus*
9 *hydrogeniformans* (**Figure 5**). Next to a CODH/ACS cluster, ANCO1 encodes for two other
10 CODHs that are likely monofunctional. One lies adjacent to a hydrogenase complex
11 (ACONDI_01147-1151). However, carboxydophilic hydrogenogenic activity was not
12 apparent from the physiological tests (no H₂ formation from CO), which means that this
13 hydrogenase is not linked with the CODH, like, for example, Ech is.

14 The annotated cytoplasmic Fe-only hydrogenases exhibit similarity to bifurcating
15 NADPH-dependent hydrogenases potentially involved in redox distribution, in particular to
16 recycle NAD(P)H and ferredoxin pools used as reductants for biosynthesis, building
17 membrane potential and anaerobic respiration (Buckel and Thauer, 2013). Several formate
18 dehydrogenases are also present, of which two (ACONDI 00243-00246 and 02919-02924)
19 show similarity with the CO-reactive hydrogenase of *Acetobacterium woodii* (HDCR type,
20 Schuchmann et al., 2013), and may be used to link hydrogen oxidation to formate production.
21 Genes encoding acetate kinase and acetyl-phosphotransferase are present, enabling acetate
22 production from acetyl-CoA and energy conservation.

23 Comparative proteomic analysis of cells grown acetogenically either with CO or
24 pyruvate was performed in order to understand more about the CO-specific metabolism of
25 ANCO1. However, it appears, that the same set of enzymes involved mostly in the Wood-

1 Ljungdahl pathway and pyruvate oxidation were equally expressed (Supplementary **Fig. S3**
2 **and Table S2**). This might indicate that both CO and pyruvate are funneled into the same
3 pathway.

4 Regarding key energy-related reactions, ANCO1 encodes for a Rnf-complex and F1F0
5 ATP synthase (both annotated as sodium-translocating). In addition, two membrane bound
6 primary sodium-translocating decarboxylases are present: glutaconyl-CoA and
7 methylmalonyl-CoA decarboxylase, which are potentially involved in the sodium-based
8 bioenergetics and a control of sodium levels and osmoregulation in anaerobic alkaliphiles
9 (Buckel, 2001; Dimroth and Schink, 1998). Further fate of the thioester products (propionyl-
10 CoA and crotonyl-CoA) of these two putative reactions is difficult to predict, but, likely, they
11 might be channeled into anabolic routes, such as amino acid or lipid biosynthesis.

12 Several proton/sodium antiporters are also encoded, including two multi-subunit Mnh
13 complexes, one independent (ACONDI 02179-02187) and another one in a large locus
14 together with the Na⁺-Rnf complex (ACONDI 00389-00396), enabling linkage of the proton
15 and sodium gradients and maintenance of pH homeostasis. Also, a secondary sodium pump -
16 potassium-stimulated Na⁺-pyrophosphatase, involved the ATP-dependent Na⁺ removal from
17 the cytoplasm is encoded by ACONDI 00187.

18 For osmoprotection, the genome predicts two potential pathways of glycine betaine
19 biosynthesis. The first is sequential glycine methylation (ACONDI 00109/00157), including
20 glycine/sarcosine and sarcosine/*N,N*-dimethylglycine methyltransferases (Roberts, 2005). The
21 second is the oxidative pathway from carnitin (ACONDI 01812/01814), including L-
22 carnithine dehydrogenase LcdH and 3-keto-5-aminohexanoate cleavage enzyme Kce
23 (Rebouche, 1998). In addition, a presence in the ANCO1 genome of genes coding for
24 multiple types of transporters for betaine/carnitin/choline import (Angelidis and Smith, 2003;
25 Ongagna-Yhombi et al., 2015; Pflüger and Müller, 2004) strongly suggest that active

1 transport of betaine and its precursors is active in the ANCO1 as well. These include the
2 following families: BCCT, Gbu, BetS, OpuAC, OpuAB, OpuE and OpuD (the full list with
3 the locus tags is present in **Supplementary Table S1**). A possible source of betaine for
4 import might be yeast extract required by ANCO1 for growth at any conditions. Furthermore,
5 potassium may play a significant role in osmoprotection too, judging from the presence of a
6 large repertoire of genes encoding potassium import/export (KefC, TrkA, TrkAH, KtrAB,
7 NhaP2, Kch) (**Supplementary Table S1**). Calculation of the total proteome acidity using the
8 IPC calculator (<http://isoelectric.org/calculate.php>) showed a bimodal distribution of the pI
9 value, with a major moderately acidic peak between pH 4 and 6 and a smaller basic peak
10 between pH 8 and 10 (**Supplementary Fig. S4**), indicating that ANCO1 is most probably
11 using the "salt-out" strategy as the dominant osmoprotection mechanism with potassium
12 import as a secondary mechanism. But for the definite conclusion, both intracellular betaine
13 and potassium accumulation needs to be determined.

14 Despite the inability to grow on sugars, the genome of ANCO1 encodes for the
15 complete pentose-phosphate pathway and glycolysis. However, it lacks sugar transporters,
16 suggesting that these pathways are most likely used for gluconeogenesis. Presence of
17 pyruvate oxidoreductase enables conversion of acetyl-CoA into pyruvate for assimilatory
18 metabolism. An incomplete TCA-cycle lacking malate dehydrogenase is encoded by the
19 genome of ANCO1.

20 Despite the absence of lipoquinones (both by direct analysis and predicted by the lack
21 of genes encoding menaquinone biosynthesis), the genome analysis confirms the potential of
22 ANCO1 to obtain energy by nitrate/nitrite ammonification and thiosulfate- and fumarate-
23 dependent respiration found in growth experiments. For DNRA, ANCO1 potentially uses an
24 unusual module encoded in a single locus consisting of an intracellular truncated Nap type
25 respiratory nitrate reductase NapAGHDF (ACONDI 01139-01143) and an exported

1 ammonifying nitrite reductase NrfA (ACONDI 01136). The locus, however, appears to lack
2 NrfH, a membrane tetraheme *c* involved in electron transport to NrfA from quinones. As the
3 NrfA present in the genome is predicted to have a single transmembrane helix and lies in the
4 same operon as the Nap nitrate reductase, this might indicate a possibility for direct electron
5 transfer from a membrane electron donor (which is still unknown) and an interaction between
6 the two complexes. On the other hand, the genome also contains another locus that encodes a
7 mosaic redox complex with an uncertain (to predict) function (ACONDI 02948-
8 02952/02956). It includes a membrane diheme b_{556} cytochrome of the PhsC/NarI type (might
9 be a missing subunit of the Phs operon, see below), followed by a putative NarG (catalytic
10 subunit of the membrane respiratory nitrate reductase). Next, a tetraheme *c* cytochrome of the
11 NapC/CymA type containing a single transmembrane loop is encoded. While not homologous
12 to the integral membrane NrfH, it still might potentially be involved in the DNRA of ANCO1.
13 The last part of this locus codes for the electron transfer complex FixXCA.

14 The genome of ANCO1 includes an operon coding for PhsAB complex (both subunits
15 are exported) potentially involved in a 2-electron reduction of thiosulfate, but apparently
16 lacking its membrane electron-transferring anchor PhsC, which might be encoded in another
17 genomic locus (see above). Subunits of fumarate reductase (FrdAB) potentially involved in
18 fumarate respiration are also present. A membrane associated NADPH oxidoreductases and a
19 formate dehydrogenase (ACONDI-00243-00246) might be involved in donation of electrons
20 to the respiration metabolism. However, the absence of respiratory lipoquinones still
21 questions how those are linked to the electron-accepting respiratory complexes. The only
22 candidate for the electron transfer encoded in the genome is a low-potential diheme *c* split-
23 Soret cytochrome (ACONDI-02684), which is exported and attached to membrane. Such
24 cytochromes serve in electron transport of sulfate-reducing bacteria (Devreese et al., 1997).

1 Despite the observed lactate production and utilization by the strain, lactate
2 dehydrogenase could not be clearly annotated. However, the genome encodes for an operon
3 encoding for a bifurcating lactate dehydrogenase, similar to an earlier reported bifurcating
4 lactate dehydrogenase complex (Weghoff et al., 2014). This complex might be involved in the
5 utilization of lactate for respiration and could additionally be involved in lactate production
6 when grown on pyruvate, which is in line with the proteomics data showed relatively high
7 abundance of this complex in cell grown on pyruvate.

8

9 DISCUSSION

10

11 Strain ANCO1 represents the first example of an extremely haloalkaliphilic CO-utilizing
12 anaerobe. It is a member of a deep-branching phylogenetic lineage in the phylum *Firmicutes*,
13 class "*Natraanaerobiiia*", which currently consists of only three genera found in soda lakes:
14 the extremely salt-tolerant genera *Natranaerobius* and *Natronovirga* (Mesbah et al., 2007;
15 Mesbah and Wiegel, 2009) and a moderately salt-tolerant genus *Natranaerobaculum*
16 (Zavarzina et al., 2013). All of them are moderately thermophilic, obligately anaerobic
17 fermentative heterotrophs with potential for anaerobic respiration, and in these the new
18 member of this branch, strain ANCO1, is most similar to *Natranaerobius thermophilus*,
19 which is also evident from the genome analysis (see **Supplementary Table S1**). However,
20 the ability to utilize CO as electron donor has not been reported for any of the ANCO1
21 relatives and the available genomes from the *Natranaerobius* species lack the respective
22 genetic potential for such metabolism. The CODH/ACS cluster in ANCO1 is similar to that of
23 other thermophilic carboxydrotrophs, like *Moorella thermoacetica* and *Carboxydotherrmus*
24 *hydrogenoformans* (**Figure 5**). The main product of CO conversion by strain ANCO1 is
25 acetate, similar to what is observed in *M. thermoacetica* (Drake and Daniel, 2004).
26 Thermophilic carboxydrotrophs *M. thermoacetica* and *C. hydrogenoformans* lack the RnF-

1 complex and employ energy converting hydrogenases (Ech) in order to generate a cation
2 gradient and produce ATP (Schuchmann and Müller, 2014; Wu et al., 2005). Although a
3 CODH-hydrogenase like gene-cluster is found in the genome of ANCO1 (**Figure 4**), the
4 absence of clear hydrogen production suggests it is not employing it. In addition, this cluster
5 does not show the typical Ech structure, indicating that in contrast to other CO-utilizing
6 thermophiles, ANCO1 uses the Rnf-complex for its energy metabolism.

7 Despite the presence of soluble hydrogenases encoded in the genome, strain ANCO1
8 was unable to grow acetogenically on H₂/CO₂ or use H₂ as electron donor for anoxic
9 respiration. The strain did produce minor amounts of H₂ when grown on pyruvate. This raises
10 the question why strain ANCO1 is able to use CO but not H₂ to drive acetogenesis. A possible
11 reason are the thermodynamic constrains imposed during H₂-dependent acetogenesis – the
12 fact that the standard redox potential of the pair 2H⁺+2e⁻/H₂ (≈-400 mV) is not sufficiently
13 negative to reduce ferredoxin, demands for bifurcation mechanisms to perform CO₂ reduction
14 in the WLP (Schuchmann & Müller, 2012; Buckel & Thauer, 2013). Utilization of CO (with a
15 substantially lower standard redox potential, i.e. E^{0'} (CO₂/CO) ≈ -520 mV) circumvents this
16 bottleneck, and ferredoxin can be directly reduced via CODH. If ANCO1 is unable to link H₂-
17 oxidation to ferredoxin reduction via a bifurcating hydrogenase, this would explain the
18 absence of acetogenic growth with H₂/CO₂. The H₂ that is produced during growth on
19 pyruvate might originate from Fe-only hydrogenases using ferredoxin as donor, and favouring
20 the hydrogen producing reaction direction (ref Adams 1990).

21 As ANCO1 encodes for a HDCR-like complex (ACONDI 00243-00246 and 02919-
22 02924), the WLP appears to be H₂-dependent in this strain. Hydrogen production via Fe-only
23 hydrogenases with ferredoxin (derived from CO oxidation) might play a role in introducing
24 the reduction equivalents via the HDCR-like complex into the WLP. Such internal cycling of
25 hydrogen by acetogens has been observed before from organic substrates such as fructose

1 (ref. Wiechmann 2020). To note that activity of HDCR-like complex in ANCO1 was not
2 confirmed experimentally in this study, it is also possible that formate alternatively originates
3 from bifurcating cytoplasmic enzymes that require NAD(P)H and ferredoxin, similar to what
4 is observed in *Clostridium autoethanogenum* (wang et al., 2013). In this case, H₂ would not
5 be required to drive the WLP, allowing operation on CO without intermediate H₂ production.
6 The potential roles for hydrogenases and their role in acetogenesis are depicted in figure 4
7 (dotted red lines). Overall, with current information, we can only speculate on the H₂
8 metabolism of strain ANCO1 and more biochemical research is required to further elucidate
9 this.

10 Regarding anaerobic respiration, the ability to utilize fumarate and thiosulfate as the
11 electron acceptors is reported for *Natranaerobius thermophilus* and *Natranaerobaculum*
12 *magadiense*, although succinate formation from fumarate was not measured. But the genome
13 of *N. thermophilus* does contain the genes coding for fumarate reductase and thiosulfate
14 reductase (Nther_2664-2665 and Nther_0643-0644, respectively) with the highest similarity
15 to the ones present in ANCO1 genome. What is questionable, however, is the universal ability
16 for the DNRA in the whole "Natranaerobiiia" members described previously. The reasons for
17 doubts are the following: i) formation of ammonia as the final product was not analyzed in
18 *Natranaerobius truperii* and *Natronovirga*, and ii) genes encoding for respiratory nitrate
19 reductases (Nar/Nap) and the NrfAH-type multiheme cytochromes *c* are not present in the
20 genomes of both *Natranaerobius* species (genomes of *Natronovirga* and *Natranaerobaculum*
21 are not available). Therefore, this important mode of anaerobic respiration currently can not
22 be assumed as a common trait? in this group. It is also questionable that as much as 20 mM
23 nitrate was used in the tests with the "Natranaerobiiia" members. Our experience with
24 ANCO1 showed, that the complete ammonification is inhibited at far lower nitrate
25 concentrations. Furthermore, the inability of ANCO1 to directly utilize nitrite for DNRA is

1 also intriguing, pointing out that there is a problem. The root of this "sluggishness" might lay
2 in the unusual modularity of the DNRA system in this Gram-positive bacterium. The classical
3 ammonifying nitrite reductase consists of the periplasmic catalytic pentaheme *c* NrfA and its
4 electron donor tetraheme *c* NrfH, which is an integral membrane quinol-dehydrogenase
5 (Simon and Klotz, 2013). In ANCO1, only the NrfA subunit is present, which is also a part of
6 a locus containing a truncated Nap nitrate reductase lacking its cytochrome *c* containing
7 electron donating NapB and NapC. Such deviations from the classical structures might be
8 related to the facts that i) ANCO1 does not have a periplasm, and ii) that it does not have the
9 respiratory quinones, which normally serve as the immediate electron donors for the
10 periplasmically located respiratory complexes. Therefore, the only available low-redox
11 potential electron donors in ANCO1 appears to be produced in the cytoplasm [ferredoxins and
12 NAD(P)H]. This could explain the difficulties with the DNRA growth we observed in
13 ANCO1, particularly that the nitrite reduction was only possible after initiation of growth
14 either by pyruvate fermentation or at fumarate-reducing conditions. This might have been
15 necessary to built up the required low redox potential electron donor pool for nitrate/nitrite
16 reduction. Regarding the ability for DNRA reported previously in the H₂/CO-utilizing
17 acetogens (REFS), it looks like that this is rather complex. At least in two documented cases
18 (*Moorella thermoacetica* and *Clostridium ljungdahlii*) the ammonification of nitrate/nitrite
19 does happen, however, no evidence for genes encoding multicytochrome *c* type of
20 ammonifying Nir (NrfA) are present in the genomes. Instead, the cytoplasmic assimilatory
21 NADH-dependent NirB seems to operate as the electron sink rather resembling "dump of
22 electrons" in fermentative prokaryotes (Emerson et al., 2018; Pierce et al., 2008; Seifritz et al.,
23 2003). Therefore, such process is fundamentally different from the respiratory DNRA.

24

25 **Taxonomy**

1 Based on the phylogenomic analysis and unique phenotypic properties we suggest to classify
2 strain ANCO1 into a new genus and species *Natranaerofaba carboxydovora*, which forms a
3 new family *Natranaerofabaceae* within the class "*Natranarobiia*".

4

5 ***Natranaerofabaceae* fam. nov.**

6 *Natr.an.ae.ro.fa.ba.ce'ae*. N.L. fem. n. *Natranaerofaba* a bacterial genus; *-aceae* ending to
7 denote a family; N.L. fem. pl. n. *Natranaerofabaceae*, the family of *Natranaerofaba*.

8

9 The family includes obligately anaerobic, acetogenic, extremely haloalkaliphilic bacteria
10 living in hypersaline soda lakes. It is a member of class "*Natranarobiia*", phylum *Firmicutes*.

11 The family consists of a single genus and species *Natranaerofaba carboxydovora*. The type
12 genus is *Natranaerofaba*.

13 ***Natranaerofaba* gen. nov.**

14 *Natr.an.ae.ro.fa'ba* N.L. neut. n. *natron*, derived from Arabic *natrun* soda (sodium carbonate);
15 Gr. pref. *an-*, not; Gr. masc. n. *aer*, air; L. fem. n. *faba*, bean; N.L. fem. n. *Natranaerofaba*,
16 bean-shaped soda loving anaerobe.

17

18 The genus includes obligately anaerobic acetogenic bacteria with bean-shaped cells. The polar
19 lipids are dominated by diethers with phosphocholine and phosphoglycerol polar heads. They
20 are extremely halophilic and obligately alkaliphilic moderate thermophiles capable of
21 anaerobic respiration. They can utilize CO and pyruvate during acetogenic growth and
22 formate and lactate in presence of thiosulfate, fumarate or nitrate as electron acceptors. Form
23 a deep-branching lineage within the class "*Natranarobiia*", phylum *Firmicutes*. The type
24 species is *Natranaerofaba carboxydovora*. Family classification: *Natranaerofabaceae*.

25

26 ***Natranaerofaba carboxydovora* sp. nov.**

27

28 *car.bo.xy.do.vo'ra* L. pref. *carboxydum*, carbon monoxide; L. v. *voro*, to devour, consume; L.
29 fem. adj. *carboxydovora*, consuming carbon monoxide

30

31 Cells possess a Gram-positive type of cell wall and are bean-shaped rods, 0.4 x 3-6 μm ,
32 motile by peritrichous flagella. Endospore formation is not observed, but the potential is
33 present in the genome. Polar lipids include phosphocholines and phosphoglycerols, with the
34 most common core components being mostly present as ether-bound C_{14:0} and plasmalogen-
35 derived aiC_{17:0}. Respiratory lipoquinones are not present. The species is strictly anaerobic and
36 heterotrophic acetogen capable of anaerobic respiration. Acetogenic growth is possible with

1 pyruvate and CO as the electron donors with production of acetate/lactate and acetate/formate
2 as products, respectively. Anaerobic respiration is possible with CO, pyruvate, formate and
3 lactate as donors and fumarate and thiosulfate (2-electron reduction) as the electron acceptors.
4 Lactate is converted to acetate. Formate also supports anaerobic growth with nitrate as
5 acceptor resulting in formation of ammonia. Yeast extract is utilized as a C-source. Obligately
6 alkaliphilic with a pH range for growth between 9 and 10.5 and an optimum at pH 9.5-9.7.
7 Extremely salt-tolerant with a total Na⁺ range for growth from 2.5 to 4.5 M (optimum 3.5-4
8 M). Moderately thermophilic with a temperature range of 35-56 °C and an optimum at 48-50
9 °C. The type strain was obtained from anaerobic sediments of a hypersaline soda lake in
10 Kulunda Steppe (Altai region, Russia). DNA G+C is 35.3 mol % (genome). Type strain is
11 ANCO1^T (DSM 24608). The EMBL/GenBank genome accession number is CP054394.

12

13 **Experimental procedures**

14

15

16 **Cultivation conditions**

17 The basic medium used for enrichment and routine cultivation of pure culture was obtained by 1:1
18 mixing of a NaCl and a sodium carbonate bases both containing 4 M total Na⁺ and sterilized separately
19 at 120°C for 40 min. The NaCl base contained (g/l): NaCl 240, KCl 5, K₂HPO₄ 2.5, NH₄Cl 0.5. The
20 pH was adjusted to 7 by titration with 10% KHPO₄ before sterilization. The carbonate base contained
21 (g/l): Na₂CO₃ 190, NaHCO₃ 30, NaCl 16, K₂HPO₄ 1 (pH 10). After sterilization, both bases were
22 supplemented with 1 ml/l each of filter-sterilized vitamin and acidic trace metal stock solutions
23 (Pfennig and Lippert, 1966), 1 ml/l of filter-sterilized alkaline W-Se solution (Plugge, 2007) and 1
24 mM MgSO₄. The alkaline base was also supplemented with 4 mM NH₄Cl from 2 M sterile stock
25 solution. Finally, the two bases were mixed 1:1 in a sterilized flask, resulting in a 4 M Na⁺ Cl-
26 carbonate alkaline medium with a final of pH 9.7. For enrichment of the potential CO-dependent
27 methyl-reducing methanogens, this medium was amended with 50 mM MeOH, 2 mM sodium acetate,
28 100 mg/l yeast extract and 1% (v/v) of autoclaved 1:1 sediment:brine suspension from Bitter-1 soda
29 lake (according to Sorokin et al., 2017). 40 ml complete medium was dispensed into 115 ml serum
30 bottles, supplemented with 0.5 mM of sodium filter-sterilized Na₂S, closed with butyl rubber stoppers
31 and made anoxic by three cycles of vacuum boiling/sterile argon flushing. Finally, the medium was

1 reduced by adding 20 μ l of sterile dithionite solution in 1 M sodium bicarbonate. Sterile CO gas was
2 added at 5-50% in the gas phase on the top of argon. The bottles were inoculated with 1 cm³ of 1:1
3 sediment-brine suspension and incubated statically with periodic hand shaking at 50°C.

4 Cultivation of pure culture was mostly done with the same basic medium, whereby CO and
5 MeOH were replaced by various electron donors with or without electron acceptors. Tests for the Cl⁻
6 dependence was done by either decreasing the proportion of NaCl base to 0, or increasing it up to a
7 maximum of 3.9 M (retaining carbonates at a minimal concentration which allowed sufficient
8 alkalinity for growth). Test for total Na⁺ range for growth was done using 1:1 mixture (based of Na⁺
9 molarity) of NaCl/Na-carbonates, creating a range from 1 to 4.5 M Na⁺ at pH 9.5. The pH range for
10 growth was tested in 3 buffer systems containing 4 M total Na⁺: for the pH from 7 to 8, 50 mM
11 HEPES-50 mM K₂HPO₄/4 M NaCl; for the pH 8-8.5, 4 M NaCl/0.5 M filter-sterilized NaHCO₃; for
12 the pH 9-11, 2 M NaCl/2 M Na-carbonate buffer with different proportions of bicarbonate and
13 carbonate.

15 **Analyses**

16 CO, CO₂, CH₄ and H₂ in the gas phase were quantified by the GC equipped with a methanator and
17 flame ionization and thermal conductivity detectors [Chromateck Crystall 5000 (Ufa, Russia);
18 column Hayesep 80-100 mesh, 2 m x 3 mm, 40 °C; carrier gas Ar]. Nitrite and sulfide were
19 quantified by colorimetric methods (Gries-Romijn-van Eck, 1966; Trüper and Schlegel, 1964). Nitrate
20 was monitored qualitatively using Merck sticks after neutralizing the culture supernatant to pH 7. The
21 organic acids in the liquid phase were analyzed by HPLC. 0.4 ml cell-free culture supernatant was
22 added to 0.6 ml 0.05 M H₂SO₄ solution containing 10 mM DMSO as internal standard. Samples were
23 run on a Shimadzu iSeries Plus (Kioto, Japan), equipped with a MetaCarb 67H column (Agilent
24 technologies, Santa Clara, CA), operated either at 30 or 45°C with 0.005 M H₂SO₄ as eluent at a flow
25 rate of 0.9 ml/min. Both, a UV and RI detector were used for detection of components. Concentrations
26 could be accurately determined down to 0.1 mM.

1 Activity of anaerobic carbon monoxide dehydrogenase (CODH) was measured via enzymatic
2 assays. Cells grown with CO were harvested from a 50 ml late exponential phase culture by
3 centrifugation in anaerobic chamber, the cell pellet was washed with anoxic 0.8 M NaHCO₃ (pH 8.5),
4 cooled on ice and resuspended in 0.5 M NaHCO₃ (pH 8.5) containing 2 mM DTT. The cells were
5 disrupted by sonication with a Bandelin Sonopuls (Berlin, Germany), under anaerobic conditions, and
6 the resulting cell free extract was transferred to rubber stoppered glass vials and kept on ice.
7 Enzymatic assays were performed in quartz cuvettes sealed with a rubber stopper. After placing the
8 rubber stopper, the gas phase was flushed twice with nitrogen to remove oxygen, and a final time with
9 CO. Cuvettes were filled with 0.9 ml 0.8 M NaHCO₃ via syringe and methyl viologen was added at 10
10 mM final concentration. Before start of the assay the absorbance in the cuvette was set between 0.1
11 and 0.3 at 578 nm using drops of 100 mM dithionite. The sample was placed in a pre-heated (50°C)
12 socket in a U-2010 spectrophotometer (Hitachi, Tokyo, Japan). The reaction was started by adding
13 100, 50 or 25 µl cell free extract. The CO-dependent methyl viologen reduction activity was measured
14 at 578 nm, while temperature was kept constant at 50°C. The background activity was determined with
15 N₂ in the headspace instead of CO. Overall background activity was 10 times lower as measured in the
16 samples fed with CO and activity ceased fast (<1 min). Protein concentrations in the cell free extract
17 was analyzed by Roti-Nanoquant protein assay (CarlRoth, Karlsruhe, Germany), according to
18 manufacturer instructions.

19 For the lipid analyses, the cells of pure culture of strain ANCO1 were grown with pyruvate at
20 50°C until late exponential phase, harvested by centrifugation, washed once with 2 M NaCl and freeze
21 dried. The extracts for core lipid analysis were hydrolyzed in HCl/MeOH (1.5 N) by refluxing for 3 h.
22 The hydrolysate was adjusted with aqueous KOH to pH 5, extracted three times with dichloromethane,
23 and dried over Na₂SO₄. Derivatization of the hydrolyzed extract and its analysis were carried out as
24 described previously (Bale et al., 2019b). Briefly, core lipid quantification was carried out on an
25 Agilent Technologies 7890B GC with a silica column (CP Sil-5, 25 × 0.32 mm) using the method
26 described in Bale et al. (2019b). Compound identification was carried out on an Agilent Technologies
27 7890A gas chromatograph (GC) coupled to an Agilent Technologies 5975C VL MSD mass
28 spectrometer (MS), operated at 70 eV, with a mass range m/z 50–800 and a scan rate of 3 scans s⁻¹.

1 The column and oven settings were the same as for the quantification GC analysis. Compounds were
2 identified based on literature data and library mass spectra. Intact polar lipids (IPLs) were extracted
3 from freeze-dried biomass using a modified Bligh-Dyer procedure and analyzed by Ultra High
4 Pressure Liquid Chromatography-High Resolution Mass Spectrometry (UHPLC-HRMS) as described
5 previously (Bale et al., 2019a). The presence of respiratory menaquinones was tested as described
6 previously (Sorokin et al., 2019).

7 For electron microscopy, cells from 1 ml of pure culture grown with pyruvate were harvested
8 by centrifugation, washed once and resuspended in 0.2 ml 2 M NaCl, pH 7 and stained for 1 min in
9 1% (w/v) uranyl acetate. The preparations were examined in JEOL 100 model transmission electron
10 microscope (Japan).

11

12 **Genome sequencing and phylogenomic analysis**

13 Genomic DNA was extracted from cells grown with pyruvate+fumarate using the MasterPure Gram
14 positive DNA extraction kit (Middleton, Wisconsin, USA), according to manufacturer instructions.
15 Genomic DNA was commercially sequenced at Novogene (Beijing, China) using the PacBio
16 technology. Genome was assembled using Flye (version 2.7.1) and checked for completeness and
17 contamination using CheckM (version 1.1.2). The assembled genome was annotated using Prokka
18 (version 1.14.0). The genome was deposited in the GenBank under accession number CP054394. The
19 identity of selected functional proteins (including mostly catabolic and pH-salt homeostasis functions,
20 see **Supplementary Table S1**) from the automatic annotation was verified by using manual Blast in
21 UniProt.

22 For 16S rRNA gene-based phylogeny, sequences were aligned using MAFFT v7.427 (G-INS-i
23 strategy) (Nakamura et al., 2018). For phylogenomic analysis the list of 120 bacterial core genes was
24 taken from Genome Taxonomy DataBase (GTDB) (Parks et al., 2018). These marker genes were
25 identified in selected genomes, aligned and concatenated using GTDBtk v1.2.0 (Chaumeil et al.,
26 2019). Alignment was automatically trimmed using trimAl 1.2rev59 by using automated1 and gt 0.95
27 options (Capella-Gutiérrez et al., 2009). The resulting alignment consisted of 21189 amino acid
28 residues. In both cases phylogenetic tree was built using IQ-TREE 1.6.12 program (Nguyen et al.,

1 2015) with SH-aLRT test (Anisimova et al., 2011) as well as ultrafast bootstrap with 1000 replicates
2 (Hoang et al., 2018) and ModelFinder to determine the best-fit model (Kalyaanamoorthy et al., 2017).

3

4 **Proteomic analysis**

5 For the shot-gun proteomic analysis, strain ANCO1 was grown either with pyruvate or CO as the
6 electron donors and yeast extract as the C-source. 20 mg pelleted cell biomass each (wet weight) were
7 lysed using B-PER reagent/TEAB buffer and bead beating followed by centrifugation at 14,000 x g
8 under cooling to collect protein supernatant. The proteins were precipitated with TCA (20% v/v final)
9 at 4°C followed by washing twice using ice cold acetone. The protein pellet was redissolved in 200
10 mM ammonium bicarbonate containing 6 M urea, reduced in a 10 mM dithiothreitol solution and
11 further alkylated using 20 mM iodoacetamide. The solution was diluted to below 1 M urea and
12 digested using Trypsin at a ratio protease to protein of 1:50. Before analysis, peptides were desalted
13 using an Oasis HLB solid phase extraction sorbent (Waters) according to the manufacturer protocols.
14 An aliquot corresponding to approx. 100 ng protein digest was analysed in duplicates using an one
15 dimensional shot-gun proteomics approach (Köcher et al., 2012). For this, 1 µL of samples were
16 analysed using a nano-liquid-chromatography system consisting of an ESAY nano LC 1200, equipped
17 with an Acclaim PepMap RSLC RP C18 separation column (50 µm x 150 mm, 2µm), and an QE plus
18 Orbitrap mass spectrometer (Thermo). The flow rate was maintained at 300 nL min⁻¹ over a linear
19 gradient from 5% to 30% solvent B over 90 min, and finally to 75% B over 25 min. Solvent A was
20 H₂O containing 0.1% formic acid, and solvent B consisted of 80% acetonitrile in H₂O and 0.1%
21 formic acid. The Orbitrap was operated in data-dependent acquisition mode acquiring peptide signals
22 from *m/z* 350 -1400, where the top 10 signals were isolated at a window of *m/z* 2.0 and fragmented
23 using an NCE of 30. The collected data were analysed against the established proteome database,
24 using PEAKS Studio 8.5 (Bioinformatics Solutions Inc) allowing 20 ppm parent ion mass error
25 tolerance, 3 missed cleavages, carbamidomethylation as fixed and methionine oxidation and N/Q
26 deamidation as variable modifications. Peptide spectrum matches were filtered against 1% false
27 discovery rate (FDR) and protein identifications were accepted as being significant when having 2
28 unique peptides minimum. Relative protein abundances were correlated to protein molecular weight

1 normalized spectral counts (Hongbin et al., 2004; Keiji and Itoh, 2008). The obtained dataset was
2 deposited at the proteomics identification database (PRIDE Archive) and are openly available through
3 the proteome exchange server (<http://www.proteomexchange.org/>, PXD020223;
4 <http://www.ebi.ac.uk/pride>, reviewer account details: reviewer26358@ebi.ac.uk/phJgNecx).

5

6

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14

15

16 **Conflict of interests**

17 The authors declare no conflict of interests

18

19

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1 **Table 1.** Utilization of various electron donors/acceptors by strain ANCO1 during
 2 anaerobic growth at 48°C in a medium containing 4 M total Na⁺, pH 9.7, supplemented
 3 with 500 mg/l of yeast extract as the carbon source.

<i>e</i> -donor	<i>e</i> -acceptor	Growth	Biomass yield increase (times)*	Products from donor	Products from acceptor
CO	-	+		Acetate/formate	-
	Fumarate	+	1.5	Acetate	Succinate (0.5 mM)
	Thiosulfate	+	1.2	nd	HS ⁻ (up to 8 mM)
Pyruvate	-	+		Acetate/lactate/H ₂	-
	Fumarate	+	2.2	Acetate/lactate	Succinate 0.5 (mM)
	Thiosulfate	+	1.6	nd	HS ⁻ (up to 11 mM)
Lactate	-	-			
	Fumarate	w	-	Acetate	Succinate (0.5 mM)
	Thiosulfate	+	-	Acetate	HS ⁻ (up to 12 mM)
Formate	-	-			
	Fumarate	+	-	CO ₂	Succinate (5.7 mM)
	Thiosulfate	+	-		HS ⁻ (up to 4 mM)
	Nitrate/nitrite	+	-		NH ₃ (up to 5 mM)

4 *In comparison to growth without acceptor; nd, not determined;

1 **Table 2:** Relative abundance (% of total) of core lipids released after acid
 2 hydrolysis of cells of ANCO1.

Alkyl carbon number	Alkyl component	Individual %	Carbon number sum %
14	C14:0 FA ¹	1.6	15.5
	C14:0 MGE ²	13.9	
15	<i>iso</i> C15:0 MGE ²	0.3	0.5
	C15:0 MGE ²	0.2	
16	C16:0 FA ¹	2.9	5.8
	C16:0 DA ³	0.3	
	C16:0 MGE ²	2.6	
17	10-Me C16:0 FA ¹	3.9	23.3
	<i>iso</i> + <i>anteiso</i> C17:0 DA ³	17.9	
	<i>iso</i> C17:0 MGE ²	0.7	
	<i>anteiso</i> C17:0 MGE ²	0.6	
	C17:0 MGE ²	0.2	
18	C18:0 FA ¹	2.4	3.1
	C18:0 DA ³	0.2	
	1-OH C18:0 alcohol	0.5	
19	<i>iso</i> C19:0 FA ¹	0.3	1.4
	<i>iso</i> C19:0 DA ³	1.1	
Dialkyls	C29:0 DGE ⁴	0.5	
	C31:0 DGE ⁴	21.1	
	C31:0 Macrocyclic DGE ⁴	1.3	
	C32:0 DGE ⁴	3.1	
	C33:0 DGE ⁴	4.4	
	C34:0 DGE ⁴	12.8	

3 Components formed during acid hydrolysis: ¹fatty acid derived from ester-bound alkyl chains;
 4 ²monoalkyl glycerol ethers, not including glycerol carbons; ³dimethyl acetals derived from
 5 alk-1-enyl ether substituents of plasmalogen lipids; ⁴dialkyl glycerol ethers (sum of two
 6 alkyl chains, not including glycerol carbons). Trace level components ($\leq 0.1\%$) are not given.
 7

1 Legend to the figures

2 **Fig. 1** Cell morphology of strain ANCO1 grown at 4 M total Na⁺, pH 9.7 and 50°C. (**a, b**),
3 phase contrast microphotographs of cells grown with CO and pyruvate, respectively; (**c**)
4 electron microphotograph of a cell showing flagellation.

5
6 **Fig. 2** Phylogeny of ANCO1 based on (**a**) 16S rRNA gene sequence analysis and (**b**) the
7 phylogenomic analysis of 120 bacterial conserved single copy protein coding genes
8 (taxonomic designations correspond to the Genome Taxonomy Data Base) (Parks et al.,
9 2018). Only sequences from the type species were used. The trees were built using IQ-TREE
10 (Nguyen et al., 2015). Bootstrap values above 80% are shown at the nodes. Bar, 0.10 changes
11 per position.

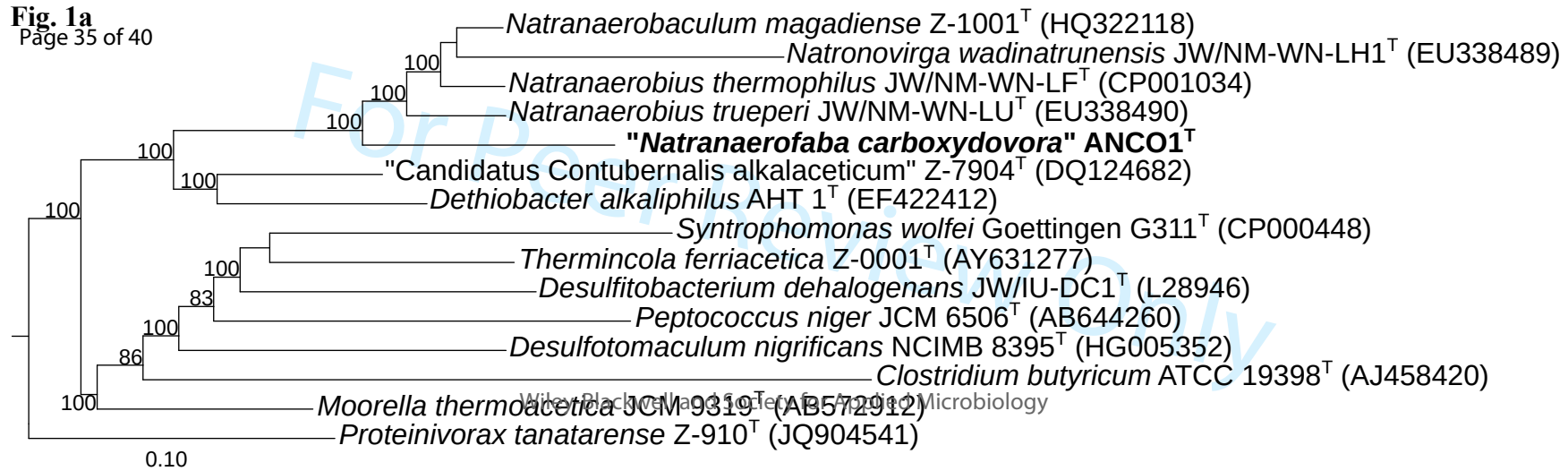
12
13 **Fig. 3** Influence of pH (at 4 M total Na⁺) (**a**) and salinity (at pH 9.5) (**b**) on growth of strain
14 ANCO1 with pyruvate+fumarate at 48°C. The data are mean from parallel duplicate cultures.

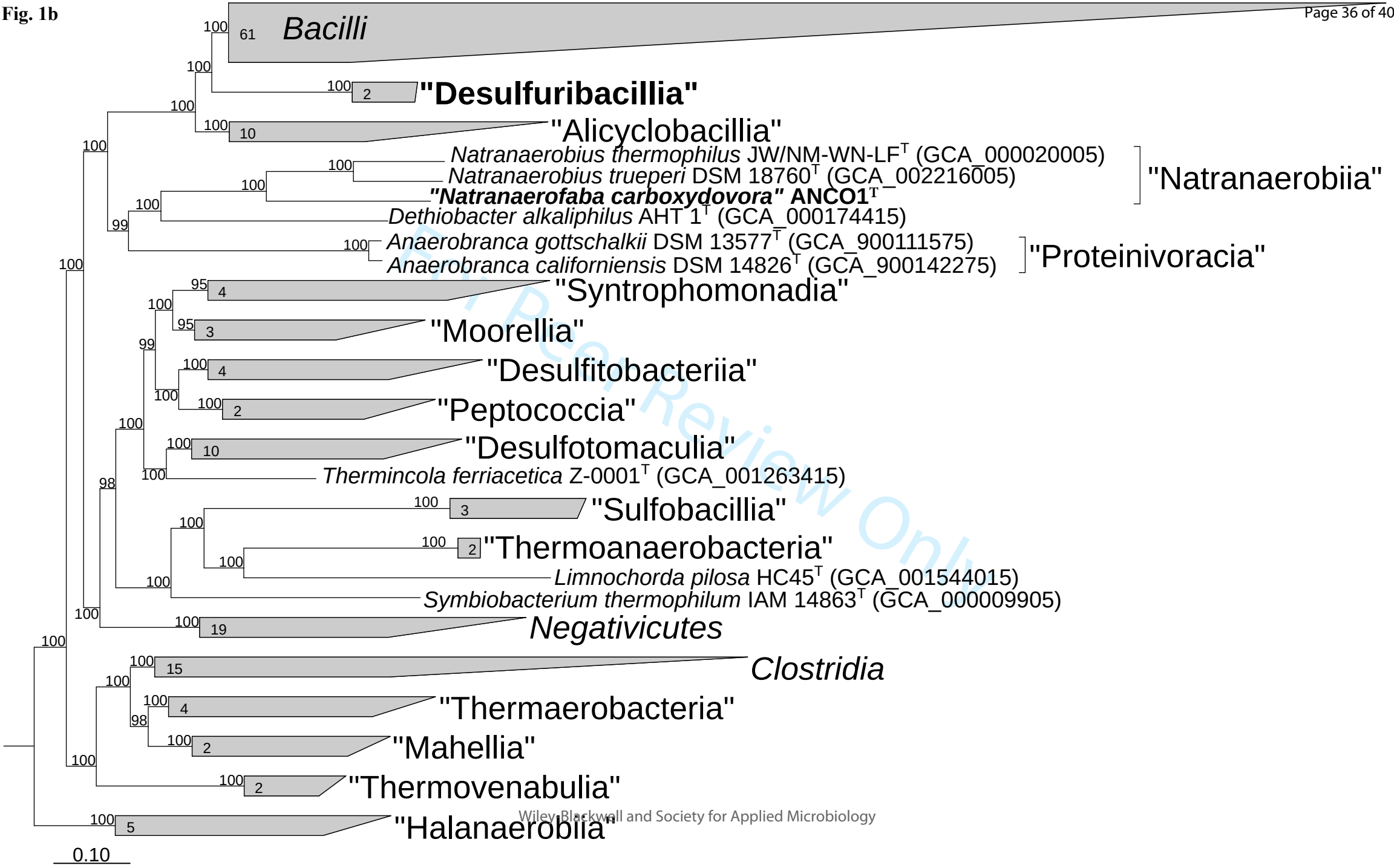
15
16 **Fig. 4** Overview of the metabolism of ANCO1, including associated locus tags. Dashed lines
17 represent multiple reactions. Dotted lines represent putative hydrogenase reactions taking
18 place in the metabolism. Subunits of complexes that are shadowed in grey could not be found.
19 Colors indicate specific pathways: CO-utilization pathways (red), Rnf-complex & ATP-
20 synthase (pink), fumarate respiratory complex (green), nitrate respiration complex (blue),
21 thiosulfate respiration complex (yellow). Abbreviations: CODH: carbon monoxide
22 dehydrogenase; ACS: acetyl-CoA synthase; fdh/HDRC: formate dehydrogenase; formyl-THF
23 synthase; metF : methylene-THF reductase; folD: formyl-THF cyclohydrolase/ methylene-
24 THF dehydrogenase; pta: phospho-acetyltransferase; ack: acetate kinase; acsA: acetyl-CoA
25 synthase; por: pyruvate oxidoreductase; ppd: phosphopyruvate decarboxylase; pps:
26 phosphoenolpyruvate synthase; pkn: pyruvate dehydrogenase; me: malic enzyme; fum:
27 fumarate hydratase; frd: fumarate reductase; sco: succinyl-CoA synthetase; kor: 2-
28 oxoglutarate oxidoreductase; idh: isocitrate dehydrogenase; aco: cis-aconitase; csy: citrate
29 synthase; mmd: methylmalonyl-CoA decarboxylase; gcd: glutaconyl-CoA decarboxylase;
30 mnh: Na⁺/H⁺ antiporter; nrfA: nitrite reductase; phs: thiosulfate reductase.

31
32 **Fig. 5** Organization of the CODH/ acetyl-CoA synthase (ACS) operon in ANCO1 compared
33 to other carboxydrotrophs. Gene abbreviations: *cooC* : CODH chaperone; *acsA* : CODH

1 ; *acs B*: ACS; *acsC* : corrinoid iron-sulfur protein large subunit; *orf7* : unknown; *acsF*: ACS
2 chaperone; *acsD* : corrinoid iron-sulfur protein small subunit; *acsE* : methyltransferase
3 A; *fhs* : formyl-THF synthase; *metF* : methylene-THF reductase; *acoL* : dihydrolipoamide
4 dehydrogenase; *fold* : formyl-THF cyclohydrolase/methylene-THF dehydrogenase.
5

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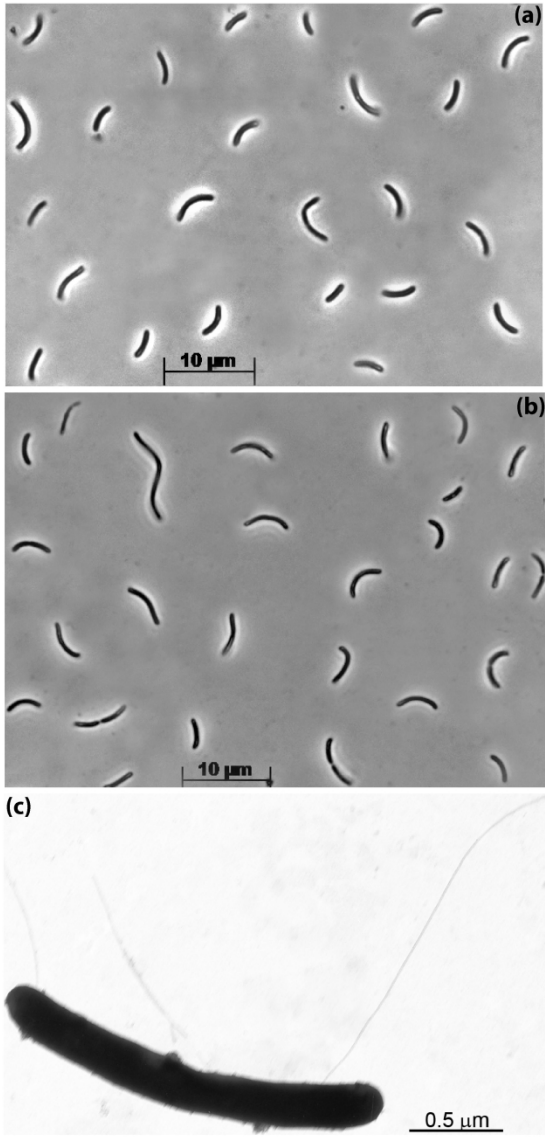


Fig. 2

fig.2

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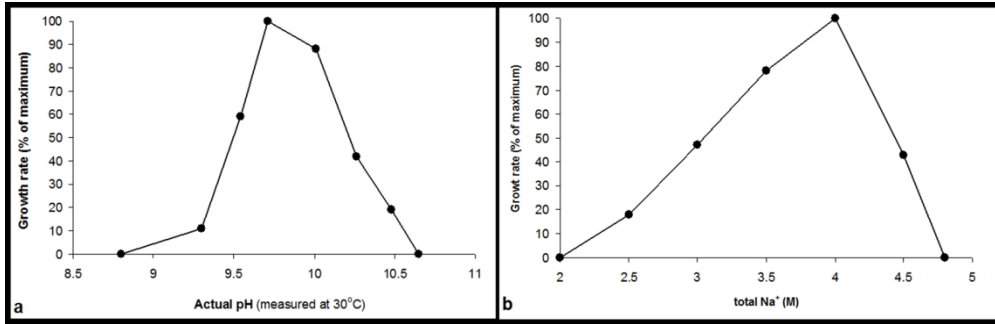


fig.3

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fig.4_rev2

ANCO1

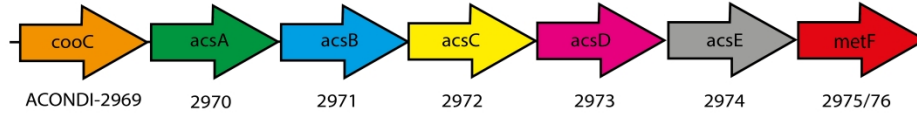
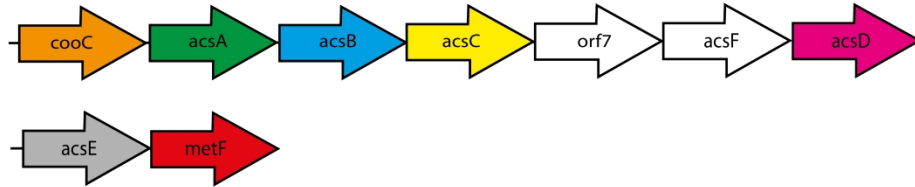
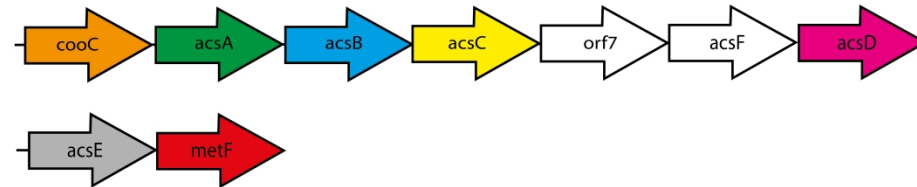
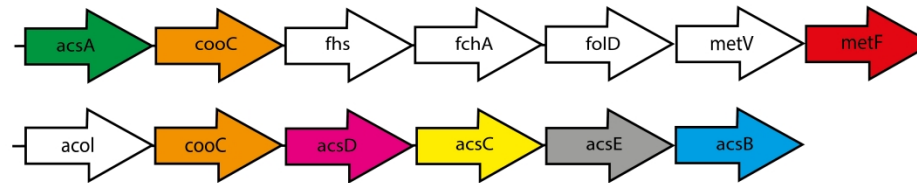
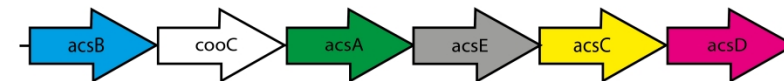
*Moorella thermoacetica**Carboxydothemus hydrogenoformans**Clostridium ljungdahlii**Acetobacterium woodii*

fig.5