

Supplementary Information

Organic matter degradation in the deep, sulfidic waters of the Black Sea: Insights into the ecophysiology of novel anaerobic bacteria

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Running title: Cultivation and physiology of novel piezotolerant bacteria from the Black Sea.

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Microbial community analysis

Firstly, we performed 16S rRNA gene amplicon sequencing to determine the diversity of the collected sulfidic water from 2,000 m depth. This contained 16S rRNA gene sequences affiliated to the phyla *Fusobacteriota*, *Cloacimonadota*, *Planctomycetota*, *Chloroflexota*, *Desulfobacterota*, *Bacteroidota*, *Marinimicrobia*, and *Omnitrophica* (Fig. 1A-i), in good agreement with the microbial diversity previously reported [1–4]. Next, we used different treatments to enrich specific microbes. The growth medium amended with simpler carbon sources like acetate (Fig. 1A-ii) and pyruvate (Fig. 1A-iii) mostly supported enrichment of members of the phyla *Bacteroidota* (55.4 and 84.4%, respectively) and *Desulfobacterota* (41.0 and 11.7%, respectively), while amino acid mixtures supported *Bacteroidota* (79.0%) and alphaproteobacterial members (18.9%; Fig. 1A-iv). A significant increase in the relative abundance of *Cloacimonadota* (~17%) and *Planctomycetota* (~30–42%) was observed in the growth media amended with propionate and chitin, respectively (Fig. 2).

The growth medium containing cellulose as the major carbon source promoted a much greater range of microbial members affiliated with *Bacteroidota*, *Clostridiales*, *Alphaproteobacteria*, *Desulfobacterota*, *Epsilonproteobacteria*, *Spirochaetota*, *Ignavibacteriota*, and *Cloacimonadota* (Fig. 1A-vii-x) (BS1; see M & M for details). Growth of such a wide variety of microbial members was expected, given that the gradual hydrolysis of cellulose gently generates a myriad of carbon sources that function as substrates for diverse physiological groups of microorganisms. Moreover, the gentle release of carbon sources in the medium is advantageous since most of the uncultivated microorganisms are not ready to cope with the sudden exposure of carbon sources [5–7]. Application of a diluted cellulose medium (BS2) incubated at a lower temperature (10°C) and higher sulfide concentration (>2 mM) promoted the growth of an even

broader range of bacteria, including *Ignavibacteriota*, *Cloacimonadota*, *Chloroflexota*, and *Planctomycetota* (Fig. 1A-ix). Further relative increases in the abundance of members of *Ignavibacteriota*, *Cloacimonadota*, and *Planctomycetota* (Fig. 1A-x) were observed in the cellulose enrichments incubated at 10°C at even higher sulfide concentrations (4 mM; BS3 medium). An increase in the abundance of members of these phyla might be possible due to their adaptation to *in-situ* conditions.

Physiology and metabolic properties of the *Psychrilyobacter piezotolerans* strain S5

Psychrilyobacter sp. strain S5 was isolated by repeatedly streaking on cellulose amended medium (see M&M for details; Fig. 1B-i), and it was found to have a 100% 16S rRNA gene sequence similarity with the *Psychrilyobacter piezotolerans* strains SD5^T and BL5 of the phylum *Fusobacteriota* previously isolated from the Black Sea [1] (Fig. S1A). The strain S5 was initially rod-shaped and Gram-negative in growth medium containing 1 mM sulfide (Fig. S1C), but at higher sulfide concentrations (>20 mM) its cell morphology transformed into a spherical shape, as previously described [1]. While strain S5 shared similar physiology and metabolic properties with *P. piezotolerans* strains SD5^T and BL5 [1], it differs from them in its inability to use chitin, differences in optimal growth temperature range (18-23 °C) and lipid profile (Table S42-S43; by relatively lower abundance of hydroxy fatty acids). Based on phenotypic and genotypic analyses this culture could represent as an additional strain of the *Psychrilyobacter piezotolerans*.

Genome characteristics, physiology, and metabolic properties of *Clostridiales* bacteria strains A1^T and A2

The genomes of *Clostridiales* bacteria strains A1^T and A2 were 4,111,109 bp and 4,116,947 bp in length, respectively. These genomes were found to be almost complete and free of contamination (Table S6). The G+C mol% of the A1^T and A2 strains were 42.88% and 42.87%,

respectively. Annotation of the genomes revealed that strain A1^T had 3,889 coding sequences, while A2 had 3,901. No CRISPR repeats were identified in either genome. Based on a BLAST search analysis of the complete 16S rRNA gene sequences (1,541 bp), strains A1^T and A2 showed 90.3% similarity with *Fusibacter paucivorans* SEBR 4211^T of the order *Clostridiales* of the phylum *Bacillota*. The genomes of both strains contained a complete set of genes encoding enzymes of the Embden-Meyerhof glycolytic pathway (Fig. S14). The pentose phosphate pathway was represented only by a non-oxidative branch, as the transaldolase gene was not found. The tricarboxylic acid cycle was incomplete due to the lack of malate dehydrogenase and was likely only used for biosynthetic purposes. Interestingly, an upper TCA cycle metabolite 2-oxoglutarate was a key intermediate in the biosynthesis of some amino acids. Both strains possessed all the genes involved in the 2-C-methyl-D-erythritol4-phosphate/1-deoxy-D-xylulose 5-phosphate (MEP/DOXP) pathway of isoprenoid biosynthesis (Fig. S14). Hydrogen was a major fermentation product in both strains and may be produced by [FeFe]-hydrogenase, which is known to couple the oxidation of reduced ferredoxin to the evolution of H₂ during carbohydrate and protein fermentation[8]. The reversible electron transfer between NADP(H) and ferredoxin could be performed by ferredoxin-NADP⁺ reductase. The presence of V-type ATPase indicates that strains A1^T and A2 may rely on substrate-level phosphorylation for ATP production.

Both strains (A1^T and A2) were strict anaerobic bacteria and did not show catalase and oxidase activity, which is consistent with their anaerobic lifestyle. However, they were able to tolerate air exposure for about an hour, indicating some level of aerotolerance. This property was supported by the presence of genes coding for superoxide reductase, superoxide dismutase, rubredoxin, and rubrerythrin that might help in oxygen detoxification. Our observation suggests that aerotolerance in these strains could be an adaptation to neutralize oxygen exposure in the

upper water column of the Black Sea. The strains could use thiosulfate and sulfur as terminal electron acceptors, but not sulfate. They were unable to oxidize sulfide but were able to grow at sulfide concentrations up to 7 mM at pH 7.0, which is 17.5-fold higher than the natural concentration. Under strict anaerobic conditions, both strains were able to grow at hydrostatic pressures up to 50 MPa. However, growth at higher pressures (>20 MPa) was slower and the doubling time was extended to 8-10 hours at 30 MPa. This observation suggests that these strains are adapted to the extreme conditions of the deep sulfidic waters of the Black Sea.

Genome characteristics, physiology, and metabolic properties of novel species of the phylum *Spirochaetota* (strains M1^T, M2 and S2)

Oceanispirochaeta sp. strains M1^T and M2 have similar genome sizes of 5.88 and 5.87 Mbp, respectively, with genome completeness of 99.73% and contamination levels of 3.73% and 4.13%, respectively (Table S6). Both strains have a G+C content of 42.89% and 56 tRNA and 98 CRISPR repeats. Strain M1^T has 5440 CDS with 2333 functional proteins, while strain M2 has 5495 CDS with 2,120 functional proteins. *Sphaerochaeta* sp. strain S2 has a genome size of 3.1 Mbp with a genome completeness of 98% and 1.2% contamination (Table S6). It has a G+C content of 46.87%, 43 tRNA, 6 rRNA, and 14 CRISPR repeats, with 3368 genes, of which 1,589 have functional assignments.

All three strains preferentially fermented glucose for their growth (Table S1, S3), which was further supported by the presence of relevant genes in their genomes (Fig. S18). While the strains possessed enzymes for the hydrolysis of polysaccharides like starch (glucoamylase), they could not utilize starch or cellobiose for their growth (Table S1). *Sphaerochaeta* sp. strain S2 stopped growing during repeated subculturing under laboratory conditions; therefore, we present the relevant findings of organic matter degradation for this strain under the Candidatus option.

The genomes of all three strains contained a complete set of genes encoding enzymes of the Embden-Meyerhof glycolytic pathway (Fig. S18). However, the pentose phosphate pathway is incomplete, lacking the transaldolase gene and only having a non-oxidative branch. The tricarboxylic acid cycle is also incomplete due to the absence of the malate dehydrogenase gene, and it is likely used only for biosynthetic purposes. The preferred carbon source for all three strains was pyruvate, as evidenced by their ability to utilize it for growth and the presence of pyruvate-formate lyase and pyruvate-ferredoxin oxidoreductase genes in their genomes (Fig. S18). Lactate dehydrogenase was also present, indicating that lactate could be produced by fermentation, along with acetate. The cytoplasmic [FeFe] group A hydrogenase can couple the oxidation of reduced ferredoxin generated by pyruvate-ferredoxin oxidoreductase to the reduction of protons to hydrogen during fermentative growth. V-type ATPase was found, indicating that these strains rely on substrate-level phosphorylation for ATP production. They can hydrolyze protein (casein) and ferment aspartate and glutamate for growth, as supported by the presence of peptidase and amino acid fermentation genes in their genomes (Fig. S18). However, aerobic, or anaerobic respiration is not possible due to the absence of related genes in the genomes. Hydrogen is a major fermentation product in all three strains, likely produced by [FeFe]-hydrogenase, which is known to couple the oxidation of reduced ferredoxin to the evolution of hydrogen during carbohydrate and protein fermentation [8].

All three strains were found to be Gram-negative bacteria (Fig. S3) and exhibited negative reactions for oxidase and catalase activities. They were able to tolerate oxygen exposure for up to 30 minutes, and the presence of superoxide reductase genes in strains M1^T and M2 supported this observation. Strain S2, on the other hand, contained catalase and superoxide dismutase genes in addition to the aforementioned genes (Fig. S18). All three strains also contained genes encoding

rubredoxin and rubrerythrin, which could act as oxygen scavengers. None of the strains could utilize sulfate, sulfite, thiosulfate, nitrate, or elemental sulfur as electron acceptors, as evidenced by the absence of the corresponding genes in their genomes. Sulfide oxidation was not possible in any of the strains. However, they were able to grow at sulfide concentrations of up to 7 mM at pH 7.0, which was 17.5-fold the natural concentration. Under strict anaerobic conditions, all three strains were able to grow at hydrostatic pressures of up to 50 MPa. However, they exhibited weak growth at elevated hydrostatic pressures (>20 MPa), and their doubling time was extended to 8-10 hours at 30 MPa (strain M1^T; Fig. 4C).

Genome characteristics, physiology, and metabolic properties of novel members of the phylum *Bacteroidota* (strains S6, L6, B1^T, B2, M2P and SYP)

We have isolated six bacterial strains (S6, L6, B1^T, B2, M2P and SYP) affiliated with phylum *Bacteroidota* from cellulose enrichments. The genome size of *Lentimicrobium* sp. strain S6 was 5,722,269 bp with a genome completeness of 98.92%, 2.24% contamination, and a G+C content of 35.04%. In comparison, *Lentimicrobium* sp. strain L6 had a smaller genome size of 5,144,386 bp with a genome completeness of 96.24%, 1.70% contamination, and a G+C content of 35.03%. Based on a BLAST search analysis of the complete 16S rRNA gene sequences, strains S6 and L6 showed 88.7% similarity with *Lentimicrobium saccharophilum* TBC1^T, which belongs to the family *Lentimicrobiaceae* of the phylum *Bacteroidota*. Due to the extremely slow growth of strains S6 and L6, we could delay the deposition of these strains to recognized culture collections. However, due to the high relevance of the findings reported here for OM degradation, we chose to use the *Candidatus* option for strain S6.

Lutibacter sp. strain B1^T had a genome size of 3,428,913 bp with a genome completeness of 97.65%, 1.10% contamination, and a lower G+C content of 30.27%. It had 3110 genes and 1929 proteins with functional assignments, along with 1,251 hypothetical proteins, 39 tRNA, and 4

rRNA. Interestingly, it also had 31 CRISPR repeats. *Lutibacter* sp. strain B2 had a slightly smaller genome size of 3,240,965 bp with a genome completeness of 97.16%, 0.35% contamination, and a G+C content of 30.83%. It had 3,155 CDS and 1,759 proteins with functional assignments, along with 1396 hypothetical proteins and 86 tRNA. One copy of 16S rRNA gene sequences was identified in the genome of strains B1^T and B2. 16S rRNA gene sequence obtained from the draft genome sequence was identical to the one retrieved with the 16S rRNA gene PCR on the DNA extracted from the strain B1^T and B2. 16S rRNA gene sequence similarity between strains B1^T and B2 was 100%. Strains B1^T and B2 showed 96.25% 16S rRNA gene sequence similarity with *Lutibacter profundus* LP1^T and <95.8% with other members of the genus *Lutibacter*.

Ancylomarina euxinus strain M3P had a genome size of 4,351,206 bp with a genome completeness of 100%, no contamination, and a higher G+C content of 35.8%. It has 3,643 genes and 1,855 proteins with functional assignments, along with 1788 hypothetical proteins, 85 tRNA, and 8 rRNA. Like *Lutibacter* sp. strain B2, it has 22 CRISPR repeats.

The genomes of all six strains contain the genes involved in both the 2-C-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate (MEP/DOXP) and mevalonate pathways of isoprenoid biosynthesis (Fig S25). Growth of all six strains was observed in medium amended with either or both fosmidomycin (a pathway inhibitor of the MEP pathway) and simvastatin (a pathway inhibitor of the mevalonate pathway), indicating that both pathways are functional for isoprenoid biosynthesis. Under a microscope, flagellar motility was observed, and all six strains grew chemoheterotrophically. Fermentation of various mono- and disaccharides (such as glucose, galactose, xylose, and maltose) as well as pyruvate, lactate, and glycerol were observed (Table S8-S9, S12). Genes for central metabolic pathways, including glycolysis, the TCA cycle, and the reductive TCA cycle, were found in the genomes, supporting the capability of the strains to ferment

glucose. However, autotrophic growth was not observed in physiological tests using H₂/CO₂ as an electron donor and carbon source. Propionate was likely formed via the methylmalonyl-CoA pathway. The ability to use pyruvate as a substrate was confirmed by the presence of a pyruvate-formate lyase, which yields acetate and formate, and a pyruvate-ferredoxin oxidoreductase, which yields acetate, CO₂, and H₂ (Fig. S25). Furthermore, fermentation of lactate and maltose was confirmed by the presence of genes encoding their respective degradation pathways.

The physiological behavior toward oxygen differed among the strains. Strains B1^T and B2 preferentially grew under microaerophilic conditions, while strains S6, L6, M2P, and SYP grew well under strict anaerobic conditions. Moreover, the genomes of strains B1^T and B2 encoded a cbb3-type cytochrome c oxidase, which is comprised of three subunits and cytochrome c oxidase. These enzymes function as terminal oxygen reductases and have been suggested to be associated with microaerophilic growth[9,10]. Genes for oxygen detoxification (i.e., superoxide dismutase, catalase, and cytochrome c551 peroxidase) were identified in the genomes of all six strains tested (Fig S25). Reduction of sulfate to sulfide, nitrate to nitrite or nitrite was not observed in the presence of glucose in all the tested strains. All the isolated strains utilized glucose, pyruvate, acetate, threonine, lysine, glutamate, and aspartate (Table S4-S10, S12) for growth. However, no growth was observed on cellulose, cellobiose, and chitin. No strains used sulfate, sulfite, thiosulfate, nitrate, fumarate, and elemental sulfur as an electron acceptor. They were also able to grow at sulfide concentrations up to 9 mM at pH 7.0, which was 22.5-fold the natural concentration. All strains were able to grow up to 50 MPa of hydrostatic pressure (Fig. 5D-5E), but they grew weakly when the hydrostatic pressure was increased beyond 20 MPa.

Genome characteristics, physiology, and metabolic properties of novel members of the phylum *Desulfobacterota* (strains S3^T and S3-i)

We have isolated two bacterial strains (*Pseudodesulfovibrio* sp. strains S3^T, and S3-i;) affiliated with phylum *Desulfobacterota*. Whole genome sequencing of strains S3^T and S3-i yielded genomes of 3,706,001 bp and 3,705,660 bp in length, respectively after assembly (Table S6). The G+C mol% of strains S3^T and S3-i was 56.6 and 56.60%, respectively. Annotation of the genome of strain S3^T and S3-i indicated 3,612 and 3,607 coding sequences, respectively. One copy of 16S rRNA gene sequences was identified in the genome of strains S3^T and S3-i. 16S rRNA gene sequence obtained from the draft genome sequence was identical to the one retrieved with the 16S rRNA gene PCR on the DNA extracted from the strain S3^T and S3-i. Based on BLAST search analysis of the 16S rRNA gene sequences, strains S3^T and S3-i showed 97.4% similarity with *Pseudodesulfovibrio indicus* J2^T of the family *Desulfovibrionaceae* of the phylum *Desulfobacterota*.

Strains S3^T and S3-i were sulfate-reducing bacteria grew on a limited number of organic substrates, with lactate, pyruvate, and acetate (Table S5). In support of such activities, secreted glycoside hydrolases were not detected in the genomes of these strains. The glycolytic pathway probably operates in the direction of gluconeogenesis, as indicated by the presence of genes coding phosphoenolpyruvate synthase and fructose-1,6-bisphosphatase which specifically perform the reverse reactions (Fig. S24). Pyruvate could be reversibly decarboxylated to acetyl-CoA by pyruvate: ferredoxin oxidoreductase. Furthermore, conversion of acetyl-CoA to acetate with the production of ATP can be performed by acetyl-CoA synthetase. Oxidation of lactate to pyruvate was probably facilitated by putative lactate dehydrogenases (LDH). The tricarboxylic acid (TCA) cycle in strains S3^T and S3-i are incomplete, lacking citrate synthase and succinyl-CoA synthetase. This finding is consistent with the observed inability of strains S3^T and S3-i to oxidize organic

substrates completely. For example, growth of strain S3^T and S3-i with formate was only possible in the presence of acetate as an auxiliary carbon source, consistent with the absence of known pathways for autotrophic C1 fixation. In particular, the Wood-Ljungdahl (reductive acetyl-CoA) pathway, frequently used by autotrophic sulfate reducers, is incomplete, lacking carbon monoxide dehydrogenase/acetyl CoA synthase.

The genomes of S3^T and S3-i contain all genes necessary for dissimilatory sulfate reduction (Fig. S24). Another linkage of sulfate-reduction enzymes to the membrane is enabled by the sulfite reductase-associated electron transfer complex DsrMKJOP with subunits DsrM and DsrP containing transmembrane domains. Four hydrogenases of the [NiFe]-family and one formate dehydrogenase are encoded by the strains S3^T and S3-i genomes. Consistently, like other members of the family *Desulfovibrionaceae*, S3^T and S3-i grew with H₂ as an energy source in the presence of acetate as a carbon source. The first hydrogenase is encoded by eight-gene operon. The [NiFe] uptake hydrogenase, could oxidize H₂, by donating the electrons to the quinone pool via the third cytochrome *b* subunits linking them to the cytoplasmic membrane. The electron transfer from this soluble periplasmic complex to the cytoplasmic membrane may be facilitated by a pool of *c*-type cytochromes present in the periplasm [11,12]. This electron transport pathway probably ends at the membrane linked Hmc complex [13]. Cytochrome *c*, HmcA, may accept electrons from periplasmic cytochromes. Genes coding for cytoplasmic hydrogen: heterodisulfide oxidoreductase, consisting of CoB-CoM heterodisulfide reductase (HdrACB) and methyl viologen-reducing hydrogenase (MvhDGA) were also detected which catalyzes the endergonic reduction of ferredoxin and the exergonic reduction of heterodisulfide, coupled to H₂ oxidation by electron bifurcation involving HdrA [14].

254 Genomes of strains S3^T and S3-i also contained four subunits of soluble cytoplasmic
255 hydrogenase. These hydrogenases are bidirectional and can re-oxidize the cofactors by using
256 protons as electron acceptors [15]. The presence of a NADPH-binding motif suggests that this
257 hydrogenase can use NADPH in hydrogen turnover reactions. The presence of formate
258 dehydrogenase explains the observed ability of strains S3^T and S3-i to use formate as an electron
259 donor. The presence of a N-terminal Tat signal peptide in FdhA suggests that it is in the periplasm.
260 Like the periplasmic uptake hydrogenase, formate dehydrogenase lacks a membrane subunit, and
261 electron transfer to the membrane is probably performed via the periplasmic cytochromes and Hmc
262 complex. Genomes of strains S3^T and S3-i also contain several other membrane-linked
263 oxidoreductases that can contribute to the generation of transmembrane ion gradient and/or the use
264 of alternative electron acceptors to sulfate. Two putative complexes similar to the bacterial
265 NADH:quinone oxidoreductase are present. Both comprise the subunits like NuoA, B, C, D, H, I,
266 J, K, L, M, and N, while the genes for the subunits NuoEFG that form the NADH dehydrogenase
267 module are missing, indicating that NADH is likely not an electron donor. The first cluster is linked
268 to genes coding for two subunits of CISM oxidoreductases of the Psr/Psh family[16]: the
269 molybdopterin-binding catalytic subunit A and the iron-sulfur electron transfer subunit B. It is
270 possible that such an arrangement indicates coupling of transmembrane proton transfer, performed
271 by the core subunits of NADH: quinone oxidoreductase, with the oxidation or reduction of sulfur
272 compounds. Oxidation of pyruvate produces reduced ferredoxin that could provide electrons to
273 this oxidoreductase. Genes encoding molybdopterin family oxidoreductase consisting of all three
274 subunits: A, B and membrane subunit C of the NrfD family. The catalytic A subunit was predicted
275 to contain a N-terminal Tat signal peptide and is phylogenetically related to thiosulfate or
276 polysulfide reductases. The presence of putative thiosulfate reductase, capable of producing sulfide

and sulfite from thiosulfate, explains the ability of strains S3^T and S3-i to use thiosulfate as an electron acceptor.

Despite the observed inability of strains S3^T and S3-i to grow by nitrite reduction, its genome contains cytochrome *c* nitrite reductase [17], comprising large NrfA and small NrfH subunits with five and four hemes, respectively. The presence of an N-terminal signal peptide in the large subunit suggests that this complex faces the periplasmic side of the membrane. The physiological role of nitrite reductase could be detoxification of nitrite, which is known as an inhibitor of sulfate-reducing organisms [18]. A similar function in detoxification of oxygen could be assigned to the cytochrome *bd* ubiquinol oxidase. Furthermore, all three strains were able to grow at sulfide concentrations up to 9 mM at pH 7.0, which was 22.5 -fold of the *in-situ* environmental conditions. All strains were able to grow up to 50 MPa of hydrostatic pressure. A significant improvement in the growth was observed in the glutamate amended medium at elevated hydrostatic pressure (30 MPa; Fig. 5F).

Genome characteristics and metabolic properties of *Mycoplasmata* bacterium strain Izemo-BS

The MAG of '*Ca Atrimarinoplasma cellobiosiphila*' strain Izemo-BS was 2,431,619 base pairs in size and has a genome completeness of 97.33%. The MAG has a low contamination rate of 1.3% and the G+C content was 31.1%. The genome contains 2,233 protein-coding genes, and out of these, 930 have functional assignments, while 1,303 are hypothetical proteins. The MAG contains 33 tRNA genes that are responsible for translating genetic information into proteins. Various genes e.g., endoglucanase, alpha-amylase (EC 3.2.1.1), amylomaltase, beta-glycosyl hydrolase, glycosyltransferase was detected in Izemo-BS which are involved in the hydrolysis of polysaccharides (cellulose and starch) supporting the capabilities to obtain energy by hydrolyzing the polysaccharides. Furthermore, the genes responsible for the degradation of cellobiose

(cellobiose phosphorylase) was also identified. Various genes involve in the glucose fermentation (Fig. S16) are also present indication fermenting lifestyles in the sulfidic waters. Various genes encoding endonuclease, exonuclease, and extracellular ribonuclease are also detected (Fig. S16) in the MAG which further which is in line with the earlier report [19,20]. Genes involved in the glycolysis were also present, however, complete absence of electron transfer chain involved in the anaerobic respiration indicates that Izemo-BS obtain their energy through the degradation of DNA and sugars. Largely, the various genes detected in genome indicated that Izemo-BS might be actively involved in the degradation of DNA and simpler carbon sources like cellobiose; a component of the organic matter sinking from upper oxic zones of Black Sea.

Genome characteristics and metabolic properties of *Cloacimonadota* bacterium strain Cloa-SY6

The MAG of the *Cloacimonadota* bacterium strain Cloa-SY6 was 3,336,172 bp in size. The genome is estimated to be 98.84% complete with low contamination (1.1%). The G+C content of the genome is 34.31%. The genome contains 2,981 protein-coding genes, of which 1,111 have functional annotations and 1,870 are hypothetical proteins. There are 184 clustered regularly interspaced short palindromic repeats (CRISPRs) in the genome, which suggests that the bacterium may have the ability to defend against viral attacks. The genome also encodes 55 transfer RNA genes a complete set of ribosomal RNA gene operon. The 16S rRNA gene sequence (1535 bp) is identical to that obtained from 16S rRNA gene amplicon analysis. The Cloa-SY6 strain has 82.72%, 83.8% and 81.9% 16S rRNA gene similarities with '*Ca* Cloacamonas acidaminovorans' strain Evry, '*Ca* Syntrophosphaera thermopropionivorans', and bin40 (16S rRNA gene sequence recovered from the bin40; *Cloacimonadota* MAG obtained from the Black Sea) respectively (Fig. S8A). Comparative analysis of Cloa-SY6 along with '*Ca* Cloacamonas acidaminovorans', '*Ca* Syntrophosphaera thermopropionivorans', and other MAGs obtained from the Black Sea (bin40,

bin80, bin85 and bin108) showed distinct differences (Table S8). The genome sequence of Cloa-SY6 is relatively larger than that of '*Ca* Cloacamonas acidaminovorans', '*Ca* Cloacamonas acidaminovorans', '*Ca* Syntrophosphaera thermopropionivorans', and other MAGs obtained from the Black Sea (bin40, bin80, bin85 and bin108)[21] indicating their independent nature with respect to various biosynthetic pathways (Fig. S21).

Members of the phylum *Cloacimonadota* have been frequently observed in both engineered and natural habitats and are mostly known for their involvement in sugar transformation[21–30]. However, the MAG of Cloa-SY6 contained a limited number of 64 CAZymes, most of which are GTs (n=45) involved in the initiation and elongation of glycan chains[31]. Only 11 CAZymes were classified as GHs, indicating limited carbohydrate utilization capability. While the increased abundance in the propionate medium suggests a preference for propionate, like '*Ca* Syntrophosphaera thermopropionivorans'. Moreover, genome of Cloa-SY6 encoded most of the genes involved in the amino acid biosynthesis (Fig. S21) which further reflects their independent nature. In contrast, *Cloacimonadota* MAGs (bin40, bin80, bin85 and bin108) obtained earlier[21] from the Black Sea lacks several genes involved in the amino acid biosynthesis.

Cloa-SY6 encodes both a rudimentary respiratory and fermentative pathway for energy generation. In the absence of a canonical electron transport chain (ETC) for generating a membrane potential, we assume that Rnf electron transport complexes are likely sources of a transmembrane ion gradient[32] in Cloa-SY6. Various enzymes related to anaerobic lifestyles were detected in the MAG of Cloa-SY6, including ribonucleoside triphosphate reductase, ferredoxin oxidoreductases, and radical S-adenosylmethionine-dependent proteins, indicating that Cloa-SY6 is well adapted to the permanently anoxic conditions of the Black Sea. However, the presence of genes related to

microaerophilic growth (such as superoxide reductase; EC 1.15.1.2), ruberythrin, and thioredoxin reductase (EC 1.8.1.9) in Cloa-SY6 suggests an adaptation to survive in the suboxic zones of the Black Sea.

Cloa-SY6 differed from previously reported MAGs due to its relatively larger genome size, presence of various genes involved in amino acid biosynthesis, and lack of many CAZymes. Additionally, it exhibits low 16S rRNA gene sequence similarity and have distinct phylogenomic differences (Fig. S8A-B), which suggests that Cloa-SY6 belongs to a novel taxon at the order level within the phylum *Cloacimonadota*.

Genome characteristics and metabolic properties of *Planctomycetota* bacterium strain Plnct-SY6

We recovered a high-quality metagenome-assembled genome (MAG) affiliated with this phylum, as shown in Table S3. The MAG was 5,418,934 base pairs in size and had a genome completeness of 97.66%, indicating that most of the expected genes were present in the genome. The genome had a low contamination rate of 1.14%. The G+C content of the genome was relatively high at 41.35%. The MAG contained 4,562 protein-coding genes, out of which 1,332 had functional assignments, while 3,230 were hypothetical proteins whose function was not yet known. The genome also contained 47 tRNA genes that were responsible for translating genetic information into proteins. A complete 23S rRNA gene was present in the genome sequence, showing 83.8% similarity with *Phycisphaerae* bacterium ST-NAGAB-D1 of the order *Sedimentisphaerales* in the phylum *Planctomycetota*. Phylogenetic analysis confirmed their affiliation with an uncultivated clade of this phylum thriving in deep marine habitats (Fig. S7A). Additionally, the genome of the '*Ca Atrisphaera chitinolytica*' strain Plnct-SY6 had 79 CRISPR repeats, which are commonly found in bacterial genomes and play a crucial role in the immune system of bacteria against phage infection.

The genes encoding metabolic pathways common for chemoorganotrophic bacteria, such as glycolysis, the citrate cycle, the pentose-phosphate pathway, and oxidative phosphorylation are present. The Plnct-SY6 has the genomic potential for synthesis of all amino acids. Two fructose-type sugar-specific subunits of the phosphotransferase system could be found in Plnct-SY6. The survey for genes related to cell division revealed that the FtsZ-encoding gene was absent, while two copies of the gene coding for DNA translocase FtsK were present in the genome of Plnct-SY6. The gene encoding the key enzyme for synthesis of N-methylated phosphorus-free ornithine membrane lipids, N-methyltransferase (OlsG), is identified in the genome of Plnct-SY6 which might help their growth under phosphate limiting conditions. Genomic analysis revealed the presence of the major components of electron transfer chain i.e., proton-translocating NADH-dehydrogenase complexes, a membrane-bound succinate dehydrogenase/ fumarate reductase, isoprenoid quinones, and a F₀F₁-type bacterial ATP synthase.

Genome characteristics and metabolic properties of *Ignavibacteriota* bacterium strain Igna-SY6

The size of '*Ca* Ponteuxinibacter sulfidophilus' strain Igna-SY6 was 4,731,990 base pairs, with a genome completeness of 100%. The G+C content of the genome was 32.95%. The genome contained 3,922 protein-coding genes, out of which 1,809 had functional assignments, while 2,113 were hypothetical proteins whose function was not yet known. The genome also contained 41 tRNA genes responsible for translating genetic information into proteins. Additionally, the MAG of '*Ca* Ponteuxinibacter sulfidophilus' strain Igna-SY6 has 112 CRISPR repeats, which are commonly found in bacterial genomes and play a crucial role in the immune system of bacteria against phages and other invading elements.

The Igna-SY6 genome encodes a complete set of genes for glycolysis, the TCA cycle, and gluconeogenesis which indicates that it can grow with glucose or other oligosaccharides as sole

399 carbon source (Fig. S22). The genome also includes genes for glycogen synthase and glycogen
400 phosphorylase, which suggests that glycogen is its major storage compound. Genes for
401 polyhydroxyalkanoate (PHA) synthesis and degradation were not detected, hence, it is assumed
402 that Igna-SY6 probably produces acetate and L-lactate as the main products when growing
403 fermentatively. Common pathways for fermentative production of propionate (via methylmalonyl-
404 CoA carboxyltransferase), ethanol (via alcohol dehydrogenase), and formate (via pyruvate formate
405 lyase) are present which indicate the fermentative growth mode. Igna-SY6 possesses genes
406 encoding two CO₂ fixing enzymes pyruvate: ferredoxin oxidoreductase (PFOR) and 2-
407 oxoglutarate: ferredoxin oxidoreductase (OFOR), which are essential for autotrophic CO₂ fixation
408 in green sulfur bacteria [33]. Because the glyoxylate cycle is not present, PFOR is probably
409 essential for the assimilation of acetate by carboxylation of acetyl-CoA to pyruvate. The Igna-SY6
410 genome encode citrate lyase, which is a key enzyme required for autotrophic CO₂ fixation by the
411 reverse TCA cycle [34]. This enzyme catalyzes the cleavage of citrate to acetate and oxaloacetate,
412 and it is involved in citrate fermentation in some organisms [35]. The operation of the reverse TCA
413 cycle for CO₂ assimilation would also depend upon the availability of electron sources to produce
414 reduced ferredoxin. The genome of Igna-SY6 includes genes necessary to take advantage of some
415 potential electron sources and to produce reduced ferredoxin from them. Thus, the gene repertoire
416 Igna-SY6 shows that the organism can probably grow mixotrophically. The Igna-SY6 genome
417 encodes different ferredoxins and a variety of electron transfer complexes, including the RNF
418 (Na⁺-translocating ferredoxin:NAD⁺ oxidoreductase) complex [36], two type-1 NADH
419 dehydrogenase complexes, and alternative complex III (ACIII). The presence of such a broad array
420 of electron transfer complexes likely reflects an ability of Igna-SY6 to utilize the different electron
421 carriers used by various redox enzymes as well as the various terminal electron acceptors that

might be available *in-situ* conditions of the Black Sea. Genome sequence of Igna-SY6 does not encodes the photosynthetic apparatus to produce the reduced ferredoxins required for carbon fixation by the reverse TCA cycle.

The Igna-SY6 has three different oxygen-dependent terminal oxidases, including cbb3-type heme-copper cytochrome c oxidases and two different cytochrome bd-quinol oxidases (Fig. S25). Both types of terminal oxidases could participate in aerobic respiration and/or protection against reactive oxygen species [37]. The cbb3 cytochrome oxidase and cytochrome bd-quinol oxidase typically have much higher affinity for O₂ than the caa3 cytochrome oxidase[38], and because of this, they are frequently involved in protecting anaerobes from reactive oxygen species. The presence of all three types of complexes in Igna-SY6 strongly suggests that Igna-SY6 experiences varying O₂ concentrations in-situ conditions of the Black Sea. The presence of these different terminal oxidases in Igna-SY6 would confer not only the ability to respire under oxic conditions but also the ability to protect oxygen-sensitive enzymes such as hydrogenase under microoxic conditions. 16S rRNA gene amplicon data analysis indicated that members of the phylum *Ignavibacteriota* are also present in the suboxic zone of the Black Sea. Genome of Igna-SY6 has genes encoding both catalase and superoxide dismutase, which protect organisms exposed to oxygen from reactive oxygen species. The genome of Igna-SY6 also encodes an oxygen-dependent protoporphyrinogen oxidase for heme biosynthesis, catabolic enzymes pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase, which are typically found in aerobes. The presence of these genes in Igna-SY6 might be an adaptation to survive under oxic conditions. The genome of Igna-SY6 also encode two different [FeFe]-hydrogenases. Fe-only hydrogenases are often associated with H₂ evolution [15]. Thus, it appears likely that Igna-SY6 could use these enzymes to establish redox balance during fermentation The genome encodes a sulfide-quinone

oxidoreductases which indicates that Igna-SY6 I can use sulfide as an electron donor which ultimately helps in the sulfide detoxification by formation of sulfur/polysulfide. The genome of Igna-SY6 is missing key genes involved in the biosynthetic pathways for several amino acids. Hence, it is expected that it might obtain them from its environment. Here, we detected them in the growth media containing yeast extract and tryptone which are rich in amino acids and oligopeptides which confirms their involvement in amino acid utilization.

Genome characteristics and metabolic properties of *Chloroflexota* bacterium strain Chflx-SY6

The *Chloroflexota* bacterium strain Chflx-SY6 has a genome size of 4,951,662 base pairs. The genome completeness is 99.09%, and the percentage of contamination is 7.27%. The G+C content of the genome is 43.12%. The genome contains 4,488 coding DNA sequences (CDS) and 1,705 proteins with functional assignments. Additionally, there are 2,783 hypothetical proteins, 40 tRNA, 1 rRNA, and 38 CRISPR repeats. The genome also has the mevalonate pathway of isoprenoid biosynthesis.

Potential for aerobic respiration via cytochrome C oxidase and the tricarboxylic acid (TCA) cycle were also identified in Chflx-SY6 (Fig. S23). However, we could not detect Chflx-SY6 in the enrichment cultures grown under aerobic or microaerophilic conditions which suggest that Chflx-SY6 could be a strict anaerobe. We identified a total of 146 genes coding for carbohydrate active enzymes in the genome sequence (Table S35). Genes coding for the hydrolysis of cellulose, xylan, and starch were present in the genome sequence, however, their low abundance in the cellulose medium indicated that such polysaccharides could not be preferred carbon sources for growth. All the genes involved in the Wood Ljungdahl (W-L) pathway were present in the genome sequence which could be involved in the carbon dioxide fixation.

Genomic analysis revealed the presence of the electron transfer chain i.e., proton-translocating NADH-dehydrogenase complexes, isoprenoid quinones, and a F₀F₁-type bacterial ATP synthase. Their presence indicates that Chflx-SY6 in the sulfidic waters have the potential to conserve energy via sugar fermentation/gluconeogenesis [39], the potentially reversible W-L pathway, pyruvate ferredoxin oxidoreductase, ATP synthase and NADH-quinone oxidoreductase (Fig. S23). Such energy metabolisms are also reported in the earlier studies from anoxic site indicate fermentation and acetogenesis as potential metabolisms in the subseafloor *Chloroflexaeota* [40,41]. Pyruvate ferredoxin oxidoreductase is also present which may provide a link between the W-L pathway and other anabolic pathways in Chflx-SY6 as suggested previously [19]. Genes coding ferredoxin and flavodoxin are likely involved as electron carriers in cellular redox reactions [42]. Peptide transporters and peptidases were also present in the genome sequence which indicate that Chflx-SY6 might be utilizing traces of proteinaceous components present in the sulfidic waters of the Black Sea.

References:

1. Yadav S, Koenen M, Bale N, Sinninghe Damsté JS, Villanueva L. The physiology and metabolic properties of a novel, low-abundance Psychrilyobacter species isolated from the anoxic Black Sea shed light on its ecological role. *Env Microbiol Rep.* 2021;13:899–910.
2. Suominen S, Dombrowski N, Sinninghe Damsté JS, Villanueva L. A diverse uncultivated microbial community is responsible for organic matter degradation in the Black Sea sulfidic zone. *Env Microbiol.* 2021;23:2709–28.
3. Suominen S, Doorenspleet K, Sinninghe Damsté JS, Villanueva L. Microbial community development on model particles in the deep sulfidic waters of the Black Sea. *Env Microbiol.* 2021;23:2729–46.

491 4. Yadav S, Villanueva L, Bale N, Koenen M, Hopmans EC, Damsté JS. Physiological,
 492 chemotaxonomic and genomic characterization of two novel piezotolerant bacteria of the family
 493 Marinifilaceae isolated from sulfidic waters of the Black Sea. *Syst Appl Microbiol*. 2020;43.

494 5. Davis KE, Joseph SJ, Janssen PH. Effects of growth medium, inoculum size, and incubation
 495 time on culturability and isolation of soil bacteria. *Appl Env Microbiol*. 2005;71:826–34.

496 6. Cannon SA, Giovannoni SJ. High-throughput methods for culturing microorganisms in very-
 497 low-nutrient media yield diverse new marine isolates. *Appl Env Microbiol*. 2002;68:3878–85.

498 7. Zengler K, Toledo G, Rappe M, Elkins J, Mathur EJ, Short JM, et al. Cultivating the uncultured.
 499 *Proc Natl Acad Sci U A*. 2002;99:15681–6.

500 8. Greening C, Biswas A, Carere CR, Jackson CJ, Taylor MC, Stott MB. Genomic and
 501 metagenomic surveys of hydrogenase distribution indicate H₂ is a widely utilized energy source
 502 for microbial growth and survival. *ISME J*. 2016;10:761–77.

503 9. Ramel F, Brasseur G, Pieulle L, Valette O, Hirschler-Réa A, Fardeau ML. Growth of the
 504 Obligate Anaerobe *Desulfovibrio vulgaris* Hildenborough under Continuous Low Oxygen
 505 Concentration Sparging: Impact of the Membrane Bound Oxygen Reductases. *PLoS ONE*.
 506 2015;10:0123455.

507 10. Mardanov AV, Panova IA, Beletsky AV, Avakyan MR, Kadnikov A VV, DV B, et al. Genomic
 508 insights into a new acidophilic, copper-resistant *Desulfosporosinus* isolate from the oxidized
 509 tailings area of an abandoned gold mine. *FEMS Microbiol Ecol*. 2016;92:111.

- 510 11. Pereira IAC, Romão CV, Xavier AV, LeGall J, Teixeira M. Electron transfer between
511 hydrogenases and mono and multiheme cytochromes in *Desulfovibrio* spp. *J Biol Inorg Chem*.
512 1998;3:494–8.
- 513 12. Matias PM, Pereira IA, Soares CM, Carrondo MA. Sulphate respiration from hydrogen in
514 *Desulfovibrio* bacteria: a structural biology overview. *Prog Biophys Mol Biol*. 2005;89:292–329.
- 515 13. Rossi M, Pollock WBR, Reij MW, Keon RG, Fu R, Voordouw G. The hmc operon of
516 *Desulfovibrio vulgaris* subsp. *vulgaris* Hildenborough encodes a potential transmembrane redox
517 protein complex. *J Bacteriol*. 1993;175:4699–711.
- 518 14. Thauer RK, Kaster AK, Seedorf H, Buckel W, Hedderich R. Methanogenic archaea:
519 ecologically relevant differences in energy conservation. *Nat Rev Microbiol*. 2008;6:579–91.
- 520 15. Vignais PM, Billoud B. Occurrence, classification, and biological function of hydrogenases:
521 an overview. *Chem Rev*. 2007;107:4206–72.
- 522 16. Rothery RA, Workun GJ, Weiner JH. The prokaryotic complex iron-sulfur molybdoenzyme
523 family. *Biochim Biophys Acta*. 2008;1778:1897–929.
- 524 17. Rodrigues ML, Oliveira TF, Pereira IA, Archer M. X-ray structure of the membrane-bound
525 cytochrome c quinol dehydrogenase NrfH reveals novel haem coordination. *EMBO J*.
526 2006;25:5951–60.
- 527 18. Greene EA, Hubert C, Nemati M, Jenneman GE, Voordouw G. Nitrite reductase activity of
528 sulfate-reducing bacteria prevents their inhibition by nitrate-reducing, sulfide-oxidizing bacteria.
529 *Env Microbiol*. 2003;5:607–17.

- 530 19. Wasmund K, Pelikan C, Schintlmeister A, Wagner M, Watzka M, Richter A, et al. Genomic
531 insights into diverse bacterial taxa that degrade extracellular DNA in marine sediments. *Nat*
532 *Microbiol.* 2021;6:885–98.
- 533 20. Zheng R, Liu R, Shan Y, Cai R, Liu G, Sun C. Characterization of the first cultured free-living
534 representative of *Candidatus Izemoplasma* uncovers its unique biology. *ISME J.* 2021;15:2676–
535 91.
- 536 21. Villanueva L, Meijenfeldt FAB, Westbye AB, Yadav S, Hopmans E, Dutilh BE, et al. Bridging
537 the membrane lipid divide: bacteria of the FCB group superphylum have the potential to synthesize
538 archaeal ether lipids. *ISME J.* 2021;15:168–82.
- 539 22. Johnson LA, Hug LA. Cloacimonadota metabolisms include adaptations in engineered
540 environments that are reflected in the evolutionary history of the phylum. *Env Microbiol Rep.*
541 2022;14:520–9.
- 542 23. Chouari R, Le Paslier D, Daegelen P, Ginestet P, Weissenbach J, Sghir A. Novel predominant
543 archaeal and bacterial groups revealed by molecular analysis of an anaerobic sludge digester. *Env*
544 *Microbiol.* 2005;7:1104–15.
- 545 24. Solli L, Håvelsrud OE, Horn SJ, Rike AG. A metagenomic study of the microbial communities
546 in four parallel biogas reactors. *Biotechnol Biofuels.* 2014;7.
- 547 25. Ahlert S, Zimmermann R, Ebling J, König H. Analysis of propionate-degrading consortia from
548 agricultural biogas plants. *Microbiology.* 2016;5:1027–37.

- 549 26. Westerholm M, Crauwels S, Houtmeyers S, Meerbergen K, Geel M, Lievens B. Microbial
550 community dynamics linked to enhanced substrate availability and biogas production of
551 electrokinetically pre-treated waste activated sludge. *Bioresour Technol.* 2016;218:761–70.
- 552 27. Calusinska M, Goux X, Fossépré M, Muller EEL, Wilmes P, Delfosse P. A year of monitoring
553 20 mesophilic full-scale bioreactors reveals the existence of stable but different core microbiomes
554 in bio-waste and wastewater anaerobic digestion systems. *Biotechnol Biofuels.* 2018;11.
- 555 28. Jankowska E, Duber A, Chwialkowska J, Stodolny M, Oleskowicz-Popiel P. Conversion of
556 organic waste into volatile fatty acids - the influence of process operating parameters. *Chem Eng*
557 *J.* 2018;345:395–403.
- 558 29. Theuerl S, Klang J, Heiermann M, Vrieze J. Marker microbiome clusters are determined by
559 operational parameters and specific key taxa combinations in anaerobic digestion. *Bioresour*
560 *Technol.* 2018;263:128–35.
- 561 30. Shakeri Yekta S, Liu T, Axelsson Bjerg M, Šafarič L, Karlsson A, Björn A, Schnürer A. Sulfide
562 level in municipal sludge digesters affects microbial community response to long-chain fatty acid
563 loads. *Biotechnol Biofuels.* 2019;12.
- 564 31. Drickamer K, Taylor ME. Evolving views of protein glycosylation. *Trends Biochem Sci.*
565 1998;23:321–4.
- 566 32. Hess V, Gallegos R, Andrew Jones J, Barquera B, Malamy MH, Müller V. Occurrence of
567 ferredoxin: NAD⁺ oxidoreductase activity and its ion specificity in several gram-positive and
568 gram-negative bacteria. *PeerJ.* 2016;4:e1515.

- 569 33. Feng X, Tang KH, Blankenship RE, Tang YJ. Metabolic flux analysis of the mixotrophic
570 metabolisms in the green sulfur bacterium *Chlorobaculum tepidum*. J Biol Chem.
571 2010;285:39544–50.
- 572 34. Wahlund TM, Tabita FR. The reductive tricarboxylic acid cycle of carbon dioxide assimilation:
573 initial studies and purification of ATP-citrate lyase from the green sulfur bacterium *Chlorobium*
574 *tepidum*. J Bacteriol. 1997;179:4859–67.
- 575 35. Meyer M, Dimroth P, Bott M. Catabolite repression of the citrate fermentation genes in
576 *Klebsiella pneumoniae*: evidence for involvement of the cyclic AMP receptor protein. J Bacteriol.
577 2001;183:5248–56.
- 578 36. Biegel E, Müller V. Bacterial Na⁺-translocating ferredoxin:NAD⁺ oxidoreductase. Proc Natl
579 Acad Sci U A. 2010;107:18138–42.
- 580 37. García-Horsman JA, Barquera B, Rumbley J, Ma J, Gennis RB. The superfamily of heme-
581 copper respiratory oxidases. J Bacteriol. 1994;176:5587–600.
- 582 38. van der Oost J, de Boer AP, de Gier JW, Zumft WG, Stouthamer AH, van Spanning RJ. The
583 heme-copper oxidase family consists of three distinct types of terminal oxidases and is related to
584 nitric oxide reductase. FEMS Microbiol Lett. 1994;121:1–9.
- 585 39. Seshadri R, Adrian L, Fouts DE, Eisen JA, Phillippy AM, Methe BA. Genome sequence of the
586 PCE-dechlorinating bacterium *Dehalococcoides ethenogenes*. Science. 2005;307:105–8.

587 40. Sewell HL, Kaster AK, Spormann AM. Homoacetogenesis in Deep-Sea Chloroflexi, as
588 Inferred by Single-Cell Genomics, Provides a Link to Reductive Dehalogenation in Terrestrial
589 Dehalococcoidetes. *mBio*. 2017;8:e02022-17.

590 41. Kaster AK, Mayer-Blackwell K, Pasarelli B, Spormann AM. Single cell genomic study of
591 Dehalococcoidetes species from deep-sea sediments of the Peruvian Margin. *ISME J*.
592 2014;8:1831–42.

593 42. Buckel W, Thauer RK. Flavin-Based Electron Bifurcation, A New Mechanism of Biological
594 Energy Coupling. *Chem Rev*. 2018;118:3862–86.

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Supplementary Figures

A

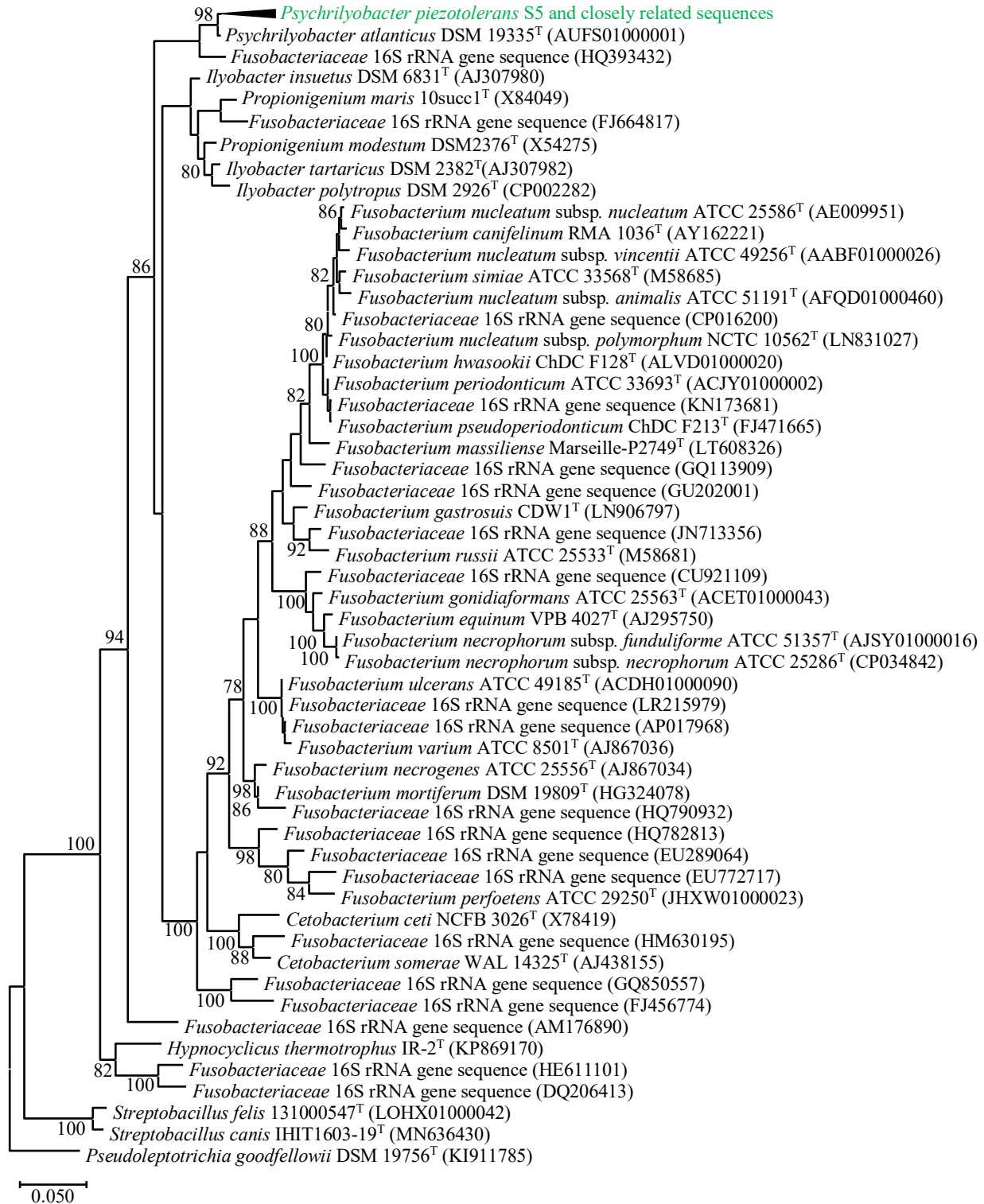


Fig. S1: (A) Phylogenetic tree based on 16S rRNA gene sequences showing the relationship of strains S5, SD5^T and BL5 within the phylum *Fusobacteriota*. The tree was reconstructed by the maximum-likelihood method using MEGAX software and was rooted by using the 16S rRNA gene sequence of *Pseudoleptotrichia goodfellowii* DSM 19756^T (KI911785) as the outgroup. Numbers at nodes represent bootstrap value (percentages, based on 1000 resamplings). GenBank accession numbers for 16S rRNA gene sequences are shown between parentheses. Bar, indicated 5 nucleotide substitutions per 100 nucleotides.

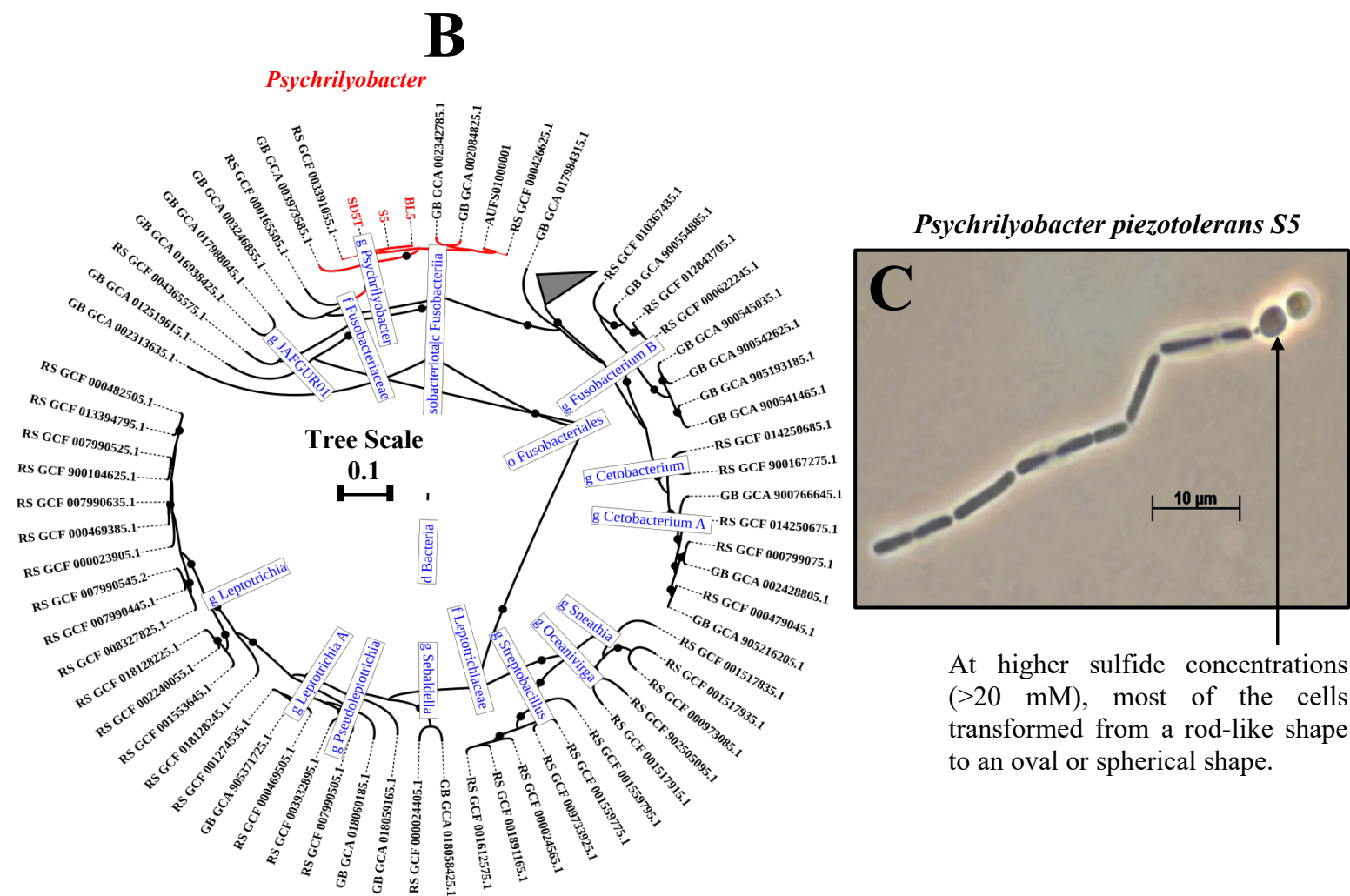
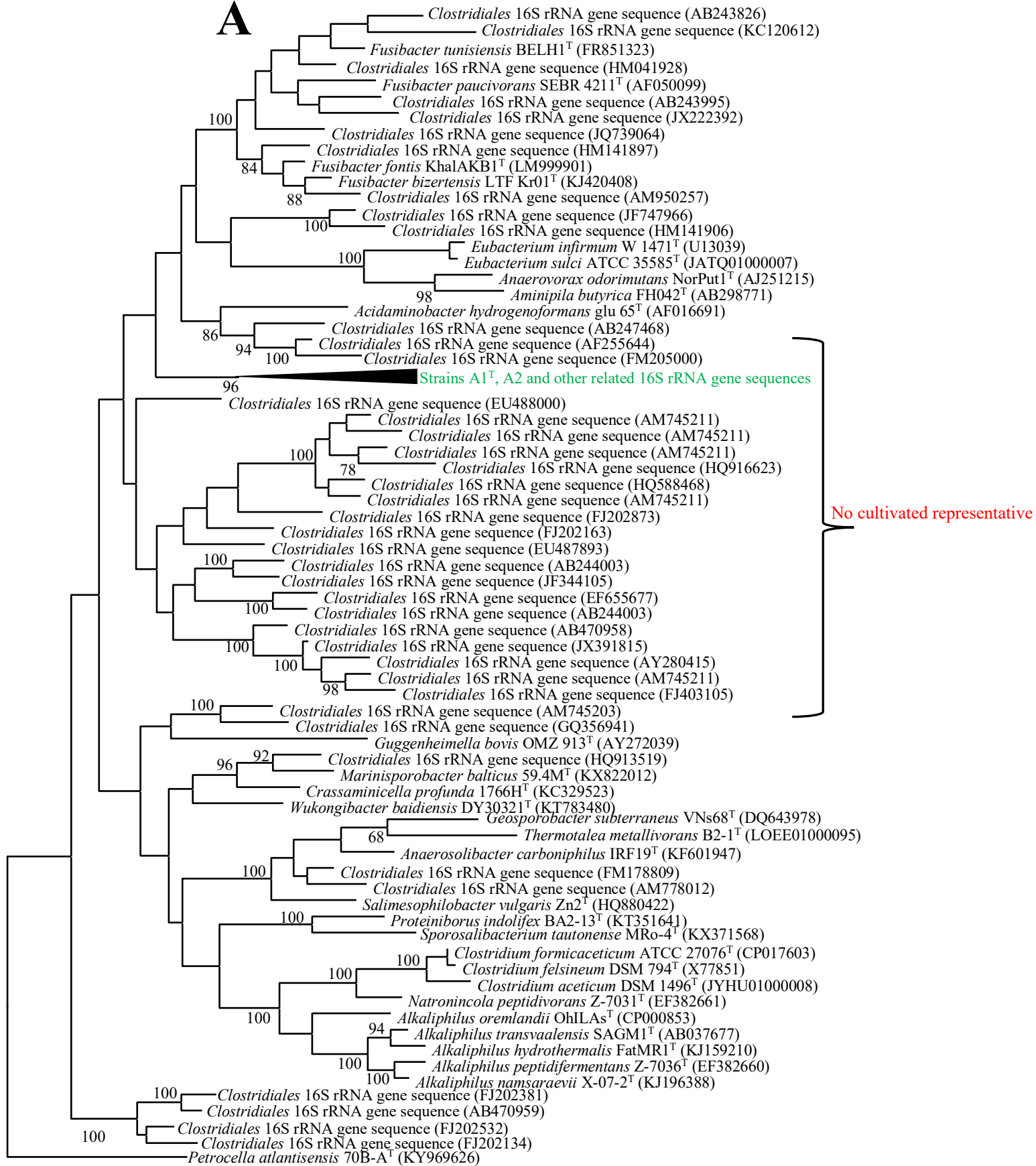


Fig. S1: (B) Subtrees of the GTDB-Tk 2.1.1 phylogenomic tree showing the affiliation of *Psychrilyobacter piezotolerans* strain S5, SD5^T and BL5 (shown in red color) with other closely related members of the phylum *Fusobacteriota*. Class names are indicated by a leading “c_,” order names by “o_,” family names by “f_,” and genus names by “g_.” The genome sequence accession numbers of the closely related taxa are shown. Black circle at nodes represents bootstrap value (100). The length of the bar indicates ten nucleotide substitutions per 100 nucleotides. **(C)** Cell morphology (phase contrast micrograph) of the *Psychrilyobacter piezotolerans* strain S5 grown at optimal growth conditions. Most of the cells appear as rod shape at optimal growth conditions while spherical cells are observed at increasing sulfide concentration.

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0.050

Fig. S2: (A) Phylogenetic tree based on 16S rRNA gene sequences showing the relationship of *Clostridiales* bacteria strains A1^T and A2 within the phylum *Bacillota*. The tree was reconstructed by the maximum-likelihood method using MEGAX software and was rooted by using the 16S rRNA gene sequence of *Petrocella atlantisensis* 70B-A^T (KY969626) as the outgroup. Numbers at nodes represent bootstrap value (percentages, based on 1000 resamplings). GenBank accession numbers for 16S rRNA gene sequences are shown in parentheses. Bar, 5 nucleotide substitutions per 100 nucleotides.

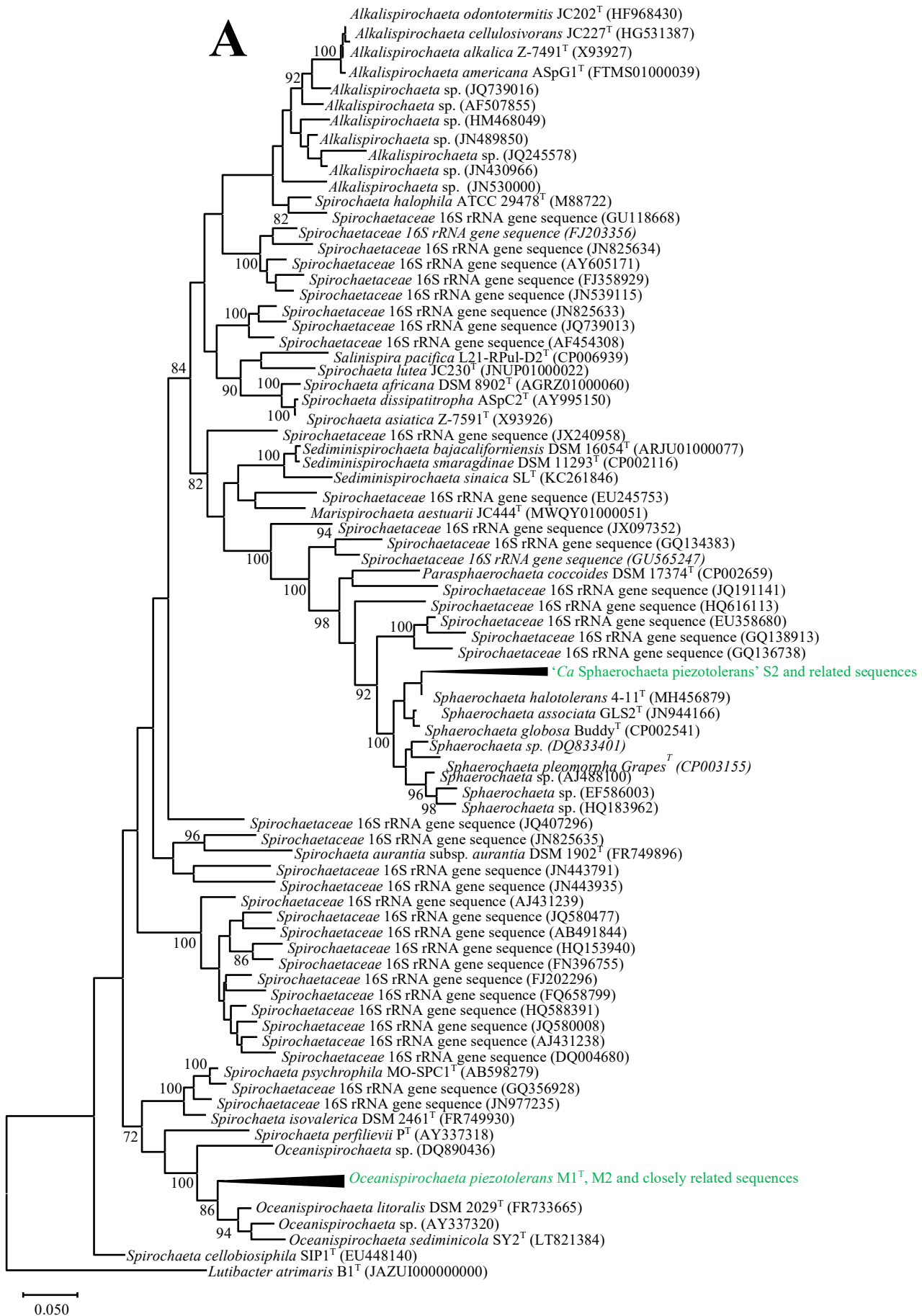


Fig. S3: (A) Phylogenetic tree based on 16S rRNA gene sequences showing the relationship of *Oceanispirochaeta* sp. strains M1^T, M2 and ‘Ca Sphaerochaeta piezotolerans’ strain S2 within the phylum *Spirochaetota*. The tree was reconstructed by the maximum-likelihood method using MEGA X software and was rooted by using the 16S rRNA gene sequence of *Lutibacter atrimaris* B1^T (JAZUI000000000) as the outgroup. Numbers at nodes represent bootstrap value (percentages, based on 1000 resamplings). GenBank accession numbers for 16S rRNA gene sequences are shown in parentheses. Bar, 5 nucleotide substitutions per 100 nucleotides.

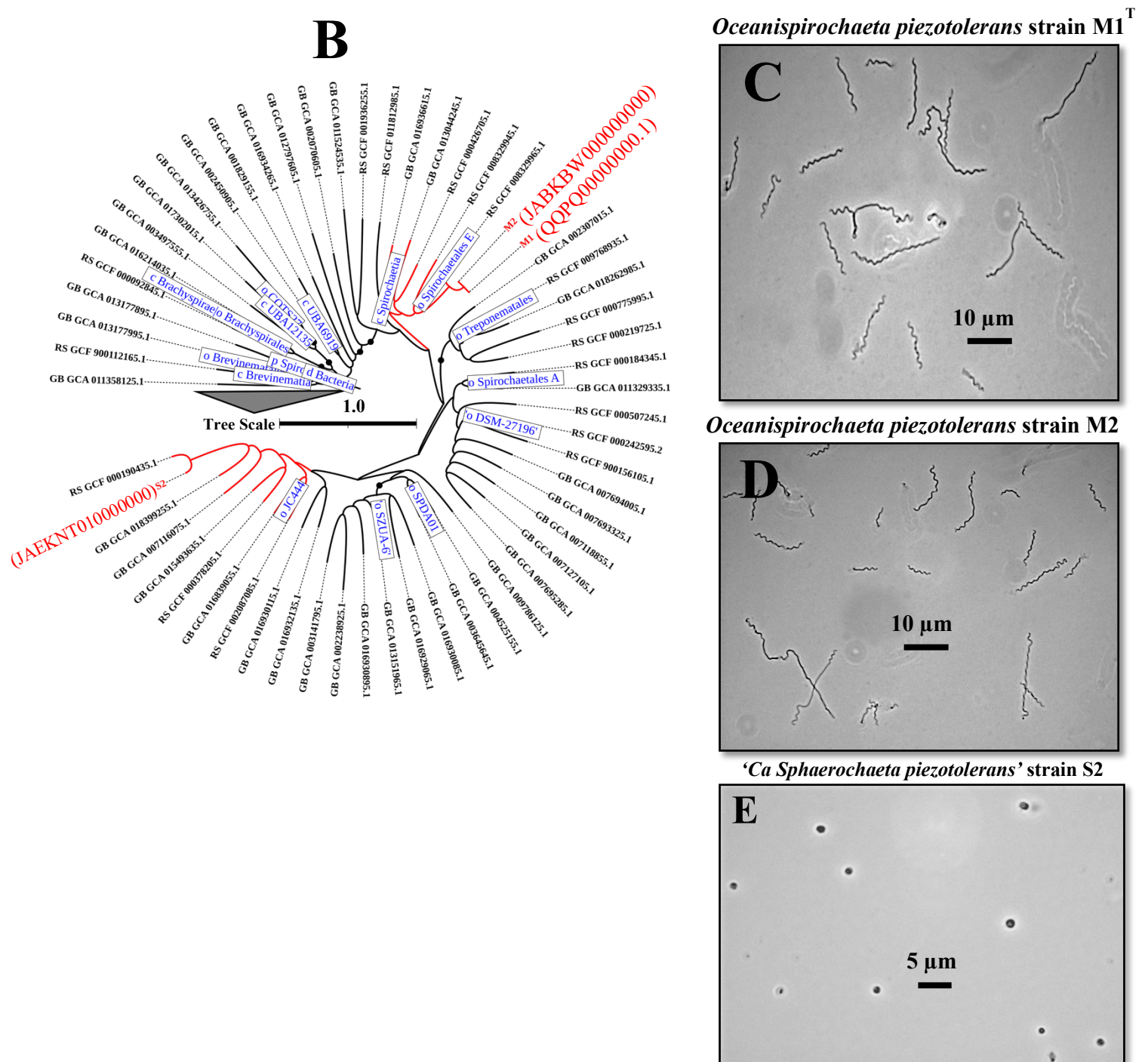


Fig. S3: (B) Subtrees of the GTDB-Tk 2.1.1 phylogenomic tree showing the affiliation of *Oceanispirochaeta piezotolerans* strains M1^T, M2 and *'Ca Sphaerochaeta piezotolerans'* strain S2 (shown in red color) with other closely related members of the phylum *Spirochaetota*. Class names are indicated by a leading “c_,” order names by “o_,” family names by “f_,” and genus names by “g_.” The genome sequence accession numbers of the closely related taxa are shown. Black circle at nodes represents bootstrap value (100). The length of the bar indicates 100 nucleotide substitutions per 100 nucleotides. (C) Cell morphology (phase contrast micrograph) of the *Oceanispirochaeta piezotolerans* strains (C) M1^T, (D) M2 and (E) *'Ca Sphaerochaeta piezotolerans'* strain S2 grown at optimal growth conditions.

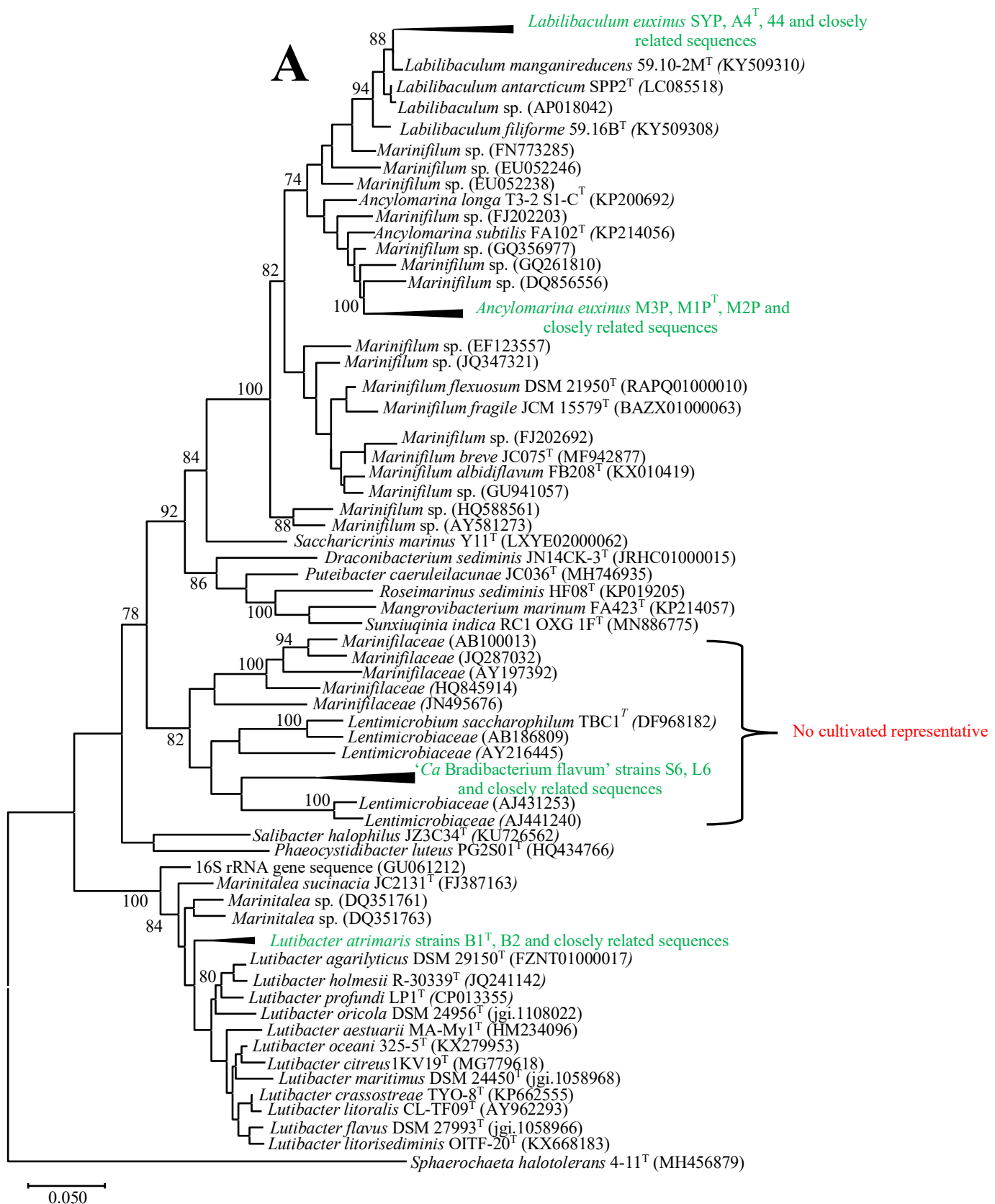


Fig. S4: (A) Phylogenetic tree based on 16S rRNA gene sequences showing the relationship of members of the phylum *Bacteroidota* (*Lutibacter* sp. strains B1^T, B2; 'Ca Bradibacterium flavum' strain S6, L6; *Ancylomarina euxinus* M2P; *Labilibaculum euxinus* SYP) isolated from deep sulfidic waters of the Black Sea. The tree was reconstructed by the maximum-likelihood method using MEGA X software and was rooted by

using the 16S rRNA gene sequence of *Sphaerochaeta halotolerans* 4-11^T (MH456879) as the outgroup. Numbers at nodes represent bootstrap value (percentages, based on 1000 resamplings). GenBank accession numbers for 16S rRNA gene sequences are shown in parentheses. Bar, 5 nucleotide substitutions per 100 nucleotides.

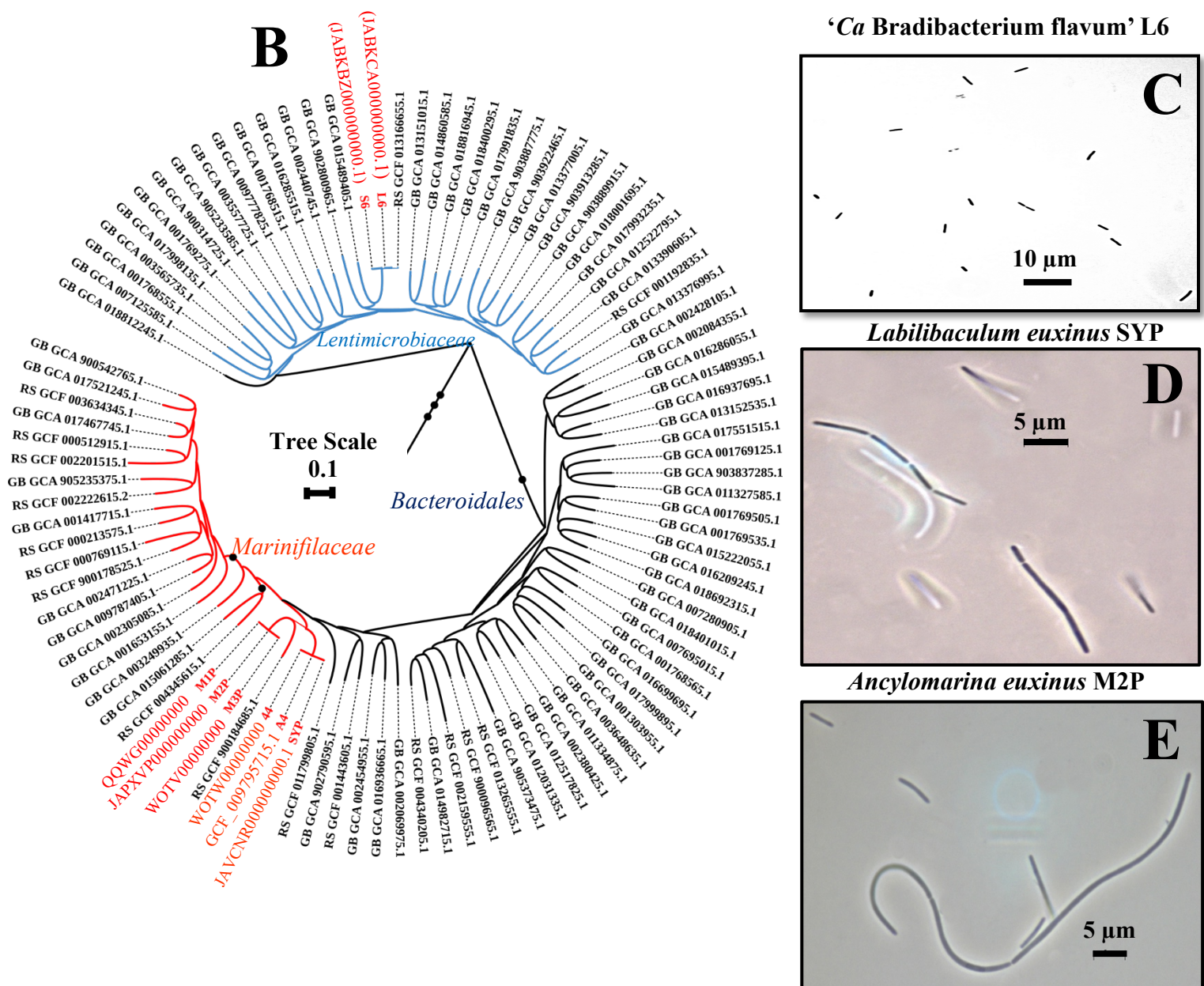


Fig. S4: (B) Subtrees of the GTDB-Tk 2.1.1 phylogenomic tree showing the affiliation of various members of the phylum *Bacteroidota* (‘*Ca* *Bradibacterium flavum*’ strains S6, L6; *Ancylomarina euxinus* strains M2P, M1P^T and M3P; *Labilibaculum euxinus* strain SYP, A4^T and 44 (shown in red color) with other closely related members of the phylum *Bacteroidota*. The genome sequence accession numbers of the closely related taxa are shown. Black circle at nodes represents bootstrap value (100). The length of the bar indicates ten nucleotide substitutions per 100 nucleotides. (C) Cell morphology (phase contrast micrograph) of the ‘*Ca* *Bradibacterium flavum*’ strain S6 (D) *Ancylomarina euxinus* strains M2P and (E) *Labilibaculum euxinus* strain SYP grown at optimal growth conditions.

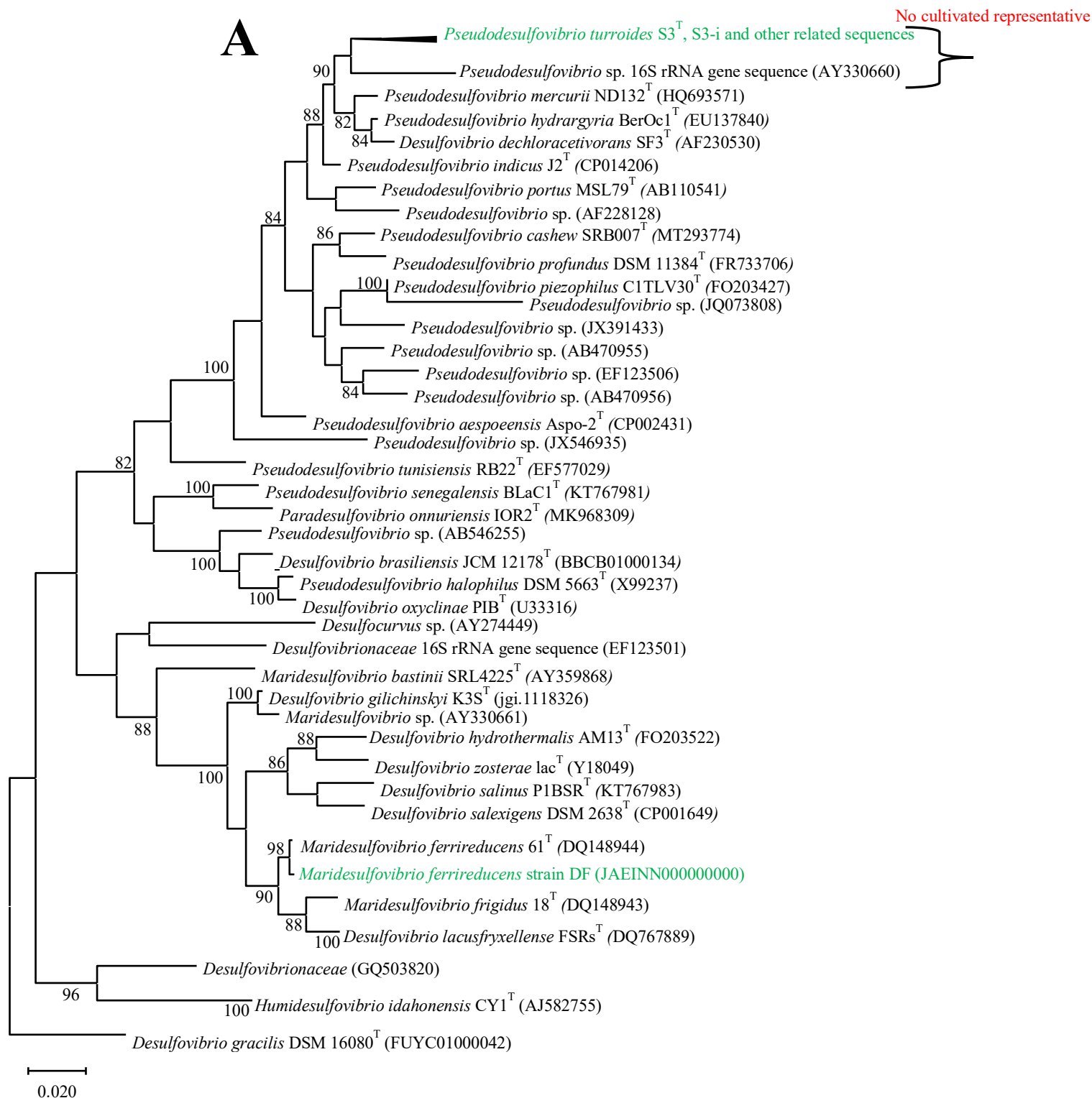


Fig. S6: (A) Phylogenetic tree based on 16S rRNA gene sequences showing the relationship of *Pseudodesulfovibrio* sp. strains S3^T, S3-i and other closely related members of the phylum *Desulfobacterota*. The tree was reconstructed by the maximum-likelihood method using MEGA X software and was rooted by using the 16S rRNA gene sequence of *Desulfovibrio gracilis* DSM 16080^T (FUYC01000042) as the outgroup. Numbers at nodes represent bootstrap value (percentages, based on 1000 resamplings). GenBank accession numbers for 16S rRNA gene sequences are shown in parentheses. Bar, 2 nucleotide substitutions per 100 nucleotides.

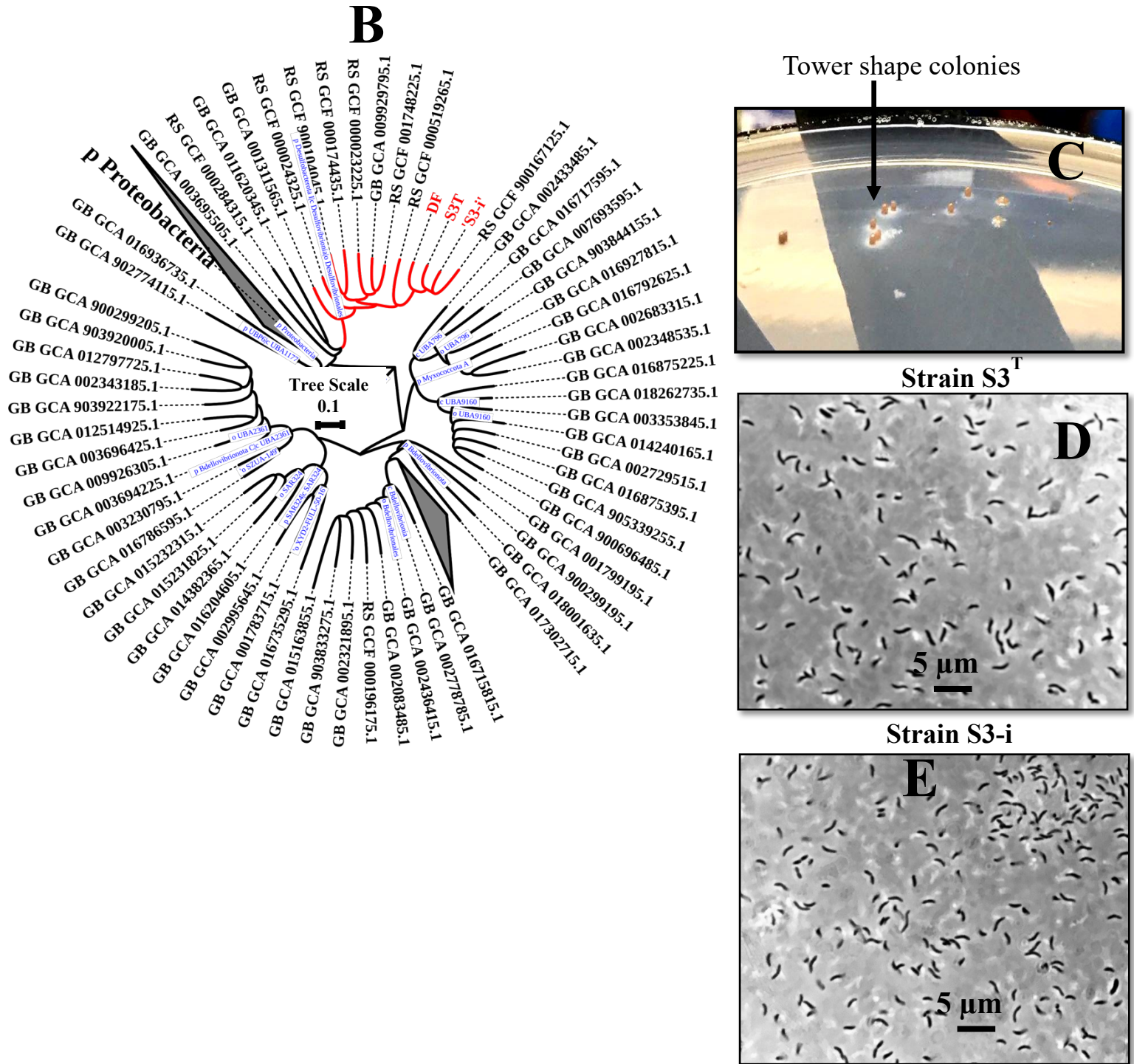


Fig. S6: (B) Subtrees of the GTDB-Tk 2.1.1 phylogenomic tree showing the affiliation of *Pseudodesulfovibrio turroides* strains S3^T, S3-i and *Maridesulfovibrio ferrireducens* strain DF (shown in red color) with other closely related members of the phylum *Desulfobacterota*. Class names are indicated by a leading “c_,” order names by “o_,” family names by “f_,” and genus names by “g_.” The genome sequence accession numbers of the closely related taxa are shown. Black circle at nodes represents bootstrap value (100). The length of the bar indicates ten nucleotide substitutions per 100 nucleotides. **(C)** Orange colored tower shape colony morphology of strain S3^T grown on agar medium (1.8%) after 1 month of incubation at 20 °C under strict anaerobic conditions at atmospheric pressure. **(D)** Cell morphology of *Pseudodesulfovibrio turroides* strains S3^T **(E)** *Pseudodesulfovibrio turroides* strains S3-i grown at optimal growth conditions.

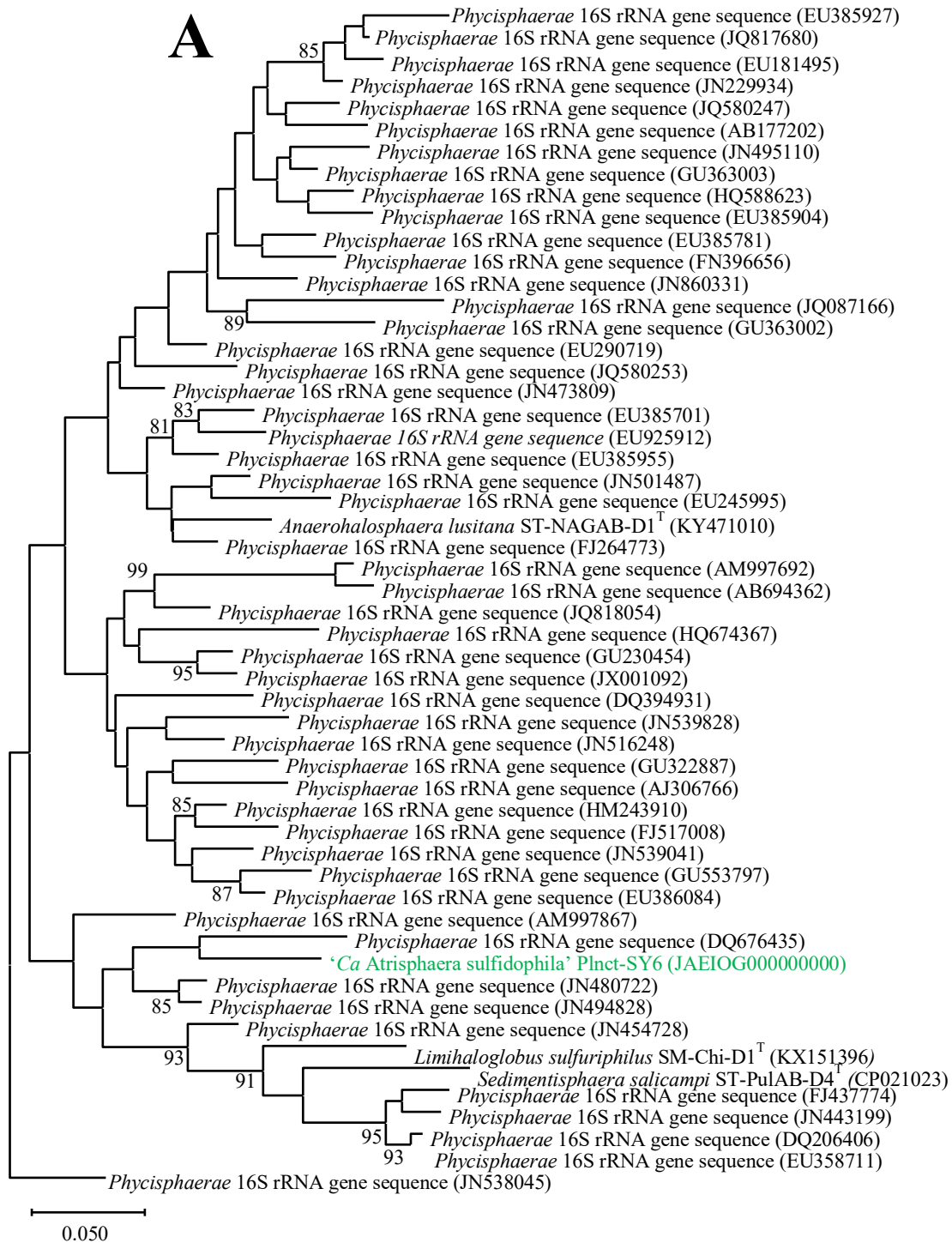


Fig. S7: (A) Phylogenetic tree based on 16S rRNA gene sequences showing the relationship of ‘*Ca Atrisphaera sulfidophila*’ Plnct-SY6 and other closely related members of the phylum *Planctomycetota*. The tree was reconstructed by the maximum-likelihood method using MEGA X software and was rooted by using *Phycisphaerae* 16S rRNA gene sequence (JN538045) as the outgroup. Numbers at nodes represent bootstrap value (percentages, based on 1000 resamplings). GenBank accession numbers for 16S rRNA gene sequences are shown in parentheses. Bar, 5 nucleotide substitutions per 100 nucleotides.

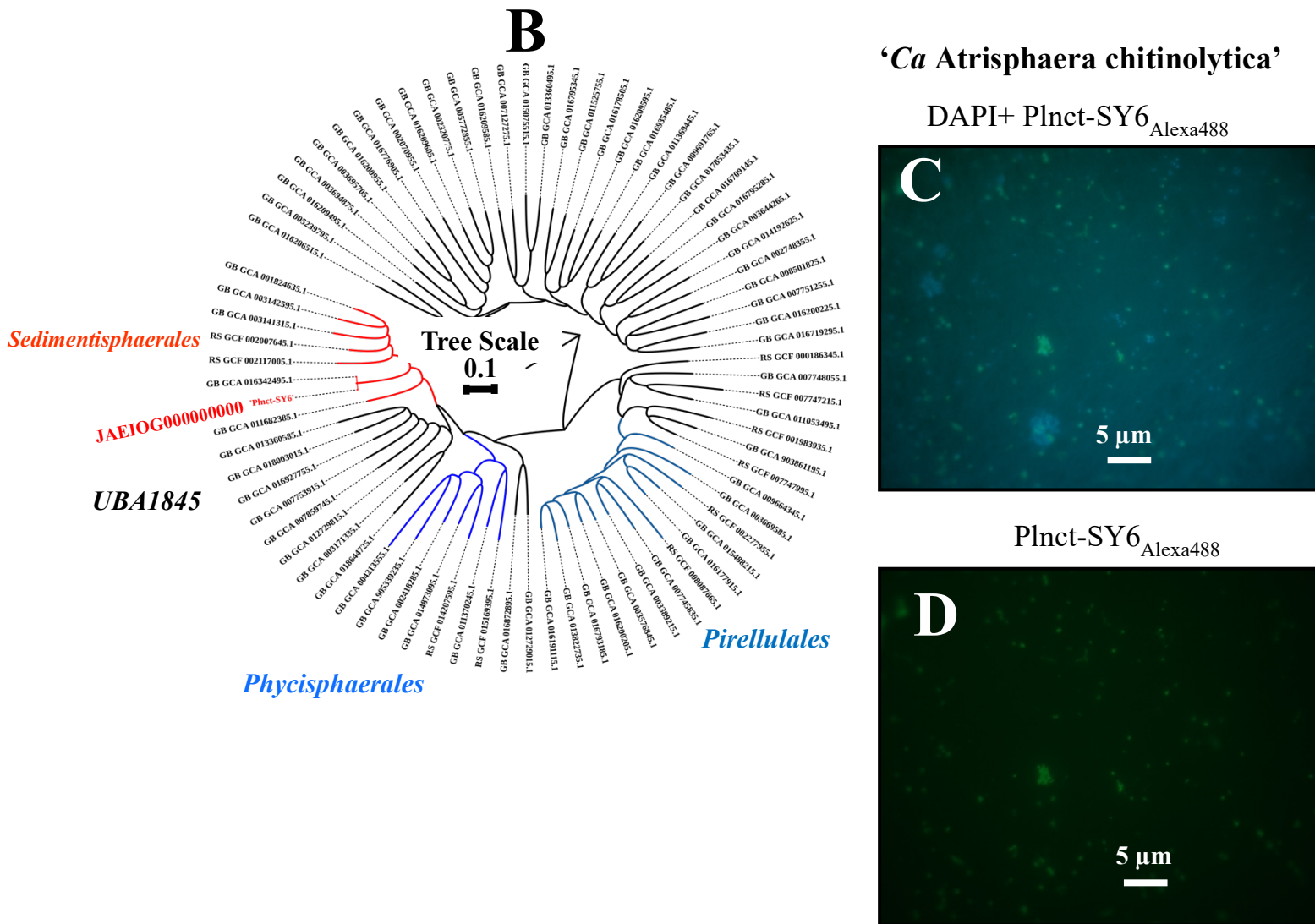


Fig. S7: (B) Subtrees of the GTDB-Tk 2.1.1 phylogenomic tree showing the affiliation of '*Ca Atrisphaera sulfidophila*' Plnct-SY6 (shown in red color) with other closely related members of the phylum *Planctomycetota*. The length of the bar indicates 10 nucleotide substitutions per 100 nucleotides. Taxa name shown in blue color are also obtained from the Black Sea. CARD-FISH microscopic analysis of '*Ca Atrimarinobacter sulfidophilus*' strain Cloa-SY6 (C) DAPI (panel C) and CARD-FISH (panel D) microscopical analysis of (C and D) the enrichment culture of '*Ca Atrisphaera sulfidophila*' Plnct-SY6 using 0.02% chitin medium. All microbial cells were made visible by staining with DAPI, while cells of '*Ca Atrisphaera sulfidophila*' Plnct-SY6 were revealed by using the specifically designed fluorescent probe PB1_{Alexa488} (see M&M for details).

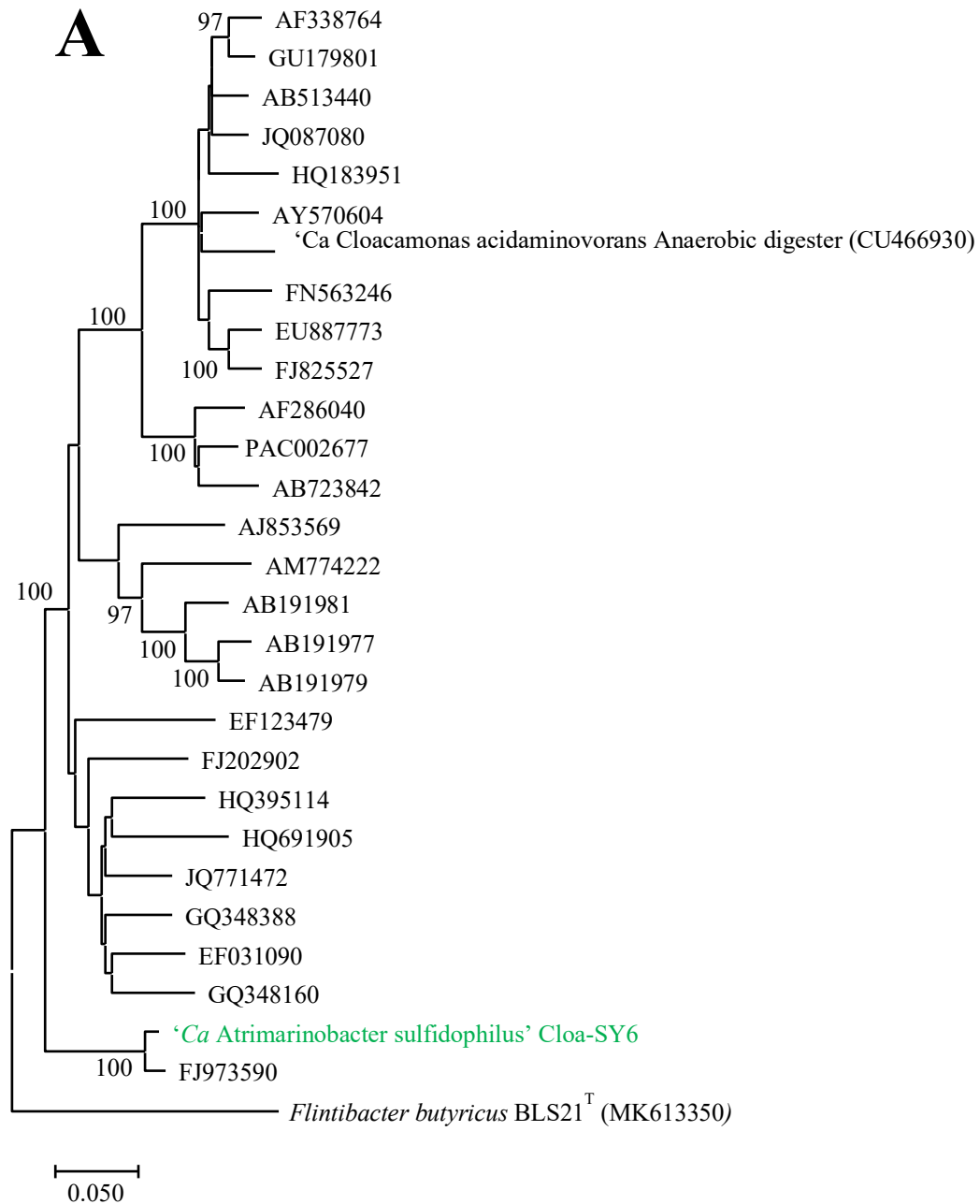
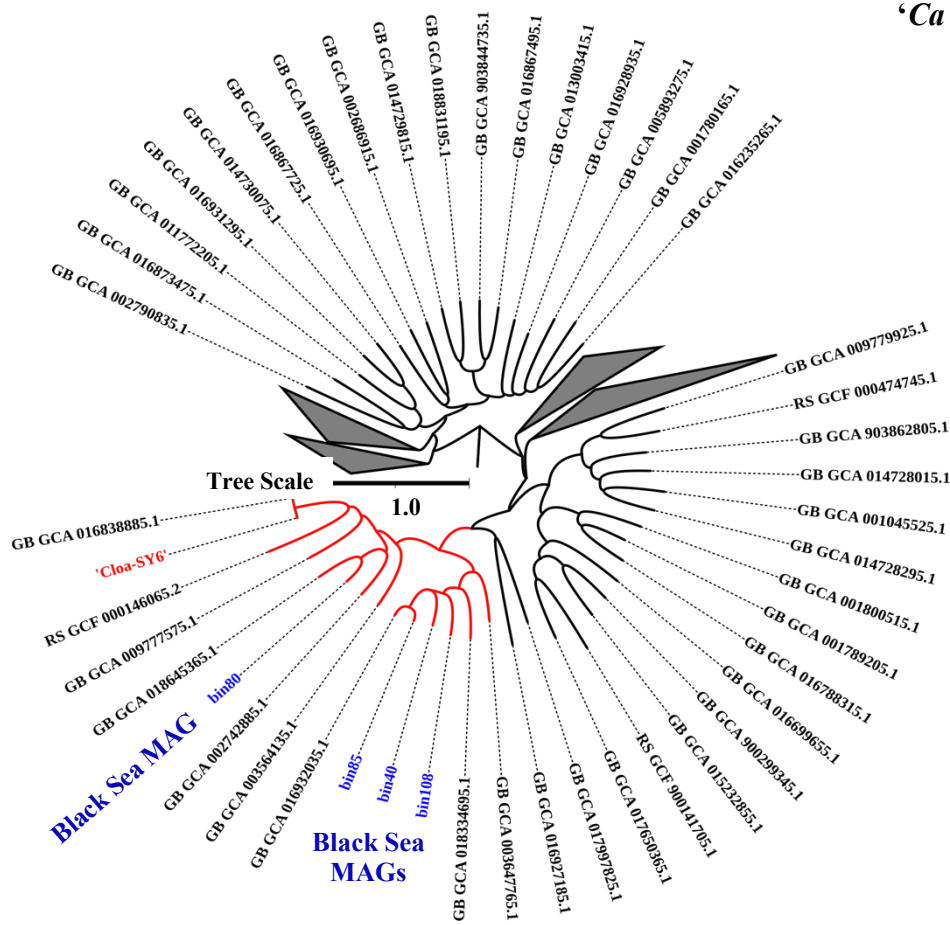
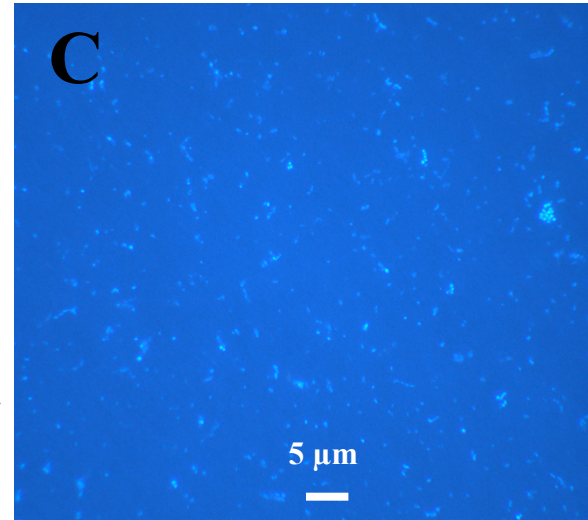


Fig. S8: (A) Phylogenetic tree based on 16S rRNA gene sequences showing the relationship of 'Ca Atrimarinobacter sulfidophilus' Cloa-SY6 and other closely related members of the phylum *Cloacimonadota*. The tree was reconstructed by the maximum-likelihood method using MEGA X software and was rooted by using the 16S rRNA gene sequence of *Flintibacter butyricus* BLS21^T (MK613350) as the outgroup. Numbers at nodes represent bootstrap value (percentages, based on 1000 resamplings). GenBank accession numbers for 16S rRNA gene sequences are shown in parentheses. Bar, 5 nucleotide substitutions per 100 nucleotides.

B

'Ca Atrimarinobacter sulfidophilus' Cloa-SY6
DAPI



Cloa-SY6
Alexa488

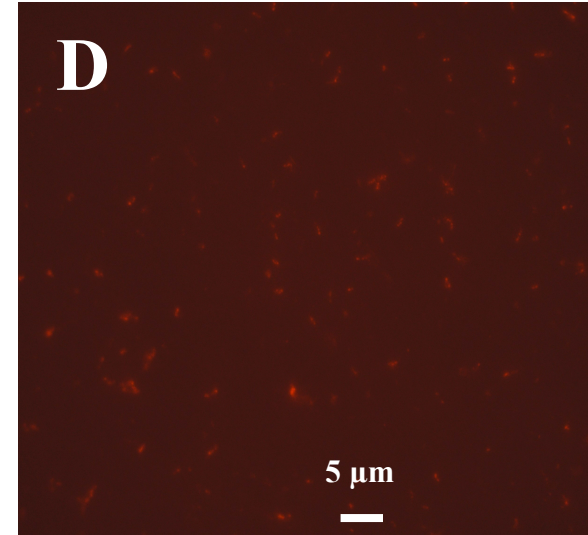


Fig. S8: (B) Subtrees of the GTDB-Tk 2.1.1 phylogenomic tree showing the affiliation of '*Ca Atrimarinobacter sulfidophilus*' Cloa-SY6 (shown in red color) within the closely related members of the phylum *Cloacimonadota*. MAGs shown in blue color obtained from the Black Sea waters. The length of the bar indicates 100 nucleotide substitutions per 100 nucleotides. CARD-FISH microscopic analysis of '*Ca Atrimarinobacter sulfidophilus*' Cloa-SY6 **(B)** DAPI **(panel B)** and CARD-FISH **(panel C)** microscopical analysis of (B and C) the enrichment culture of '*Ca Atrimarinobacter sulfidophilus*' Cloa-SY6 using 0.02% propionate medium. All microbial cells were made visible by staining with DAPI, while cells of '*Ca Atrimarinobacter sulfidophilus*' Cloa-SY6 were revealed by using the specifically designed fluorescent probe PB1_{Alexa488} (see M&M for details).

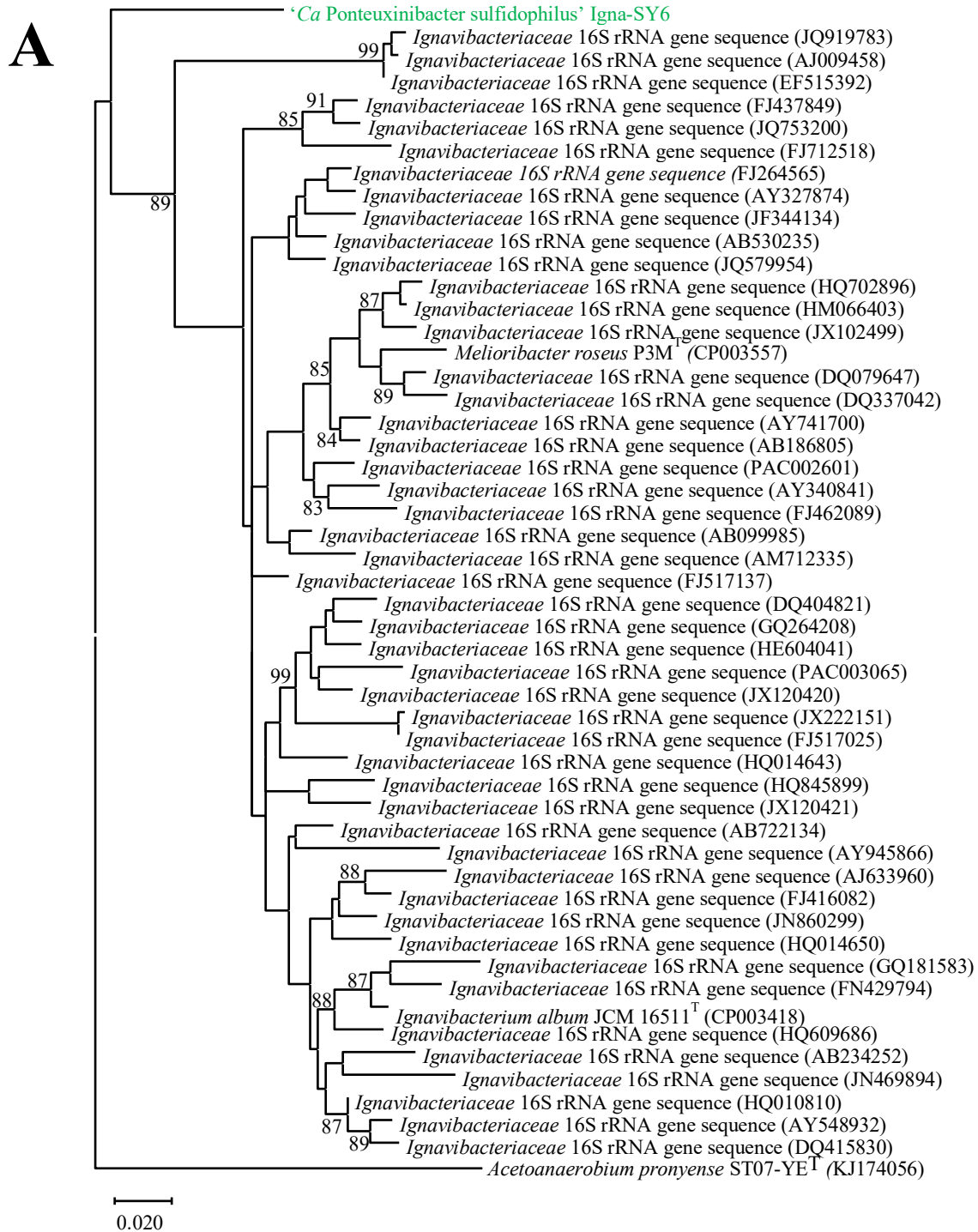


Fig. S9: (A) Phylogenetic tree based on 16S rRNA gene sequences showing the relationship of '*Ca Ponteuxinibacter sulfidophilus*' Igna-SY6 and other closely related members of the phylum *Ignavibacteriota*. The tree was reconstructed by the maximum-likelihood method using MEGA X software and was rooted by using *Phycisphaerae* 16S rRNA gene sequence (JN538045) as the outgroup. Numbers at nodes represent bootstrap value (percentages, based on 1000 resamplings). GenBank accession numbers for 16S rRNA gene sequences are shown in parentheses. Bar, 2 nucleotide substitutions per 100 nucleotides.

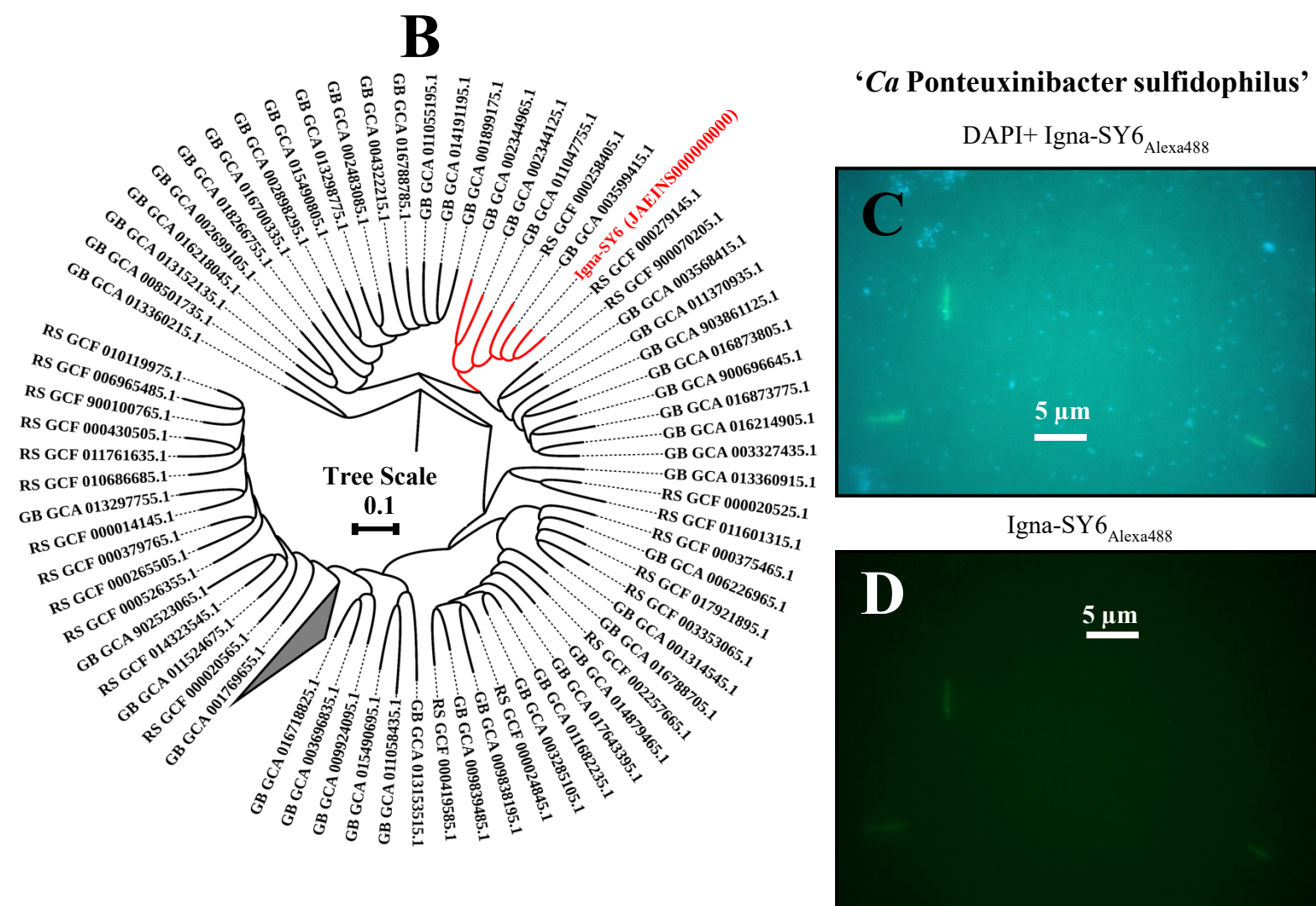


Fig. S9: (B) Subtrees of the GTDB-Tk 2.1.1 phylogenomic tree showing the affiliation of ‘*Ca Ponteuxinibacter sulfidophilus*’ Igna-SY6 (shown in red color) within the closely related members of the phylum *Ignavibacteriota*. The length of the bar indicates ten nucleotide substitutions per 100 nucleotides. CARD-FISH microscopic analysis of ‘*Ca Ponteuxinibacter sulfidophilus*’ strain Igna-SY6 (**B**) DAPI (**panel B**) and CARD-FISH (**panel C**) microscopical analysis of (B and C) the enrichment culture of ‘*Ca Ponteuxinibacter sulfidophilus*’ strain Igna-SY6 using 0.02% cellulose medium. All microbial cells were made visible by staining with DAPI, while cells of ‘*Ca Ponteuxinibacter sulfidophilus*’ strain Igna-SY6 were revealed by using the specifically designed fluorescent probe PB1_{Alexa488} (see M&M for details).

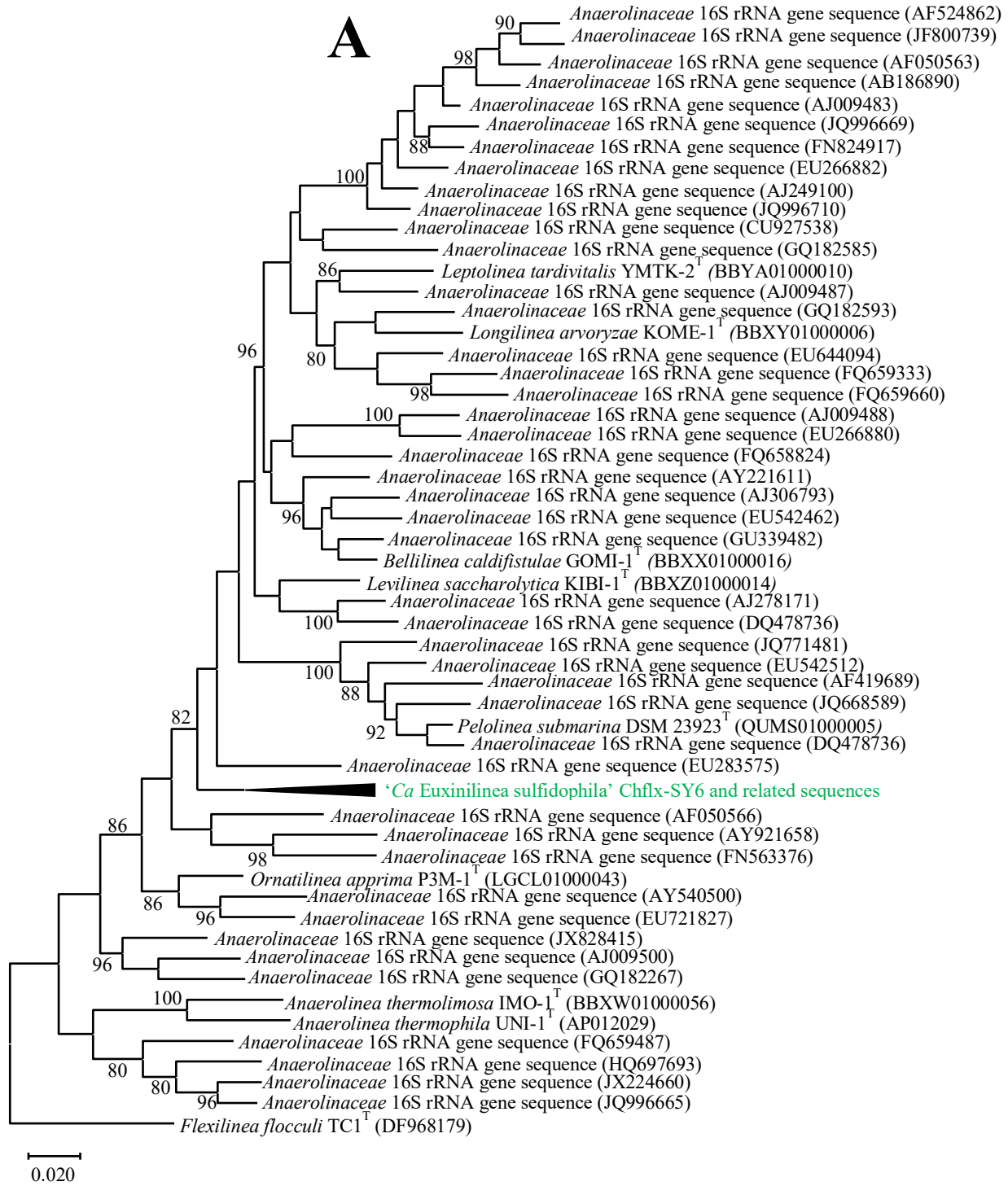


Fig. S10: (A) Phylogenetic tree based on 16S rRNA gene sequences showing the relationship of *'Ca Euxinilinea sulfidophila'* strain Chflx-SY6 and other closely related members of the phylum *Chloroflexota*. The tree was reconstructed by the maximum-likelihood method using MEGA X software and was rooted by using the 16S rRNA gene sequence of *Flexilinea flocculi* TC1^T (DF968179) as the outgroup. Numbers at nodes represent bootstrap value (percentages, based on 1000 resamplings). GenBank accession numbers for 16S rRNA gene sequences are shown in parentheses. Bar, 2 nucleotide substitutions per 100 nucleotides.

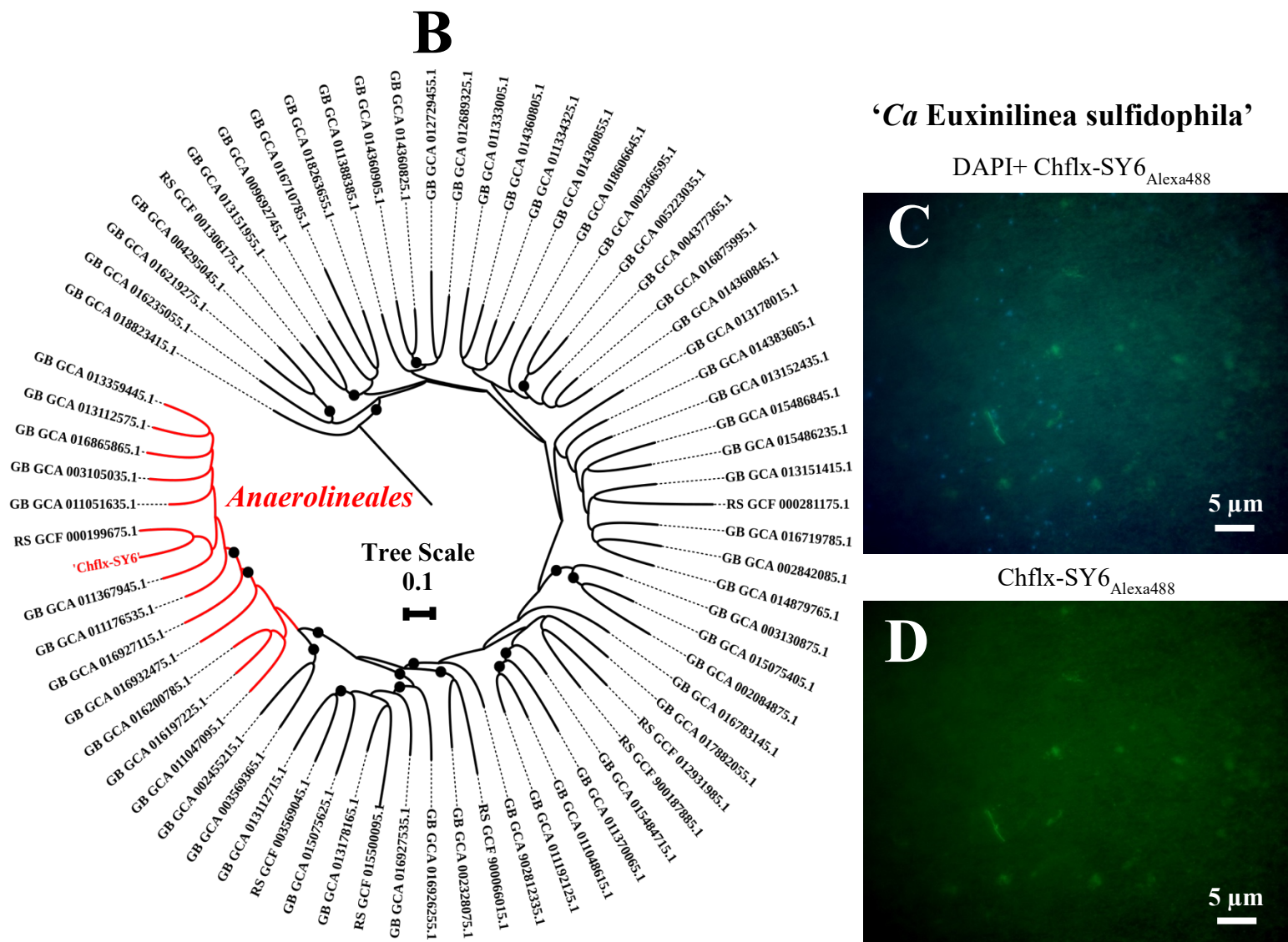


Fig. S10: (B) Subtrees of the GTDB-Tk 2.1.1 phylogenomic tree showing the affiliation of ‘*Ca Euxinilinea sulfidophila*’ Chflx-SY6 (labeled in red color) within the closely related members of the phylum *Chloroflexota*. Black circle at the nodes represents the bootstrap value (100). The length of the bar indicates 10 nucleotide substitutions per 100 nucleotides. CARD-FISH microscopic analysis of ‘*Ca Euxinilinea sulfidophila*’ strain Chflx-SY6 **(B)** DAPI **(panel B)** and CARD-FISH **(panel C)** microscopical analysis of (B and C) the enrichment culture of ‘*Ca Euxinilinea sulfidophila*’ strain Chflx-SY6 using 0.02% cellulose medium. All microbial cells were made visible by staining with DAPI, while cells of ‘*Ca Euxinilinea sulfidophila*’ strain Chflx-SY6 were revealed by using the specifically designed fluorescent probe PB1_{Alexa488} (see M&M for details).

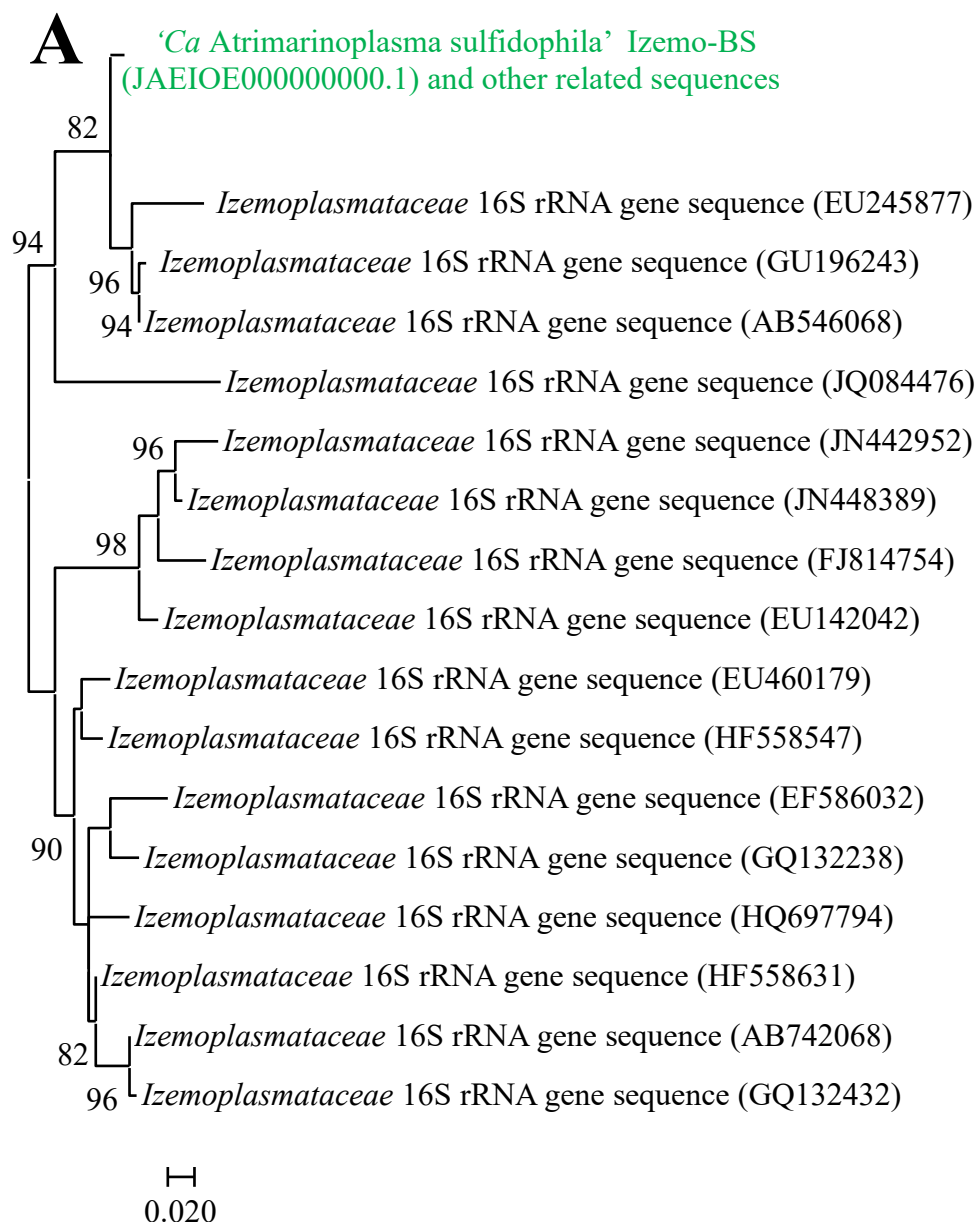


Fig. S11: (A) Phylogenetic tree based on 16S rRNA gene sequences showing the relationship of *'Ca Atrimarinoplasma sulfidophila' Izemo-BS* and other closely related members of the phylum *Mycoplasmatota*. The tree was reconstructed by the maximum-likelihood method using MEGA X software and was rooted by using *Izemoplasmataceae* 16S rRNA gene sequence (GQ132432) as the outgroup. Numbers at nodes represent bootstrap value (percentages, based on 1000 resamplings). GenBank accession numbers for 16S rRNA gene sequences are shown in parentheses. Bar, 2 nucleotide substitutions per 100 nucleotides.

B

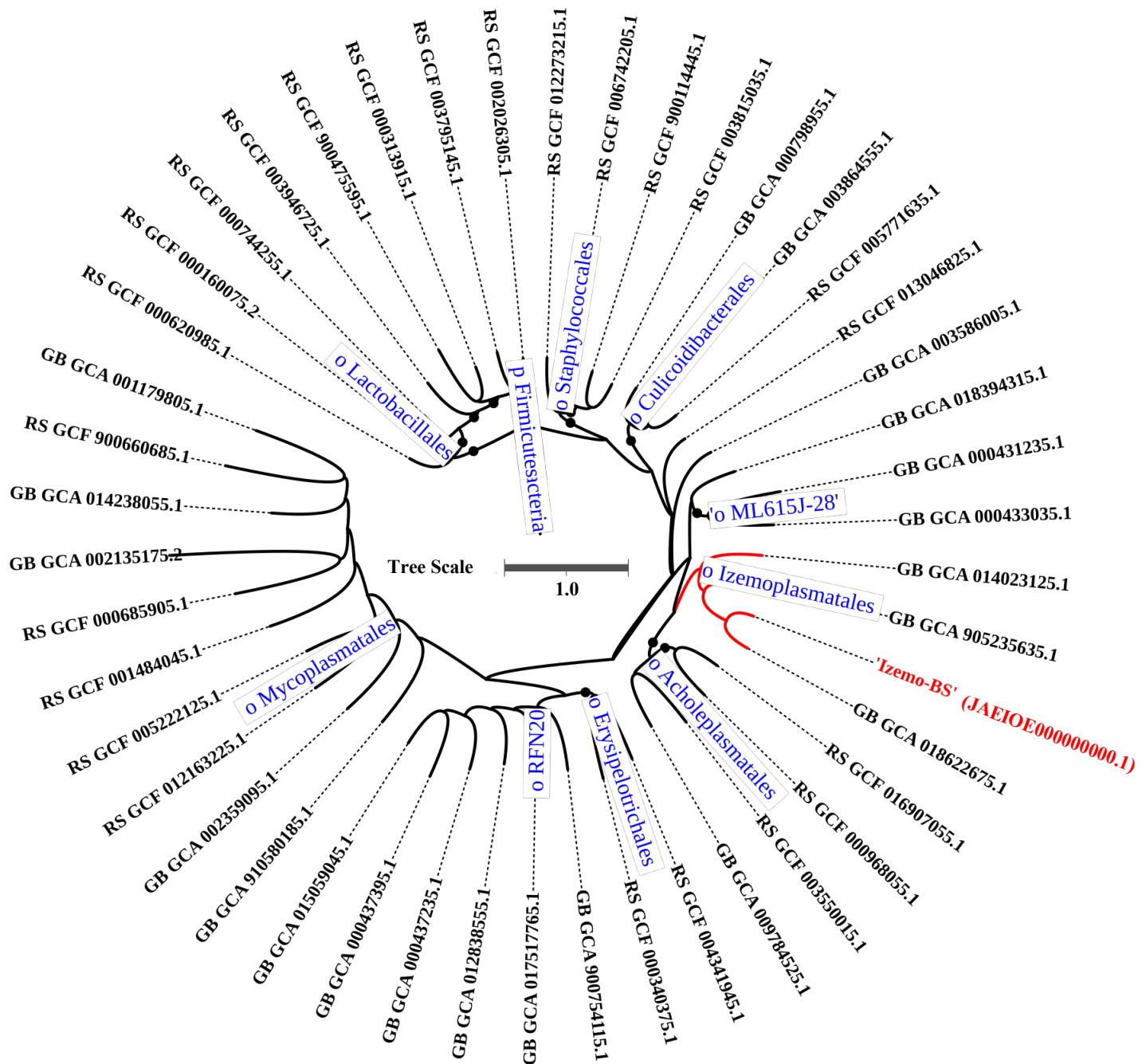


Fig. S11: (B) Subtrees of the GTDB-Tk 2.1.1 phylogenomic tree showing the affiliation of ‘*Ca Atrimarinoplasma cellobiosiphila*’ Izemo-BS (shown in red color) with other closely related members of the phylum *Mycoplasmata*. Black circle at the nodes represents the bootstrap value (100). The length of the bar indicates 100 nucleotide substitutions per 100 nucleotides.

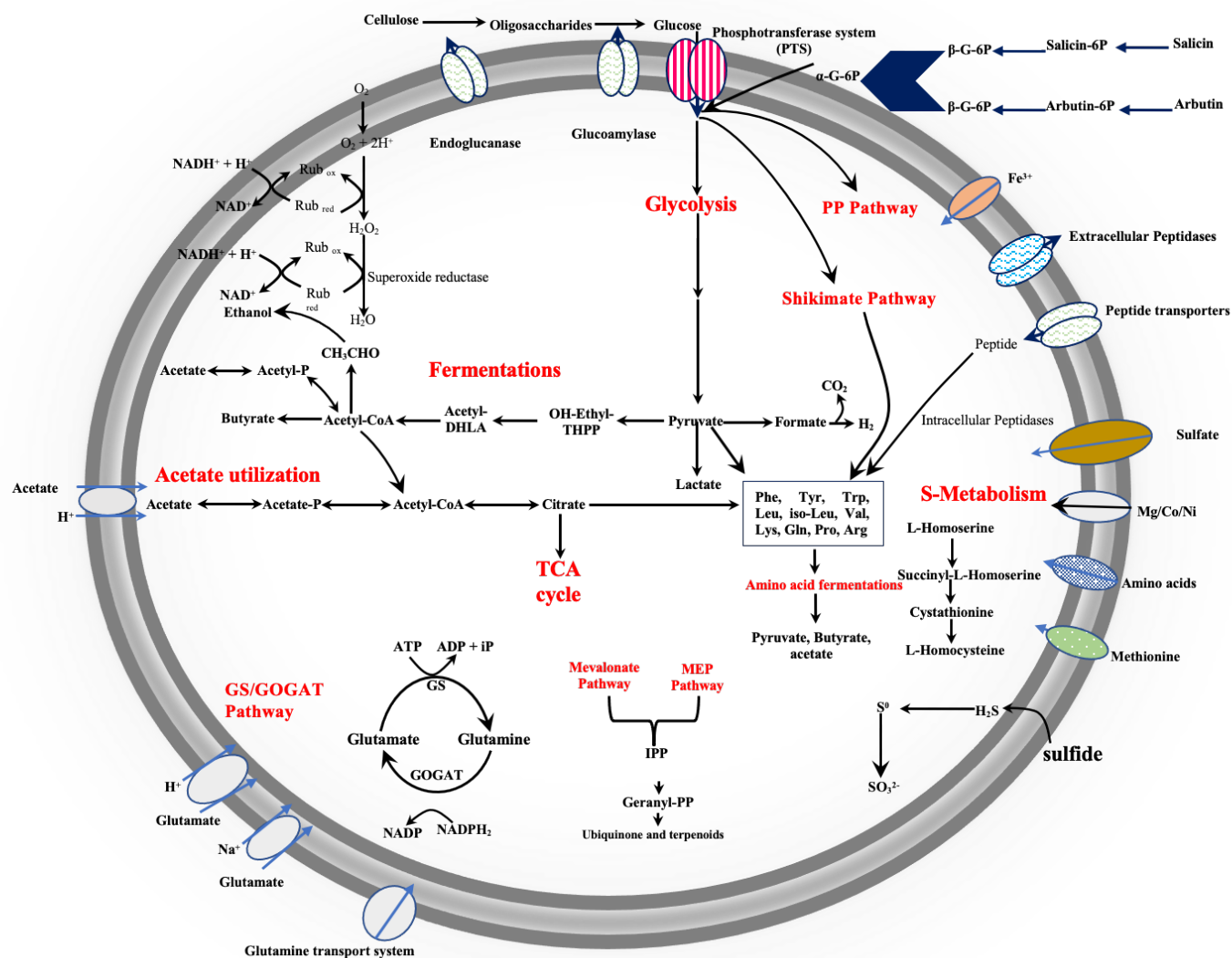


Fig. S12: Reconstruction of the central metabolic pathway of *Psychrilyobacter piezotolerans* strain S5 based on different physiological analyses and by the presence of various genes identified in the genome sequence. IPP, isopentenyl pyrophosphate; PP Pathway, pentose phosphate pathway; geranyl-PP, geranyl pyrophosphate; GS, glutamine synthetase; glutamate synthase (GOGAT).

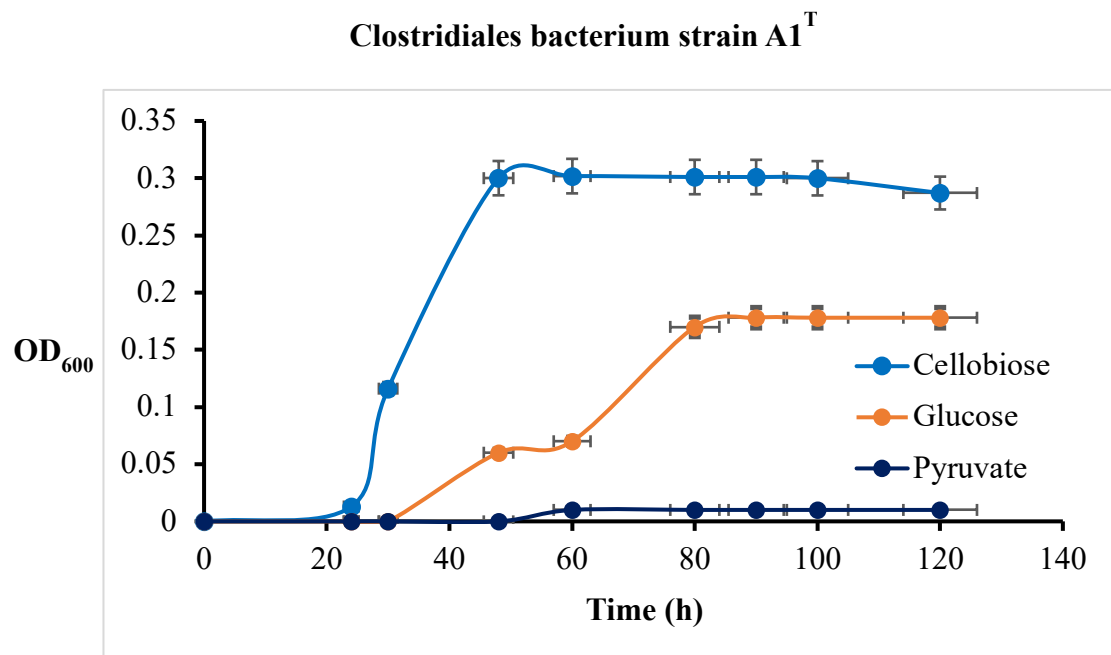


Fig. S13: Growth of *Clostridiales* bacterium strain A1^T at various carbon substrate at 20 °C under strict anaerobic condition.

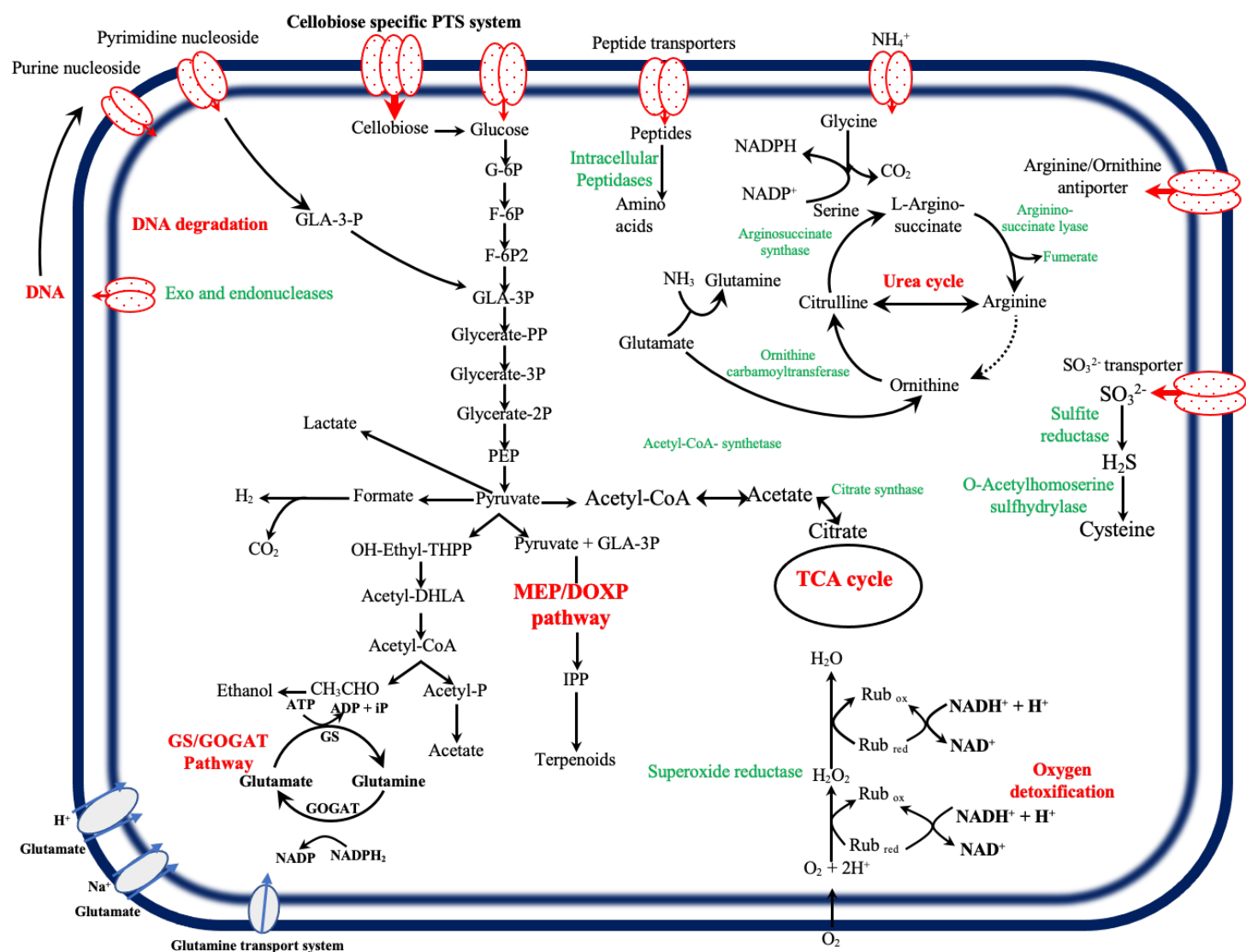


Fig. S14: Reconstruction of the central metabolic pathway of *Clostridiales* bacteria strains A1^T and A2 based on different physiological analyses and by the presence of various genes identified in the genome sequence. IPP, isopentenyl pyrophosphate; G-6P, glucose-6-phosphate; F-6P, fructose-6-phosphate; GLA-3P, glyceraldehyde-3-phosphate; PEP, phosphoenol-pyruvate; GS, glutamine synthetase; glutamate synthase (GOGAT).

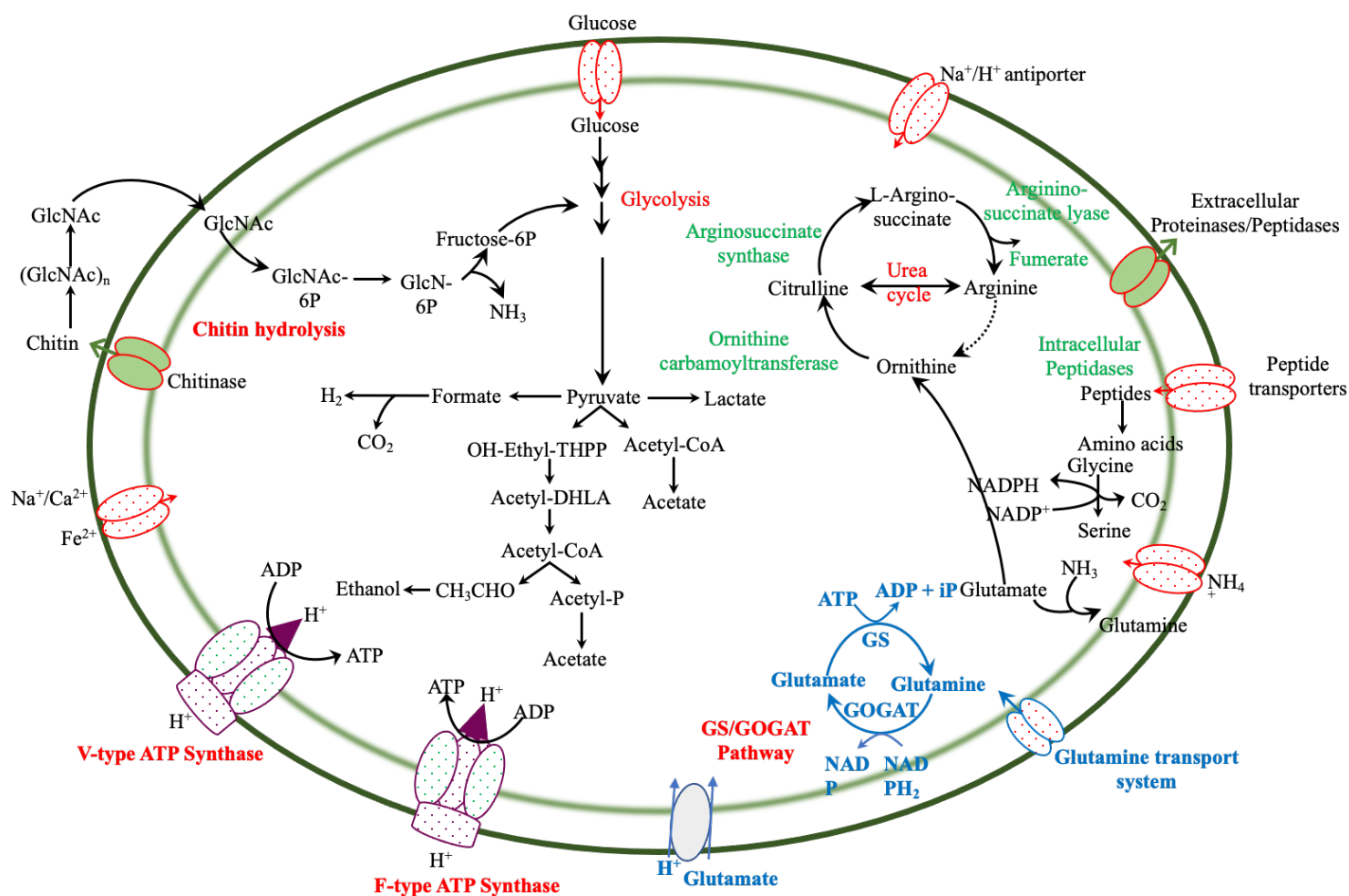


Fig. S15: Reconstruction of the central metabolic pathway of '*Ca Atrisphaera chitinolytica*' strain Plnct-SY6 based on different physiological analyses and by the presence of various genes identified in the MAG. GlcNAc, *N*-acetyl-D-glucosamine; GlcNAc-6P, *N*-acetyl-D-glucosamine-6-phosphate; GS, glutamine synthetase; glutamate synthase (GOGAT).

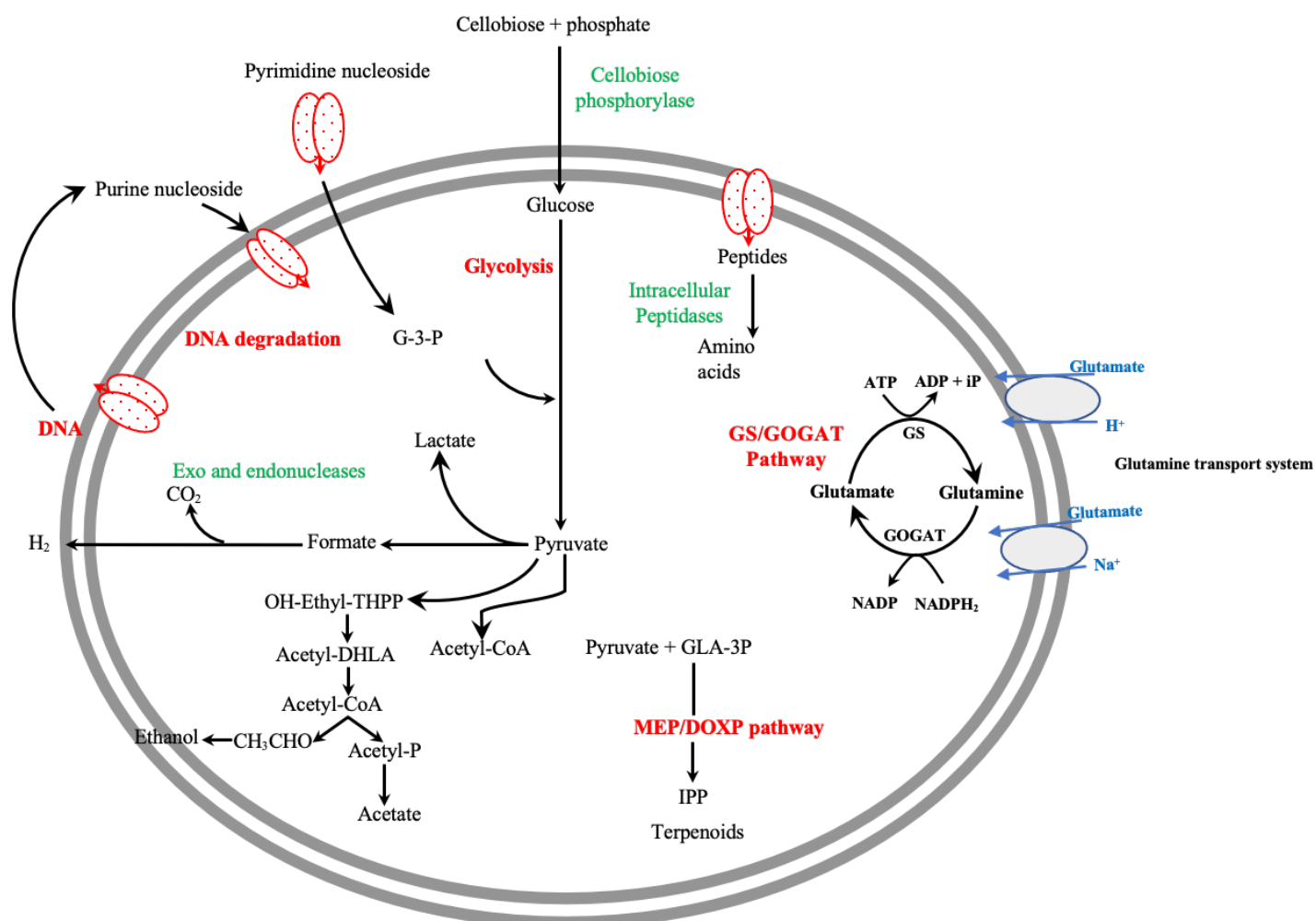


Fig. S16: Reconstruction of the central metabolic pathway of '*Ca Atrimarinoplasma sulfidophila*' Izemo-BS based on different physiological analyses and by the presence of various genes identified in the genome sequence. GS, glutamine synthetase; glutamate synthase (GOGAT).

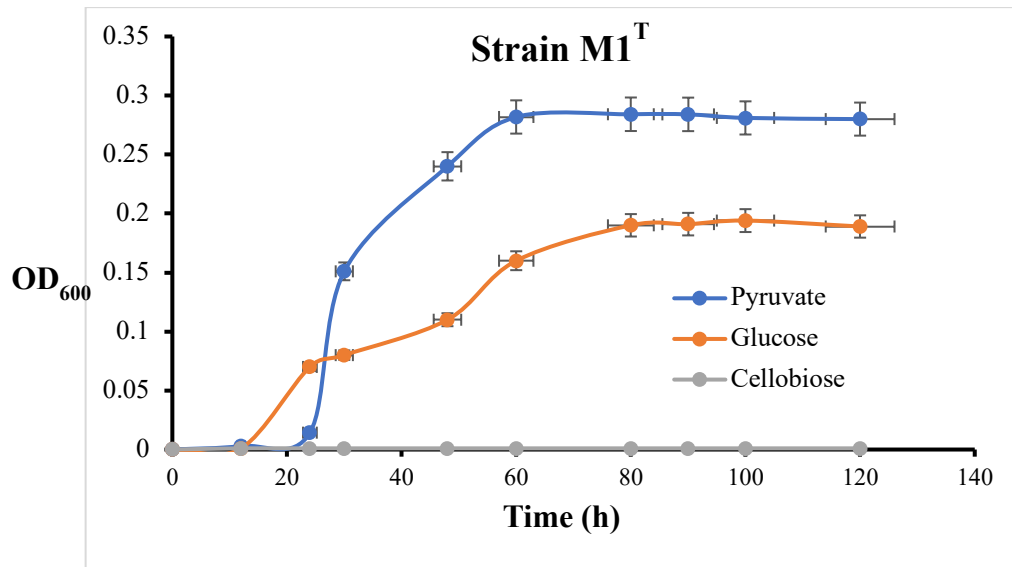


Fig. S17: Growth of *Oceanispirochaeta piezotolerans* strain M1^T at various carbon substrate at 20 °C under strict anaerobic condition.

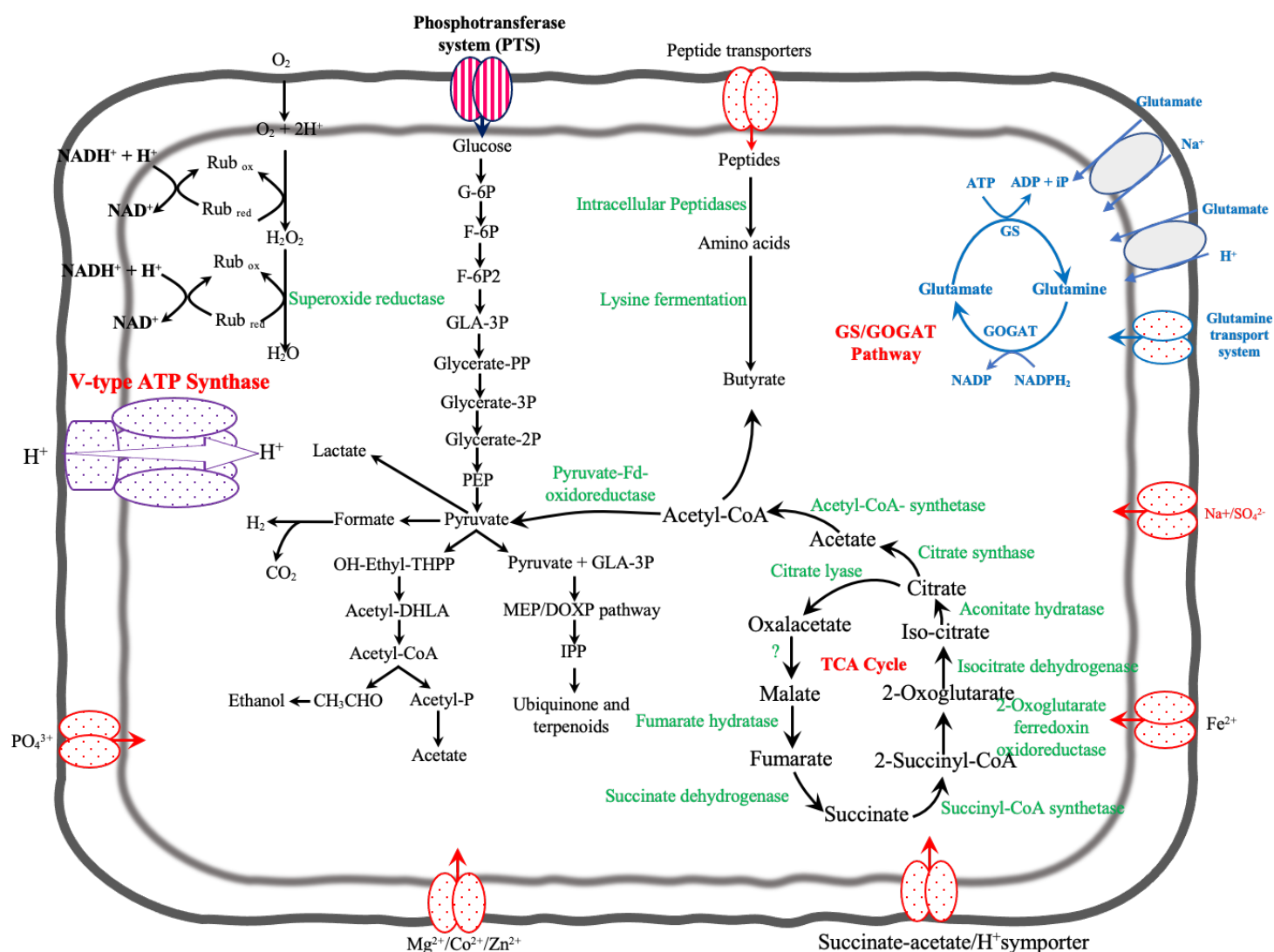


Fig. S18: Reconstruction of the central metabolic pathway of *Oceanispirochaeta piezotolerans* strain M1^T and M2 based on different physiological analyses and by the presence of various genes identified in the genome sequence. G-6P, glucose-6-phosphate; F-6P, fructose-6-phosphate; GLA-3P, glyceraldehyde-3-phosphate; PEP, phosphoenol-pyruvate; GS, glutamine synthetase; glutamate synthase (GOGAT).

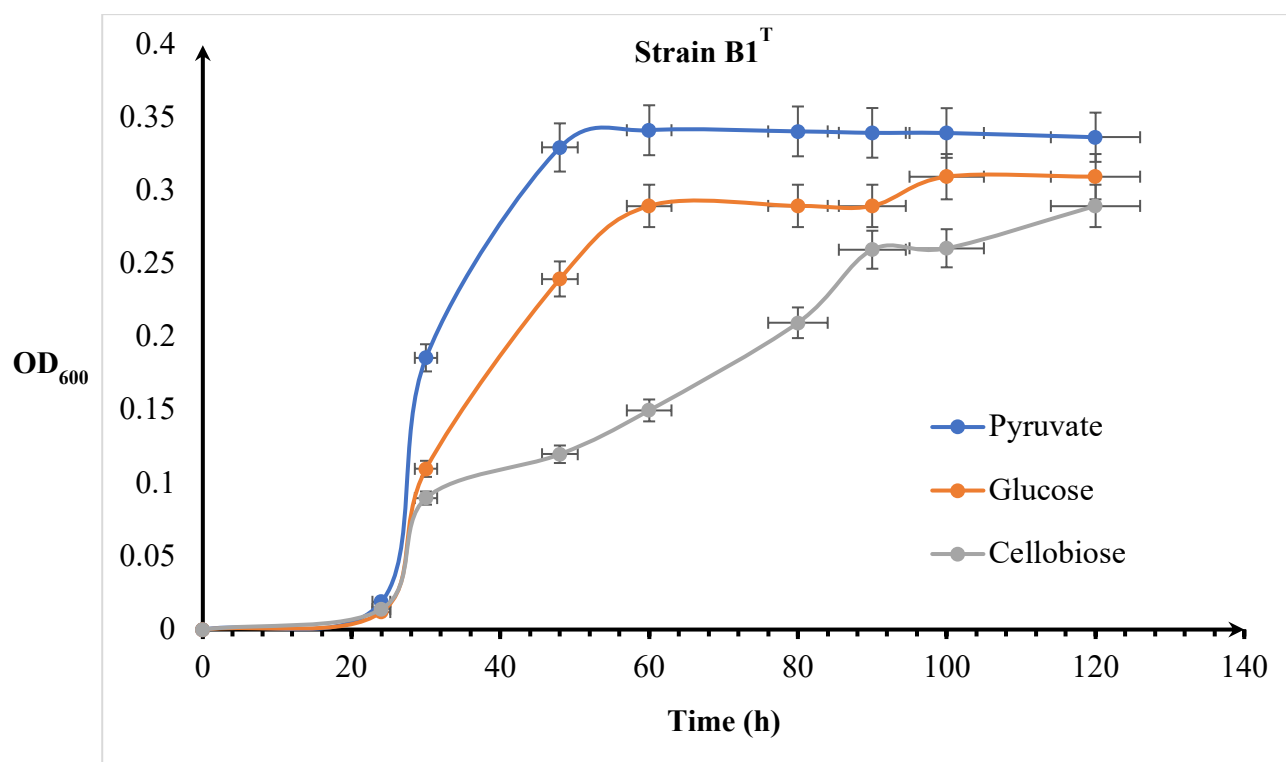


Fig. S19: Growth of *Lutibacter atrimaris* strain B1^T at various carbon substrates at 20 °C under microaerophilic condition.

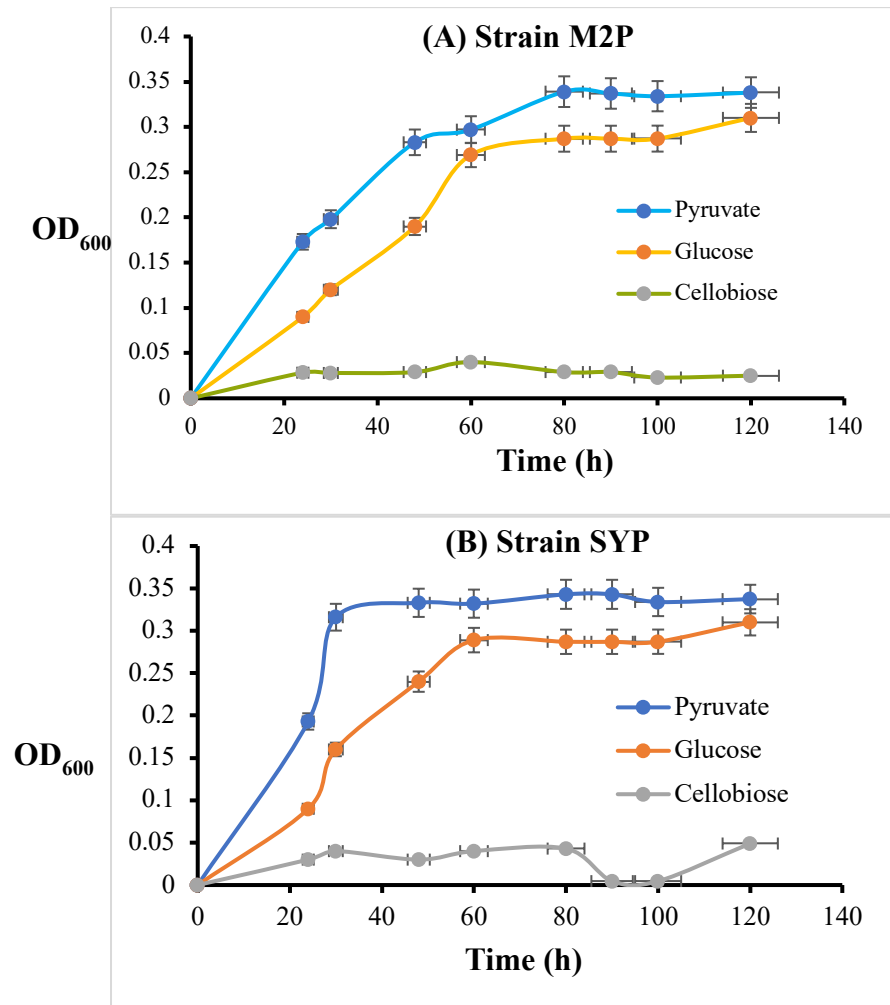


Fig. S20: (A) Growth of *Ancylomarina euxinus* strain M2P and (B) *Labilibaculum euxinus* strain SYP on various carbon substrates at 20 °C under strict anaerobic condition.

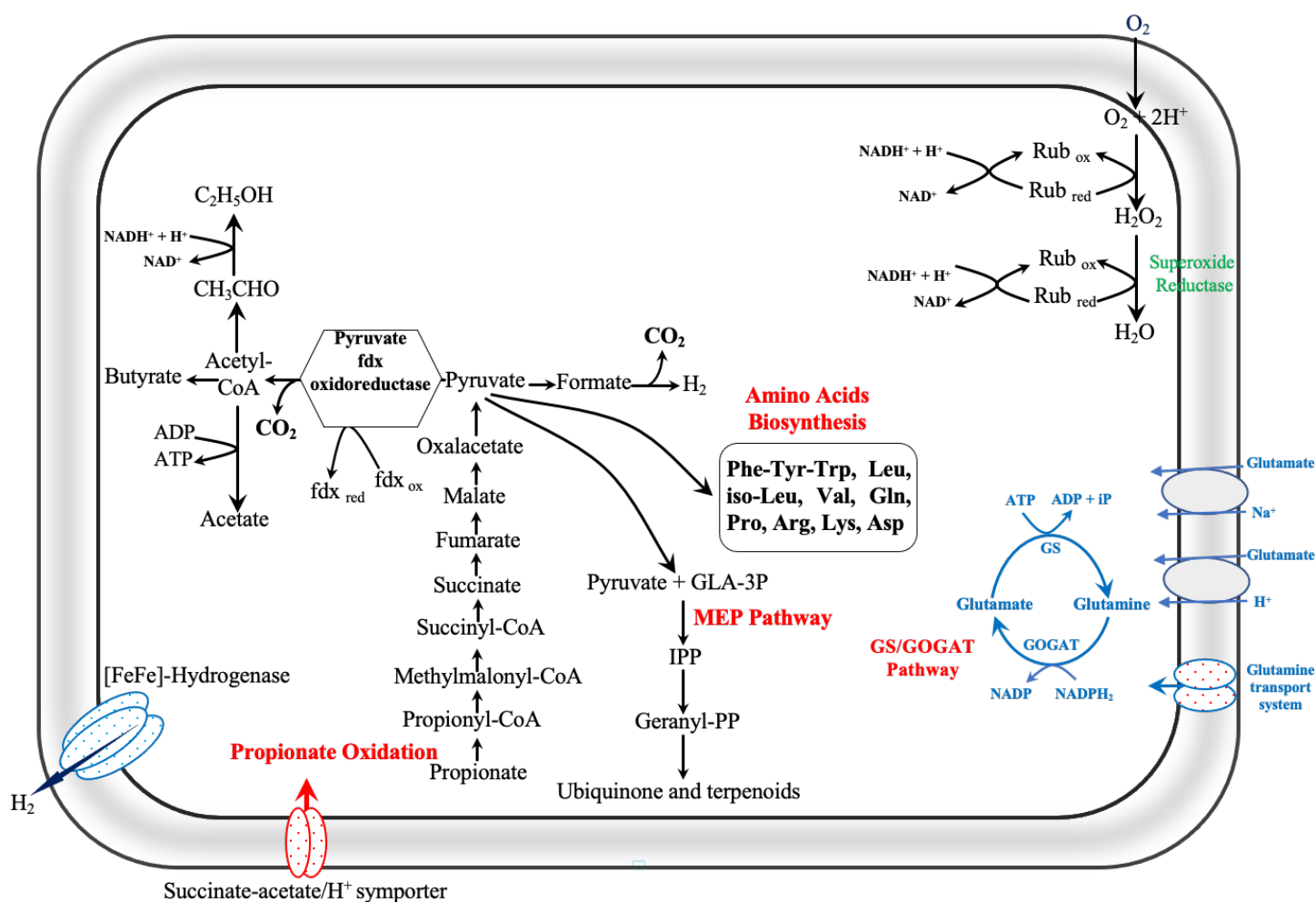


Fig. S21: Reconstruction of the central metabolic pathway of '*Ca Atrimarinobacter sulfidophilus*' Cloa-SY6 based on different physiological analyses and by the presence of various genes identified in the MAG. GS, glutamine synthetase; glutamate synthase (GOGAT).

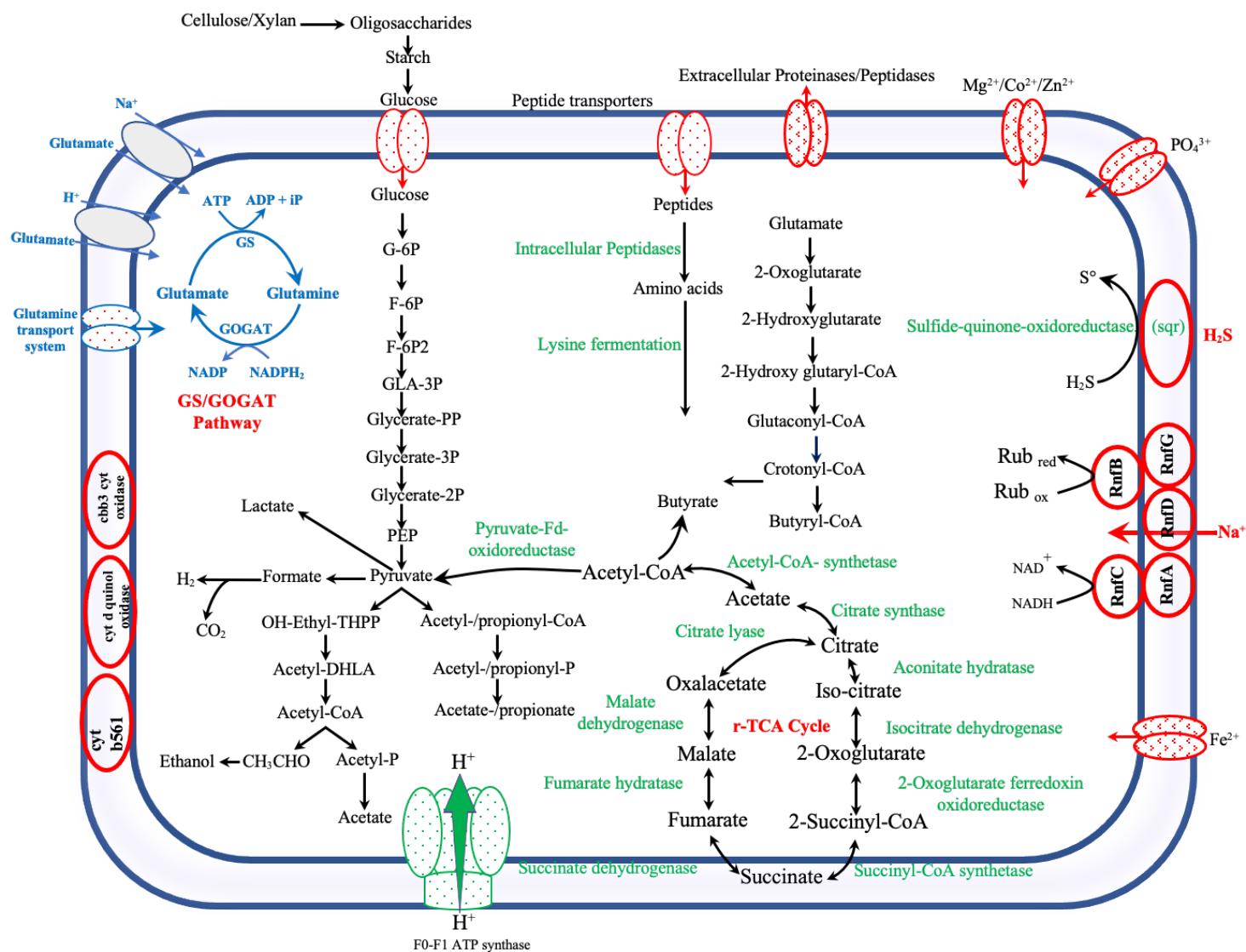


Fig. S22: Reconstruction of the central metabolic pathway of ‘*Ca Pontauxinibacter sulfidophilus*’ strain Igna-SY6 based on different physiological analyses and by the presence of various genes identified in the MAG. G-6P, glucose-6-phosphate; F-6P, fructose-6-phosphate; GLA-3P, glyceraldehyde-3-phosphate; PEP, phosphoenol-pyruvate; GS, glutamine synthetase; glutamate synthase (GOGAT).

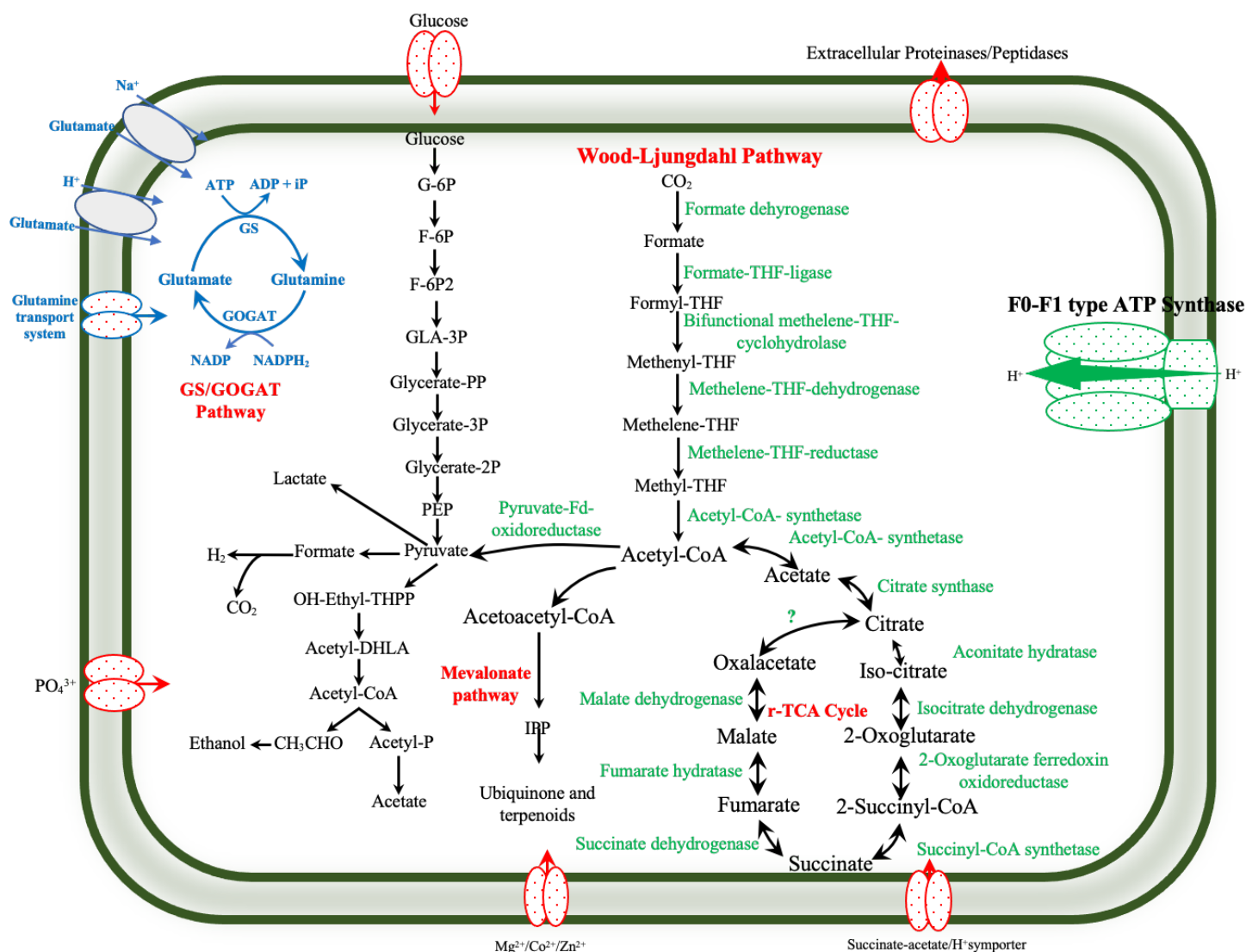


Fig. S23: Reconstruction of the central metabolic pathway of ‘*Ca Euxinilinea sulfidophila*’ Chflx-SY6 based on different physiological analyses and by the presence of various genes identified in the MAG. G-6P, glucose-6-phosphate; F-6P, fructose-6-phosphate; GLA-3P, glyceraldehyde-3-phosphate; PEP, phosphoenol-pyruvate; GS, glutamine synthetase; glutamate synthase (GOGAT).

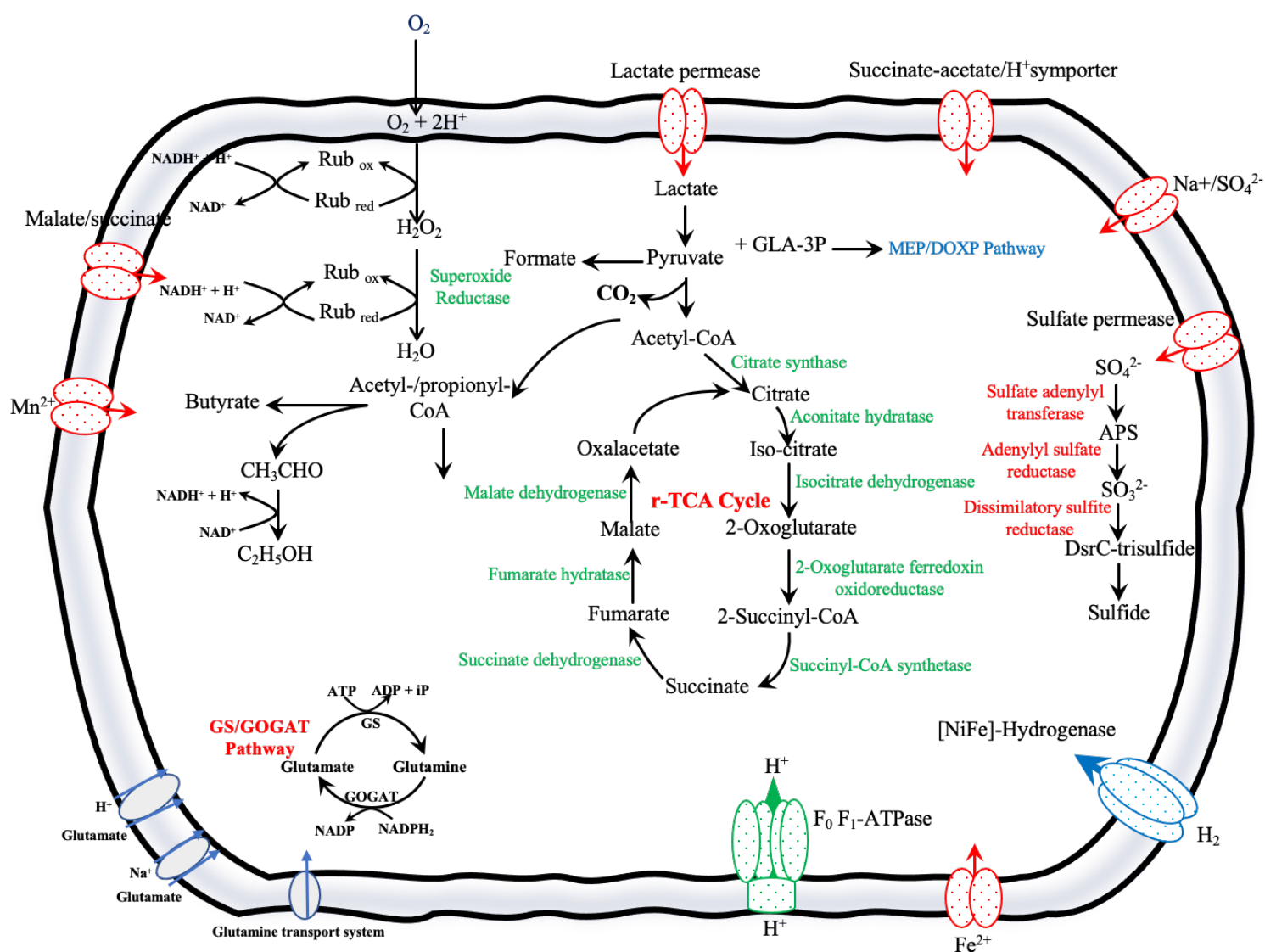


Fig. S24: Reconstruction of the central metabolic pathway of *Pseudodesulfovibrio turroides* strain S3^T and S3-i based on different physiological analyses and by the presence of various genes identified in the MAG. GLA-3P, glyceraldehyde-3-phosphate; GS, glutamine synthetase; glutamate synthase (GOGAT).

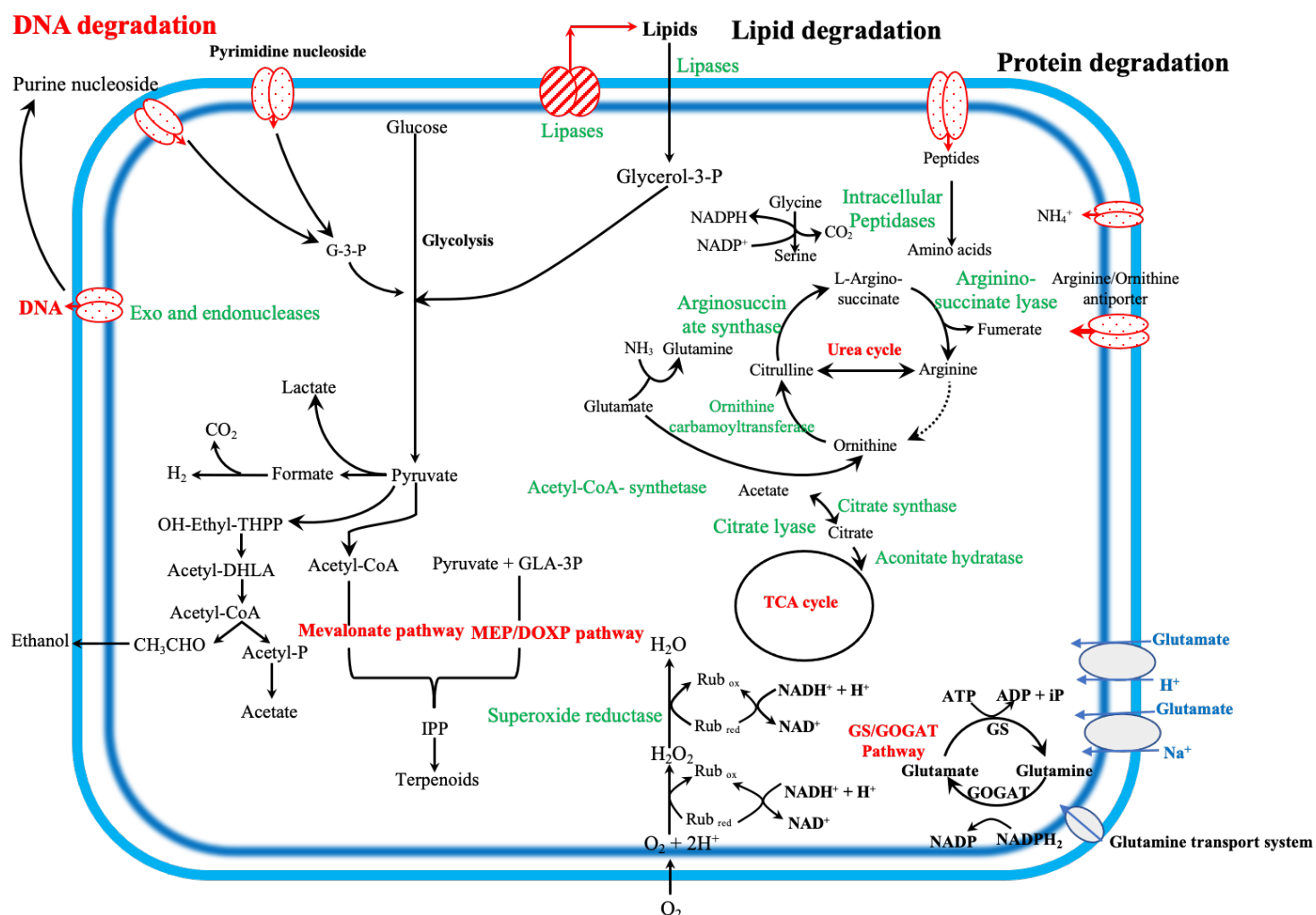


Fig. S25: Reconstruction of the central metabolic pathway of members of the phylum *Bacteroidota* (*Lutibacter atrimar* strain B1^T, B2, ‘*Ca* Bradibacterium flavus strains S6 and L6; *Ancylomarina euxinus* strain M2P, M1P^T and M3P; *Labilibaculum euxinus* strains SYP, A4^T and 44) based on different physiological analyses and by the presence of various genes identified in the MAG. G-3-P, glycerol-3-phosphate; GLA-3P, glyceraldehyde-3-phosphate; GS, glutamine synthetase; glutamate synthase (GOGAT).