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Comparative genomics analyses indicate differential methylated amine utilisation trait within members of the genus *Gemmobacter*

Running title:

Methylated amine utilisation trait in *Gemmobacter*

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Abstract

Methylated amines are ubiquitous in the environment and play a role in regulating the earth's climate via a set of complex biological and chemical reactions. Microbial degradation of these compounds is thought to be a major sink. Recently we isolated a facultative methylotroph, *Gemmobacter* sp. LW-1, an isolate from the unique environment Movile Cave, Romania, which is capable of methylated amine utilisation as a carbon source. Here, using a comparative genomics approach, we investigate how widespread methylated amine utilisation trait is within the member of the bacterial genus *Gemmobacter*. Five genomes of different *Gemmobacter* species isolated from diverse environments, such as activated sludge, fresh water, sulphuric cave waters (Movile Cave) and the marine environment were available from the public repositories and used for the analysis. Our results indicate that some members of the genus *Gemmobacter*, namely *G. aquatilis*, *G. caeni* and *G.* sp. LW-1 have the genetic potential of methylated amine utilisation while others (*G. megaterium* and *G. nectariphilus*) have not.

Introduction

Methylated amines (MAs) are ubiquitous in the environment with a variety of natural and anthropogenic sources including the oceans, vegetation, sediments and organic-rich soils, animal husbandry, food industry, pesticides, sewage, and automobiles, to mention only a few [1-3]. Methylated amines are also known to influence earth's climate, via a series of complex biological and chemical interactions [4]. Some of the most abundant methylated amines found in the atmosphere are trimethylamine (TMA), dimethylamine (DMA) and monomethylamine (MMA) [1]. Microbial

metabolism of methylated amines involves both aerobic and anaerobic microorganisms, e.g. some methanogenic archaea such as *Methanosarcina* and *Methanomicrobium* can use MAs to produce methane [5-7] while Gram-positive and Gram-negative methylotrophic bacteria can use MAs as carbon and nitrogen source [8]. Previously, MAs were typically associated with marine ecosystems as they are by-products of degradation of osmolytic chemicals such as glycine betaine, carnitine, choline and trimethylamine N-oxide [8]. However, recent studies have reported the detection and activity of aerobic methylotrophic bacteria that utilise MAs in a variety of natural and engineered environments [1, 9-12] and could play a major role in global C and N budgets.

Aerobic methylotrophs are a polyphyletic group of microorganisms capable of utilising one-carbon (C_1) compounds such as methane, methanol or methylated amines as their sole source of carbon and energy [9, 13, 14]. Methylotrophs can degrade TMA to DMA by using the enzymes TMA dehydrogenase, TMA monooxygenase or TMA methyltransferase (under anaerobic conditions by methylotrophic methanogens), encoded by the genes *tdm*, *tmm* and *mtt*, respectively [15-18]. The enzymes DMA dehydrogenase (*dmd*) or DMA monooxygenase (*dmmDABC*) modulate the conversion of DMA to MMA [14, 15]. Two distinct pathways have been characterised for the oxidation of MMA [10]. The direct MMA-oxidation pathway mediated by a single enzyme (MMA dehydrogenase in Gram-negative bacteria and MMA oxidase in Gram-positive bacteria) converts MMA to formaldehyde and releases ammonium [19, 20]. The alternate pathway, referred to as the *N*-methylglutamate (NMG) pathway or indirect MMA-oxidation pathway, is mediated by three individual enzymes via the oxidation of MMA to gamma-

glutamylmethylamide (GMA) and its further degradation to *N*-methylglutamate (NMG) and 5,10-methylenetetrahydrofolate (CH_2 = H_4F) [3, 10]. A stepwise conversion of MMA in the NMG pathway is modulated by the enzymes GMA synthetase (*gmaS*), NMG synthase (*mgsABC*) and NMG dehydrogenase (*mgdABCD*) [3, 8]. The capability to use MMA not only as a source for carbon but also for nitrogen is widespread in bacteria. Notably, the NMG pathway is not only restricted to methylotrophs but also present in non-methylotrophic bacteria that use MMA as a nitrogen but not as a carbon source [15, 21, 22].

In a recent study, we isolated an alphaproteobacterial facultative methylotrophic bacterium, *Gemmobacter* sp. LW-1 (recently renamed from *Catellibacterium* [23]) from the Movile Cave ecosystem (Mangalia, Romania) [24] that can use methylated amines as both carbon and nitrogen source [12] and subsequently obtained its genome sequence [25]. Using a ^{13}C-MMA DNA based stable-isotope probing (SIP) experiment we also showed that *Gemmobacter* sp. LW-1 was indeed an active MMA utiliser in microbial mats from this environment [12]. This was the first report of methylated amine utilisation in a member of the bacterial genus *Gemmobacter*. However, growth on C_1 compounds (methanol and formate) has been reported for the genus *Gemmobacter*, e.g. in *G. caeni* [26]. The genus *Gemmobacter* (family *Rhodobacteraceae*) currently comprises ten validated species: *Gemmobacter megaterium* [27], *G. nectariphilum* [23, 28], *G. aquatilis* [29], *G. caeni* [23, 26], *G. aquaticus* [23, 30], *G. nanjingense* [23, 31], *G. intermedius* [32], *G. lanyuensis* [33], *G. tilapiae* [34] and *G. fontiphilus* [23]. These species were isolated from a wide range of environments including fresh water environments (freshwater pond [29, 34], freshwater spring [23, 33]), coastal planktonic seaweed [27], white stork nestling [32],

waste water and activated sludge [26, 28, 31], suggesting that members of the genus *Gemmobacter* are widely distributed in engineered and natural environments.

Here, using a comparative genomics approach we study how widespread methylated amine utilisation trait (i.e. metabolic potential) is within the members of the genus *Gemmobacter*. We used five isolate genomes for members within the genus *Gemmobacter* (*G*. sp. LW-1, *G. caeni*, *G. aquatilis*, *G. nectariphilus* and *G. megaterium)* alongside genomes of four closely related organisms within the family *Rhodobacteraceae* to show that the methylated amine utilisation trait is not common within the members of the genus *Gemmobacter*.

Materials and Methods

Genome data acquisition

Five *Gemmobacter* genomes (*G. caeni*, *G. aquatilis*, *G. nectariphilus*, *G. megaterium*, *Gemmobacter* sp. LW-1) available through the Integrated Microbial Genomes (IMG) database (https://img.jgi.doe.gov/) were used for comparative genome analysis [35]. Accession numbers and genome characteristics are listed in Supplementary Table S1.

Phylogenetic analysis

Phylogenetic relatedness between the different members of the genus *Gemmobacter* was determined using phylogenetic trees constructed from 16S rRNA gene sequences (nucleotide) and metabolic gene sequences (*gmaS* and *mauA*; amino acids) involved in MMA utilisation. RNAmmer [36] was used to retrieve 16S rRNA gene sequences from the genome sequences. Multiple sequence alignment of 16S rRNA gene sequences was performed using the SINA alignment service via SILVA [37, 38] and

subsequently imported into MEGA7 [39] to construct a maximum-likelihood nucleotide-based phylogenetic tree [40]. Bootstrap analysis was performed with 1000 replicates to provide confidence estimates for phylogenetic tree topologies [41].

To determine phylogenetic affiliations for the protein encoding genes *gmaS* and *mauA*, gene sequences retrieved from the genome sequences were aligned to homologous sequences retrieved from the NCBI Genbank database using Basic Local Alignment Search Tool (BLAST, blastx) [42] and curated *gmaS* sequences used for primer design in our previous study [12]. Amino acid sequences were aligned in MEGA7 [39] using ClustalW [43] and the alignment was subsequently used to construct maximum likelihood phylogenetic trees based on the JTT matrix-based model [44]. Bootstrap analysis was performed with 1000 replicates to provide confidence estimates for phylogenetic tree topologies [41].

We investigated the phylogeny of nine bacterial species – *Gemmobacter aquatilis*, *Gemmobacter caeni*, *Gemmobacter sp. LW-1*, *Gemmobacter megabacterium* DSM-26375, *Gemmobacter nectariphilus* DSM-15620, *Paracoccus denitrificans* PD1222, *Citreicella* sp. SE45, *Roseovarius* sp. TM1035 and *Rhodobacter sphaeroides* 241 – using the protein sequences of 831 orthologs present in single copy in all species identified using OrthoFinder [45] with default settings. For each set of orthologous proteins, multiple alignments were produced using MAFFT [46] with the --auto settings. Subsequently, conserved alignment blocks were identified using trimal [47] with the option -automated1. The phylogenetic reconstruction analysis was based in a final concatenated alignment consisting of 265,536 amino acid positions, which was the input for RAxML [48] with the option PROTCATLGF. The selection of best

amino acid substitution model was performed after implementing the script ProteinModelSelection.pl provided by the RAxML package. In order to assess the environmental distribution of the genus *Gemmobacter*, we used MAPseq [49] (https://beta.microbeatlas.org) to survey the relative abundance based on 16S rRNA gene sequences (query sequence: *Gemmobacter aquatilis* DSM3857 (NR_104740.1 at 97% cut-off).

Comparative genomic analyses

CGView Comparison Tool (CCT) was used to visually compare the genomes within the genus *Gemmobacter* [50]. CCT utilises BLAST to compare the genomes and the BLAST results are presented in a DNA-based graphical map [50]. Average Nucleotide Identity (ANI) [51] between different genomes was estimated using one-way ANI (best hit) and two-way ANI (reciprocal best hit) based on Goris *et al*. [52]. In addition the whole-genome based average nucleotide identity (gANI) and the $p_r^{\text{intra-species}}$ value were determined for *G.* sp. LW-1 and *G. caeni* (these two genomes revealed the closest ANI) based on Konstantinidis and Tiedje [53] via the Joint Genome Institute (JGI) platform (https://ani.jgi-psf.org/html/home.php; Version 0.3, April 2014). In order to determine if two genomes belong to the same species, the computation of empirical probabilities ($p_r^{\text{intra-species}}$) can be calculated as follows,

$$p_r^{intra-species}[AF = a, ANI = b] = p_r^{intra-species}[AF = a] * p_r^{intra-species}[ANI = b|AF = a]$$

AF represents alignment fraction.

Pan-genome analysis including average amino acid identity (AAI) analysis, pan-genome tree construction and determination of core and dispensable genes and singletons (unique genes) was carried out using the Efficient Database framework for

comparative Genome Analyses using BLAST score Ratios (EDGAR) platform [54]. Estimation of genomic completeness and contamination was carried out using the CheckM program [55].

In order to compare the genetic potential for methylated amine utilisation within the available *Gemmobacter* genomes, known protein sequences involved in methylated amine utilisation pathways [3, 15] were used as query sequences through the BLAST (blastp) program [42] available within the Rapid Annotation using Subsystem Technology (RAST) server [56]. The list of protein queries used is given in Supplementary Table S2.

Results and discussion

Analysis of phylogenetic relatedness

The phylogenetic relatedness of the five members within the genus *Gemmobacter* (*G.* sp. LW-1, *G. caeni*, *G. aquatilis*, *G. nectariphilus* and *G. megaterium*) was resolved based on 16S rRNA gene sequences (Figure 1). Three members of the genus *Gemmobacter* (*G.* sp. LW-1, *G. caeni*, and *G. aquatilis)* clustered together with several other related *Gemmobacter* and *Rhodobacter* 16S rRNA gene sequences retrieved from fresh water, soil and sediment and activated sludge environments (Figure 1). *G. nectariphilus* and *G. megaterium* sequences clustered together with *Paracoccus kawasakiensis* and other related *Gemmobacter* sequences from marine, fresh water and activated sludge environments (Figure 1). Based on the 16S rRNA gene sequences retrieved from public database and MAPseq analyses, we observed that the members of the genus *Gemmobacter* are widely distributed in engineered (such as activated sludge and clinical environments) and natural environments i.e.

fresh water, soil and sediment, and marine environments (Figure 1 and Supplementary Figure S3). Phylogenomic analysis based on single copy marker genes (Supplementary Figure S4) revealed that *G.* sp LW1, *G. caeni and G. aquatilis* were closely related to *Rhodobacter sphaeroides* 241 whereas *G. megabacterium and G. nectariphilus* to *Paracoccus denitrificans.*

GMA synthetase, a key enzyme in the NMG pathway, is encoded by the gene *gmaS*. *gmaS* sequences retrieved from the isolate genomes along with other ratified *gmaS* sequences were used to construct an amino acid-based phylogenetic tree (Figure 2). *gmaS* gene sequences retrieved from genomes of *G.* sp. LW-1, *G. caeni* and *G. aquatilis* clustered within Group I of alphaproteobacterial *gmaS* sequences containing sequences from marine and non-marine bacteria within the orders *Rhodobacterales* and *Rhizobiales* as described in Wischer et al. [12] and were closely related to *Paracoccus yeei*, *P.* sp. 1W-5 and *Rhodobacter* sp. 1W-5 (Figure 2). Whilst *gmaS* gene sequences were detected in three of the five investigated *Gemmobacter* genomes, *mauA* gene sequences were identified only in the genomes of *G. caeni* and *G.* sp. LW-1 (Supplementary Figure S1). It has been suggested that the NMG pathway for MMA utilisation is more universally distributed and more abundant across proteobacterial methylotrophs than the direct MMA oxidation pathway [57]. However, it should be noted that genes encoding for the enzymes within the NMG pathway (*gmaS*) can not only be detected in methylotrophs but also in non-methylotrophic bacteria that use MMA as a nitrogen source, but not as a carbon source [12, 15].

A comparative genome analysis of members within the genus Gemmobacter

At the time of the analysis, five *Gemmobacter* genomes obtained from isolates from different environments were available (Figure 1 and Table 2). *Gemmobacter* genome sizes range from ~3.96 Mb to ~5.14 Mb with GC contents between 64.71% to 66.19% and genome completeness between 98.61% to 99.69% (Table 2; Supplementary Table 1). Analysis of sequence annotations revealed that on average 91.13% of the genomes consist of coding sequences.

The genomes were compared using the CGView comparison tool [50] (Figure 3). *Gemmobacter* sp. LW-1, isolated from the Movile Cave ecosystem was used as the reference genome and the results of the BLAST comparison with other *Gemmobacter* genomes are represented as a BLAST ring for each genome (Figure 3). Similarities between segments of the reference genome sequence and the other genome sequences are shown by a coloured arc beneath the region of similarity indicating the percentage of similarity as a colour code. Our analysis (Figure 3) revealed low identity levels (mostly <88%) between *Gemmobacter* sp. LW-1 and *G. aquatilis*, *G. nectariphilus* and *G. megaterium* across the genomes. Moreover, the analysis suggested several sites of potential insertion/deletion events in the genome of *Gemmobacter* sp. LW-1. Possible insertion/deletion regions can be identified as those gaps in the map where no homology is detected. For example, the region between 2200-2300 kbp (Figure 3) where a gap can be found in the otherwise contiguous homologous regions between the reference genome *G.* sp. LW-1 and the first of the query genomes (*G. caeni*). This might likely be due to a lack of hits or hits with low identity that can be spurious matches. Since it covers a large region we could possibly rule out that it is not an artefact arising from a lack of sensitivity in the BLAST analysis. Even though the genomes of *G.* sp. LW-1 and *G. caeni* are closely related, our analysis demonstrates

that their genomes are not completely identical. Despite the fact that the majority of their genomes indicate very high identity levels (mostly >96-98% as shown by the dominance of dark red colours of the circle representing the BLAST hit identity between *G.* sp. LW-1 and *G. caeni*), many segments appear to be exclusive to *G.* sp. LW-1.

In order to further resolve the similarity between these genomes we calculated the average nucleotide identity (ANI) [51] (Supplementary Table S3 and Supplementary Figure S2A-D) and the average amino acid identity,(AAI; Supplementary Figure S2E). It is generally accepted that an ANI value of >95-96% can be used for species delineation [58, 59]. Our analysis revealed that *Gemmobacter* sp. LW-1 and *Gemmobacter caeni* share an ANI value of 98.62 (Supplementary Table S3) implying that both are in fact the same species. The genome-based average nucleotide identity (gANI) between *G.* sp. LW-1 and *G. caeni* was calculated as 98.70. The AF was calculated to be 0.91, which would result in a computed probability of 0.98 suggesting that both genomes might belong to the same species. However, it should be noted that these are draft genomes and a more in depth characterization of their physiology and phenotype is required to delineate these organisms at the level of strain.

Pan-genome analysis, carried out using the EDGAR platform [54], identified metabolic genes present in all *Gemmobacter* species (core genes), two or more *Gemmobacter* species (accessory or dispensable genes), and unique *Gemmobacter* species (singleton genes). A pan-genome tree was constructed (Figure 4A) based on the pan-genome dataset and neighbor-joining method [40]. As with the 16S-rRNA

gene based phylogenetic tree (Figure 1), the five *Gemmobacter* species formed two main clusters in the pan-genome tree analysis (Figure 4A). The pan-genome tree also confirmed the phylogenetic closeness between *Gemmobacter caeni* and *Gemmobacter* sp. LW-1 (Figure 4A). According to pan-genome analysis of the five *Gemmobacter* genomes, a total of 9,286 genes were identified, consisting of 1,806 core genes, 3085 dispensable genes and 305, 1,072, 896, 1,165 and 957 singletons for *G*. sp. LW-1, *G. caeni*, *G. aquatilis*, *G. nectariphilus* and *G. megaterium*, respectively (Figure 4B).

Methylated amine utilisation, N assimilation and C_1 oxidation

Investigation of the methylated amine utilisation pathways in five *Gemmobacter* species revealed the presence of the genes encoding enzymes TMA dehydrogenase (*tmd*), TMA monooxygenase (*tmm*), TMAO demethylase (*tdm*) and DMA monooxygenase in genomes of *G*. sp. LW-1, *G. caeni* and *G. aquatilis* while none of these genes were detected in *G. nectariphilus* or *G. megaterium* (Figure 5). These findings are supported by results from a previous study which showed growth of *G.* sp. LW-1 on TMA as a carbon and nitrogen source [12]. *G*. sp. LW-1, *G. caeni* and *G. aquatilis* could potentially use the TMA oxidation pathway to convert TMA to DMA. Based on the genome sequences, it can be suggested that these three species could use the enzyme DMA monooxygenase (*dmmDABC*) to oxidize DMA to MMA but not the DMA dehydrogenase since the corresponding protein encoding gene (*dmd*) was not found (Figure 5).

We also compared the distribution of the direct MMA-oxidation and the NMG pathways in the genomes of five *Gemmobacter* species (Figure 5) and gene arrangement (Supplementary Figure S5). The direct MMA-oxidation pathway (*mauA*-

dependent) is so far only known to be present in methylotrophic bacteria that can use MMA as a carbon source. Whereas the NMG pathway (*gmaS*-dependent) has been shown to be present in non-methylotrophic bacteria that can use MMA as a nitrogen source [12, 21, 57, 60]. Analysis of the genome sequences revealed that both *G*. sp. LW-1 and *G. caeni* possess genes for both MMA oxidation pathways (Figure 5). We have previously shown that *Gemmobacter* sp. LW-1 can use MMA and TMA as both a carbon and nitrogen source [12]. Genome sequence of *G. aquatilis* indicated the presence of genes involved only in the NMG pathway. In the facultative methylotroph *Methylobacterium extorquens* AM1 it has been shown that the NMG pathway is advantageous compared to the direct MMA-oxidation pathway [60]. NMG pathway enables facultative methylotrophic bacteria to switch between using MMA as a nitrogen source or as a carbon and energy source whereas the direct MMA oxidation pathway allows for rapid growth on MMA only as the primary energy and carbon source [60]. This could suggest that *G. aquatilis* might use the NMG pathway for utilising MMA as both nitrogen and carbon source. However, growth assays are required to confirm whether *G. aquatilis* can use MMA as a carbon source. We did not detect genes for either MMA oxidation pathways in the genome sequences of *G. nectariphilus* and *G. megaterium* suggesting the lack of genetic potential of these organisms to use MMA as either C or N source.

The C_1 units derived from methylated amines need to be further oxidized when the nitrogen is sequestered without assimilation of the carbon from the methylated amines. Genome analysis confirmed that all five *Gemmobacter* species possess the genetic capability for C_1 oxidation and also indicate that tetrahydrofolate (H_4F) is the C_1 carrier (Figure 5). The bifunctional enzyme 5,10-methylene-tetrahydrofolate

dehydrogenase/ cyclohydrolase, encoded by the gene *folD*, was detected in all the *Gemmobacter* genomes (Figure 5/ Table 1). Genes encoding key enzymes in the C_1 oxidation pathway via tetrahydromethanopterin (H_4MPT) were not detected. [10]. The formate-tetrahydrofolate ligase, encoded by the gene *fhs* (Figure 5), provides C_1 units for biosynthetic pathways [15]. However, the oxidation of formyl-H_4F (CHO-H_4F) can also be facilitated by *purU*, the gene encoding for the formyl-H_4F deformylase. The formate dehydrogenase (*fdh*) mediates the last step of the C_1 oxidation pathway, the oxidation of formate to CO_2. The genes for the C_1 oxidation pathway via H_4F were detected in all five *Gemmobacter* genomes.

The *fae* gene, encoding the formaldehyde-activating enzyme that catalyses the reduction of formaldehyde with H_4MPT was not detected in any of the five *Gemmobacter* genomes confirming that these members of the genus *Gemmobacter* lack the H_4MPT pathway for formaldehyde oxidation (Figure 5/ Table 1). Investigation of the nitrogen assimilation pathway revealed the presence of the genes encoding glutamine synthetase (GS; *gluL*) and glutamine synthase (GOGAT; *glxB*) in all five *Gemmobacter* genomes. In bacteria this pathway is essential for glutamate synthesis at low ammonium concentrations [15].

Using comparative genome analysis we provide genome-based evidence that the two *Gemmobacter* isolates *G.* sp. LW-1 and *G. caeni* are capable of generating energy from complete oxidation of methylated amines via the H_4F-dependent pathway using either the NMG pathway or the direct MMA oxidation pathway. *Gemmobacter aquatilis* is genetically capable of methylated amine degradation to yield formaldehyde and only encodes the genes for the NMG pathway, which indicates that

G. aquatilis could use this pathway to use MMA as a nitrogen source. Both *G. nectariphilus* and *G. megaterium* genomes indicate the lack of potential to use methylated amines (Figure 5/Table 1).

Gemmobacter sp. LW-1 was isolated from the Movile Cave ecosystem [12]. Microbial mats and lake water within the cave have been shown to harbor a wide diversity of methylated amine-utilising bacteria [12, 61]. Whilst the mechanism of MAs production within the system has to be elucidated, it can be speculated that degradation of floating microbial mats (i.e. organic matter) could result in MAs [12]. Similarly, *G. caeni* isolated from activated sludge [26] could possibly use the MAs generated from organic matter degradation. Interestingly, whilst *G. megaterium* was isolated from a marine environment (seaweed [27]) possibly encountering MAs from the degradation of osmolytes such as glycine betaine (*N,N,N*-trimethylglycine) we did not detect metabolic genes involved in methylated amine utilisation. Our genome-based analyses suggest that the potential for methylated amine utilisation is differential within the members of the genus *Gemmobacter* that requires confirmation using growth/activity assays.

Conclusions

In summary, three of the five investigated *Gemmobacter* genomes (*G.* sp. LW-1, *G. caeni* and *G. aquatilis*) indicated metabolic potential to utilise methylated amines, of which only two (*G.* sp. LW-1 and *G. caeni*) possess the genes for both MMA oxidation pathways, the NMG pathway and the direct MMA oxidation pathway. *G.* sp. LW-1 and *G. caeni* are facultative methylotrophs which could potentially use these pathways to utilise MMA as both a carbon and nitrogen source, while

potentially *G. aquatilis* could only use the NMG pathway as a nitrogen source. Furthermore, the genomes of *G.* sp. LW-1 and *G. caeni* showed a high similarity to each other (>98%) suggesting that both belong to the same species. *G. megaterium* and *G. nectariphilus* genomes indicated no metabolic potential to utilise MAs. Phylogenetic, pan-genome and ANI analyses revealed that *G.* sp. LW-1 and *G. caeni* are closely related, although they were isolated from different environments. Overall, our results suggest that the trait for methylated amine utilisation could be independent from the habitat and localised factors or selection pressures could influence the ability of these organisms to use methylated amines. Access to *Gemmobacter* isolates with or without the genetic potential for methylated amine utilisation trait will allow us to perform physiological experiments in future to test how this trait can affect fitness of closely related organisms.

Conflict of Interest

The authors declare no conflict of interest.

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499 changlensis comb. nov., Catellibacterium aquatile as Gemmobacter
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Figures and Tables

Figure 1. Phylogenetic tree based on 16S rRNA gene sequences. The tree was constructed using the maximum likelihood method for clustering and the Tamura-Nei

model for computing evolutionary distances. Numbers at branches are bootstrap percentages >50% of 1000 replicates. Star represents the *Gemmobacter* species used for comparative genome analysis. Coloured boxes represent the habitat where the sequence was retrieved: blue (fresh water), orange (soil and sediment), green (activated sludge), grey (marine), purple (clinical source). Triangles represent sequences that are listed as *Catellibacterium* in the NCBI database, which have been recently reclassified to *Gemmobacter* [23]. Scale bar: 0.02 substitutions per nucleotide position.

Figure 2. Maximum-likelihood phylogenetic tree based on *gmaS* sequences. The tree was constructed using amino acid sequences (GmaS) using the maximum-likelihood method based on the JTT matrix-based model. Members of the genus *Gemmobacter* used for genome comparison are represented with a star. Numbers at branches are bootstrap percentages >50% of 1000 replicates. Amino acid sequences of the glutamine synthetase type III (GlnA) were used as out-group. Scale bar: 0.1 substitutions per amino acid position. MRC, marine *Roseobacter* clade.

Figure 3. DNA BLAST map of *Gemmobacter* genomes. *Gemmobacter* sp. LW-1 was used as a reference genome against *Gemmobacter megaterium* (inner ring), *Gemmobacter aquatilis* (second inner ring), *Gemmobacter nectariphilus* (third ring), and *Gemmobacter caeni* (fourth ring). The fifth and sixth ring (outer rings) represent the CDS (blue), tRNA (maroon), and rRNA (purple) on the reverse and forward strand, respectively. The colour scale (inset) shows the level of sequence identity with the respective sequences from *G. megaterium, G. aquatilis, G. nectariphilus* and *G.*

caeni. The locations of genes involved in methylotrophy are indicated at the outside of the map.

Figure 4. Pan-genome analysis. (A) Pan-genome tree consisting of five *Gemmobacter* species was constructed using the neighbour-joining method within the EDGAR platform. **(B)** Number of core, dispensable, and specific genes (singletons) of each *Gemmobacter* species.

Figure 5. Metabolic pathways involved in methylated amine utilisation and one-carbon utilisation annotated with presence/absence of specific genes in the genomes of *Gemmobacter*. The analysis was based on a five-way comparison among *Gemmobacter* sp. LW-1 (L), *Gemmobacter caeni* (C), *Gemmobacter aquatilis* (A), *Gemmobacter nectariphilus* (N) and *Gemmobacter megaterium* (M). The color-coded boxes next to the genes indicate the presence (green) or absence (orange) of a gene in each genome.

Table 1. Comparative genomic analysis of methylated amine-utilising genes in genomes-sequenced *Gemmobacters* in comparison to selected marine *Roseobacter* clade bacteria. Shown is the presence (+) or absence (-) of specific genes in the genome sequences.

Supplementary Figure S1. Maximum-likelihood phylogenetic tree (JTT matrix-based) of *mauA* sequences. Sequences from the genus *Gemmobacter* are marked with a star. Amino acid sequences (MauA) were aligned using the ClustalW algorithm. Numbers at branches are bootstrap percentages >50% of 1000 replicates. Scale bar:

0.1 substitutions per amino acid. Coloured boxes indicate *Alphaproteobacteria (*yellow), *Gammaproteobacteria (*red) and *Betaproteobacteria (*black).

Supplementary Figure S2. (A-D) Average nucleotide identity (ANI) analysis of *Gemmobacter* sp. LW-1 and *Gemmobacter caeni*, *Gemmobacter aquatilis*, *Gemmobacter nectariphilus* and *Gemmobacter megaterium* and (E) AAI analysis between those species

Supplementary Figure S3. Distribution of *Gemmobacter* in different ecosystems

Supplementary Figure S4. Maximum-likelihood phylogenetic tree of concatenated single copy marker genes from genomes of *Gemmobacter* and closely related organisms.

Supplementary Figure S5. Arrangement of genes involved in methylated amine utilisation

Supplementary Table S1. Genome characteristics of the five *Gemmobacter* isolate genomes used in this study.

Supplementary Table S2. List of protein queries used for the genome comparison with their accession number.

Supplementary Table S3. Average nucleotide identity (ANI) values between *Gemmobacter* sp. LW-1 and *Gemmobacter caeni*, *Gemmobacter aquatilis*,

Gemmobacter nectariphilus, *Gemmobacter megaterium*, *Rhodobacter sphaeroides* and *Paracoccus denitrificans*

Figure 1

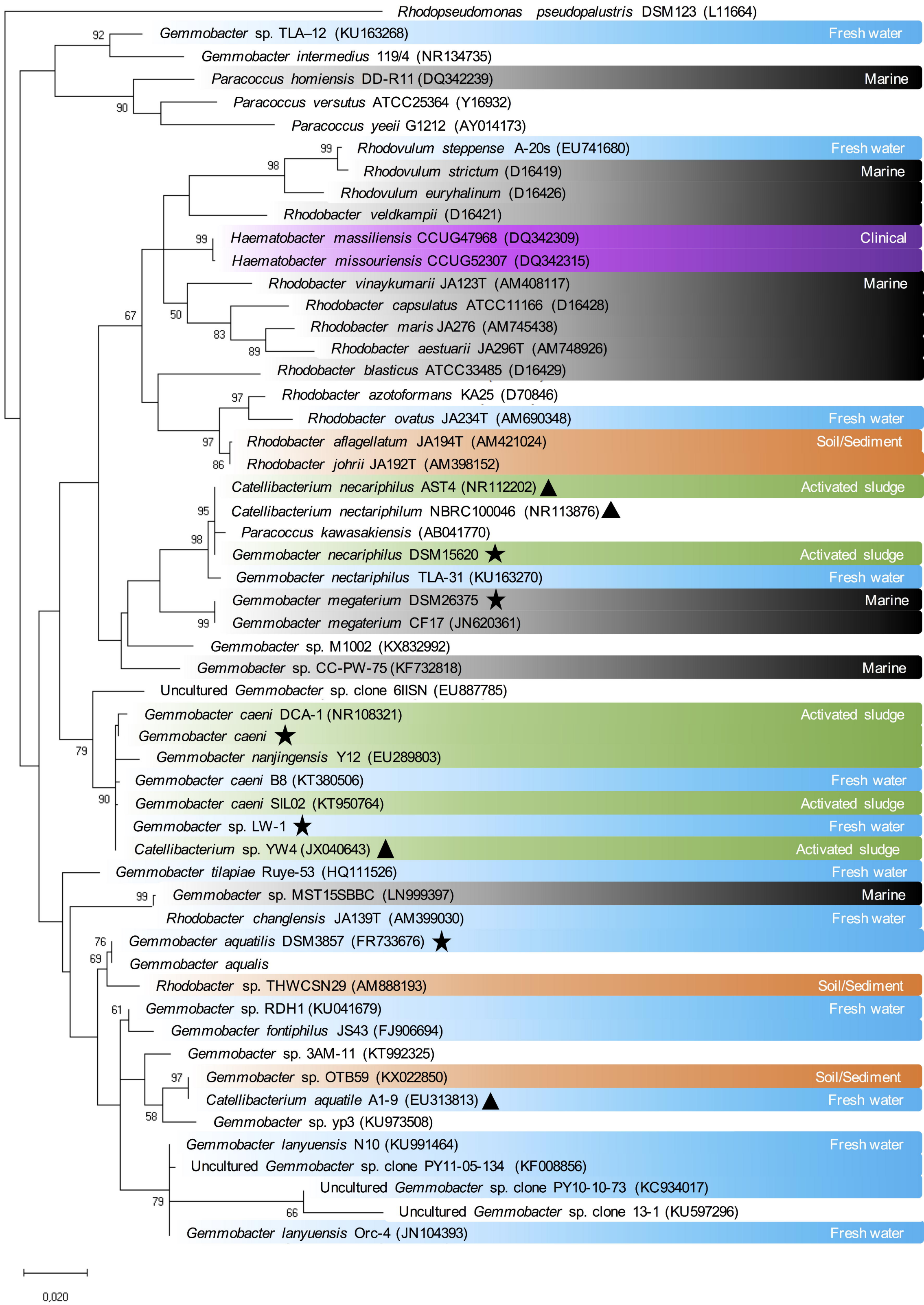
Rhodopseudomonas pseudopalustris DSM123 (L11664)
Gemmobacter sp. TLA–12 (KU163268)
Fresh water
Gemmobacter intermedius 119/4 (NR134735)
Paracoccus homiensis DD-R11 (DQ342239)
Marine
Paracoccus versutus ATCC25364 (Y16932)
Paracoccus yeeii G1212 (AY014173)
Rhodovulum steppense A-20s (EU741680)
Fresh water
Rhodovulum strictum (D16419)
Marine
Rhodovulum euryhalinum (D16426)
Rhodobacter veldkampii (D16421)
Haematobacter massiliensis CCUG47968 (DQ342309)
Clinical
Haematobacter missouriensis CCUG52307 (DQ342315)
Rhodobacter vinaykumarii JA123T (AM408117)
Marine
Rhodobacter capsulatus ATCC11166 (D16428)
Rhodobacter maris JA276 (AM745438)
Rhodobacter aestuarii JA296T (AM748926)
Rhodobacter blasticus ATCC33485 (D16429)
Rhodobacter azotoformans KA25 (D70846)
Rhodobacter ovatus JA234T (AM690348)
Fresh water
Rhodobacter aflagellatum JA194T (AM421024)
Soil/Sediment
Rhodobacter johrii JA192T (AM398152)
Catellibacterium necariphilus AST4 (NR112202)
Activated sludge
Catellibacterium nectariphilum NBRC100046 (NR113876)
Paracoccus kawasakiensis (AB041770)
Gemmobacter necariphilus DSM15620
Activated sludge
Gemmobacter nectariphilus TLA-31 (KU163270)
Fresh water
Gemmobacter megaterium DSM26375
Marine
Gemmobacter megaterium CF17 (JN620361)
Gemmobacter sp. M1002 (KX832992)
Gemmobacter sp. CC-PW-75 (KF732818)
Marine
Uncultured Gemmobacter sp. clone 6IISN (EU887785)
Gemmobacter caeni DCA-1 (NR108321)
Activated sludge
Gemmobacter caeni
Gemmobacter nanjingensis Y12 (EU289803)
Gemmobacter caeni B8 (KT380506)
Fresh water
Gemmobacter caeni SIL02 (KT950764)
Activated sludge
Gemmobacter sp. LW-1
Fresh water
Catellibacterium sp. YW4 (JX040643)
Activated sludge
Gemmobacter tilapiae Ruye-53 (HQ111526)
Fresh water
Gemmobacter sp. MST15SBBC (LN999397)
Marine
Rhodobacter changlensis JA139T (AM399030)
Fresh water
Gemmobacter aquatilis DSM3857 (FR733676)
Gemmobacter aqualis
Rhodobacter sp. THWCSN29 (AM888193)
Soil/Sediment
Gemmobacter sp. RDH1 (KU041679)
Fresh water
Gemmobacter fontiphilus JS43 (FJ906694)
Gemmobacter sp. 3AM-11 (KT992325)
Gemmobacter sp. OTB59 (KX022850)
Soil/Sediment
Catellibacterium aquatile A1-9 (EU313813)
Fresh water
Gemmobacter sp. yp3 (KU973508)
Gemmobacter lanyuensis N10 (KU991464)
Fresh water
Uncultured Gemmobacter sp. clone PY11-05-134 (KF008856)
Uncultured Gemmobacter sp. clone PY10-10-73 (KC934017)
Uncultured Gemmobacter sp. clone 13-1 (KU597296)
Gemmobacter lanyuensis Orc-4 (JN104393)
Fresh water
92
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Figure 2

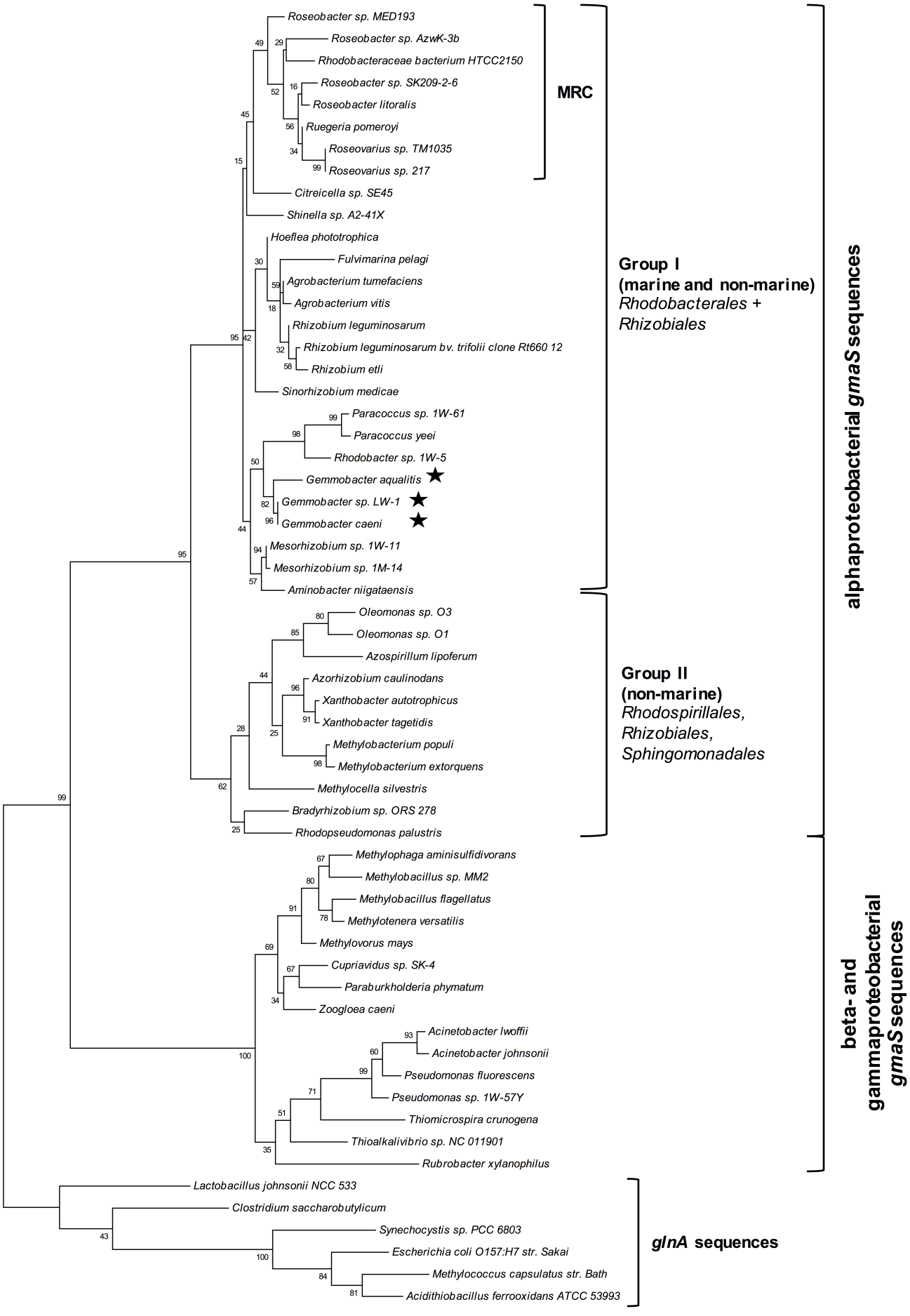
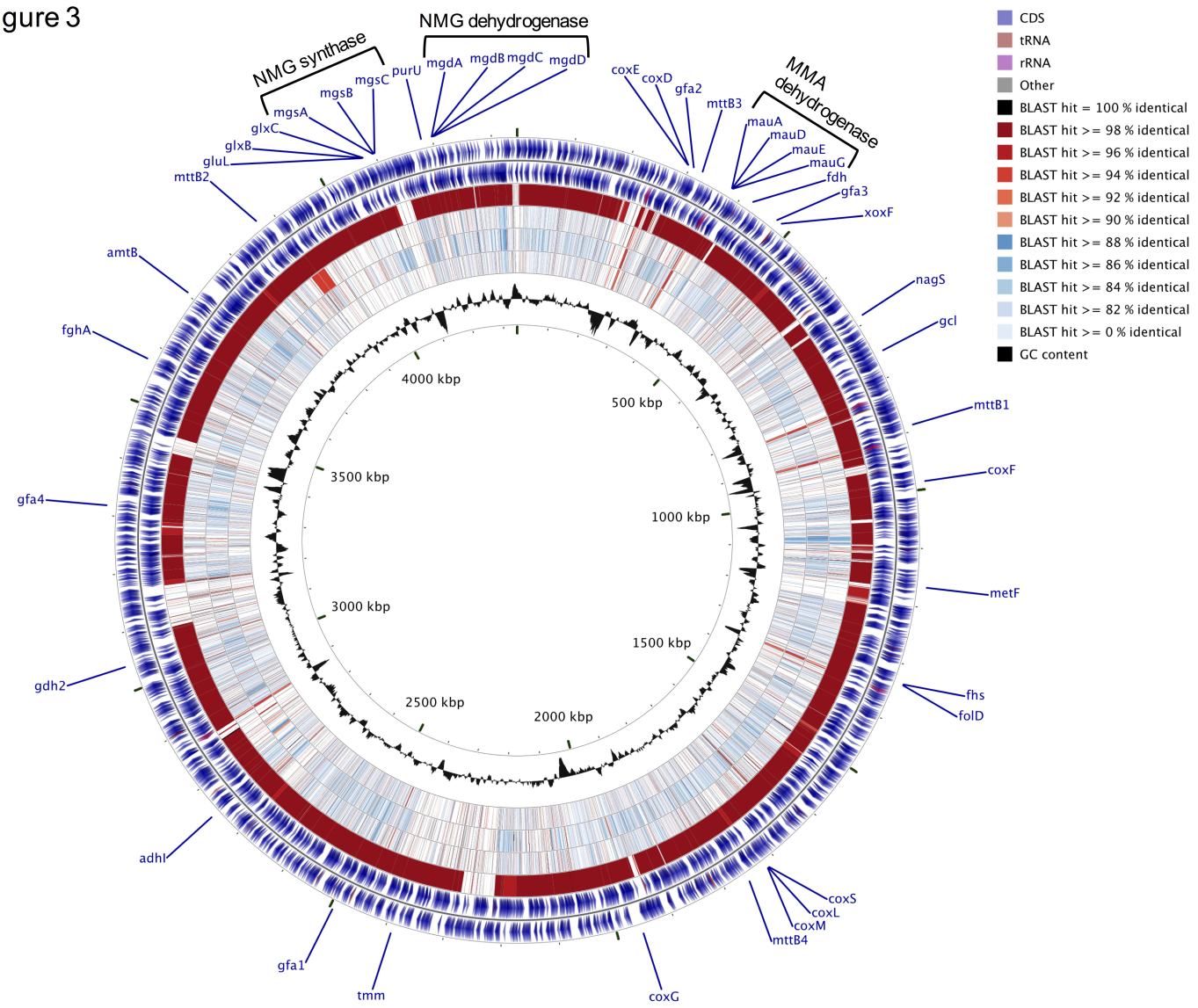

Roseobacter sp. MED193
Roseobacter sp. AzwK-3b
Rhodobacteraceae bacterium HTCC2150
Roseobacter sp. SK209-2-6
Roseobacter litoralis
Ruegeria pomeroyi
Roseovarius sp. TM1035
Roseovarius sp. 217
MRC
Citreicella sp. SE45
Shinella sp. A2-41X
Hoeflea phototrophica
Fulvimarina pelagi
Agrobacterium tumefaciens
Agrobacterium vitis
Rhizobium leguminosarum
Rhizobium leguminosarum bv. trifolii clone Rt660 12
Rhizobium etli
Sinorhizobium medicae
Paracoccus sp. 1W-61
Paracoccus yeei
Rhodobacter sp. 1W-5
Gemmobacter aqualitis
Gemmobacter sp. LW-1
Gemmobacter caeni
Mesorhizobium sp. 1W-11
Mesorhizobium sp. 1M-14
Aminobacter niigataensis
Group I
(marine and non-marine)
Rhodobacterales +
Rhizobiales
Oleomonas sp. O3
Oleomonas sp. O1
Azospirillum lipoferum
Azorhizobium caulinodans
Xanthobacter autotrophicus
Xanthobacter tagetidis
Methylobacterium populi
Methylobacterium extorquens
Methylocella silvestris
Bradyrhizobium sp. ORS 278
Rhodopseudomonas palustris
Group II
(non-marine)
Rhodospirillales,
Rhizobiales,
Sphingomonadales
alphaproteobacterial gmaS sequences
Methylophaga aminisulfidivorans
Methylobacillus sp. MM2
Methylobacillus flagellatus
Methylotenera versatilis
Methylovorus mays
Cupriavidus sp. SK-4
Paraburkholderia phymatum
Zoogloea caeni
Acinetobacter lwoffii
Acinetobacter johnsonii
Pseudomonas fluorescens
Pseudomonas sp. 1W-57Y
Thiomicrospira crunogena
Thioalkalivibrio sp. NC 011901
Rubrobacter xylanophilus
beta- and
gammaproteobacterial
gmaS sequences
Lactobacillus johnsonii NCC 533
Clostridium saccharobutylicum
Synechocystis sp. PCC 6803
Escherichia coli O157:H7 str. Sakai
Methylococcus capsulatus str. Bath
Acidithiobacillus ferrooxidans ATCC 53993
glnA sequences
0,1

Figure 3



Protein	Corresponding gene(s)
TMA monooxygenase	tmm
TMA methyltransferase	mttB1, mttB2, mttB3, mttB4
MMA dehydrogenase	mauA, mauD, mauE, mauG
NMG synthase	mgsA, mgsB, mgsC
NMG dehydrogenase	mgdA, mgdB, mgdC, mgdD
HCHO-activating enzyme	gfa1, gfa2, gfa3, gfa4
GSH-dependent HCHO dehydrogenase	adhI
S-formyl-GSH hydrolase	fghA
Glutamine synthetase (GS)	gluL
Glutamate synthase (GOGAT)	glxB, glxC
Methylene-H_4F-dehydro-genase/cyclohydrolase	folD
Formate-H_4F ligase	fhs
Formyl-H_4F deformylase	purU
Formate dehydrogenase	fdh
Carbon monoxide dehydrogenase	coxD, coxE, coxF,coxG, coxL, coxM, coxS
Methanol dehydrogenase	xoxF
N-acetylglutamate synthase	nagS
Gamma-glutamylcysteine synthetase	gcl
Methylenetetrahydrofolate reductase	metF
Glutamate dehydrogenase	gdh2
Ammonium transporter	amtB

Figure 4A

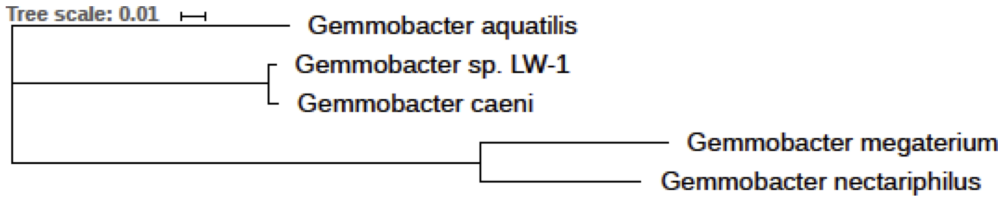


Figure 4B

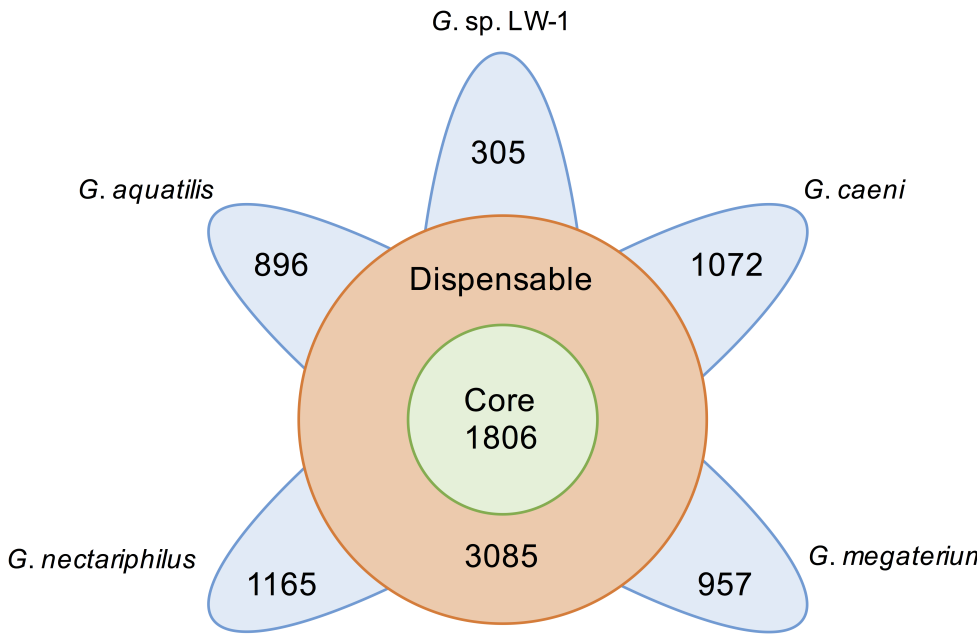


Figure 5

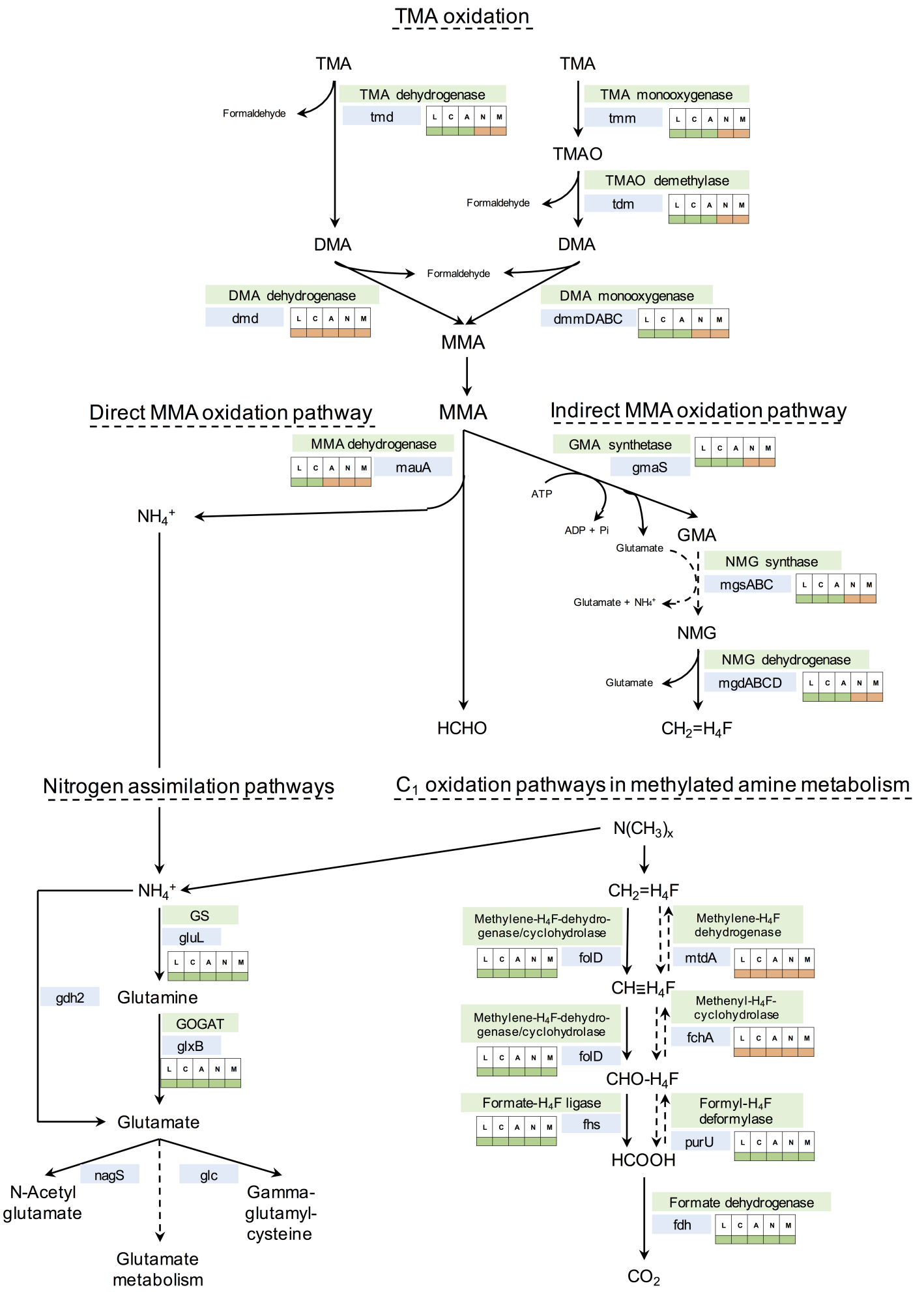

TMA oxidation
TMA
TMA
Formaldehyde
TMA dehydrogenase
tmd
TMA monooxygenase
tmm
TMAO
TMAO demethylase
tdm
Formaldehyde
DMA
DMA
Formaldehyde
DMA dehydrogenase
dmd
DMA monooxygenase
dmmDABC
MMA
MMA
Direct MMA oxidation pathway
Indirect MMA oxidation pathway
MMA dehydrogenase
mauA
GMA synthetase
gmaS
ATP
ADP + Pi
Glutamate
GMA
NH4+
NMG synthase
mgsABC
Glutamate + NH4+
NMG
NMG dehydrogenase
mgdABCD
Glutamate
HCHO
CH2=H4F
Nitrogen assimilation pathways
C1 oxidation pathways in methylated amine metabolism
N(CH3)x
NH4+
CH2=H4F
GS
gluL
Methylene-H4F-dehydro-genase/cyclohydrolase
folD
Methylene-H4F dehydrogenase
mtdA
gdh2
Glutamine
CH≡H4F
GOGAT
glxB
Methylene-H4F-dehydro-genase/cyclohydrolase
folD
Methenyl-H4F-cyclohydrolase
fchA
CHO-H4F
Glutamate
Formate-H4F ligase
fhs
Formyl-H4F deformylase
purU
HCOOH
nagS
glc
N-Acetyl glutamate
Gamma-glutamyl-cysteine
Glutamate metabolism
Formate dehydrogenase
fdh
CO2

Table 1. Comparative genomic analysis of methylamine-utilizing genes in genome-sequenced Gemmobacters in comparison to selected marine *Roseobacter* clade bacteria. Shown is the presence (+) or absence (-) of the corresponding genes in the genome sequences.

Organism	*gmaS*	*tmm*	*xoxF*	*fae*	*foldD*	*purU*	*fhs (ftfL)*	*gfa*	*adhI (flhA)*	*fghA*	*fdh*	*amtB*	*mtdA/ mtdB*	*mchA*	*ftr*	*fmdA /B/C*	*mauA*	*cox*
Citreicella sp. SE45	+	+	+	-	+	+	+	+	+	+	+	+	-/-	-	-	-	-	+
Roseovarius sp. TM1035	+	+	+	-	+	-	+	-	+	+	+	+	-/-	-	-	-	-	+
Paracoccus denitrificans PD1222	-	-	+	-	+	+	+	+	+	+	+	+	-/-	-	-	-	-	-
Rhodobacter sphaeroides 241	-	+	+	-	+	+	+	+	+	+	+	+	-/-	-	-	-	-	+
Gemmobacter caeni	+	+	+	-	+	+	+	+[a]	+	+	+	+	-/-	-	-	-	+	+
Gemmobacter sp. LW-1	+	+	+	-	+	+	+	+[a]	+	+	+	+	-/-	-	-	-	+	+
Gemmobacter aquatilis	+	+	+	-	+	+	+	-	+	+	+	+	-/-	-	-	-	-	+
Gemmobacter nectariphilus DSM-15620	-	-	-	-	+	+	+	+[a]	+	+	+	+	-/-	-	-	-	-	+
Gemmobacter megaterium DSM-26375	-	-	-	-	+	+	+	+[a]	+	+	+	+	-/-	-	-	-	-	+

[a] gfa-like protein-encoding gene