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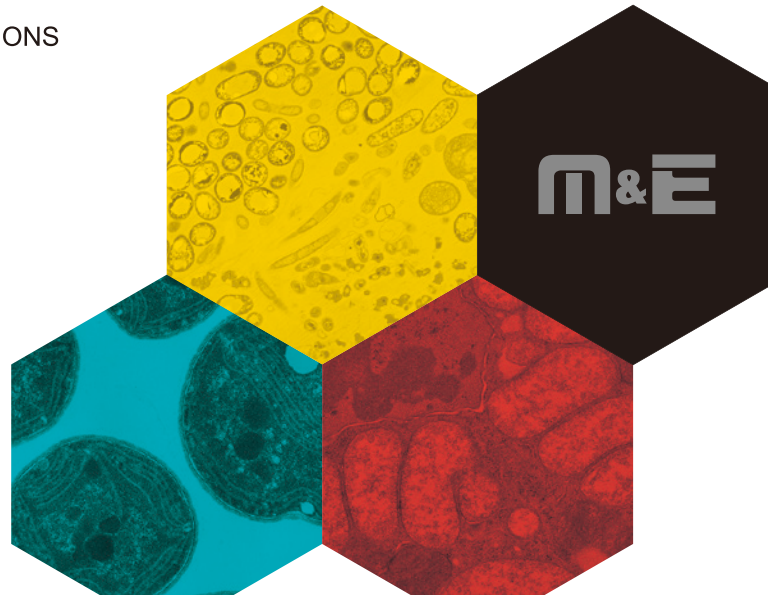


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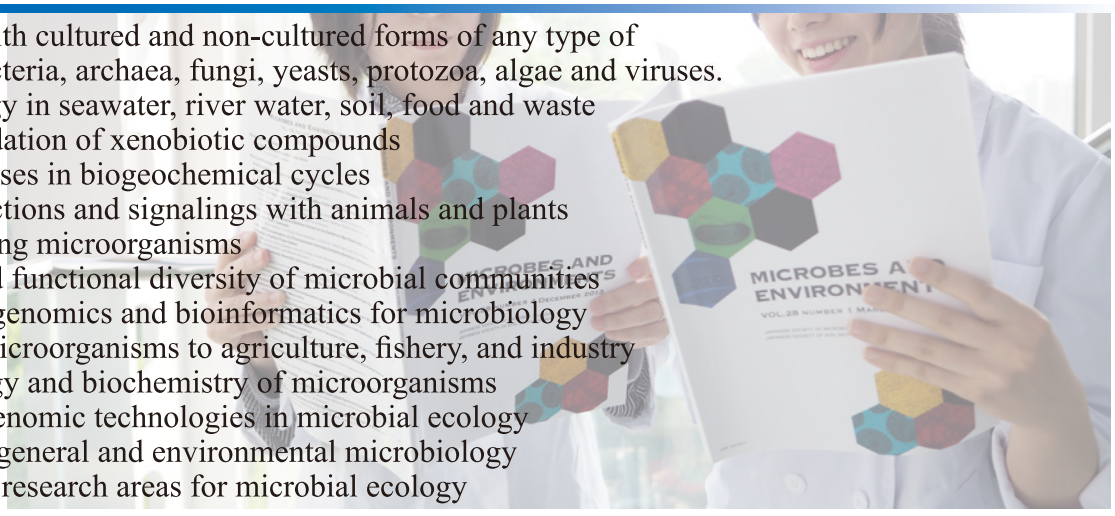

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MOST VALUABLE PAPER OF THE YEAR 2017

*Short Communication*

# Predominant but Previously-overlooked Prokaryotic Drivers of Reductive Nitrogen Transformation in Paddy Soils, Revealed by Metatranscriptomics

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Waterlogged paddy soils possess anoxic zones in which microbes actively induce reductive nitrogen transformation (RNT). In the present study, a shotgun RNA sequencing analysis (metatranscriptomics) of paddy soil samples revealed that most RNT gene transcripts in paddy soils were derived from *Deltaproteobacteria*, particularly the genera *Anaeromyxobacter* and *Geobacter*. Despite the frequent detection of the rRNA of these microbes in paddy soils, their RNT-associated genes have rarely been identified in previous PCR-based studies. This metatranscriptomic analysis provides novel insights into the diversity of RNT microbes present in paddy soils and the ecological function of *Deltaproteobacteria* predominating in these soils.

**Key words:** paddy soils, metatranscriptomics, denitrification, dissimilatory nitrate reduction to ammonium, nitrogen fixation

Paddy soils are characterized by temporal anaerobic conditions caused by waterlogging, and the active occurrence of anaerobic biogeochemical processes (9). Among these active processes, biological reductive nitrogen transformation (RNT), *i.e.*, denitrification ($NO_3^-$→$NO_2^-$→NO→$N_2O$→$N_2$), dissimilatory nitrate reduction to ammonium (DNRA; $NO_3^-$→$NO_2^-$→$NH_4^+$), and nitrogen fixation ($N_2$→$NH_4^+$) contribute to less leaching of nitrogen pollutants ($NO_3^-$, $NO_2^-$, and $N_2O$) into the environment and the greater retention of nitrogen-based nutrients ($NH_4^+$) for rice plants in waterlogged paddy soils than in upland soils (8, 22). Therefore, the identification of microbial drivers of RNT in paddy soils is important for successful rice production with minimal environmental nitrogen burden.

However, a comprehensive understanding of the RNT microbial community has not yet been achieved. In order to investigate RNT microbes in paddy soils, genes encoding the enzymes that catalyze each reaction have been assessed via PCR-based culture-independent methods, as represented by a clone library analysis (13, 24). Recent studies based on bacterial genomics reported that the diversity of microbes harboring RNT genes is greater than previously considered; PCR-based methods have underrepresented this diversity because of mismatches in the sequences of the primers used (5, 10, 21), indicating the need for alternative methods without a PCR bias. Furthermore, simultaneous assessments of microbes involved in denitrification, DNRA, and nitrogen fixation in a single paddy field have not yet been performed. Moreover, limited information is available on the transcriptional profiles *in situ* of RNT microbes in paddy soils because of the small number of field studies conducted based on soil RNA, which directly implicates RNT microbial activity. In the present study, we investigated RNT-associated microbial diversity in paddy soils via a shotgun RNA sequencing analysis without any prior PCR preparation (metatranscriptomics).

In order to obtain a more complete understanding of paddy soils with various biogeochemical properties spatially and seasonally (9, 12), soil RNA extracted from paddy soils in shallow (S1, S3) and deep (S2, S4) layers under waterlogged (S1, S2) and drained (S3, S4) conditions (Fig. S1) were subjected to a metatranscriptomic analysis using an Illumina MiSeq sequencer (Illumina, San Diego, CA, USA). The sequences of RNT genes were retrieved from the metatranscriptomic libraries obtained and taxonomically annotated through a tandem similarity search with the blat and blast programs (full methods in *Supplementary information*).

Four reactions crucial to denitrification are catalyzed by the following enzymes: $NO_3^-$ reductase (Nar), $NO_2^-$ reductase (Nir), NO reductase (Nor), and $N_2O$ reductase (Nos). The *nar* transcripts detected in all soil samples using metatranscriptomics were related to those of *Deltaproteobacteria*, *Betaproteobacteria*, *Alphaproteobacteria*, *Gammaproteobacteria*, and *Acidobacteria* (Fig. 1A), suggesting that these bacterial groups are involved in the reduction of $NO_3^-$ to $NO_2^-$. The *nir* transcripts were mostly derived from *Betaproteobacteria*, *Gammaproteobacteria*, and *Alphaproteobacteria* (Fig. 1A), the members of which include common denitrifiers (5); these were also frequently detected in the same paddy soils in our previous PCR-based survey (24). Furthermore, *nor* and *nos* transcripts were predominantly detected in *Deltaproteobacteria* (Fig. 1A), the transcripts of which were rarely detected via previous PCR assays (2, 24). Successive denitrification steps were considered to be associated with common denitrifiers, such as *Betaproteobacteria*, *Gammaproteobacteria*, *Alphaproteobacteria*, and *Actinobacteria*, which harbor *nir*, *nor*, and/or *nos* (18). However, the metatranscriptomic data obtained in the present study suggested that the reduction of $NO_2^-$ into NO was driven by these denitrifiers, and that the reduction of NO and $N_2O$ was mainly progressed by non-denitrifiers such as

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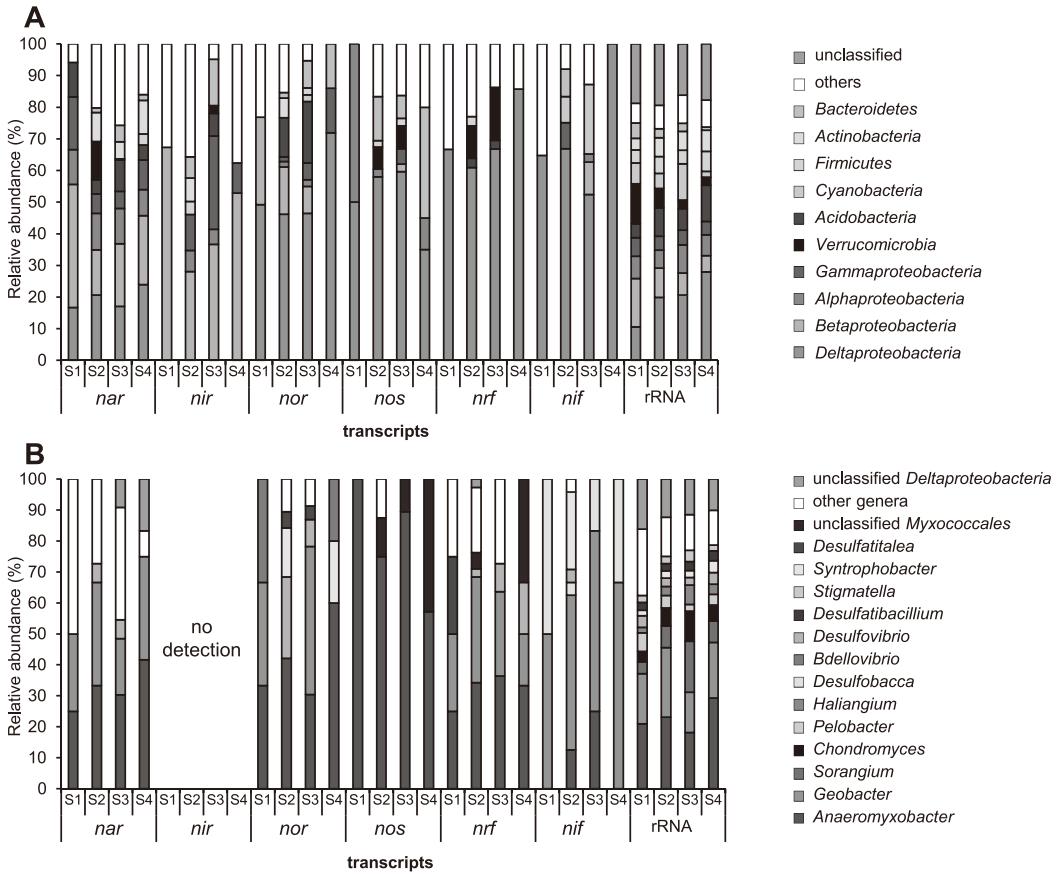


**Fig. 1.** Microbial diversity of RNT gene transcripts and rRNA. Taxonomic distribution of *nar*, *nir*, *nor*, *nos*, *nrf*, and *nif* transcripts, and rRNA at the phylum and proteobacterial class level (A), and deltaproteobacterial genus level (B). Sample IDs indicate data derived from paddy soils in shallow (S1, S3) and deep (S2, S4) layers under waterlogged (S1, S2) and drained (S3, S4) conditions. Data represent the mean of triplicates.

*Deltaproteobacteria*, *Bacteroidetes*, *Acidobacteria*, and *Verrucomicrobia*, which harbor *nor* and/or *nos*, but not *nir* (5). Thus, paddy soil denitrification appears to be a cooperative process by each nitrogen oxide reducer, *i.e.*, $NO_2^-$ reducers (denitrifiers) and $NO/N_2O$ reducers (non-denitrifiers), similar to nitrification ($NH_4^+ \rightarrow NO_2^- \rightarrow NO_3^-$) orchestrated by $NH_4^+$-oxidizing bacteria/archaea and $NO_2^-$-oxidizing bacteria (6). These inferences in the denitrification process may be verified using co-culture experiments on denitrifiers and non-denitrifiers.

DNRA, another $NO_3^-$ reduction process, is catalyzed by Nar and $NH_4^+$-forming $NO_2^-$ reductase (Nrf). Most of the *nrf* transcripts belong to *Deltaproteobacteria*, while some belong to *Verrucomicrobia* (Fig. 1A). Together with the frequent detection of *nar* transcripts derived from *Deltaproteobacteria* as described above, *Deltaproteobacteria* appear to mainly contribute to DNRA dynamics in paddy soils. Although DNRA has been geochemically detected in paddy soils (1, 23), limited information is available on DNRA microbial diversity. To the best of our knowledge, the present study is the first to attempt to identify the key player groups in DNRA in paddy soils.

Diazotrophs harboring nitrogenase (Nif) drive nitrogen fixation. The taxonomic composition of *nif* transcripts was dominated by *Deltaproteobacteria* (Fig. 1A), indicating that *Deltaproteobacteria* represents a key player group in nitrogen fixation. Rhizospheric *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria* and phototrophic *Cyanobacteria* were considered to be key diazotrophs in paddy soils (13, 18). However, we detected more *nif* transcripts in *Deltaproteobacteria* than in these well-known diazotrophs; our results were consistent with a recent metatranscriptomic analysis based on a microcosm study on Italian paddy soils (11).

The abundance of RNT genes derived from *Deltaproteobacteria*, as described above, was also demonstrated in a shotgun DNA sequencing analysis (metagenomics) (Fig. S2A). Additionally, the microbial community structure based on rRNA gene/transcript sequences showed that *Deltaproteobacteria* is a major group in paddy soil microbes (Fig. 1A, S2A). These results support *Deltaproteobacteria* being a key player group driving RNT in paddy soils.

Further analyses on *Deltaproteobacteria* at the genus level revealed the consistent detection of RNT gene transcripts in metatranscriptomic data derived from the genera *Anaeromyxobacter* and *Geobacter* (Fig. 1B), as well as their RNT genes in metagenomic data (Fig. S2B). These genera represent obligate anaerobes and metal reducers predominating in paddy soils (7, 20) and exhibit some RNT activities *in vitro* (summarized in Table S1). Although the nitrogen fixation activity of *Anaeromyxobacter* has yet to be characterized, the genomes of some *Anaeromyxobacter* spp. conserve a similar *nif* cluster to that of *Geobacter* spp. exhibiting nitrogen fixation activity (Fig. S3). Together with the detection of the *nif* transcripts of *Anaeromyxobacter* in this study, it is plausible that *Anaeromyxobacter* spp. perform nitrogen fixation.

However, in contrast to *Anaeromyxobacter* and *Geobacter* rRNA genes, their RNT genes have rarely been detected in paddy soil samples using PCR-based techniques (4, 24). Thus, the putative role of these genera in the RNT process has received little attention despite their predominance in paddy soils. The limited coverage of RNT gene-specific PCR primers used in previous studies may have led to the oversight of these genera (10, 17); additionally, the GC content may be implicated because the *nor*/*nos*/*nrf*/*nif* of *Anaeromyxobacter* spp. showed markedly higher GC contents than the rRNA genes and *nor*/*nos*/*nrf*/*nif* of other bacteria (Table S2). Even improved *nos* universal primers, which have enabled lower rates of sequence mismatches, were unable to amplify *Anaeromyxobacter nos* (10). Therefore, a metatranscriptomic analysis represents a more effective approach to examine the diversity of functional microbes, without any PCR bias arising from the high GC content of target genes as well as primer limitations.

*Anaeromyxobacter* and *Geobacter*, which have frequently been detected in Japanese, Chinese, and Italian paddy soils (4, 11, rRNA data in Fig. 1B), predominate more in paddy soils than in upland soils, as confirmed by the present study (Fig. 2A, B; Table S3). Their universal distribution and predominance in paddy soils support *Anaeromyxobacter* and *Geobacter* being the key RNT players in paddy soils. Furthermore, the predominance of these genera was found in river sediments (Fig. 2A, B; Table S3); the RNT genes of *Anaeromyxobacter* were frequently and globally detected in upland soil environments in recent shotgun metagenomics studies (14, 15), indicating the contribution of these bacteria to RNT not only in paddy soils, but also in other environments. The further application of metatranscriptomics across different environments will expand our knowledge on the diversity of RNT microbes in nature as well as the ecological function of *Deltaproteobacteria* in soil environments.

Previous studies on paddy soils identified the predominance of *Deltaproteobacteria* and their ecological roles in dissimilatory metal reduction, sulfur/sulfate reduction, and hydrogen production (7, 9, 19). Although genomic studies showed the ubiquitous possession of the RNT genes of *Deltaproteobacteria*, the association of RNT with *Deltaproteobacteria* has not been considered because of the rare detection of their RNT genes in soil environments through PCR-based analyses. The present study revealed the novel ecological functions of *Anaeromyxobacter* and *Geobacter* within *Deltaproteobacteria* dominating in paddy soils, namely, RNT, denitrification support, and $NH_4^+$ production via DNRA and nitrogen fixation (Fig. 3).

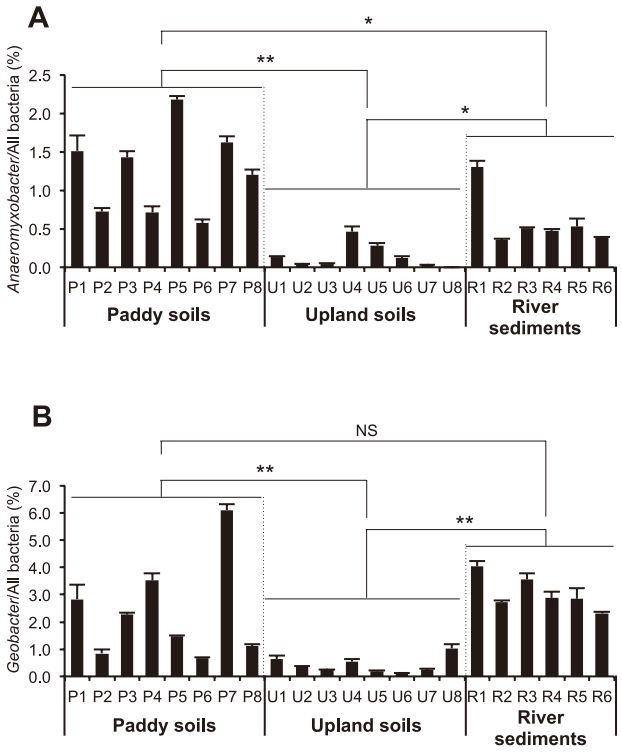


**Fig. 2.** Distribution of *Anaeromyxobacter* and *Geobacter* in various soil environments. Proportions of *Anaeromyxobacter* (A) and *Geobacter* (B) against all bacteria estimated by a quantitative PCR method. The mean±SD is shown ($n$=3). The paddy soil sample P3 was collected from the same paddy field used for the metatranscriptomic analysis in this study. Asterisks indicate significant differences (Mann-Whitney $U$ test; *, $p$<0.01; **, $p$<0.001); NS, not significant. Details of soil samples and qPCR data are summarized in Table S3.

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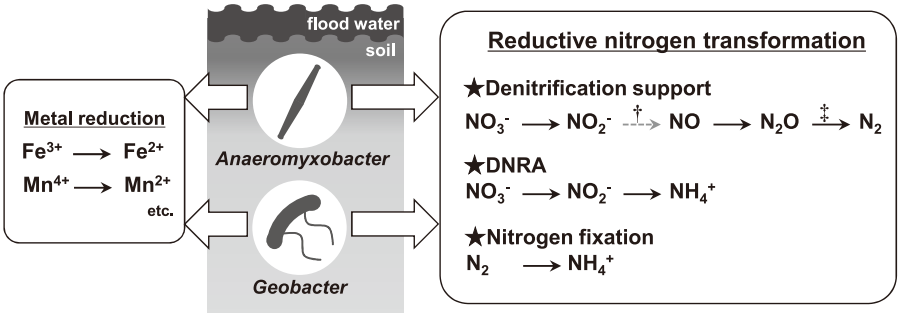


**Fig. 3.** Ecological functions of *Anaeromyxobacter* and *Geobacter*, belonging to *Deltaproteobacteria*, predominant in paddy soils, expanded by metatranscriptomics in this study. *Anaeromyxobacter* and *Geobacter*, ubiquitously predominant in paddy soils, are key player groups in the reduction of iron and manganese, which actively progresses in paddy soils soon after waterlogging (3, 4, 7, 9, 11, 20, Fig. 1B, S2B). Metatranscriptomics in this study suggested that these genera also associate with reductive nitrogen transformation, *i.e.*, denitrification, DNRA, and nitrogen fixation. Sketches of *Anaeromyxobacter* and *Geobacter* are based on electron microscope photographs published elsewhere (3, 16). †, $NO_2^-$ reduction to NO is driven by common denitrifiers within *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria* (5), not by *Anaeromyxobacter* and *Geobacter* within *Deltaproteobacteria*; ‡, *Anaeromyxobacter* reduce $N_2O$ to $N_2$, whereas *Geobacter* do not (Table S1).

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## References

1. Buresh, R.J., and W.H. Patrick. 1978. Nitrate reduction to ammonium in anaerobic soil. Soil Sci. Soc. Am. J. 42:913–918.
2. Chen, Z., J. Liu, M. Wu, X. Xie, J. Wu, and W. Wei. 2012. Differentiated response of denitrifying communities to fertilization regime in paddy soil. Microb. Ecol. 63:446–459.
3. Childers, S.E., S. Ciufo, and D.R. Lovley. 2002. *Geobacter metallireducens* accesses insoluble Fe (III) oxide by chemotaxis. Nature 416:767–769.
4. Ding, L.J., J.Q. Su, H.J. Xu, Z.J. Jia, and Y.G. Zhu. 2015. Long-term nitrogen fertilization of paddy soil shifts iron-reducing microbial community revealed by RNA-$^{13}$C-acetate probing coupled with pyrosequencing. ISME J. 9:721–734.
5. Graf, D.R., C.M. Jones, and S. Hallin. 2014. Intergenomic comparisons highlight modularity of the denitrification pathway and underpin the importance of community structure for $N_2O$ emissions. PLoS One 9:e114118.
6. Hirsch, P.R., and T.H. Mauchline. 2015. The importance of the microbial N cycle in soil for crop plant nutrition. Adv. Appl. Microbiol. 93:45–71.
7. Hori, T., A. Muller, Y. Igarashi, R. Conrad, and M.W. Friedrich. 2010. Identification of iron-reducing microorganisms in anoxic rice paddy soil by $^{13}$C-acetate probing. ISME J. 4:267–278.
8. Ishii, S., S. Ikeda, K. Minamisawa, and K. Senoo. 2011. Nitrogen cycling in rice paddy environments: past achievements and future challenges. Microbes Environ. 26:282–292.
9. Itoh, H., S. Ishii, Y. Shiratori, K. Oshima, S. Otsuka, M. Hattori, and K. Senoo. 2013. Seasonal transition of active bacterial and archaeal communities in relation to water management in paddy soils. Microbes Environ. 28:370–380.
10. Jones, C.M., D.R. Graf, D. Bru, L. Philippot, and S. Hallin. 2013. The unaccounted yet abundant nitrous oxide-reducing microbial community: a potential nitrous oxide sink. ISME J. 7:417–426.
11. Kim, Y., and W. Liesack. 2015. Differential assemblage of functional units in paddy soil microbiomes. PLoS One 10:e0122221.
12. Kögel-Knabner, I., W. Amelung, Z. Cao, S. Fiedler, P. Frenzel, R. Jahn, K. Kalbitz, A. Kölbel, and M. Schloter. 2010. Biogeochemistry of paddy soils. Geoderma 157:1–14.
13. Mårtensson, L., B. Díez, I. Wartiainen, W. Zheng, R. El-Shehawy, and U. Rasmussen. 2009. Diazotrophic diversity, *nifH* gene expression and nitrogenase activity in a rice paddy field in Fujian, China. Plant and Soil 325:207–218.
14. Nelson, M.B., A.C. Martiny, and J.B. Martiny. 2016. Global biogeography of microbial nitrogen-cycling traits in soil. Proc. Natl. Acad. Sci. U.S.A. 113:8033–8040.
15. Orellana, L.H., L.M. Rodriguez-R, S. Higgins, J.C. Chee-Sanford, R.A. Sanford, K.M. Ritalahti, F.E. Löffler, and K.T. Konstantinidis. 2014. Detecting nitrous oxide reductase (*nosZ*) genes in soil metagenomes: method development and implications for the nitrogen cycle. MBio. 5:e01193–14.
16. Sanford, R.A., J.R. Cole, and J.M. Tiedje. 2002. Characterization and description of *Anaeromyxobacter dehalogenans* gen. nov., sp. nov., an aryl-halorespiring facultative anaerobic myxobacterium. Appl. Environ. Microbiol. 68:893–900.
17. Sanford, R.A., D.D. Wagner, Q. Wu, *et al.* 2012. Unexpected nondenitrifier nitrous oxide reductase gene diversity and abundance in soils. Proc. Natl. Acad. Sci. U.S.A. 109:19709–19714.
18. Shapleigh, J.P. 2006. The denitrifying prokaryotes, p. 769–792. *In* M. Dworkin, S. Falkow, E. Rosenberg, K.H. Schleifer, and E. Stackebrandt (ed.), The Prokaryotes. Springer, New York.
19. Shrestha, P.M., M. Kube, R. Reinhardt, and W. Liesack. 2009. Transcriptional activity of paddy soil bacterial communities. Environ. Microbiol. 11:960–970.
20. Weber, K.A., L.A. Achenbach, and J.D. Coates. 2006. Microorganisms pumping iron: anaerobic microbial iron oxidation and reduction. Nat. Rev. Microbiol. 4:752–764.
21. Wei, W., K. Isobe, T. Nishizawa, L. Zhu, Y. Shiratori, N. Ohte, K. Koba, S. Otsuka, and K. Senoo. 2015. Higher diversity and abundance of denitrifying microorganisms in environments than considered previously. ISME J. 9:1954–1965.
22. Xiong, Z.Q., T.Q. Huang, Y.C. Ma, G.X. Xing, and Z.L. Zhu. 2010. Nitrate and ammonium leaching in variable- and permanent-charge paddy soils. Pedosphere 20:209–216.
23. Yin, S.X., D. Chen, L.M. Chen, and R. Edis. 2002. Dissimilatory nitrate reduction to ammonium and responsible microorganisms in two Chinese and Australian paddy soils. Soil Biol. Biochem. 34(8):1131–1137.
24. Yoshida, M., S. Ishii, D. Fujii, S. Otsuka, and K. Senoo. 2012. Identification of active denitrifiers in rice paddy soil by DNA- and RNA-based analyses. Microbes Environ. 27:456–461.

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MOST VALUABLE PAPER OF THE YEAR 2018

*Short Communication*

# Nitric Oxide Production from Nitrite Reduction and Hydroxylamine Oxidation by Copper-containing Dissimilatory Nitrite Reductase (NirK) from the Aerobic Ammonia-oxidizing Archaeon, *Nitrososphaera viennensis*

Shun Kobayashi[1], Daisuke Hira[2], Keitaro Yoshida[3], Masanori Toyofuku[3], Yosuke Shida[4], Wataru Ogasawara[4], Takashi Yamaguchi[5], Nobuo Araki[1], and Mamoru Oshiki[1]*

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Aerobic ammonia-oxidizing archaea (AOA) play a crucial role in the global nitrogen cycle by oxidizing ammonia to nitrite, and nitric oxide (NO) is a key intermediate in AOA for sustaining aerobic ammonia oxidation activity. We herein heterologously expressed the NO-forming, copper-containing, dissimilatory nitrite reductase (NirK) from *Nitrososphaera viennensis* and investigated its enzymatic properties. The recombinant protein catalyzed the reduction of $^{15}NO_2^-$ to $^{15}NO$, the oxidation of hydroxylamine ($^{15}NH_2OH$) to $^{15}NO$, and the production of $^{14\text{-}15}N_2O$ from $^{15}NH_2OH$ and $^{14}NO_2^-$. To the best of our knowledge, the present study is the first to document the enzymatic properties of AOA NirK.

**Key words:** nitrite reduction, hydroxylamine oxidation, nitrous oxide production, ammonia oxidizing archaea, *Nitrososphaera viennensis*

Aerobic ammonia oxidation, a rate-limiting step of nitrification, drives the global nitrogen cycle (24, 40), which involves aerobic ammonia-oxidizing archaea and bacteria (AOA and AOB, respectively) and complete ammonia oxidizers (comammox) (9, 44). Of these, AOA primarily contribute to aerobic ammonia oxidation in natural environments including soil and open ocean (19, 31, 46). AOA are affiliated with the phylum *Thaumarchaeota*, which includes phylogenetically and physiologically diverse members (6) and the soil-inhabiting archaeon *Nitrososphaera viennensis* (41). The biochemistry of aerobic ammonia oxidation by AOA has received a great deal of interest because ammonia oxidation to nitrite ($NO_2^-$) proceeds in a different manner to that of AOB. AOA oxidize ammonia to hydroxylamine by ammonia monooxygenase (Amo) as well as AOB (43), while hydroxylamine is further oxidized to $NO_2^-$ by an unidentified enzyme (17). All known AOA genomes lack the gene encoding hydroxylamine dehydrogenase (Hao), and the involvement of a copper-protein complex has been proposed (40, 45). In parallel with the oxidation of ammonia to $NO_2^-$, AOA produce nitric oxide (NO) (22). NO is a key intermediate in AOA cells because this highly reactive molecule is essential for sustaining aerobic ammonia oxidation activity (17, 33, 36, 47). To date, the following 2 pathways have been reported as a source of prokaryotic NO formation: $NO_2^-$ reduction to NO by copper-containing and cytochrome $cd_1$-type dissimilatory nitrite reductases (NirK and NirS, respectively) (38) and $NH_2OH$ oxidation to NO by hydroxylamine oxidoreductase (Hao) (4, 21). Although neither *nirS* nor *hao* are found in AOA genomes (6), AOA commonly possess *nirK*, which is transcribed and expressed during aerobic ammonia oxidation (8, 15, 20, 37). These findings suggest that NirK are involved in NO formation in AOA cells. However, $NO_2^-$ reduction to NO by AOA NirK has never been demonstrated.

Bacterial NirK have been characterized as homotrimeric enzymes, and each subunit has 2 Cu-binding sites (Type 1 and 2 Cu-binding sites). Type 1 Cu-binding sites receive an electron from an electron donor, such as type 1 Cu proteins (single-domain cupredoxins) and/or cytochrome *c*, and the electron is then further transferred to a type 2 Cu-binding site that is the catalytic center of NirK (14, 25). Bacterial NirK have been classified into 2 phylogenetically distinct groups (class 1 and class 2 groups) based on sequence similarities, and the NirK of the class 1 group contains linker loop and tower loop regions in the amino acid sequence (3). AOA NirK, including *Ns. viennensis* NirK, are affiliated with a distinct clade of bacterial class 1 and 2 groups (Fig. 1A). Lund *et al.* (20) reported that AOA NirK may be further classified into several phylogenetic clades showing specific geographic distributions. *Ns. viennensis* NirK has amino acid residues consistent with those of type 1 and 2 Cu-binding sites ($His_{106}$, $His_{140}$, and $His_{316}$ for type 1 Cu-binding sites and $His_{101}$, $Cys_{141}$, $His_{152}$, and $Met_{157}$ for type 2 Cu-binding sites) as well as the linker and tower loop regions, whereas the C terminus has unusual extensions of ~26 residues (Fig. 1B). These phylogenetic affiliations of and structural variations in *Ns. viennensis* NirK raise concerns regarding its enzymatic properties, such as specific enzymatic activity, affinity for $NO_2^-$, and products of $NO_2^-$ reduction.

Based on its unique sequence and lack of biochemical information, the purpose of the present study was to charac-

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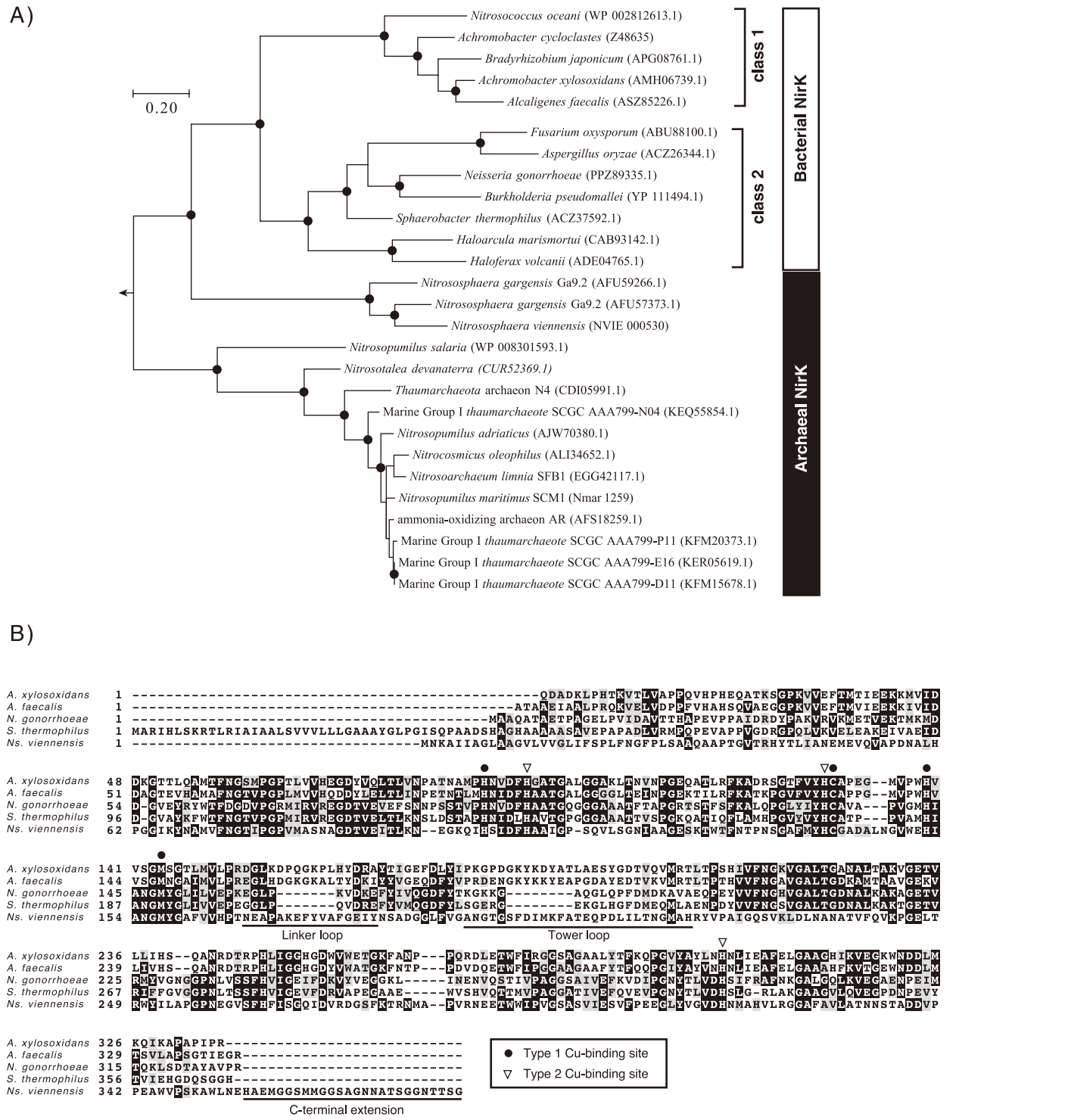


**Fig. 1.** Phylogeny (A) and sequence alignments (B) of prokaryotic NirK. A) A phylogenetic tree of prokaryotic NirK was constructed by the maximum likelihood method with the Jones-Taylor-Thornton model using the protein sequence of multicopper oxidase type 3 of *Nitrososphaera viennensis* (accession number; AIC14243.1) as an outgroup. Branching points that support a probability >80% in bootstrap analyses (based on 500 replicates) are shown as filled circles. The scale bar represents 10% sequence divergence. Sequence accession numbers are indicated in parentheses. B) Protein sequence alignment of *nirK*. NirK sequences were aligned using ClustalW software. Circles and triangles correspond to the amino acid residues of type 1 and 2 Cu-binding sites, respectively. Linker, Tower loop (3), and C-terminal extension regions are underlined. Abbreviations of microorganisms are as follows: *Nitrosomonas europaea* is *N. europaea*, *A. xylosoxidans* is *Achromobacter xylosoxidans*, *A. faecalis* is *Alcaligenes faecalis*, *N. gonorrhoeae* is *Neisseria gonorrhoeae*, *S. thermophilus* is *Sphaerobacter thermophilus*, and *Ns. viennensis* is *Nitrososphaera viennensis*.

terize *Ns. viennensis* NirK. Prior to the present study, we aimed to isolate *Ns. viennensis* NirK from a batch culture of *Ns. viennensis* as a native enzyme. However, the activity of aerobic ammonia oxidation often disappeared when we scaled up the cultures (data not shown). Additionally, a slow growth rate ($\mu_{max}$ 0.024 $h^{-1}$) (41) and low biomass concentration in the culture (*ca.* $10^{7\sim8}$ cells $mL^{-1}$) further precluded the preparation of the biomass required for protein purification. Since

recombinant NirK proteins have been successfully used to previously examine several enzymatic properties (7, 16, 32), the authors decided to heterologously express *Ns. viennensis* NirK in *Escherichia coli*, and investigate its enzymatic properties. The *nirK* gene located in the *Ns. viennensis* genome (accession number; CP007536.1) was cloned into the expression vector pCold I (Takara Bio, Shiga, Japan) with the 6×His tag using the Mighty cloning reagent set (Takara Bio), and transformed into *E. coli* strain BL21(DE3) (Takara Bio). The N-terminal region of *Ns. viennensis* NirK was predicted to be the signal peptide sequence ($Met_1$ to $Ala_{24}$), and *nir*K without the signal peptide sequence was amplified by PCR using ExTaq polymerase (Takara Bio) and specific forward (5′-GGCATATGGCCCCGACTGGTGTCACTAGACACTAT-3′) and reverse (5′-GGAAGCTTAACCAGAGGTGGTGTTGC CACCGGAGG-3′) oligonucleotide primers. The restriction sites of NdeI and HindIII in the forward and reverse primers above are underlined. Genomic DNA extracted from *Ns. viennensis* cells (JCM19564) was used as the DNA template for PCR. The constructed plasmid was subjected to Sanger sequencing, and no mutations were found in the sequence. Regarding the expression of the recombinant protein in *E. coli* cells, the expression culture was aerobically cultivated at 37°C in Luria-Bertani media containing 100 ng $\mu L^{-1}$ ampicillin. When the $OD_{600}$ of the culture increased to 0.4, the culture was transferred to 15°C and held for 30 min, and protein expression was then induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 0.1 mM. After being incubated at 15°C for 24 h, cells were harvested by centrifugation at 8,500×*g* at 4°C for 10 min. The harvested cells were suspended in buffer containing 20 mM Tris HCl (pH 8), 200 mM NaCl, and 10% glycerol. The cells were disrupted using a sonifier 250 (Branson) (output 20, duty 20% for 60 s, 6 cycles), and centrifuged at 13,000×*g* at 4°C for 1 h. The supernatant was recovered as a soluble protein fraction, and the recombinant protein was purified using His-tag affinity chromatography. The recombinant protein was bound to His60 Ni Superflow resin (Takara Bio), and washed with washing buffer containing 20 mM Tris HCl (pH 8), 200 mM NaCl, 10% glycerol, and 20 mM imidazole. The bound recombinant protein was eluted with elution buffer containing 20 mM Tris HCl (pH 8), 200 mM NaCl, 10% glycerol, and 300 mM imidazole. Protein concentrations were measured using the DC-protein assay kit (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as previously described (26), and purity was evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% polyacrylamide gel as previously described (28). As shown in Fig. 2A, a single protein band appeared at a molecular mass of 40 kDa, which closely matched the molecular mass deduced from amino acid sequences of the recombinant protein (*i.e.*, 39.7 kDa). The protein band was excised from the gel, and subjected to a matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) analysis after in-gel tryptic digestion for protein identification (The detailed methodology is described in the Supplementary text). The MALDI-TOF MS analysis confirmed that the protein band corresponded to *Ns. viennensis* NirK (Fig. S1). Regarding the reconstitution of Cu-binding sites of the recombinant protein, the purified recombinant protein was dialyzed against buffer containing 20 mM Tris HCl (pH 8), 300 mM NaCl, and 0.5 mM $CuSO_4$ at 4°C for 57 h. The protein solution was dialyzed again using the above Tris buffer without $CuSO_4$ at 4°C for 6 h. The dialyzed recombinant protein was concentrated using a Vivaspin column (MWCO; 30 kDa) (GE Healthcare Japan, Tokyo, Japan). The recombinant protein was loaded onto a gel-filtration HiLoad 16/600 Superdex 200 pg column (GE Healthcare) to assess the molecular mass of the recombinant protein, which was 105±1.3 kDa (Fig. 2B). Since the deduced molecular mass of *Ns. viennensis* NirK was 39.7 kDa, the molecular mass obtained by gel filtration indicated that the recombinant protein forms a homotrimeric structure, similar to canonical NirK.

NirK have been characterized as metalloproteins showing a blue or green color spectrum, and exhibit absorption peaks at approximately 450 and/or 600 nm (3). Bacterial NirK, which belong to the class 1 group, often show a maximum absorption peak at approximately 450 nm, although an exception (*Achromobacter xylosoxidans* NirK) that shows a peak at 593 nm has been previously reported (16). The purified recombinant protein was pale blue in color, and showed an absorption peak at 590 nm (Fig. 2C). This feature indicated that *Ns. viennensis* NirK is affiliated with the subgroup of NirK showing a blue color spectrum. The blue or green color spectrum of NirK is derived from a copper atom in the type 1 Cu-binding site (14), while the type 2 Cu-binding site does not contribute to the UV or visible spectrum. The type 2 Cu-binding site shows a characteristic electron spin resonance (ESR) spectrum (7, 16); therefore, an ESR analysis was performed using a JES-FA200 spectrometer (JEOL, Tokyo, Japan) to test for the presence of the type 2 Cu-binding site in the recombinant protein. An axial type 2 Cu signal ($g_{//}$=2.24, $A_{//}$=18.31 mT, and $g_{\perp}$=2.06) was found in the ESR measurement (Fig. 2D), indicating that the recombinant protein has a type 2 Cu-binding site coordinating with a copper atom. Additionally, we assessed the copper content of the recombinant protein by inductively coupled plasma mass spectrometry (ICP-MS). The copper content was found to be 2.9 atoms per subunit of the recombinant protein, indicating that Cu was fully incorporated into the recombinant protein. Overall, the recombinant protein shared the structural and spectroscopic features of class 1 and 2 bacterial NirK, which is consistent with sequencing information.

The kinetics of $NO_2^-$ reduction were examined by anoxically incubating the recombinant protein at 25°C and pH 6.5 with $^{15}NO_2^-$ and artificial electron donors as previously described (7). All of the buffers and stock solutions were prepared anoxically as previously described (27). Two milliliters of reaction buffer (20 mM phosphate buffer, 0.1 to 1.6 mM $Na^{15}NO_2^-$, 0.5 mM benzyl viologen (BV), and 0.24 mM sodium dithionite) was dispensed into a 1-cm sealable quartz cuvette and placed in an anaerobic chamber in which the $O_2$ concentration was maintained at lower than 1 ppm. BV was used as an artificial electron donor because it has been employed to examine the kinetics of the $NO_2^-$ reduction of bacterial NirK (7, 13). The cuvette was set in a UV-VIS spectrometer UV-2700 (Shimadzu, Kyoto, Japan), and the initial absorbance of the prepared reaction mixture at a wavelength of 550 nm was approximately 2.0. The reaction was initiated by adding the recombinant protein (50 μL containing 250 μg of protein) using a gastight syringe, and the oxidation rate of reduced BV (molecular extinction coefficient, 10.4 $mM^{-1}$ $cm^{-1}$) (13) was monitored at 550 nm.

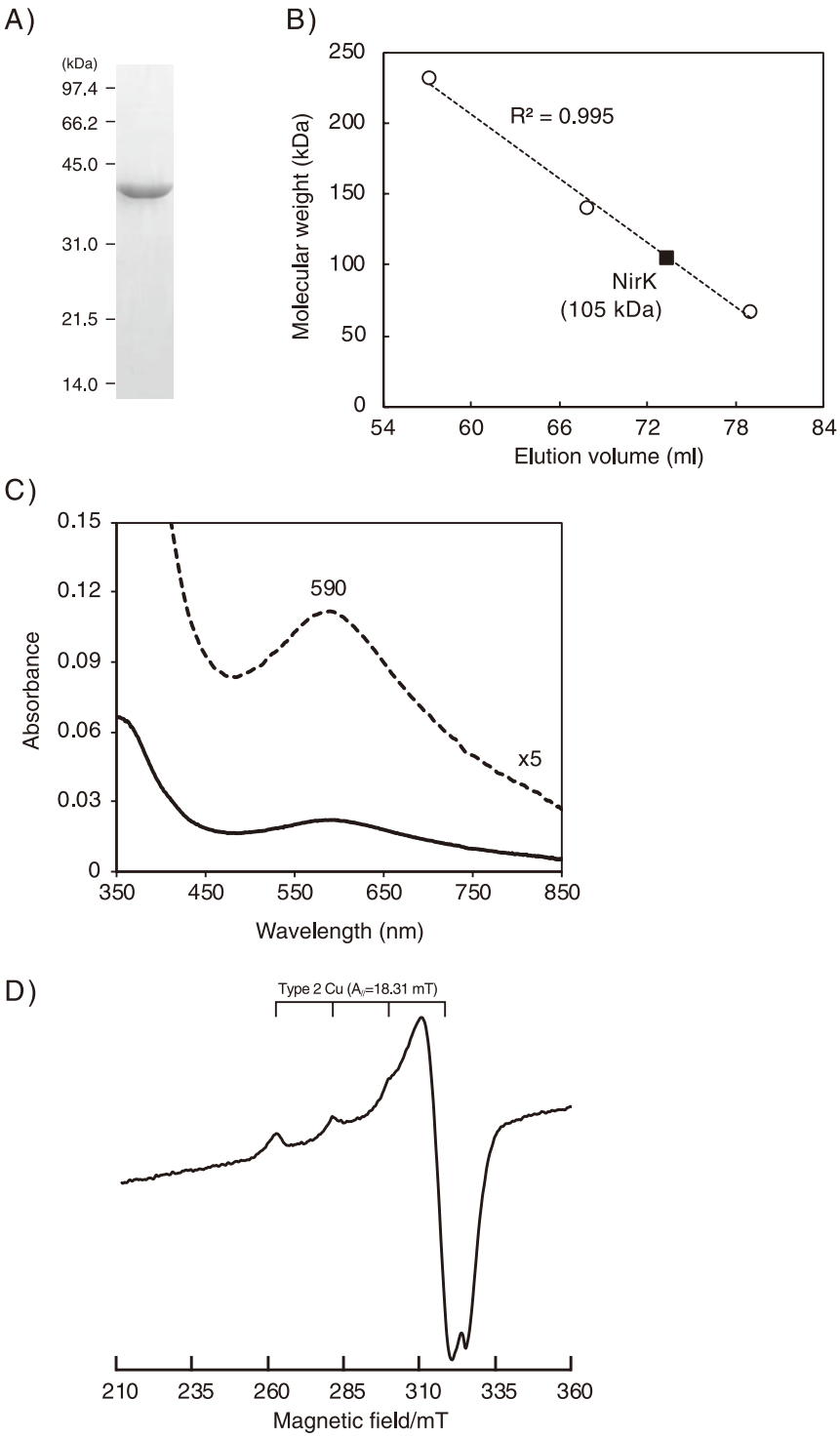


**Fig. 2.** Characterization of recombinant *Nitrososphaera viennensis* NirK. A) SDS-PAGE of the recombinant protein purified by His-tag affinity chromatography. B) Assessment of the molecular mass of the recombinant protein by gel filtration chromatography. Catalase from bovine liver (232 kDa), lactate dehydrogenase (140 kDa), and bovine serum albumin (66 kDa) were used to prepare a standard calibration curve. C) UV-VIS absorption spectra. The measurement was performed in a 20 mM Tris buffer (pH 8) containing 300 mM NaCl at 25°C. The solid line indicates the recombinant protein (1 mL $mL^{-1}$) oxidized with air. A 5×enlarged spectrum is also shown as a dashed line. D) ESR spectra. The measurement was performed using the recombinant protein (4.9 mg $mL^{-1}$) at –253°C.

The recombinant protein reduced $NO_2^-$ by oxidizing BV, whereas no significant BV oxidation was found in the cuvette without the recombinant protein. The turnover number and $K_m$ value for $NO_2^-$ reduction by the recombinant protein were 3.1 $s^{-1}$ and 287 μM, respectively (Table 1), and the turnover number and affinity constant were markedly lower and higher, respectively, than those of other canonical NirK proteins, including those from AOB. The product of $NO_2^-$ reduction by the recombinant protein was examined using phenazine methosulfate (PMS) as the electron donor instead of BV.

**Table 1.** Enzymatic properties of archaeal and bacterial copper-containing nitrite reductase (NirK). ND; not determined.

| Organisms | MW* (kDa) | Cu content† (atom per subunit) | Absorption (nm) | Activity‡ Turnover ($s^{-1}$) | $K_m$ (μM) | Reference |
|---|---|---|---|---|---|---|
| Archaeal NirK | | | | | | |
| *Nitrososphaera viennensis* | 105±1.5 | 2.9 | 590 | | | |
| $NO_2^-$ reduction | | | | 3.1 | 287 | This study |
| $NH_2OH$ oxidation | | | | 0.039 | 97 | This study |
| Bacterial NirK ($NO_2^-$ reduction) | | | | | | |
| *Nitrosomonas europaea* | 96 | ND | 450, 597 | 288 | ND | 18 |
| *Nitrosococcus oceani* | 114 | 1.67 | 455, 575 | 1,600 | 52 | 16 |
| *Achromobacter xylosoxidans* | 110 | 1.99 | 595 | 172 | 35 | 14, 32 |
| *Candidatus* Jettenia caeni | 101 | ND | 449, 598 | 319 | 250 | 7 |

* Molecular weight (MW) of a trimeric NirK. The MW of *Ca.* Jettenia caeni NirK was calculated from amino acid sequences without a signal peptide sequence. † Copper contents previously assessed by chemical analyses were shown. ‡ The following electron donors were used to evaluate the turnover number of $NO_2^-$ reduction; methyl viologen for *N. europaea* and *Nc. oceani*, pseudoazurine for *A. xylosoxidans*, and benzyl viologen for *Ns. viennensis* and *Ca.* Jettenia caeni NirK.

When BV was used as the electron donor, $NO_2^-$ was reduced to NO, and further reduced to ammonia (approx. 60% of consumed $^{15}NO_2^-$) as observed in a previous study in which the $NO_2^-$ reduction activity of *A. xylosoxidans* NirK was examined using methyl viologen (MV) as the electron donor (1). BV and MV have low redox potentials (–350 and –440 mV, respectively) (23), resulting in the reduction of NO to $NH_3$; therefore, PMS with a higher redox potential (+80 mV) was used in the present study. The recombinant protein was incubated as described above in a 1.8-mL gas-tight vial with the addition of 0.5 mM PMS and 5 mM ascorbic acid instead of BV and dithionite, and the production of $^{15}N$-labeled gaseous compounds (*i.e.*, $N_2$, NO, and $N_2O$) in the headspace was examined by gas chromatography mass spectrometry (GC/MS) as previously described (27). The diluted gases of $^{15\text{-}15}N_2$ (Cambridge Isotope Laboratories, Tewksbury, MA, USA), $^{14}NO$, and $^{14\text{-}14}N_2O$ (GL Science, Tokyo, Japan) were also analyzed to prepare standard curves for quantification. The recombinant protein reduced $^{15}NO_2^-$ with the oxidation of PMS, and 38 and 48% of consumed $^{15}NO_2^-$ were converted to $^{15}NO$ and $^{15\text{-}15}N_2O$, respectively. This is direct evidence to show that the recombinant protein is a NO-forming nitrite reductase. We found that the production of $^{15\text{-}15}N_2O$ was equal to the production of $^{15}NO$, which likely results from the reduction of $^{15}NO_2^-$ to $H^{15}NO$ (*i.e.*, $NO_2^- + 2e^- + 3H^+ \rightarrow HNO + H_2O$) and the chemical formation of $^{15\text{-}15}N_2O$ from the formed $H^{15}NO$ (*i.e.*, $2HNO \rightarrow N_2O + H_2O$) (35), as previously observed for a sulfide-linked nitrite reductase (34).

Aside from $NO_2^-$ reduction, $NH_2OH$ oxidation was also investigated using the recombinant protein because $NH_2OH$ is produced as an intermediate during aerobic ammonia oxidation by AOA. The kinetics of $NH_2OH$ oxidation were examined by aerobically incubating the recombinant protein (245 μg $mL^{-1}$) at 30°C and pH 7.5 with 0.5 mM $NH_2OH$, with dissolved oxygen being available as an oxidant. The reaction was initiated by the addition of $NH_2OH$ solution, and the concentration of $NH_2OH$ was assessed colorimetrically (5). The concentration of $H_2O_2$, which may be produced by the oxidase activity of NirK (12), was also evaluated colorimetrically using horseradish peroxidase (Wako, Osaka, Japan) and 3,3′,5,5′-tetramethylbenzidine (TMBZ) (Dojindo, Kumamoto, Japan) (2). As shown in Fig. S2, the recombinant protein oxidized $NH_2OH$ with the production of $H_2O_2$. No $NH_2OH$ oxidation or $H_2O_2$ production was observed when the incubation was repeated without the addition of the recombinant protein.

The values for the turnover number and affinity constant for $NH_2OH$ oxidation were 0.039 $s^{-1}$ and 97 μM (Table 1), respectively, and the value for the turnover number was two orders of magnitude lower than that observed for $NO_2^-$ reduction; therefore, the recombinant protein catalyzed $NO_2^-$ reduction more efficiently. The addition of cytochrome *c* from equine heart (1 mg $mL^{-1}$) or BV (0.5 mM) did not result in an increase in the reaction rate or affinity for $NH_2OH$ oxidation. The product of $NH_2OH$ oxidation by the recombinant protein was examined in a $^{15}NH_2OH$ tracer experiment (29). The recombinant protein was incubated in a 1.8-mL gas-tight vial with the addition of 0.5 mM $^{15}NH_2OH$ (Cambridge Isotope Laboratories) instead of $^{14}NH_2OH$. After a 2-h incubation, the concentrations of the $^{15}N$-labeled gaseous products were assessed by GC/MS. The recombinant protein oxidized $^{15}NH_2OH$ and produced $^{15}NO$, $^{15\text{-}15}N_2O$, and $^{15\text{-}15}N_2$ gases quantitatively (Fig. 3), whereas the production of $NO_2^-$ and $NH_3$ was not detectable (detection limits: 50 and 100 μM, respectively). The oxidation of $NH_2OH$ to NO has been

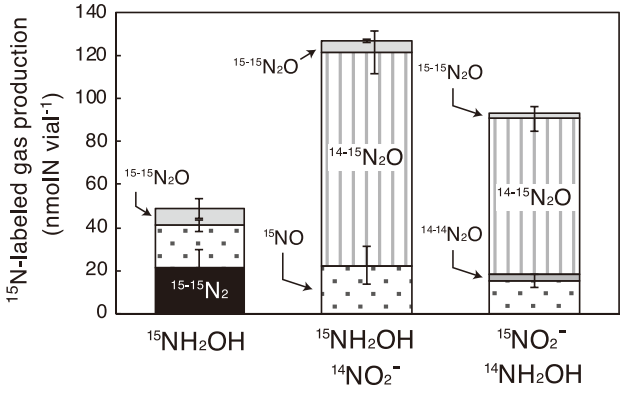


**Fig. 3.** $NH_2OH$ oxidation by recombinant *Nitrososphaera viennensis* NirK. The recombinant protein was incubated at 30°C and pH 7.5 in 1.8-mL vials (volume of the headspace: 1.5 mL), with i) 0.5 mM $^{15}NH_2OH$, ii) $^{15}NH_2OH$ and $^{14}NO_2^-$ (each 0.5 mM), or iii) $^{14}NH_2OH$ and $^{15}NO_2^-$. The production of $N_2$, NO, and $N_2O$ in the headspace was examined by gas chromatography mass spectrometry (GC/MS). $NH_3$ and $NO_2^-$ concentrations were also measured; however, they were not detectable during the incubation. During a 2-h incubation, i) 63±35 (mean±SD), ii) 149±1, and iii) 120±1 nmol N of $NH_2OH$ were consumed in the liquid phase, resulting in 75–137% of the $^{15}N$-labeled nitrogen mass balance in the vials. Error bars represent the SD derived from triplicate incubations, and the graph bars represent the mean values. $NH_2OH$ oxidation was not found in the vials without the addition of the recombinant protein.

described in bacterial Hao (21); however, to the best of our knowledge, this is the first description of $NH_2OH$ oxidation by NirK. We also observed $^{15\text{-}15}N_2O$ production from $^{15}NH_2OH$ oxidation, which likely resulted from the oxidation of $^{15}NH_2OH$ to $H^{15}NO$ and abiotic coupling of $H^{15}NO$, as previously described. Notably, $^{15\text{-}15}N_2$ was the major product of $^{15}NH_2OH$ oxidation by the recombinant protein. Hydroxylamine disproportionation (30) may not be responsible for $^{15\text{-}15}N_2$ production because $NH_3$ production was not detectable in the liquid phase. The molecular mechanisms underlying the oxidation of $^{15}NH_2OH$ to $^{15\text{-}15}N_2$ by the recombinant protein warrant further studies.

We repeated the above incubation with the addition of $NH_2OH$ and $NO_2^-$ because both compounds are available in AOA cells during aerobic ammonia oxidation. Therefore, the above incubation was repeated with the addition of $^{15}NH_2OH$ and $^{14}NO_2^-$ (each 0.5 mM) or $^{14}NH_2OH$ and $^{15}NO_2^-$ (Cambridge Isotope Laboratories) (each 0.5 mM). In both cases, $^{14\text{-}15}N_2O$ was the major product (Fig. 3), indicating that the recombinant protein produces $N_2O$ by oxidizing $NH_2OH$ using $NO_2^-$ as an electron acceptor. $N_2O$ production by the denitrifier NirK from $NH_2OH$ and $NO_2^-$ has been previously described (10), and the N-nitrosation reaction is involved in $N_2O$ production (39). Notably, *Ns. viennensis* cells produce $N_2O$ when they are incubated aerobically with $NH_3$ and $NO_2^-$ (42), although the *Ns. viennensis* genome lacks the gene encoding nitric oxide reductase (*nor*) that is involved in $N_2O$ production from nitrifier-denitrification. Stieglmeier *et al.* (42) suggested the involvement of *Ns. viennensis* NirK in the production of $N_2O$ in an *Ns. viennensis* culture, and our results support this hypothesis. Although the catalytic efficiency of *Ns. viennensis* NirK for $NH_2OH$ oxidation was markedly lower than that of $NO_2^-$ reduction (Table 1), *Ns. viennensis* NirK may act as an $NH_2OH$ oxidase in *Ns. viennensis* cells and produce $N_2O$ under oxic growth conditions. Aside from $^{14\text{-}15}N_2O$ production, the production of $^{15}NO$ and $^{15\text{-}15}N_2O$ was also observed when the recombinant protein was incubated with $^{14}NH_2OH$ and $^{15}NO_2^-$ (Fig. 3).

Although the recombinant protein catalyzes $NO_2^-$ reduction and $NH_2OH$ oxidation, the catalytic efficiency of both reactions was low, as shown in Table 1. AOA *nirK* transcripts are abundant in the transcriptome (8, 11, 20, 37), suggesting the strong expression of AOA NirK in cells. NirK was the 225$^{th}$ most abundant protein of the 1,503 proteins detected in the proteome of the late exponential phase of *Ns. viennensis* cells aerobically oxidizing ammonia (15). The strong expression of NirK appears to support the activity of $NO_2^-$ reduction to NO as well as $NH_2OH$ oxidation to NO by the low efficiency catalytic enzyme. *Ns. viennensis* NirK may function as a bifunctional enzyme that supplies NO molecules from 2 different sources (*i.e.*, $NH_2OH$ and $NO_2^-$), which provides *Ns. viennensis* cells with a competitive advantage. In the present study, the enzymatic kinetics of recombinant *Ns. viennensis* NirK for $NO_2^-$ reduction were examined using artificial electron donors; further studies are needed to identify physiological electron donors in *Ns. viennensis* cells. Bacterial NirK may accept electrons supplied from single-domain cupredoxin and cytochrome *c* (14, 25). A number of genes encoding single-domain cupredoxin were found in the *Ns. viennensis* genome (Table S1), whereas the ortholog of the gene encoding cytochrome *c* was not. To date, the biochemistry of AOA cupredoxin has not been investigated using natural enzymes and recombinant proteins, and our study provides basic information that furthers our understanding of the biochemistry of AOA.

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**References**

1. Abraham, Z.H.L., D.J. Lowe, and B.E. Smith. 1993. Purification and characterization of the dissimilatory nitrite reductase from *Alcaligenes xylosoxidans* subsp. *xylosoxidans* (N.C.I.M.B. 11015): Evidence for the presence of both type 1 and type 2 copper centres. Biochem. J. 296:587–593.
2. Bos, E.S., A.A. van der Doelen, N. van Rooy, and A.H.W.M. Schuurs. 1981. 3,3′,5,5′-Tetramethylbenzidine as an Ames test negative chromogen for horse-radish peroxidase in enzyme-immunoassay. J. Immunoassay 2:187–204.
3. Boulanger, M.J., and M.E. Murphy. 2002. Crystal structure of the soluble domain of the major anaerobically induced outer membrane protein (AniA) from pathogenic *Neisseria*: a new class of copper-containing nitrite reductases. J. Mol. Biol. 315:1111–1127.
4. Carantoa, J.D., and K.M. Lancaster. 2017. Nitric oxide is an obligate bacterial nitrification intermediate produced by hydroxylamine oxidoreductase. Proc. Natl. Acad. Sci. U.S.A. 114:8217–8222.
5. Frear, D.S., and R.C. Burrell. 1955. Spectrophotometric method for determining hydroxylamine reductase activity in higher plants. Anal. Chem. 27:1664–1665.
6. Hatzenpichler, R. 2012. Diversity, physiology, and niche differentiation of ammonia-oxidizing archaea. Appl. Environ. Microbiol. 78:7501–7510.
7. Hira, D., H. Toh, C.T. Migita, H. Okubo, T. Nishiyama, M. Hattori, K. Furukawa, and T. Fujii. 2012. Anammox organism KSU-1 expresses a NirK-type copper-containing nitrite reductase instead of a NirS-type with cytochrome $cd_1$. FEBS Lett. 586:1658–1663.
8. Hollibaugh, J.T., S. Gifford, S. Sharma, N. Bano, and M.A. Moran. 2011. Metatranscriptomic analysis of ammonia-oxidizing organisms in an estuarine bacterioplankton assemblage. ISME J. 5:866–878.
9. Isobe, K., and N. Ohte. 2014. Ecological perspectives on microbes involved in N-cycling. Microbes Environ. 29:4–16.
10. Iwasaki, H., and T. Matsubara. 1972. A nitrite reductase from *Achromobacter cycloclast*. J. Biochem. 71:645–652.
11. Jung, M.Y., S.J. Park, D. Min, J.S. Kim, W.I.C. Rijpstra, J.S. Sinninghe Damsté, G.J. Kim, E.L. Madsen, and S.K. Rhee. 2011. Enrichment and characterization of an autotrophic ammonia-oxidizing archaeon of mesophilic Crenarchaeal group I.1a from an agricultural soil. Appl. Environ. Microbiol. 77:8635–8647.
12. Kakutani, T., H. Watanabe, K. Arima, and T. Beppu. 1981. A blue protein as an inactivating factor for nitrite reductase from *Alcaligenes faecalis* strain S-6. J. Biochem. 89:463–472.
13. Kataoka, K., H. Furusawa, K. Takagi, K. Yamaguchi, and S. Suzuki. 2000. Functional analysis of conserved aspartate and histidine residues located around the type 2 copper site of copper-containing nitrite reductase. J. Biochem. 127:345–350.
14. Kataoka, K., K. Yamaguchi, M. Kobayashi, T. Mori, N. Bokui, and S. Suzuki. 2004. Structure-based engineering of *Alcaligenes xylosoxidans* copper-containing nitrite reductase enhances intermolecular electron transfer reaction with pseudoazurin. J. Biol. Chem. 279:53374–53378.
15. Kerou, M., P. Offre, L. Valledor, S.S. Abby, M. Melcher, M. Nagler, W. Weckwerth, and C. Schleper. 2016. Proteomics and comparative genomics of *Nitrososphaera viennensis* reveal the core genome and adaptations of archaeal ammonia oxidizers. Proc. Natl. Acad. Sci. U.S.A. 113:E7937–E7946.

16. Kondo, K., K. Yoshimatsu, and T. Fujiwara. 2012. Expression, and molecular and enzymatic characterization of Cu-containing nitrite reductase from a marine ammonia-oxidizing gammaproteobacterium, *Nitrosococcus oceani*. Microbes Environ. 27:407–412.
17. Kozlowski, J.A., M. Stieglmeier, C. Schleper, M.G. Klotz, and L.Y. Stein. 2016. Pathways and key intermediates required for obligate aerobic ammonia-dependent chemolithotrophy in bacteria and Thaumarchaeota. ISME J. 10:1836–1845.
18. Lawton, T.J., K.E. Bowen, L.A. Sayavedra-Soto, D.J. Arp, and A.C. Rosenzweig. 2013. Characterization of a nitrite reductase involved in nitrifier denitrification. J. Biol. Chem. 288:25575–25583.
19. Leininger, S., T. Urich, M. Schloter, L. Schwark, J. Qi, G.W. Nicol, J.I. Prosser, S.C. Schuster, and C. Schleper. 2006. Archaea predominate among ammonia-oxidizing prokaryotes in soils. Nature 442:806–809.
20. Lund, M.B., J.M. Smith, and C.A. Francis. 2012. Diversity, abundance and expression of nitrite reductase (*nirK*)-like genes in marine thaumarchaea. ISME J. 6:1966–1977.
21. Maalcke, W.J., A. Dietl, S.J. Marritt, J.N. Butt, M.S.M. Jetten, J.T. Keltjens, T.R.M. Barends, and B. Kartal. 2014. Structural basis of biological NO generation by octaheme oxidoreductases. J. Biol. Chem. 289:1228–1242.
22. Martens-Habbena, W., W. Qin, R.E. Horak, *et al.* 2015. The production of nitric oxide by marine ammonia-oxidizing archaea and inhibition of archaeal ammonia oxidation by a nitric oxide scavenger. Environ. Microbiol. 17:2261–2274.
23. Nagashima, K.V.P. 2009. Redox titration for electron transfer proteins. Low Temp. Sci. 67:545–550 (in Japanese).
24. Nelson, M.B., A.C. Martiny, and J.B.H. Martiny. 2016. Global biogeography of microbial nitrogen-cycling traits in soil. Proc. Natl. Acad. Sci. U.S.A. 113:8033–8040.
25. Nojiri, M., H. Koteishi, T. Nakagami, K. Kobayashi, T. Inoue, and K.Y.S. Suzuki. 2009. Structural basis of inter-protein electron transfer for nitrite reduction in denitrification. Nature 462:117–120.
26. Oshiki, M., T. Awata, T. Kindaichi, H. Satoh, and S. Okabe. 2013. Cultivation of planktonic anaerobic ammonium oxidation (anammox) bacteria by using membrane bioreactor. Microbes Environ. 28:436–443.
27. Oshiki, M., S. Ishii, K. Yoshida, N. Fujii, M. Ishiguro, H. Satoh, and S. Okabe. 2013. Nitrate-dependent ferrous iron oxidation by anaerobic ammonium oxidation (anammox) bacteria. Appl. Environ. Microbiol. 79:4087–4093.
28. Oshiki, M., R. Takagi, M. Hatamoto, T. Yamaguchi, and N. Araki. 2016. High-cell-density cultivation of *Nitrosomonas europaea* in a membrane bioreactor for performing protein purification and characterization studies. J. Gen. Appl. Microbiol. 62:330–333.
29. Oshiki, M., M. Ali, K. Shinyako-Hata, H. Satoh, and S. Okabe. 2016. Hydroxylamine-dependent anaerobic ammonium oxidation (anammox) by "*Candidatus* Brocadia sinica". Environ. Microbiol. 18:3133–3143.
30. Pacheco, A.A., J. McGarry, J. Kostera, and A. Corona. 2011. Techniques for investigating hydroxylamine disproportionation by hydroxylamine oxidoreductases. Methods Enzymol. 486:447–463.
31. Prosser, J.I., and G.W. Nicol. 2008. Relative contributions of archaea and bacteria to aerobic ammonia oxidation in the environment. Environ. Microbiol. 10:2931–2941.
32. Prudêncio, M., R.R. Eady, and G. Sawers. 1999. The blue copper-containing nitrite reductase from *Alcaligenes xylosoxidans*: cloning of the *nirA* gene and characterization of the recombinant enzyme. J. Bacteriol. 181:2323–2329.
33. Sauder, L.A., A.A. Ross, and J.D. Neufeld. 2016. Nitric oxide scavengers differentially inhibit ammonia oxidation in ammonia-oxidizing archaea and bacteria. FEMS Microbiol. Lett. 363:fnw052.
34. Sawhney, V., and D.J.D. Nicholas. 1978. Sulphide-linked nitrite reductase from *Thiobacillus denitrificans* with cytochrome oxidase activity: purification and properties. J. Gen. Microbiol. 106:119–128.
35. Shafirovich, V., and S.V. Lymar. 2002. Nitroxyl and its anion in aqueous solutions: Spin states, protic equilibria, and reactivities toward oxygen and nitric oxide. Proc. Natl. Acad. Sci. U.S.A. 99:7340–7345.
36. Shen, T., M. Stieglmeier, J. Dai, T. Urich, and C. Schleper. 2013. Responses of the terrestrial ammonia-oxidizing archaeon *Ca. Nitrososphaera viennensis* and the ammonia-oxidizing bacterium *Nitrosospira multiformis* to nitrification inhibitors. FEMS Microbiol. Lett. 344:121–129.
37. Shi, Y., G.W. Tyson, J.M. Eppley, and E.F. DeLong. 2011. Integrated metatranscriptomic and metagenomic analyses of stratified microbial assemblages in the open ocean. ISME J. 5:999–1013.
38. Simon, J., and M.G. Klotz. 2013. Diversity and evolution of bioenergetic systems involved in microbial nitrogen compound transformations. Biochim. Biophys. Acta 1827:114–135.
39. Spott, O., R. Russow, and C.F. Stange. 2011. Formation of hybrid $N_2O$ and hybrid $N_2$ due to codenitrification: First review of a barely considered process of microbially mediated N-nitrosation. Soil Biol. Biochem. 43:1995–2011.
40. Stahl, D.A., and J.R. de la Torre. 2012. Physiology and diversity of ammonia-oxidizing archaea. Annu. Rev. Microbiol. 66:83–101.
41. Stieglmeier, M., A. Klingl, R.J.E. Alves, S.K.-M.R. Rittmann, M. Melcher, N. Leisch, and C. Schleper. 2014. *Nitrososphaera viennensis* gen. nov., sp. nov., an aerobic and mesophilic, ammonia-oxidizing archaeon from soil and a member of the archaeal phylum *Thaumarchaeota*. Int. J. Syst. Evol. Microbiol. 64:2738–2752.
42. Stieglmeier, M., M. Mooshammer, B. Kitzler, W. Wanek, S. Zechmeister-Boltenstern, A. Richter, and C. Schleper. 2014. Aerobic nitrous oxide production through N-nitrosating hybrid formation in ammonia-oxidizing archaea. ISME J. 8:1135–1146.
43. Vajrala, N., W. Martens-Habbena, L.A. Sayavedra-Soto, A. Schauer, P.J. Bottomley, D.A. Stahl, and D.J. Arp. 2013. Hydroxylamine as an intermediate in ammonia oxidation by globally abundant marine archaea. Proc. Natl. Acad. Sci. U.S.A. 110:1006–1011.
44. van Kessel, M.A.H.J., D.R. Speth, M. Albertsen, P.H. Nielsen, H.J.M. Op den Camp, B. Kartal, and M.S.M. Jetten. 2015. Complete nitrification by a single microorganism. Nature 528:555–559.
45. Walker, C.B., J.R. de la Torre, M.G. Klotz, *et al.* 2010. *Nitrosopumilus maritimus* genome reveals unique mechanisms for nitrification and autotrophy in globally distributed marine crenarchaea. Proc. Natl. Acad. Sci. U.S.A. 107:8818–8823.
46. Wuchter, C., B. Abbas, M.J.L. Coolen, *et al.* 2006. Archaea nitrification in the ocean. Proc. Natl. Acad. Sci. U.S.A. 103:12317–12322.
47. Yan, J., S.C.M. Haaijer, H.J.M. Op den Camp, *et al.* 2012. Mimicking the oxygen minimum zones: stimulating interaction of aerobic archaeal and anaerobic bacterial ammonia oxidizers in a laboratory-scale model system. Environ. Microbiol. 14:3146–3158.

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*Short Communication*

# Metagenomics Reveals a Novel Virophage Population in a Tibetan Mountain Lake

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Virophages are parasites of giant viruses that infect eukaryotic organisms and may affect the ecology of inland water ecosystems. Despite the potential ecological impact, limited information is available on the distribution, diversity, and hosts of virophages in ecosystems. Metagenomics revealed that virophages were widely distributed in inland waters with various environmental characteristics including salinity and nutrient availability. A novel virophage population was overrepresented in a planktonic microbial community of the Tibetan mountain lake, Lake Qinghai. Our study identified coccolithophores and coccolithovirus-like phycodnaviruses in the same community, which may serve as eukaryotic and viral hosts of the virophage population, respectively.

**Key words:** virophage, metagenomics, microbial ecology, Tibetan lake

Virophages are small (<100 nm), icosahedral, double-stranded DNA viruses that replicate using the replication machinery of giant viruses (7, 14, 19). Virophages parasitize giant viruses, which is reflected in the formation of the abnormal particles of giant viruses and the increased survival rates of eukaryotic hosts when co-cultured (9, 14). Virophages have the ability to regulate members of *Mimivirus*, including *Acanthamoeba polyphaga mimivirus*, *Moumouvirus*, and *Megavirus chilensis*, which infect *A. polyphaga* (10). *Acanthamoeba* are commonly found in soil, fresh water, and seawater and are predators of various bacteria. Virophages control the populations of phototrophic algae by attenuating the infectivity of phycodnaviruses, which, in turn, may affect algal blooms (29). Thus, elucidating the distribution and host-virus interactions of virophages has important implications for nutrient cycling and food webs in natural aquatic ecosystems.

Only a few studies are available on virophage isolates and their host-virus interactions. Sputnik, the first virophage identified, was discovered with *A. polyphaga mimivirus*, which was isolated from a water sample taken from a cooling tower in France (14). Sputnik and its closest relatives (Sputnik2 and Suptnik3, the genome sequences of which share >99% nucleotide identity with the Sputnik genome) have a broad range of viral hosts among *Mimiviridae* that infect *Amoeba* (6, 10, 15). Zamilon is closely related to Sputnik (76% genome sequence identity) and has the ability to multiply in members of *Mimiviridae*. Zamilon and the giant virus Mont1 were co-isolated in a soil sample from Tunisia (11). Mavirus was isolated with the *Cafeteria roenbergensis* virus isolated from coastal waters in the USA (9). The Mavirus infection led to a decrease in the infectivity of the giant virus to the eukaryotic host, a marine phagotrophic flagellate.

Other virophages such as those found in Organic Lake (OLV), Yellowstone Lake (YSLV1 through YSLV4), and Ace Lake (ALM) were investigated by analyzing their genomes recovered from metagenomic datasets (29, 30). These studies provided insights into the viral and eukaryotic hosts of virophages and their co-evolution (*e.g.*, horizontal gene transfer among virophages, giant viruses, and eukaryotic hosts). Previous metagenomic studies documented the occurrence of virophages in diverse environments (fresh water, ocean, and soil) (5, 30) and the phylogenetic diversity of virophages in marine environments and Antarctic lakes (29). However, it has not yet been determined whether virophages are widely distributed in inland water environments, and, if so, what types of virophages are present. Therefore, addressing these issues is important in order to infer the ecological impact of virophages in inland water environments, given that characterized virophages are known to interact with different viral and eukaryotic hosts.

The metagenomic datasets of microbial communities sampled from the surface waters of ten lakes (Lanier, Mendota, Spark, Trout, Damariscotta, Vattern, Ekoln, Erken, Qinghai, and Yellowstone) and a river (Amazon) were collected (Table S1). Two Antarctic lakes (Ace and Organic) and a freshwater lagoon (Albufera) derived from seawater were also included. Major capsid protein (MCP) sequences are often used to classify viruses (3) and virophages (5, 24). Nine MCP sequences from all of the available virophage genomes were aligned using ClustalW (26). The sequence alignment identified a conserved region (amino acid positions 345–404; based on Sputnik) in the MCP sequences (Fig. S1). The metagenomic reads of the 13 datasets were searched against the conserved region using BLASTx (2) with cut-offs of >30% amino acid identity and >55 match length. The analysis revealed 134 virophage-like metagenomic reads originating from the majority ($n$=8) of the metagenomic datasets, indicating the widespread occurrence of virophages in inland waters with environmental gradients of salinity (freshwater to hypersaline) and nutrient availability (oligotrophic to eutrophic) (Table S1). The structure-based phylogenetic diversity of the virophages was investigated using the 134 virophage-like sequences

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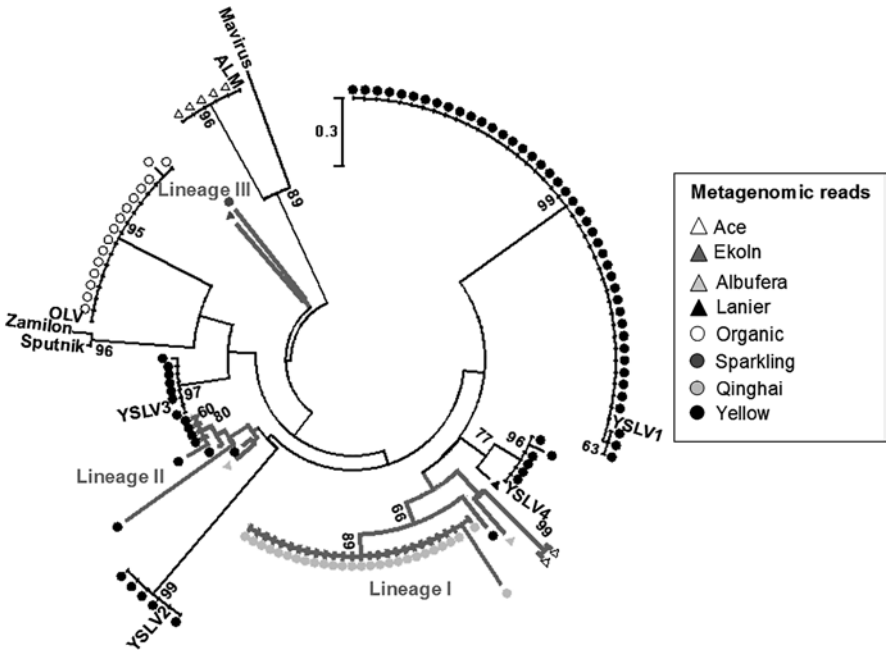


**Fig. 1.** Occurrence and diversity of virophage populations in diverse terrestrial aquatic environments. The phylogenetic tree was built based on the maximum likelihood method with the Jones-Taylor-Thornton model using MEGA 6.0 (25). Bootstrap support values (higher than 50) from 100 replicates are shown on the nodes of the tree. The three lineages (Lineages I, II, and III) that were not closely related to the previously characterized virophages were highlighted.

(Fig. 1). While 92 sequences were closely related to those of the six previously characterized virophages (OLV, YSLV1 through 4, and ALM), the remaining 42 sequences corresponded to three distinct lineages (Lineages I, II, and III). Most of the newly identified virophage-like sequences (Lineages I and II) were more closely related (38 to 62% amino acid identity) to phycodnavirus-associated virophages (YSLVs and OLV) than Sputnik, Zamilon, and Mavirus.

Since many ($n$=26) of the virophage-like sequences of Lineage I identified in Qinghai were identical (100% identity) (Fig. 1), we attempted to reconstruct the genome sequence of the virophage population, designated as the Qinghai Lake virophage (QLV). The Qinghai metagenomic dataset was trimmed and assembled, as described previously (18). A search of all assembled contig sequences against the nine MCP sequences using BLASTx identified one MCP-encoding contig (contig_0049). Qinghai metagenomic reads were recruited to all assembled contigs using BLASTn with cut-offs of >95% nucleotide identity and >50% query length coverage, as described previously (16, 17). The read recruitment analysis identified metagenomic reads that simultaneously mapped on two distinct contigs. This approach was able to identify three additional contigs that were consecutively connected from contig_0049, based on the mapping patterns of the metagenomic reads. The metagenomic reads and four contigs were combined and re-assembled together using Newbler 2.8, which generated a single contig. The metagenomic reads were successively overlapped between the end and beginning of the single contig (Fig. S2), indicating that the single contig represents a circular genome, as with other virophages reported previously. Metagenomic read recruitment showed 9 to 100× coverage (56× on average) at 95–100% identities over the entire contig region. More than 93% of the recruited reads showed 98±2% identity, which was higher than the 90–95% average identities frequently used for phage species demarcation (1). These results suggest that the reconstructed QLV genome represents that of a species-like population, which has an intrapopulation genetic variation of approximately 2% (Fig. S2).

The length and G+C content of the QLV genome were 23,379 bp and 33.2% (Fig. 2), respectively. These genomic features were similar to those of other virophages reported previously as genome sizes of 17 to 28 kbp and G+C contents of 26 to 38% (9, 11, 14, 29, 30). The QLV genome encoded 25 open reading frames (ORFs) (Fig. 2 and Table S2). A gene content analysis identified genes commonly conserved between QLV and other virophage genomes: FtsK-HerA family ATPase (QLV1), cysteine protease (QLV6), MCP (QLV18), minor capsid protein (QLV19), and DNA helicase/primase/polymerase (QLV23) genes. The gene products of the core genes are known to carry out essential functions such as DNA replication and packaging in the virophage life cycle (11, 30). Phylogenetic trees were built using the full-length MCP, FtsK-HerA family ATPase, and cysteine protease genes (Fig. S3). The phylogenetic relationships observed based on the three different protein sequences were congruent with those shown in Fig. 1. These results confirmed the higher evolutionary relatedness of QLV to OLV-like virophages (OLV and YSLVs) than Sputnik and Mavirus, which corroborated the conserved region of the MCP sequences used in Fig. 1 serving as a robust genetic marker for a phylogenetic analysis.

QLV shared 7 (41% of the average amino acid identity), 8 (39%), 9 (40%), and 11 (46%) gene homologues with YSLV3, OLV, YSLV1, and YSLV4, respectively. QLV showed <35% average amino acid identity and <6 gene homologues with Sputnik, Zamilon, Mavirus, and ALM. QLV encoded 11 QLV-specific genes that were not found in other virophages

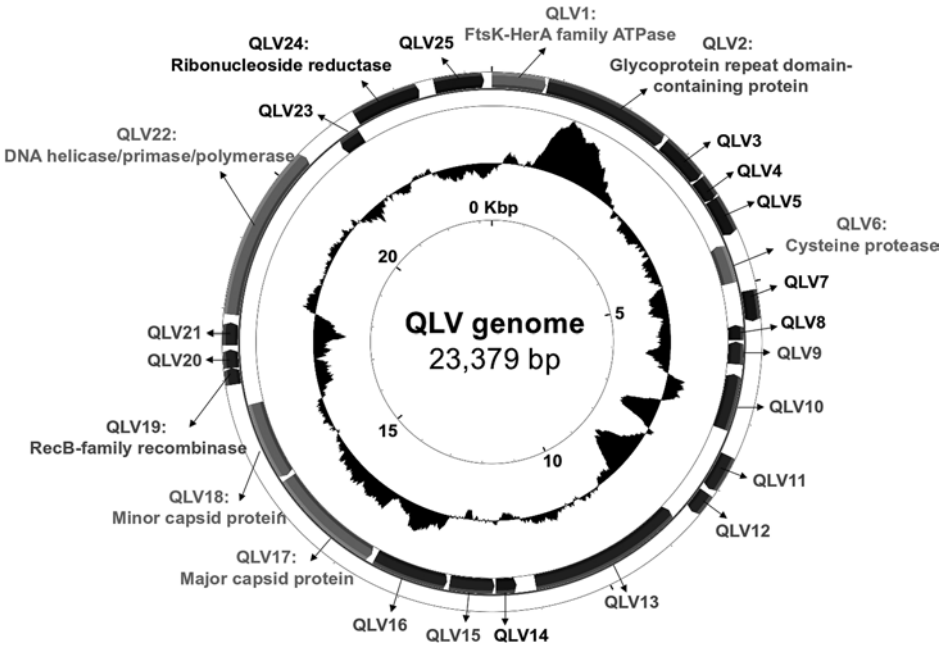


**Fig. 2.** Circular map of the Qinghai Lake virophage (QLV) genome. Inwards: ORFs of the two DNA strands (red, blue, and black representing core, specific, and others, respectively) and G+C content. Protein-coding genes on the genome were predicted using GeneMark.hmm with the heuristic model (4). The protein-coding genes were functionally annotated by searching the amino acid sequences against the non-redundant protein database (nr) and metagenomic protein (env_nr) database, respectively, using BLASTp with >30% amino acid identity and >50% query length coverage.

when using cut-offs of 30% amino acid identity and 50% query length coverage (Fig. 2). While many of the predicted functions of the QLV-specific genes were hypothetical, QLV2 and QLV19 encoded a glycoprotein repeat domain-containing protein and RecB-family recombinase, respectively (Table S2). RecB is a subunit of the RecBCD enzyme that salvages double strand breaks in DNA through recombinational DNA repair (8); however, its exact role in virophages has not yet been investigated. Glycoproteins participate in the formation of the extracellular envelope, adhesion processes, and protein-protein interactions between viruses and their hosts (14, 29). A search of the QLV2 amino acid sequence against the non-redundant protein database (nr) showed the best hits (>48% amino acid identity) to phycodnaviruses (*Paramecium bursaria* and *Acanthocystis turfacea* Chlorella virus), which are known to infect unicellular green algae. Gene homologues conserved between virophages and their giant viruses were subjected to genetic exchange through the virophage-giant virus interaction (14, 29). Overall, the evolutionary relatedness of QLV to OLV-like virophages (Fig. 1 and S2) and the gene homology (QLV2) between QLV and phycodnaviruses (Table S2) collectively suggested that QLV presumably prey on phycodnaviruses.

The occurrence of QLV in the planktonic microbial community of Lake Qinghai may be interpreted by considering the filter size used for sampling and the size of virophages. Microbial cells smaller than 5 μm (in length) and larger than 0.22 μm were collected from a surface water sample taken from Lake Qinghai in a previous study (18). Since virophages are less than 0.1 μm in diameter, they may be underrepresented among the microbial cells collected using the above sample preparation. However, the QLV genome was overrepresented in the metagenomic dataset in terms of genome coverage (56×) relative to that of the other contigs assembled (<30×). Thus, it was speculated that QLV were sampled with their viral and/or eukaryotic hosts, in addition to those attached to and collected with suspended solids.

The Qinghai metagenomic reads were searched against the small subunit ribosomal RNA (SSU rRNA) gene (V9 hypervariable region) database (13) using BLASTn with cut-offs of >70% nucleotide identity and >90% target length coverage. More than 99% of the total SSU rRNA gene sequences retrieved were bacterial or archaeal, whereas four chloroplast 16S rRNA and two 18S rRNA gene sequences were identified (Fig. S4). The chloroplast 16S rRNA gene sequences were closely related (>98% identity) to coccolithophores (more specifically, *Isochrysis* spp. and *Emiliania huxleyi*). Coccolithophores are unicellular phytoplanktons that play a key role in nutrient cycling and food webs in water environments (21, 28). While *E. huxleyi* form extensive blooms from tropical to subpolar oceans, they are often found in oligotrophic environments. Some mechanisms (efficient cellular nitrogen utilization and ATP synthesis in nitrogen-limiting conditions) confer a fitness advantage to *E. huxleyi* in oligotrophic environments (22). Phytoplanktons have the ability to use alkaline phosphatase, which hydrolyzes extracellular inorganic phosphate, and this facilitates cellular uptake under phosphorous-limited conditions. The alkaline phosphatase of *E. huxleyi* exhibits maximum activity at approximately pH 9 (28). The surface waters (depth of 0.5 meters) of Lake Qinghai are oligotrophic (<1 mg $L^{-1}$ of total nitrogen and <0.02 mg $L^{-1}$ of total phosphorus) and alkaline (pH 9.3) (27). While *E. huxleyi* are commonly found in marine environments, they may have also been favored in Lake Qinghai due to, at least in part, the oligotrophic and alkaline conditions. Furthermore, we performed a metagenomic survey to ascertain the presence of

coccolithophore-infecting viruses within the same microbial community. The alignment of MCP sequences from phycodnavirus genomes using ClustalW identified a conserved region (amino acid positions 433–492; based on Organic Lake phycodnavirus 1) in the MCP sequences. A search of the Qinghai metagenomic reads against the conserved MCP region using BLASTx with cut-offs of >30% identity and >90% target length coverage identified three MCP-encoding metagenomic reads. The three MCP sequences showed higher amino acid identities (31–40%) to *E. huxleyi* viruses than other phycodnaviruses (Fig. S5). These results suggest that phycodnavirus populations in the same microbial community were evolutionarily more closely related to *E. huxleyi* viruses, the parasites of the *E. huxleyi* observed in Fig. S4.

A PCR and metagenomic approach recently revealed a virophage population (DSLV1) in the surface waters of Lake Dishui in East China (12). Since DSLV1 showed genomic relatedness to YSLV3, it was associated with algae and algae-infecting large dsDNA viruses. QLV showed 35–51% amino acid identities on five gene homologues with DSLV1, suggesting marked genomic divergence between two virophage populations. The PCR amplification of DNA samples using MCP gene-specific primers revealed the occurrence of the DSLV1 population in Lake Dishui over a one-year period (12). Although PCR amplification is useful for identifying virophages closely related to the reference virophage strains used for primer design, it may not be useful for successfully detecting environmental virophages with marked genomic divergence (Fig. 1), as revealed in this study.

The development of a marker gene with high resolution within a specific viral group is essential for examining its diversity, distribution, and relative abundance in the environment, while viruses share no universal marker genes (*e.g.*, 16S rRNA genes in prokaryotes). A previous study searched metagenomic sequences using a cut-off of $<10^{-5}$ E-value against "virophage-specific marker genes," defined as those that had no hits to the nr database (30). In contrast, we selected a MCP gene as a genetic marker because it was one of the five core genes conserved among all characterized virophages that occurred in a single copy in a virophage genome and contained a 60-amino-acid-long region with the longest consecutive segment. We then searched raw metagenomic reads against the MCP alignment using cut-offs of >30% amino acid identity and >55 match length. Although our marker gene survey used a target region (60 amino acids), the amino acid length and cut-offs used were similar to the threshold (29–31% identities in 55–60 amino acids) for inferring structural homology between two proteins (23). In contrast to the $10^{-3}$–$10^{-6}$ E-value cut-offs used in previous studies (5, 30), the 30% amino acid identity criterion was employed in the present study because it is considered to be more stringent and E-values change depending on the size of the database used (20). The 134 metagenomic reads (Fig. 1) retrieved in the present study showed best hits to the nine virophage MCP genes in the nr database, strongly suggesting that the 134 reads retrieved were of a virophage origin.

It is important to note that the occurrence and diversity of virophages (Fig. 1) in inland waters revealed in this study are underestimated because many of the sample preparations (Table S1) for metagenome sequencing were conducted using filter sizes larger than the typical size of virophages. Nevertheless, our metagenomic study detected a large number of virophage sequences in metagenomic datasets from many terrestrial aquatic environments using the robust genetic marker developed in the present study. Notably, we uncovered a novel virophage population particularly overrepresented in the planktonic microbial community of Lake Qinghai (Fig. 1 and 2). Distinctive from previous studies, our bioinformatic results detected eukaryotic and phycodnaviral populations as well as virophages in the same microbial community, which further implies that QLV are associated with coccolithophores and coccolithovirus-like phycodnaviruses. Therefore, we encourage future experiments determining the infectivity and host specificity of the novel virophage, which will contribute to more accurate assessments of the ecological consequences of the virophage population in the Tibetan mountain lake ecosystem.

## Nucleotide sequence accession numbers

The genome sequence of QLV was deposited in GenBank under the accession number KJ854379.1

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### References

1. Adriaenssens, E.M., L. Van Zyl, P. De Maayer, E. Rubagotti, E. Rybicki, M. Tuffin, and D.A. Cowan. 2014. Metagenomic analysis of the viral community in Namib Desert hypoliths. Environ. Microbiol. 17:480–495.
2. Altschup, S.F., W. Gish, W. Miller, E.W. Myers, and D.J. Lipman. 1990. Basic local alignment search tool. J. Mol. Biol. 215:403–410.
3. Bamford, D.H., J.M. Grimes, and D.I. Stuart. 2005. What does structure tell us about virus evolution? Curr. Opin. Struct. Biol. 15:655–663.
4. Besemer, J., and M. Borodovsky. 1999. Heuristic approach to deriving models for gene finding. Nucleic Acids Res. 27:3911–3920.
5. Desnues, C., M. Boyer, and D. Raoult. 2012. Sputnik, a virophage infecting the viral domain of life. Adv. Virus Res. 82:63–89.
6. Desnues, C., B. La Scola, N. Yutin, *et al.* 2012. Provirophages and transpovirons as the diverse mobilome of giant viruses. Proc. Natl. Acad. Sci. U.S.A. 109:18078–18083.
7. Desnues, C., and D. Raoult. 2012. Virophages question the existence of satellites. Nat. Rev. Microbiol. 10:234.
8. Dillingham, M.S., and S.C. Kowalczykowski. 2008. RecBCD enzyme and the repair of double-stranded DNA breaks. Microbiol. Mol. Biol. Rev. 72:642–671.
9. Fischer, M.G., and C.A. Suttle. 2011. A virophage at the origin of large DNA transposons. Science 332:231–234.
10. Gaia, M., I. Pagnier, A. Campocasso, G. Fournous, D. Raoult, and B. La Scola. 2013. Broad spectrum of Mimiviridae virophage allows its isolation using a Mimivirus reporter. PLoS ONE 8:e61912.
11. Gaia, M., S. Benamar, M. Boughalmi, I. Pagnier, O. Croce, P. Colson, D. Raoult, and B. La Scola. 2014. Zamilon, a novel virophage with *Mimiviridae* host specificity. PLoS ONE 9:e94923.
12. Gong, C., W. Zhang, X. Zhou, H. Wang, G. Sun, J. Xiao, Y. Pan, S. Yan, and Y. Wang. 22 January 2016. Novel virophages discovered in a freshwater lake in China. Front. Microbiol. doi: 10.3389/fmicb.2016.00005.

13. Huse, S.M., D.B. Mark Welch, A. Voorhis, A. Shipunova, H.G. Morrison, A.M. Eren, and M.L. Sogin. 2014. Vamps: a website for visualization and analysis of microbial population structures. BMC Bioinformatics 15:41.
14. La Scola, B., C. Desnues, I. Pagnier, *et al.* 2008. The virophage as a unique parasite of the giant mimivirus. Nature 455:100–104
15. La Scola, B., A. Campocasso, R. N'Dong, G. Fournous, L. Barrassi, C. Flaudrops, and D. Raoult. 2010. Tentative characterization of new environmental giant viruses by MALDI-TOF mass spectrometry. Intervirology 53:344–353.
16. Oh, S., M. Tandukar, S.G. Pavlostathis, P.S.G. Chain, and K.T. Konstantinidis. 2013. Microbial community adaptation to quaternary ammonium biocides as revealed by metagenomics. Environ. Microbiol. 15:2850–2864.
17. Oh, S., Z. Kurt, D. Tsementzi, M.R. Weigand, M. Kim, J.K. Hatt, M. Tandukar, S.G. Pavlostathis, J.C. Spain, and K.T. Konstantinidis. 2014. Microbial community degradation of widely used quaternary ammonium disinfectants. Appl. Environ. Microbiol. 80:5892–5900.
18. Oh, S., R. Zhang, Q. Wu, and W. Liu. 2014. Draft genome sequence of a novel SAR11 clade species abundant in a Tibetan lake. Genome Announc. 2:10–11.
19. Pearson, H. 2008. 'Virophage' suggests viruses are alive. Nature 454:677.
20. Pearson, W.R. 2013. An introduction to sequence similarity ("homology") searching. 2013. Curr Protoc Bioinformatics. 42:3.1.1–3.1.8.
21. Read, B.A., J. Kegel, M.J. Klute, *et al.* 2013. Pan genome of the phytoplankton *Emiliania* underpins its global distribution. Nature 499:209–213.
22. Rokitta, S.D., P. Von Dassow, B. Rost, and U. John. 2014. *Emiliania huxleyi* endures N-limitation with an efficient metabolic budgeting and effective ATP synthesis. BMC Genomics 15:1051.
23. Sander, C., and R. Schneider. 1991. Database of homology-derived protein structures and the structural meaning of sequence alignment. Proteins: Struct. Funct. Genet. 9:56–68.
24. Sun, S., B. La Scola, V.D. Bowman, C.M. Ryan, J.P. Whitelegge, D. Raoult, and M.G. Rossmann. 2010. Structural studies of the Sputnik virophage. J. Virol. 84:894–897.
25. Tamura, K., G. Stecher, D. Peterson, A. Filipski, and S. Kumar. 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30:2725–2729.
26. Thompson, J.D., D.G. Higgins, and T.J. Gibson. 1994. Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22:4673–4680.
27. Wu, Q.L., A. Chatzinotas, J. Wang, and J. Boenigk. 2009. Genetic diversity of eukaryotic plankton assemblages in eastern Tibetan lakes differing by their salinity and altitude. Microb. Ecol. 58:569–581.
28. Xu, Y., T.M. Wahlund, L. Feng, Y. Shaked, and F.M.N. Morel. 2006. A novel alkaline phosphatase in the coccolithophore *Emiliania huxleyi* (prymnesiophyceae) and its regulation by phosphorus. J. Phycol. 42:835–844.
29. Yau, S., F.M. Lauro, M.Z. DeMaere, *et al.* 2011. Virophage control of Antarctic algal host-virus dynamics. Proc. Natl. Acad. Sci. U.S.A. 108:6163–6168.
30. Zhou, J., W. Zhang, S. Yan, J. Xiao, Y. Zhang, B. Li, Y. Pan, and Y. Wang. 2013. Diversity of virophages in metagenomic data sets. J. Virol. 87:4225–4236.

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*Minireview*

# Metagenomics and Bioinformatics in Microbial Ecology: Current Status and Beyond

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Metagenomic approaches are now commonly used in microbial ecology to study microbial communities in more detail, including many strains that cannot be cultivated in the laboratory. Bioinformatic analyses make it possible to mine huge metagenomic datasets and discover general patterns that govern microbial ecosystems. However, the findings of typical metagenomic and bioinformatic analyses still do not completely describe the ecology and evolution of microbes in their environments. Most analyses still depend on straightforward sequence similarity searches against reference databases. We herein review the current state of metagenomics and bioinformatics in microbial ecology and discuss future directions for the field. New techniques will allow us to go beyond routine analyses and broaden our knowledge of microbial ecosystems. We need to enrich reference databases, promote platforms that enable meta- or comprehensive analyses of diverse metagenomic datasets, devise methods that utilize long-read sequence information, and develop more powerful bioinformatic methods to analyze data from diverse perspectives.

**Key words:** metagenomics, bioinformatics, database, meta-analysis, long-read sequencer

### *Data deluge in microbial ecology*

Although microbes play fundamental roles in various ecosystems, most have not yet been characterized in detail. Bioinformatics, which aims to discover new biological concepts and laws based on large-scale data, is now expected to accelerate discovery in unexamined areas of the microbial universe. The *data deluge* has made bioinformatics indispensable in modern research; recent innovative technologies are producing large amounts of data at an unprecedented pace. Observations are key to science; for example, optical and electron microscopies are important methods of observation combined with various staining methods. Among recent observational technologies, high-throughput DNA sequencing technologies have rapidly produced vast amounts of genetic information at low cost, making available thousands of microbial genomes. These genome sequences provide a comprehensive catalog of the microbial genetic elements underlying diverse microbial physiology, and also assist in weaving a massive tapestry of microbial evolutionary histories (72, 154).

In microbial ecology, research has been hindered because the majority of environmental microbes are uncultivable. A large number of studies across diverse natural environments have identified many microbial groups with no axenic culture (110, 113, 133, 138). In order to overcome this fundamental difficulty, culture-independent approaches, including DNA hybridization (*e.g.*, microarray and fluorescent *in situ* hybridization), DNA cloning, and PCR have been used to detect specific members and/or functional genes in microbial communities (3, 8, 9, 32, 34, 47, 56, 61, 74, 157, 174, 185). High-throughput sequencing technologies have recently popularized shotgun metagenomic and (typically 16S ribosomal RNA [rRNA] gene) amplicon sequencing methods, which identify members and/or functional genes at a greater scale and in more detail. Their use in diverse environments has revealed the presence of extremophiles (27, 33, 92), uncovered relationships between microbes and human diseases (10, 44, 55, 57, 85, 87, 96, 134, 147), and characterized the nutrition systems involved in symbiosis (68, 174, 177). Even more applications of these methods are used in agriculture (93), food science and pharmaceuticals (32), and forensics (49, 79, 82, 182). Many large-scale metagenomic projects are now generating comprehensive microbial sequence collections for different environments (*e.g.*, human-associated [116, 167], soil [54, 171], and ocean environments [17, 142]). Since microbial communities change as they interact with other organisms and as the environment changes, time-series analyses have also become common (21, 24, 77, 115, 172).

Several bioinformatic tools have been developed and popularized to analyze metagenomic and amplicon sequence data. Web servers, such as MG-RAST (104), IMG/M (97), EBI Metagenomics (69), and SILVAngs (135), and pipelines, such as MEGAN (70), QIIME (25), and Mothur (145), now allow researchers to perform integrated metagenomic analyses and visualize results without command-line operations or strong computational knowledge. Since there are already several introductory articles on these popular tools (36, 88, 103, 123, 146), we herein addressed how can we examine large datasets in detail in order to obtain a deeper understanding of the ecology and evolution of microbes in the environment beyond existing approaches that are already popular (Fig. 1).

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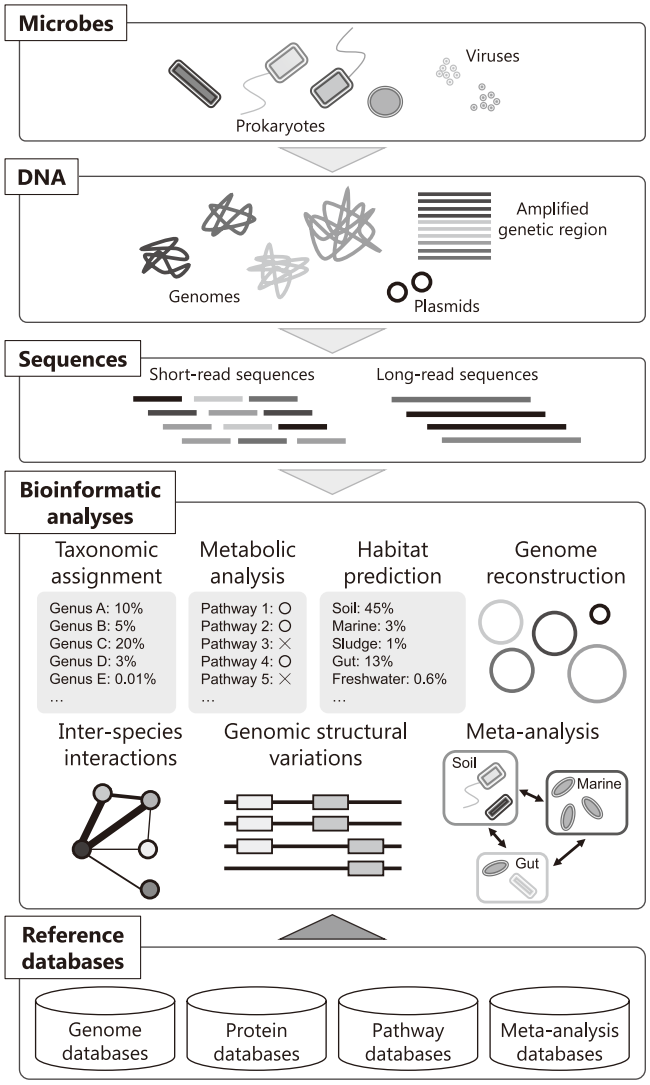


**Fig. 1.** Schematic figure of metagenomic and bioinformatic analyses in microbial ecology. The illustration covers topics that are already popular, that need further development, and that will become important in the future. At the bottom of the illustration, reference databases lay foundations for various bioinformatic analyses.

### *Toward better taxonomic assignments*

A fundamental step in microbial ecology is to describe the taxonomic distribution of microbial community members. Thus, the precise taxonomic assignment of sequencing reads is one of the most important issues in the analysis of metagenomic and amplicon sequencing data. Reference-based methods are frequently used for this purpose, in which taxonomic assignments are based on straightforward sequence similarity searches against reference genomes (*e.g.*, RefSeq [163]) or 16S rRNA sequence databases (*e.g.*, Greengenes [101], SILVA [135], RDP [31], and Ez-Taxon [29]). These databases typically contain the sequences of previously isolated and taxonomically classified strains, whereas they also contain environmental clone sequences. Many bioinformatic tools, such as TANGO (6), MetaPhlAn (148), and Kraken (176), have been developed to improve the computational efficiency, accuracy, and sensitivity of taxonomic assignments. Although these tools perform well for many applications, discriminating between closely related species is sometimes difficult, particularly in cases of highly conserved genes (*e.g.*, 16S rRNA genes). Additionally, genes that undergo horizontal gene transfer (HGT) between different taxa may cause incorrect taxonomic assignments. A more fundamental issue is taxonomic bias in reference databases, which leads to biased taxonomic assignments. A previous study reported that taxonomic assignments markedly change when different versions of reference databases are used (128). Therefore, even in this era of data deluge, the further taxonomic enrichment of reference databases is key to the improvement of reference-based methods. It is important to note that this issue is more crucial in the analysis of fungal and viral sequences because fewer reference sequences are available and their taxonomy is under debate. In order to overcome this obstacle, several projects are now attempting to obtain a number of genomic sequences to enrich databases (58, 180). In cases in which amplicon sequencing data are analyzed, the filtering of chimeric sequences formed during PCR is very important for precise analyses (63). Several bioinformatic tools, such as AmpliconNoise (136), ChimeraSlayer (63), and UCHIME (41), have been proposed and commonly used to remove chimeric sequences.

Reference-free methods may be used (*e.g.*, CD-HIT [50], UCLUST [40], and UPARSE [42]) as an alternative to reference-based methods. These methods use clustering to group marker genes, such as 16S rRNA, ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), ammonia monooxygenase (*amoA*), sulfate thioesterase/thiohydrolase (*soxB*), and methyl-coenzyme M reductase genes (*mcrA*), into unique representative sequences that serve as operational taxonomic units (OTUs) (27). 16S rRNA genes are used to study the general composition of a microbial community, while RuBisCO, *amoA*, *soxB*, and *mcrA* genes are typically used to investigate microbes that play critical roles in carbon, nitrogen, sulfur, and methane cycles, respectively. In addition to traditional genes, useful marker genes may be found and used by comprehensively profiling metagenomic datasets (159). In reference-free methods, OTUs often cannot be assigned to known taxa. In order to estimate phylogenetic information for these OTUs, PhylOTU (150), pplacer (98), and PhyloSift (35) couple reference-free methods with phylogenetic analyses.

### *Toward the cultivation-free reconstruction of genomic sequences*

Most metagenomic studies currently focus at the level of individual genes ("gene-centric" metagenomics [132]). In contrast, in some pioneering research on "genome-centric" metagenomics, microbial genomes that include those of important uncultivated taxonomic groups were successfully reconstructed by metagenomic binning and assembly from various environments including oceans, groundwater, soil, hypersaline lakes, and acid mine drainage (4, 18, 45, 95, 112, 168). Although amplification bias still poses a non-negligible difficulty, single-cell genomic sequencing is expected to accelerate direct genome reconstruction from environmental samples (43, 90, 140), in which the combination of single cell genomic and metagenomic approaches may be a promising approach (102).

Metagenomic assembly is an important step for revealing the ecology and physiology of environmental microbes, in which the fundamental concepts of metagenomic assembly from short-read sequences have already been described in detail (36, 88, 103, 123, 146). Several tools have been developed for metagenomic assembly, and are classified into reference-based (*e.g.*, AMOS [130]) and *de novo* methods (*e.g.*, MetaVelvet-SL [2], SPAdes [119], and IDBA-UD [127]). In the case of *de novo* assembly, users need to consider chimeric contigs because similar genetic regions may be shared by different genomes (100, 129, 170). In order to improve the performance of *de novo* metagenomic assembly, composition-based methods use specific sequence features in a metagenomic dataset to split reads into different species. For example, CONCOCT (5), metaBAT (81), and MaxBin (178) bin sequences based on their tetra-nucleotide frequency composition and coverages. These composition-based approaches are computationally intensive, particularly in their memory usage. Thus, a fast-clustering approach using matrix decomposition with streaming singular value decomposition may be combined (30). On the other hand, sequence coverage information across different DNA extraction methods may also be used to effectively split sequences into species because the numbers of sequence reads from the same genome need to be similar regardless of the extraction method (4). A related approach bins co-abundant sequences across a series of metagenomic samples from similar environments (*e.g.*, human gut microbiome) to identify co-abundance gene groups (117).

Another information source that may improve the performance of metagenomic assembly is long-range contiguity. The recent development of methods to investigate long-range chromatin interactions (*e.g.*, Carbon-Copy Chromosome Conformation Capture [5C] [38] and Hi-C [11]) may also contribute to metagenomic assembly because these methods ligate sequences from two different genomic regions that are in the same cell (20). The Irys system (BioNano Genomics, San Diego, USA), which also detects long-range contiguity with fluorescently labeled DNA, may be used to obtain long contigs (64).

### *Toward a more reliable estimation of community metabolism*

Microbial genomes are affected by the environment during their evolution. Metabolic processes encoded in the genome, from biosynthesis to biodegradation, directly link microbial communities to the environment. Since most microbes are uncultivable, the direct estimation of community-scale metabolic pathways is also targeted by a metagenomic analysis. The most straightforward approach is to conduct sequence-similarity searches against pathway databases, such as KEGG (80), MetaCyc (22), and SEED (124), and use the findings obtained to annotate metabolic genes. Since many pathways with component genes that are only partially found in given metagenome data are typically detected with this naïve approach, MAPLE (160), MinPath (183), MetaNetSam (75), and HUMAnN (1) quantitatively or probabilistically evaluate whether these pathways likely function, enabling comparisons between samples. Significant biases in the databases of known pathways need to be taken into consideration when interpreting the findings of these methods. If shotgun metagenome data are unavailable, "virtual metagenomes" or functional gene abundance may be estimated using 16S rRNA amplicon sequencing data (89, 121). This approach takes advantage of closely related genomes being more likely to have a similar gene content, and, thus, given the 16S rRNA sequence, the gene content of its host genome may be estimated (at least, to some extent) if a closely related genome is already sequenced. It is important to note that such estimations may become difficult when applied to microbial groups with genomes that are rarely available and also that genomic variations within closely related microbial groups cannot be precisely considered. Despite these difficulties, this approach is very cost-effective and more easily applicable to large-scale comparative analyses.

### *Toward a community-level analysis of genomic structural variations and dynamics*

Operon structures, which are unique to prokaryotic genomes, reflect the function of their encoded genes and need to be associated with microbial ecological strategies. Thus, if we observe systematic variations in the gene order (or gene cluster structures) due to gene losses, fusions, duplications, inversions, translocations, and HGTs from an analysis of metagenome data, these variations may provide important clues for linking microbial communities to the environment (Fig. 2A). Although difficulties are sometimes associated with distinguishing variations under selection pressure from those because of population changes, MaryGold (118) is a tool for the visual inspection of such variations. Variations in the gene order for genes in the tryptophan pathway were identified within contigs assembled from the Sargasso Sea metagenome (78). Since the availability of long sequences that encompass multiple genes greatly facilitates a gene-order analysis, DNA cloning may also be used if the targeted pathways are efficiently enriched by colony selection (51, 158). On a larger scale, gene order may be affected by genome replication mechanisms. Most prokaryotic genomes are circular with one replication origin; thus, genes close to the origin may physically exist in multiple copies, particularly during an active growth phase. Thus, the detection of these regions from metagenomic sequences may reveal the growth dynamics of microbes in a community (86).

Among various sources of genomic variations and dynamics, HGT is of particular interest because it may help microbes adapt quickly to different environments (120, 139, 156). Although many comparative genomic studies have analyzed HGT (26, 73, 153), its role in microbial communities has not yet been elucidated in detail. A classical approach to detect HGT within communities is based on DNA cloning, which is particularly effective if colony selection is applied to the targeted genes (162, 175). In metagenomics, assembled contigs may be used to comprehensively identify HGT events by analyzing phylogenetic incongruence and gene order differences (62, 125, 161). In addition, gene gains via plasmids are also important driving forces that accelerate microbial adaptation to their environment. In accordance with this hypothesis, plasmids are frequently reported to contain genes that may contribute to fitness, such as detoxification genes (151, 152, 165) and antibiotic resistance genes (13). Notably, plasmid-specific metagenomics, or a *plasmidome* analysis, is

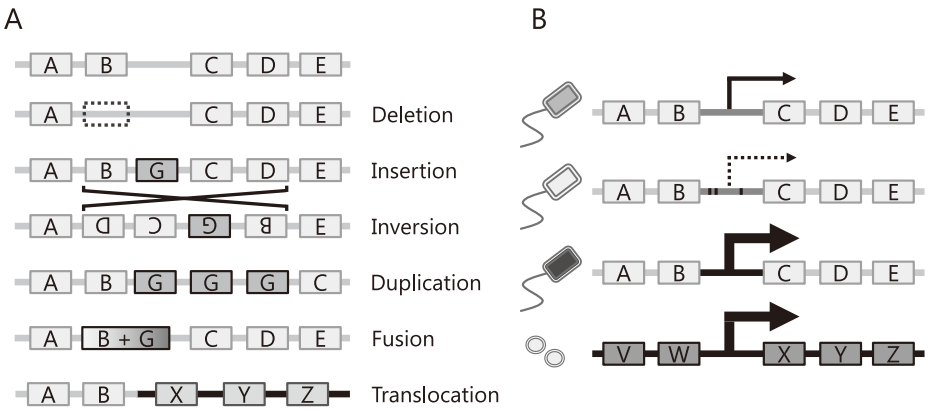


**Fig. 2.** Schematic figures of genomic variations in environmental microbes. Each box represents a protein-coding gene, in which the letters indicate homology. Boxes and thick lines of different brightnesses represent genes and genomic fragments, respectively, that originated from different genomic areas or genomes. Dashed lines represent lost genes or expression. A: Types of genomic structural variations. B: Variations in regulatory sequences. Mutations (black dots) and the horizontal transfer of intergenic regulatory sequences (thick lines in black) both affect the strength of gene expression.

now being conducted to directly investigate environmental plasmids without culturing or cloning (37, 173). For example, the bovine rumen plasmidome was previously reported to contain genes that may confer advantages to their hosts (19). Rat gut (76) and wastewater (149) plasmidomes have also been investigated.

In addition to genes, regulatory sequences in intergenic regions are related to the function of nearby genes. Thus, variations in the comprehensive set of regulatory sequences, or the regulome, for example, by promoter propagation, may play important roles in microbial adaptation (99, 122). We envision direct investigations of regulomes in microbial communities, or a "metaregulome" analysis, becoming an attractive research field as technical advances occur in long-read metagenomics. In a metaregulome analysis, variations and/or possible transfers of regulatory sequences, in addition to those of coding genes, may be identified from metagenomic datasets (Fig. 2B) (48, 122).

### *Toward a comprehensive analysis of inter-species interactions*

Inter-species interactions, including mutualism and parasitism, are of general interest in microbial ecology (16). Using abundant information from large-scale metagenomic datasets, co-occurrences (or anti-occurrences) among microbes, hosts, and/or viruses have been studied, and, for example, species interaction networks have been identified (12, 23, 28, 46, 53, 94, 114, 155, 164, 184). Recent large-scale projects include the *Tara* Oceans project, which revealed interactions among all three domains and viruses (17). Since environmental samples were revealed to contain *environmental DNA* shed from large organisms in addition to microbial DNA (107), a combinatorial analysis of microbial and environmental DNA is expected to accelerate the analysis of interactions between microbes and larger organisms.

The viral metagenome is called the *metavirome* or simply the *virome*. Viruses also play fundamental roles in ecosystems; therefore, a virome analysis is becoming an important field. To date, viral communities in hypersaline (143, 144) and human gut (105) environments have been extensively studied, and antibiotic viruses have also been of interest (108). A novel bacteriophage present in the majority of published human fecal metagenomes was recently reconstructed (39), and phage-bacteria ecological networks were suggested to protect gut microflora from antibiotic stress (108). Since viruses are classified into different types of DNA and RNA viruses, different approaches must be combined for comprehensive analyses (169). The use of targeted sequence capture techniques to efficiently increase the proportion of viral reads in metagenomic samples may also be considered (179). The largest limitation in bioinformatic analyses of viromes is insufficient reference genome data. Similarity searches using viral sequences often result in no significant hits, suggesting that there are many unknown viruses. In order to overcome this limitation, several bioinformatic tools have been developed and used for virome studies, such as ViromeScan (137) for taxonomy assignment and Metavir 2 (141) for viral genome reconstruction. Another difficulty is that in contrast to prokaryotes that have universal marker genes for a phylogenetic analysis (*i.e.*, 16S rRNA), there is no such gene for viral studies. An analysis of clustered regularly interspaced short palindromic repeats (CRISPRs) is a related emerging field because these repeats represent previous exposures to (or attacks from) viruses (15, 109, 131). CRISPRs are found in approximately 40% of bacteria and approximately 90% of archaea (59), and, thus, a metagenomic analysis of CRISPRs will contribute to advancing the field toward a comprehensive analysis of viral-microbial interactions.

### *Toward a meta-analysis of metagenomes*

Abundant metagenomic datasets containing dozens of terabytes of sequence data are currently found in the Short Read Archive database at NCBI, and its content is increasing daily (84). Whereas each metagenomic dataset provides a snapshot of the microbial community at the time of sampling, a comprehensive analysis (or *meta-analysis*) of many datasets is expected to reveal general patterns or laws that determine how microbes interact with their environments and how their genomes have been shaped. It is important to note that different datasets have been constructed with different experimental methods and conditions.

Regarding global correlations between environments and microbial genomes, correlations involving genomic GC contents (66) and genome sizes (14) have been reported. MetaMetaDB (181) was developed for a meta-analysis of different environments inhabited by a microbe and the factors that contribute to adaptation. This database may be used to predict all possible habitats of microbes by searching for the presence of microbes in metagenomic and 16S rRNA amplicon sequencing datasets derived from diverse environments. Given a metagenomic or 16S rRNA amplicon sequencing dataset, researchers may find environments with microbial community structures that are similar to that dataset using MetaMetaDB (181). A meta-analysis of metagenomic datasets was also performed to examine microbial adaptation to environments in terms of metabolic flexibility (52, 60) and to investigate specific functional genes that facilitate adaptation to extreme habitats, such as heavy metal resistance genes (65, 106) and salt-stress responsive genes (166). In a meta-analysis, associations were found between membrane protein variations and oceanographic variables in a global ocean sampling expedition (126). Microbial interactions between humans and the indoor environment have also been investigated (91).

### *Toward metagenomics with long-read sequencers*

Sequencers with the ability to produce long-read data are currently being developed, such as the PacBio RS II (Pacific Biosciences, Menlo Park, USA) and nanopore-based sequencers (Oxford Nanopore Technologies, Oxford, UK). Long reads are already contributing to many types of bioinformatic analyses, including the high-quality *de novo* assembly of bacterial and viral genomes (7, 67) and the detection of genomic structural variations, such as large-scale insertions/deletions or HGTs in microbial communities (71). Long reads are expected to be helpful for reconstructing genomes from metagenome data, directly observing genomic structural variations, and analyzing metaregulomes in various microbial communities. High-density microbial habitats, such as biofilms and gut communities, may be interesting targets because their genomic structures may be changed by the frequent exchange of genetic materials.

Long-read metagenomics will be an emerging field, but there are still limitations to be considered. Although PacBio RS II with P6-C4 chemistry may generate reads with an average length of approximately 15 kb, less than 50,000 reads are generated per SMART cell (*i.e.*, less than 1 Gb in each SMART cell). This throughput is markedly smaller than that of the so-called massively parallel sequencers (*e.g.*, approximately 15 Gb in each run of MiSeq [Illumina]) and may be insufficient for describing taxonomically diverse microbial communities. In addition, the low accuracy of PacBio RS II reads (approximately 85%) may hinder a bioinformatic analysis, unless highly redundant sequencing (*e.g.*, more than 50X coverage) is performed to reach high accuracy in the ensemble. Along with the development of new bioinformatic methods, protocols also need to be optimized to avoid DNA fragmentation during extraction (83, 111).

### *Concluding remarks*

Metagenomic and bioinformatic approaches are already common in microbial ecology and have been used to investigate whole communities containing many types of uncultivable microbes (Fig. 1). However, to date, most analyses have depended on straightforward sequence similarity searches against reference databases. This may not be satisfactory because microbial genomes need to be the fundamental basis for microbial ecology and evolution. The enrichment of reference sequences (for microbial taxa and functional genes) is one of the fundamental issues for promoting various kinds of analyses. Platforms that enable a meta-analysis of diverse metagenomic datasets will allow us to discover the hidden laws of the microbial ecosystem from publicly available data. Long-read sequence information will open up the possibility of studies that focus on subjects that have not yet been examined in detail by using short-read sequences. Furthermore, more powerful bioinformatic methods for analyzing data from diverse perspectives are required in order to advance past routine metagenomic analyses.

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**References**

1. Abubucker, S., N. Segata, J. Goll, *et al.* 2012. Metabolic reconstruction for metagenomic data and its application to the human microbiome. PLoS Comput. Biol. 8:e1002358.
2. Afiahayati, K. Sato, and Y. Sakakibara. 2015. MetaVelvet-SL: an extension of the Velvet assembler to a *de novo* metagenomic assembler utilizing supervised learning. DNA Res. 22:69–77.
3. Akkermans, A.D.L., M.S. Mirza, H.J.M. Harmsen, H.J. Blok, P.R. Herron, A. Sessitsch, and W.M. Akkermans. 1994. Molecular ecology of microbes: a review of promises, pitfalls, and true progress. FEMS Microbiol. Rev. 15:185–194.
4. Albertsen, M., P. Hugenholtz, A. Skarshewski, K.L. Nielsen, G.W. Tyson, and P.H. Nielsen. 2013. Genome sequences of rare, uncultured bacteria obtained by differential coverage binning of multiple metagenomes. Nat. Biotechnol. 31:533–538.
5. Alneberg, J., B.S. Bjarnason, I. de Bruijn, *et al.* 2014. Binning metagenomic contigs by coverage and composition. Nat. Methods 11:1144–1146.
6. Alonso-Alemany, D., A. Barré, S. Beretta, P. Bonizzoni, M. Nikolski, and G. Valiente. 2014. Further steps in TANGO: improved taxonomic assignment in metagenomics. Bioinformatics. 30:17–23.
7. Alquezar-Planas, D.E., T. Mourier, C.A.W. Bruhn, *et al.* 2013. Discovery of a divergent HPIV4 from respiratory secretions using second and third generation metagenomic sequencing. Sci. Rep. 3:2468.
8. Banik, J.J., and S.F. Brady. 2008. Cloning and characterization of new glycopeptide gene clusters found in an environmental DNA megalibrary. Proc. Natl. Acad. Sci. U.S.A. 105:17273–17277.
9. Béjà, O., M.T. Suzuki, E.V. Koonin, *et al.* 2000. Construction and analysis of bacterial artificial chromosome libraries from a marine microbial assemblage. Environ. Microbiol. 2:516–529.
10. Belda-Ferre, P., L.D. Alcaraz, R. Cabrera-Rubio, H. Romero, A. Simón-Soro, M. Pignatelli, and A. Mira. 2012. The oral metagenome in health and disease. ISME J. 6:46–56.
11. Belton, J.M., R.P. McCord, J.H. Gibcus, N. Naumova, Y. Zhan, and J. Dekker. 2012. Hi-C: a comprehensive technique to capture the conformation of genomes. Methods 58:268–276.

12. Beman, J.M., J.A. Steele, and J.A. Fuhrman. 2011. Co-occurrence patterns for abundant marine archaeal and bacterial lineages in the deep chlorophyll maximum of coastal California. ISME J. 5:1077–1085.
13. Bennett, P.M. 2008. Plasmid encoded antibiotic resistance: acquisition and transfer of antibiotic resistance genes in bacteria. Br. J. Pharmacol. 153:S347–S357.
14. Bentkowski, P., C. Van Oosterhout, and T. Mock. 2015. A model of genome size evolution for prokaryotes in stable and fluctuating environments. Genome Biol. Evol. 7:2344–2351.
15. Bolotin, A., B. Quinquis, A. Sorokin, and S.D. Ehrlich. 2005. Clustered regularly interspaced short palindrome repeats (CRISPRs) have spacers of extrachromosomal origin. Microbiology 151:2551–2561.
16. Boon, E., C.J. Meehan, C. Whidden, D.H.J. Wong, M.G.I. Langille, and R.G. Beiko. 2014. Interactions in the microbiome: communities of organisms and communities of genes. FEMS Microbiol. Rev. 38:90–118.
17. Bork, P., C. Bowler, C. de Vargas, G. Gorsky, E. Karsenti, and P. Wincker. 2015. Tara Oceans studies plankton at planetary scale. Science 348:873.
18. Brown, C.T., L.A. Hug, B.C. Thomas, *et al.* 2015. Unusual biology across a group comprising more than 15% of domain Bacteria. Nature 523:208–211.
19. Brown Kav, A., G. Sasson, E. Jami, A. Doron-Faigenboim, I. Benhar, and I. Mizrahi. 2012. Insights into the bovine rumen plasmidome. Proc. Natl. Acad. Sci. U.S.A. 109:5452–5457.
20. Burton, J.N., I. Liachko, M.J. Dunham, and J. Shendure. 2014. Species-level deconvolution of metagenome assemblies with Hi-C-based contact probability maps. G3 4:1339–1346.
21. Caporaso, J.G., C.L. Lauber, E.K. Costello, *et al.* 2011. Moving pictures of the human microbiome. Genome Biol. 12:R50.
22. Caspi, R., T. Altman, R. Billington, *et al.* 2014. The MetaCyc database of metabolic pathways and enzymes and the BioCyc collection of Pathway/Genome Databases. Nucleic Acids Res. 42:D459–D471.
23. Chaffron, S., H. Rehrauer, J. Pernthaler, and C. Mering. 2010. A global network of coexisting microbes from environmental and whole-genome sequence data. Genome Res. 2010:947–959.
24. Chaillou, S., A. Chaulot-Talmon, H. Caekebeke, *et al.* 2015. Origin and ecological selection of core and food-specific bacterial communities associated with meat and seafood spoilage. ISME J. 9:1105–1118.
25. Chen, H.M., and C.H. Lifschitz. 1989. Preparation of fecal samples for assay of volatile fatty acids by gas-liquid chromatography and high-performance liquid chromatography. Clin. Chem. 35:74–76.
26. Chen, J., and R.P. Novick. 2009. Transfer of toxin genes. Science 323:139–141.
27. Chen, Y., L. Wu, R. Boden, A. Hillebrand, D. Kumaresan, H. Moussard, M. Baciu, Y. Lu, and J.C. Murrell. 2009. Life without light: microbial diversity and evidence of sulfur- and ammonium-based chemolithotrophy in Movile Cave. ISME J. 3:1093–1104.
28. Chow, C.-E.T., D.Y. Kim, R. Sachdeva, D.A. Caron, and J.A. Fuhrman. 2014. Top-down controls on bacterial community structure: microbial network analysis of bacteria, T4-like viruses and protists. ISME J. 8:816–829.
29. Chun, J., J.-H. Lee, Y. Jung, M. Kim, S. Kim, B.K. Kim, and Y.-W. Lim. 2007. EzTaxon: a web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences. Int. J. Syst. Evol. Microbiol. 57:2259–2261.
30. Cleary, B., I.L. Brito, K. Huang, D. Gevers, T. Shea, S. Young, and E.J. Alm. 2015. Detection of low-abundance bacterial strains in metagenomic datasets by eigengenome partitioning. Nat. Biotechnol. 33:1053–1060.
31. Cole, J.R., Q. Wang, J.A. Fish, *et al.* 2014. Ribosomal Database Project: data and tools for high throughput rRNA analysis. Nucleic Acids Res. 42:D633–D642.
32. Coughlan, L.M., P.D. Cotter, C. Hill, and A. Alvarez-Ordóñez. 2015. Biotechnological applications of functional metagenomics in the food and pharmaceutical industries. Front. Microbiol. 6:672.
33. Cowan, D.A., J.B. Ramond, T.P. Makhalanyane, and P. De Maayer. 2015. Metagenomics of extreme environments. Curr. Opin. Microbiol. 25:97–102.
34. Culligan, E.P., J.R. Marchesi, C. Hill, and R.D. Sleator. 2012. Mining the human gut microbiome for novel stress resistance genes. Gut Microbes 3:394–397.
35. Darling, A.E., G. Jospin, E. Lowe, F.A. Matsen, H.M. Bik, and J.A. Eisen. 2014. PhyloSift: phylogenetic analysis of genomes and metagenomes. PeerJ 2:e243.
36. Di Bella, J.M., Y. Bao, G.B. Gloor, J.P. Burton, and G. Reid. 2013. High throughput sequencing methods and analysis for microbiome research. J. Microbiol. Methods 95:401–414.
37. Dib, J.R., M. Wagenknecht, M.E. Farías, and F. Meinhardt. 2015. Strategies and approaches in plasmidome studies-uncovering plasmid diversity disregarding of linear elements? Front. Microbiol. 6:463.
38. Dostie, J., and J. Dekker. 2007. Mapping networks of physical interactions between genomic elements using 5C technology. Nat. Protoc. 2:988–1002.
39. Dutilh, B.E., N. Cassman, K. McNair, *et al.* 2014. A highly abundant bacteriophage discovered in the unknown sequences of human faecal metagenomes. Nat. Commun. 5:4498.
40. Edgar, R.C. 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26:2460–2461.
41. Edgar, R.C., B.J. Haas, J.C. Clemente, C. Quince, and R. Knight. 2011. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27:2194–2200.
42. Edgar, R.C. 2013. UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nat. Methods 10:996–998.
43. Eloe-Fadrosh, E.A., D. Paez-Espino, J. Jarett, *et al.* 2016. Global metagenomic survey reveals a new bacterial candidate phylum in geothermal springs. Nat. Commun. 7:10476.
44. Erickson, A.R., B.L. Cantarel, R. Lamendella, *et al.* 2012. Integrated metagenomics/metaproteomics reveals human host-microbiota signatures of Crohn's disease. PLoS One 7:e49138.
45. Evans, P.N., D.H. Parks, G.L. Chadwick, S.J. Robbins, V.J. Orphan, S.D. Golding, and G.W. Tyson. 2015. Methane metabolism in the archaeal phylum Bathyarchaeota revealed by genome-centric metagenomics. Science 350:434–438.
46. Faust, K., and J. Raes. 2012. Microbial interactions: from networks to models. Nat. Rev. Microbiol. 10:538–550.
47. Felczykowska, A., S.K. Bloch, B. Nejman-Faleńczyk, and S. Barańska. 2012. Metagenomic approach in the investigation of new bioactive compounds in the marine environment. Acta Biochim. Pol. 59:501–505.
48. Fernandez, L., J.M. Mercader, M. Planas-Fèlix, and D. Torrents. 2014. Adaptation to environmental factors shapes the organization of regulatory regions in microbial communities. BMC Genomics 15:877.
49. Fierer, N., C.C.L. Lauber, N. Zhou, D. McDonald, E.K. Costello, and R. Knight. 2010. Forensic identification using skin bacterial communities. Proc. Natl. Acad. Sci. U.S.A. 107:6477–6481.
50. Fu, L., B. Niu, Z. Zhu, S. Wu, and W. Li. 2012. CD-HIT: accelerated for clustering the next-generation sequencing data. Bioinformatics 28:3150–3152.
51. Gatte-Picchi, D., A. Weiz, K. Ishida, C. Hertweck, and E. Dittmann. 2014. Functional analysis of environmental DNA-derived microviridins provides new insights into the diversity of the tricyclic peptide family. Appl. Environ. Microbiol. 80:1380–1387.
52. Gianoulis, T.A., J. Raes, P.V. Patel, *et al.* 2009. Quantifying environmental adaptation of metabolic pathways in metagenomics. Proc. Natl. Acad. Sci. U.S.A. 106:1374–1379.
53. Gilbert, J.A. 2012. Defining seasonal marine microbial community dynamics. ISME J. 6:298–308.
54. Gilbert, J.A., J.K. Jansson, and R. Knight. 2014. The Earth Microbiome project: successes and aspirations. BMC Biol. 12:69.
55. Gill, S.R., M. Pop, R.T. Deboy, *et al.* 2006. Metagenomic analysis of the human distal gut microbiome. Science 312:1355–1359.
56. Gillespie, D.E., S.F. Brady, A.D. Bettermann, N.P. Cianciotto, M.R. Liles, M.R. Rondon, J. Clardy, R.M. Goodman, and J. Handelsman. 2002. Isolation of antibiotics turbomycin A and B from a metagenomic library of soil microbial DNA. Appl. Environ. Microbiol. 68:4301–4306.
57. Greenblum, S., P.J. Turnbaugh, and E. Borenstein. 2012. Metagenomic systems biology of the human gut microbiome reveals topological shifts associated with obesity and inflammatory bowel disease. Proc. Natl. Acad. Sci. U.S.A. 109:594–599.
58. Grigoriev, I.V., R. Nikitin, S. Haridas, *et al.* 2014. MycoCosm portal: gearing up for 1000 fungal genomes. Nucleic Acids Res. 42:D699–D704.

59. Grissa, I., G. Vergnaud, and C. Pourcel. 2007. The CRISPRdb database and tools to display CRISPRs and to generate dictionaries of spacers and repeats. BMC Bioinformatics 8:172.
60. Grzymski, J.J., A.E. Murray, B.J. Campbell, *et al.* 2008. Metagenome analysis of an extreme microbial symbiosis reveals eurythermal adaptation and metabolic flexibility. Proc. Natl. Acad. Sci. U.S.A. 105:17516–17521.
61. Guazzaroni, M.E., V. Morgante, S. Mirete, and J.E. González-Pastor. 2013. Novel acid resistance genes from the metagenome of the Tinto River, an extremely acidic environment. Environ. Microbiol. 15:1088–1102.
62. Guo, J., Q. Wang, X. Wang, F. Wang, J. Yao, and H. Zhu. 2015. Horizontal gene transfer in an acid mine drainage microbial community. BMC Genomics 16:496.
63. Haas, B.J., D. Gevers, A.M. Earl, *et al.* 2011. Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons. Genome Res. 21:494–504.
64. Hastie, A.R., L. Dong, A. Smith, *et al.* 2013. Rapid genome mapping in nanochannel arrays for highly complete and accurate *de novo* sequence assembly of the complex *Aegilops tauschii* Genome. PLoS One 8:e55864.
65. Hemme, C.L., Y. Deng, T.J. Gentry, *et al.* 2010. Metagenomic insights into evolution of a heavy metal-contaminated groundwater microbial community. ISME J. 4:660–672.
66. Hildebrand, F., A. Meyer, and A. Eyre-walker. 2010. Evidence of selection on genomic GC content in bacteria. PLoS Genet. 6:e1001107.
67. Hiraoka, S., A. Machiyama, M. Ijichi, K. Inoue, K. Oshima, M. Hattori, S. Yoshizawa, K. Kogure, and W. Iwasaki. 2016. Genomic and metagenomic analysis of microbes in a soil environment affected by the 2011 Great East Japan Earthquake tsunami. BMC Genomics 17:53.
68. Hongoh, Y. 2010. Diversity and genomes of uncultured microbial symbionts in the termite gut. Biosci. Biotechnol. Biochem. 74:1145–1151.
69. Hunter, S., M. Corbett, H. Denise, *et al.* 2014. EBI metagenomics—a new resource for the analysis and archiving of metagenomic data. Nucleic Acids Res. 42:D600–D606.
70. Huson, D.H., and N. Weber. 2013. Microbial community analysis using MEGAN. Methods Enzymol. 531:465–485.
71. Ikuta, T., Y. Takaki, Y. Nagai, *et al.* 2016. Heterogeneous composition of key metabolic gene clusters in a vent mussel symbiont population. ISME J. 10:990–1001.
72. Iwasaki, W., and T. Takagi. 2007. Reconstruction of highly heterogeneous gene-content evolution across the three domains of life. Bioinformatics 23:i230–i239.
73. Iwasaki, W., and T. Takagi. 2009. Rapid pathway evolution facilitated by horizontal gene transfers across prokaryotic lineages. PLoS Genet. 5:e1000402.
74. Jackson, S.A., E. Borchert, F. O'Gara, and A.D.W. Dobson. 2015. Metagenomics for the discovery of novel biosurfactants of environmental interest from marine ecosystems. Curr. Opin. Biotechnol. 33:176–182.
75. Jiao, D., Y. Ye, and H. Tang. 2013. Probabilistic inference of biochemical reactions in microbial communities from metagenomic sequences. PLoS Comput. Biol. 9:e1002981.
76. Jørgensen, T.S., Z. Xu, M.A. Hansen, S.J. Sørensen, and L.H. Hansen. 2014. Hundreds of circular novel plasmids and DNA elements identified in a rat cecum metamobilome. PLoS One 9:e87924.
77. Jung, J.Y., S.H. Lee, J.M. Kim, M.S. Park, J.W. Bae, Y. Hahn, E.L. Madsen, and C.O. Jeon. 2011. Metagenomic analysis of kimchi, a Traditional Korean fermented food. Appl. Environ. Microbiol. 77:2264–2274.
78. Kagan, J., I. Sharon, O. Beja, and J.C. Kuhn. 2008. The tryptophan pathway genes of the Sargasso Sea metagenome: new operon structures and the prevalence of non-operon organization. Genome Biol. 9:R20.
79. Kakizaki, E., Y. Ogura, S. Kozawa, S. Nishida, T. Uchiyama, T. Hayashi, and N. Yukawa. 2012. Detection of diverse aquatic microbes in blood and organs of drowning victims: first metagenomic approach using high-throughput 454-pyrosequencing. Forensic Sci. Int. 220:135–146.
80. Kanehisa, M., S. Goto, Y. Sato, M. Furumichi, and M. Tanabe. 2012. KEGG for integration and interpretation of large-scale molecular data sets. Nucleic Acids Res. 40:D109–D114.
81. Kang, D.D., J. Froula, R. Egan, and Z. Wang. 2015. MetaBAT, an efficient tool for accurately reconstructing single genomes from complex microbial communities. PeerJ 3:e1165.
82. Khodakova, A.S., R.J. Smith, L. Burgoyne, D. Abarno, and A. Linacre. 2014. Random whole metagenomic sequencing for forensic discrimination of soils. PLoS One 9:e104996.
83. Kim, S.W., W. Suda, S. Kim, K. Oshima, S. Fukuda, H. Ohno, H. Morita, and M. Hattori. 2013. Robustness of gut microbiota of healthy adults in response to probiotic intervention revealed by high-throughput pyrosequencing. DNA Res. 20:241–253.
84. Kodama, Y., M. Shumway, and R. Leinonen. 2012. The sequence read archive: explosive growth of sequencing data. Nucleic Acids Res. 40:D54–D56.
85. Kong, H.H., J. Oh, C. Deming, *et al.* 2012. Temporal shifts in the skin microbiome associated with disease flares and treatment in children with atopic dermatitis. Genome Res. 22:850–859.
86. Korem, T., D. Zeevi, J. Suez, *et al.* 2015. Growth dynamics of gut microbiota in health and disease inferred from single metagenomic samples. Science 349:1101–1106.
87. Kostic, A.D., D. Gevers, C.S. Pedamallu, *et al.* 2012. Genomic analysis identifies association of *Fusobacterium* with colorectal carcinoma. Genome Res. 22:292–298.
88. Kunin, V., A. Copeland, A. Lapidus, K. Mavromatis, and P. Hugenholtz. 2008. A bioinformatician's guide to metagenomics. Microbiol. Mol. Biol. Rev. 72:557–578.
89. Langille, M.G., J. Zaneveld, J.G. Caporaso, *et al.* 2013. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nat. Biotechnol. 31:814–821.
90. Lasken, R.S. 2012. Genomic sequencing of uncultured microorganisms from single cells. Nat. Rev. Microbiol. 10:631–640.
91. Lax, S., D.P. Smith, J. Hampton-Marcell, *et al.* 2014. Longitudinal analysis of microbial interaction between humans and the indoor environment. Science 345:1048–1052.
92. Lewin, A., A. Wentzel, and S. Valla. 2013. Metagenomics of microbial life in extreme temperature environments. Curr. Opin. Biotechnol. 24:516–525.
93. Li, R.W. 2011. Metagenomics and Its Applications in Agriculture, Biomedicine, and Environmental Studies. Nova Science Publisher's.
94. Lozupone, C., K. Faust, J. Raes, J.J. Faith, D.N. Frank, J. Zaneveld, J.I. Gordon, and R. Knight. 2012. Identifying genomic and metabolic features that can underlie early successional and opportunistic lifestyles of human gut symbionts. Genome Res. 22:1974–1984.
95. Luo, C., D. Tsementzi, N.C. Kyrpides, and K.T. Konstantinidis. 2012. Individual genome assembly from complex community short-read metagenomic datasets. ISME J. 6:898–901.
96. Manichanh, C., L. Rigottier-Gois, E. Bonnaud, *et al.* 2006. Reduced diversity of faecal microbiota in Crohn's disease revealed by a metagenomic approach. Gut 55:205–211.
97. Markowitz, V.M., I.M.A. Chen, K. Chu, *et al.* 2014. IMG/M 4 version of the integrated metagenome comparative analysis system. Nucleic Acids Res. 42:D568–D573.
98. Matsen, F.A., R.B. Kodner, and E.V. Armbrust. 2010. pplacer: linear time maximum-likelihood and Bayesian phylogenetic placement of sequences onto a fixed reference tree. BMC Bioinformatics 11:538.
99. Matus-Garcia, M., H. Nijveen, and M.W.J. Van Passel. 2012. Promoter propagation in prokaryotes. Nucleic Acids Res. 40:10032–10040.
100. Mavromatis, K., N. Ivanova, K.W. Barry, *et al.* 2007. Use of simulated data sets to evaluate the fidelity of metagenomic processing methods. Nat. Methods 4:495–500.
101. McDonald, D., M.N. Price, J. Goodrich, E.P. Nawrocki, T.Z. DeSantis, A. Probst, G.L. Andersen, R. Knight, and P. Hugenholtz. 2012. An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. ISME J. 6:610–618.
102. Mende, D.R., F.O. Aylward, J.M. Eppley, T.N. Nielsen, and E.F. DeLong. 2016. Improved environmental genomes via integration of metagenomic and single-cell assemblies. Front. Microbiol. 7:143.
103. Mendoza, M.L.Z., T. Sicheritz-Pontén, and M.P.T. Gilbert. 2014. Environmental genes and genomes: understanding the differences and challenges in the approaches and software for their analyses. Brief. Bioinform. 16:745–758.
104. Meyer, F., D. Paarmann, M. D'Souza, *et al.* 2008. The metagenomics RAST server—a public resource for the automatic phylogenetic and functional analysis of metagenomes. BMC Bioinformatics 9:386.

105. Minot, S., R. Sinha, J. Chen, H. Li, S.A. Keilbaugh, G.D. Wu, J.D. Lewis, and F.D. Bushman. 2011. The human gut virome: inter-individual variation and dynamic response to diet. Genome Res. 21:1616–1625.
106. Mirete, S., C.G. De Figueras, and J.E. González-Pastor. 2007. Novel nickel resistance genes from the rhizosphere metagenome of plants adapted to acid mine drainage. Appl. Environ. Microbiol. 73:6001–6011.
107. Miya, M., Y. Sato, T. Fukunaga, *et al.* 2015. MiFish, a set of universal PCR primers for metabarcoding environmental DNA from fishes: detection of more than 230 subtropical marine species. R. Soc. Open Sci. 2:150088.
108. Modi, S.R., H.H. Lee, C.S. Spina, and J.J. Collins. 2013. Antibiotic treatment expands the resistance reservoir and ecological network of the phage metagenome. Nature 499:219–222.
109. Mojica, F.J.M., C. Díez-Villaseñor, J. García-Martínez, and E. Soria. 2005. Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements. J. Mol. Evol. 60:174–182.
110. Mori, K., and Y. Kamagata. 2014. The Challenges of studying the anaerobic microbial world. Microbes Environ. 29:335–337.
111. Morita, H., T. Kuwahara, K. Ohshima, H. Sasamoto, K. Itoh, M. Hattori, T. Hayashi, and H. Takami. 2007. An improved DNA isolation method for metagenomic analysis of the microbial flora of the human intestine. Microbes Environ. 22:214–222.
112. Narasingarao, P., S. Podell, J.A. Ugalde, C. Brochier-Armanet, J.B. Emerson, J.J. Brocks, K.B. Heidelberg, J.F. Banfield, and E.E. Allen. 2012. *De novo* metagenomic assembly reveals abundant novel major lineage of Archaea in hypersaline microbial communities. ISME J. 6:81–93.
113. Narihiro, T., and Y. Kamagata. 2013. Cultivating yet-to-be cultivated microbes: the challenge continues. Microbes Environ. 28:163–165.
114. Navarrete, A.A., S.M. Tsai, L.W. Mendes, K. Faust, M. De Hollander, N.A. Cassman, J. Raes, J.A. Van Veen, and E.E. Kuramae. 2015. Soil microbiome responses to the short-term effects of Amazonian deforestation. Mol. Ecol. 24:2433–2448.
115. Neelakanta, G., and H. Sultana. 2013. The use of metagenomic approaches to analyze changes in microbial communities. Microbiol. Insights 6:37–48.
116. Nelson, K.E., J.L. Peterson, and S. Garges. 2011. Metagenomics of the human body, p. 1–351. *In* K.E. Nelson (ed.), Metagenomics of the Human Body. Springer.
117. Nielsen, H.B., M. Almeida, A.S. Juncker, *et al.* 2014. Identification and assembly of genomes and genetic elements in complex metagenomic samples without using reference genomes. Nat. Biotechnol. 32:822–828.
118. Nijkamp, J.F., M. Pop, M.J.T. Reinders, and D. De Ridder. 2013. Exploring variation-aware contig graphs for (comparative) metagenomics using MARYGOLD. Bioinformatics 29:2826–2834.
119. Nurk, S., A. Bankevich, D. Antipov, *et al.* 2013. Assembling single-cell genomes and mini-metagenomes from chimeric MDA products. J. Comput. Biol. 20:714–737.
120. Ochman, H., J.G. Lawrence, and E.A. Groisman. 2000. Lateral gene transfer and the nature of bacterial innovation. Nature 405:299–304.
121. Okuda, S., Y. Tsuchiya, C. Kiriyama, M. Itoh, and H. Morisaki. 2012. Virtual metagenome reconstruction from 16S rRNA gene sequences. Nat. Commun. 3:1203.
122. Oren, Y., M.B. Smith, N.I. Johns, *et al.* 2014. Transfer of noncoding DNA drives regulatory rewiring in bacteria. Proc. Natl. Acad. Sci. U.S.A. 111:16112–16117.
123. Oulas, A., C. Pavloudi, P. Polymenakou, G.A. Pavlopoulos, N. Papanikolaou, G. Kotoulas, C. Arvanitidis, and I. Iliopoulos. 2015. Metagenomics: tools and insights for analyzing next-generation sequencing data derived from biodiversity studies. Bioinform. Biol. Insights 9:75–88.
124. Overbeek, R., T. Begley, R.M. Butler, *et al.* 2005. The subsystems approach to genome annotation and its use in the project to annotate 1000 genomes. Nucleic Acids Res. 33:5691–5702.
125. Palenik, B., Q. Ren, V. Tai, and I.T. Paulsen. 2009. Coastal *Synechococcus* metagenome reveals major roles for horizontal gene transfer and plasmids in population diversity. Environ. Microbiol. 11:349–359.
126. Patel, P.V., T.A. Gianoulis, R.D. Bjornson, K.Y. Yip, D.M. Engelman, and M.B. Gerstein. 2010. Analysis of membrane proteins in metagenomics: networks of correlated environmental features and protein families. Genome Res. 20:960–971.
127. Peng, Y., H.C.M. Leung, S.M. Yiu, and F.Y.L. Chin. 2012. IDBA-UD: A *de novo* assembler for single-cell and metagenomic sequencing data with highly uneven depth. Bioinformatics 28:1420–1428.
128. Pignatelli, M., G. Aparicio, I. Blanquer, V. Hernández, A. Moya, and J. Tamames. 2008. Metagenomics reveals our incomplete knowledge of global diversity. Bioinformatics 24:2124–2125.
129. Pignatelli, M., and A. Moya. 2011. Evaluating the fidelity of *de novo* short read metagenomic assembly using simulated data. PLoS One 6:e19984.
130. Pop, M., A. Phillippy, and A.L. Delcher. 2004. Comparative genome assembly. Bioinformatics 5:237–248.
131. Pourcel, C., G. Salvignol, and G. Vergnaud. 2005. CRISPR elements in Yersinia pestis acquire new repeats by preferential uptake of bacteriophage DNA, and provide additional tools for evolutionary studies. Microbiology 151:653–663.
132. Prosser, J.I. 2015. Dispersing misconceptions and identifying opportunities for the use of "omics" in soil microbial ecology. Nat. Rev. Microbiol. 13:439.
133. Puspita, I.D., Y. Kamagata, M. Tanaka, K. Asano, and C.H. Nakatsu. 2012. Are uncultivated bacteria really uncultivable? Microbes Environ. 27:356–366.
134. Qin, J., Y. Li, Z. Cai, *et al.* 2012. A metagenome-wide association study of gut microbiota in type 2 diabetes. Nature 490:55–60.
135. Quast, C., E. Pruesse, P. Yilmaz, J. Gerken, T. Schweer, P. Yarza, J. Peplies, and F.O. Glöckner. 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 41:D590–D596.
136. Quince, C., A. Lanzen, R.J. Davenport, and P.J. Turnbaugh. 2011. Removing noise from pyrosequenced amplicons. BMC Bioinformatics 12:38.
137. Rampelli, S., M. Soverini, S. Turroni, S. Quercia, E. Biagi, P. Brigidi, and M. Candela. 2016. ViromeScan: a new tool for metagenomic viral community profiling. BMC Genomics 17:165.
138. Rappé, M.S., and S.J. Giovannoni. 2003. The uncultured microbial majority. Annu. Rev. Microbiol. 57:369–394.
139. Roberts, A.P., and J. Kreth. 2014. The impact of horizontal gene transfer on the adaptive ability of the human oral microbiome. Front Cell Infect Microbiol. 4:124.
140. Rodrigue, S., R.R. Malmstrom, A.M. Berlin, B.W. Birren, M.R. Henn, and S.W. Chisholm. 2009. Whole genome amplification and de novo assembly of single bacterial cells. PLoS One 4:e6864.
141. Roux, S., J. Tournayre, A. Mahul, D. Debroas, and F. Enault. 2014. Metavir 2: new tools for viral metagenome comparison and assembled virome analysis. BMC Bioinformatics 15:76.
142. Rusch, D.B., A.L. Halpern, G. Sutton, *et al.* 2007. The sorcerer II global ocean sampling expedition: northwest atlantic through eastern tropical pacific. PLoS Biol. 5:0398–0431.
143. Santos, F., P. Yarza, V. Parro, C. Briones, and J. Antón. 2010. The metavirome of a hypersaline environment. Environ. Microbiol. 12:2965–2976.
144. Santos, F., P. Yarza, V. Parro, I. Meseguer, R. Rosselló-Móra, and J. Antón. 2012. Culture-independent approaches for studying viruses from hypersaline environments. Appl. Environ. Microbiol. 78:1635–1643.
145. Schloss, P.D., S.L. Westcott, T. Ryabin, *et al.* 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl. Environ. Microbiol. 75:7537–7541.
146. Scholz, M.B., C.C. Lo, and P.S.G. Chain. 2012. Next generation sequencing and bioinformatic bottlenecks: the current state of metagenomic data analysis. Curr. Opin. Biotechnol. 23:9–15.
147. Schwabe, R.F., and C. Jobin. 2013. The microbiome and cancer. Nat. Rev. Cancer 13:800–812.
148. Segata, N., L. Waldron, A. Ballarini, V. Narasimhan, O. Jousson, and C. Huttenhower. 2012. Metagenomic microbial community profiling using unique clade-specific marker genes. Nat. Methods 9:811–814.
149. Sentchilo, V., A.P. Mayer, L. Guy, *et al.* 2013. Community-wide plasmid gene mobilization and selection. ISME J. 7:1173–1186.
150. Sharpton, T.J., S.J. Riesenfeld, S.W. Kembel, J. Ladau, J.P. O'Dwyer, J.L. Green, J.A. Eisen, and K.S. Pollard. 2011. PhyLOTU: a high-throughput procedure quantifies microbial community diversity and resolves novel taxa from metagenomic data. PLoS Comput. Biol. 7:e1001061.

151. Silver, S., and T.K. Misra. 1988. Plasmid-mediated heavy metal resistances. Annu. Rev. Microbiol. 42:717–743.
152. Smalla, K., and P.A. Sobecky. 2002. The prevalence and diversity of mobile genetic elements in bacterial communities of different environmental habitats: insights gained from different methodological approaches. FEMS Microbiol. Ecol. 42:165–175.
153. Smillie, C.S., M.B. Smith, J. Friedman, O.X. Cordero, L.A. David, and E.J. Alm. 2011. Ecology drives a global network of gene exchange connecting the human microbiome. Nature 480:241–244.
154. Snel, B., P. Bork, and M.A. Huynen. 2002. Genomes in flux: the evolution of Archaeal and Proteobacterial gene content. Genome Res. 12:17–25.
155. Soffer, N., J. Zaneveld, and R.V. Thurber. 2015. Phage-bacteria network analysis and its implication for the understanding of coral disease. Environ. Microbiol. 17:1203–1218.
156. Soucy, S.M., J. Huang, and J.P. Gogarten. 2015. Horizontal gene transfer: building the web of life. Nat. Rev. Genet. 16:472–482.
157. Su, C., L. Lei, Y. Duan, K.Q. Zhang, and J. Yang. 2012. Culture-independent methods for studying environmental microorganisms: methods, application, and perspective. Appl. Microbiol. Biotechnol. 93:993–1003.
158. Suenaga, H., Y. Koyama, M. Miyakoshi, R. Miyazaki, H. Yano, M. Sota, Y. Ohtsubo, M. Tsuda, and K. Miyazaki. 2009. Novel organization of aromatic degradation pathway genes in a microbial community as revealed by metagenomic analysis. ISME J. 3:1335–1348.
159. Sunagawa, S., D.R. Mende, G. Zeller, *et al.* 2013. Metagenomic species profiling using universal phylogenetic marker genes. Nat. Methods 10:1196–1199.
160. Takami, H., T. Taniguchi, Y. Moriya, T. Kuwahara, M. Kanehisa, and S. Goto. 2012. Evaluation method for the potential functionome harbored in the genome and metagenome. BMC Genomics 13:699.
161. Tamames, J., and A. Moya. 2008. Estimating the extent of horizontal gene transfer in metagenomic sequences. BMC Genomics 9:136.
162. Tasse, L., J. Bercovici, S. Pizzut-Serin, *et al.* 2010. Functional metagenomics to mine the human gut microbiome for dietary fiber catabolic enzymes. Genome Res. 20:1605–1612.
163. Tatusova, T., S. Ciufo, B. Fedorov, K. O'Neill, and I. Tolstoy. 2014. RefSeq microbial genomes database: new representation and annotation strategy. Nucleic Acids Res. 42:D553–D559.
164. Toju, H., P.R. Guimarães, J.M. Olesen, and J.N. Thompson. 2014. Assembly of complex plant–fungus networks. Nat. Commun. 5:5273.
165. Top, E.M., W.E. Holben, and L.J. Forney. 1995. Characterization of diverse 2,4-dichlorophenoxyacetic acid-degradative plasmids isolated from soil by complementation. Appl. Environ. Microbiol. 61:1691–1698.
166. Trabelsi, D., A. Mengoni, M.E. Aouani, M. Bazzicalupo, and R. Mhamdi. 2010. Genetic diversity and salt tolerance of sinorhizobium populations from two tunisian soils. Ann. Microbiol. 60:541–547.
167. Turnbaugh, P.J., R.E. Ley, M. Hamady, C.M. Fraser-Liggett, R. Knight, and J.I. Gordon. 2007. Feature the human microbiome project. Nature 449:804–810.
168. Tyson, G.W., J. Chapman, P. Hugenholtz, *et al.* 2004. Community structure and metabolism through reconstruction of microbial genomes from the environment. Nature 428:37–43.
169. Urayama, S., Y. Yoshida-Takashima, M. Yoshida, Y. Tomaru, H. Moriyama, K. Takai, and T. Nunoura. 2015. A new fractionation and recovery method of viral genomes based on nucleic acid composition and structure using tandem column chromatography. Microbes Environ. 30:199–203.
170. Vázquez-Castellanos, J.F., R. García-López, V. Pérez-Brocal, M. Pignatelli, and A. Moya. 2014. Comparison of different assembly and annotation tools on analysis of simulated viral metagenomic communities in the gut. BMC Genomics 15:37.
171. Vogel, T.M., P. Simonet, J.K. Jansson, *et al.* 2009. TerraGenome: a consortium for the sequencing of a soil metagenome. Nat. Rev. Microbiol. 7:252.
172. Voigt, A.Y., P.I. Costea, J.R. Kultima, S.S. Li, G. Zeller, S. Sunagawa, and P. Bork. 2015. Temporal and technical variability of human gut metagenomes. Genome Biol. 16:73.
173. Walker, A. 2012. Welcome to the plasmidome. Nat. Rev. Microbiol. 467:379.
174. Warnecke, F., P. Luginbühl, N. Ivanova, *et al.* 2007. Metagenomic and functional analysis of hindgut microbiota of a wood-feeding higher termite. Nature 450:560–565.
175. Wichmann, F., N. Udikovic-Kolic, S. Andrew, and J. Handelsman. 2014. Diverse antibiotic resistance genes in dairy cow manure. MBio 5:e01017–13.
176. Wood, D.E., and S.L. Salzberg. 2014. Kraken: ultrafast metagenomic sequence classification using exact alignments. Genome Biol. 15:R46.
177. Woyke, T., H. Teeling, N.N. Ivanova, *et al.* 2006. Symbiosis insights through metagenomic analysis of a microbial consortium. Nature 443:950–955.
178. Wu, Y.-W., Y.-H. Tang, S.G. Tringe, B.A. Simmons, and S.W. Singer. 2014. MaxBin: an automated binning method to recover individual genomes from metagenomes using an expectation-maximization algorithm. Microbiome 2:26.
179. Wylie, T.N., K.M. Wylie, B.N. Herter, G.A. Storch, and T.N. Wylie. 2015. Enhanced virome sequencing using targeted sequence capture. Genome 25:1910–1920.
180. Yamazaki, Y., R. Akashi, Y. Banno, *et al.* 2010. NBRP databases: databases of biological resources in Japan. Nucleic Acids Res. 38:D26–D32.
181. Yang, C.C., and W. Iwasaki. 2014. MetaMetaDB: a database and analytic system for investigating microbial habitability. PLoS One 9:e87126.
182. Yang, Y., B. Xie, and J. Yan. 2014. Application of next-generation sequencing technology in forensic science. Genomics Proteomics Bioinformatics 12:190–197.
183. Ye, Y., and T.G. Doak. 2011. A parsimony approach to biological pathway reconstruction/inference for metagenomes. PLoS Comput. Biol. 5:e1000465.
184. Zhang, Y., C.Y. Lun, and S.K.W. Tsui. 2015. Metagenomics: a newway to illustrate the crosstalk between infectious diseases and host microbiome. Int. J. Mol. Sci. 16:26263–26279.
185. Zoetendal, E.G., E.E. Vaughan, and W.M. De Vos. 2006. A microbial world within us. Mol. Microbiol. 59:1639–1650.

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# *Candidatus* Methanogranum caenicola: a Novel Methanogen from the Anaerobic Digested Sludge, and Proposal of *Methanomassiliicoccaceae* fam. nov. and *Methanomassiliicoccales* ord. nov., for a Methanogenic Lineage of the Class *Thermoplasmata*

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The class *Thermoplasmata* harbors huge uncultured archaeal lineages at the order level, so-called Groups E2 and E3. A novel archaeon Kjm51a affiliated with Group E2 was enriched from anaerobic sludge in the present study. Clone library analysis of the archaeal 16S rRNA and *mcrA* genes confirmed a unique archaeal population in the enrichment culture. The 16S rRNA gene-based phylogeny revealed that the enriched archaeon Kjm51a formed a distinct cluster within Group E2 in the class *Thermoplasmata* together with *Methanomassiliicoccus luminyensis* B10$^T$ and environmental clone sequences derived from anaerobic digesters, bovine rumen, and landfill leachate. Archaeon Kjm51a showed 87.7% 16S rRNA gene sequence identity to the closest cultured species, *M. luminyensis* B10$^T$, indicating that archaeon Kjm51a might be phylogenetically novel at least at the genus level. In fluorescence *in situ* hybridization analysis, archaeon Kjm51a was observed as coccoid cells completely corresponding to the archaeal cells detected, although bacterial rod cells still coexisted. The growth of archaeon Kjm51a was dependent on the presence of methanol and yeast extract, and hydrogen and methane were produced in the enrichment culture. The addition of 2-bromo ethanesulfonate to the enrichment culture completely inhibited methane production and increased hydrogen concentration, which suggested that archaeon Kjm51a is a methanol-reducing hydrogenotrophic methanogen. Taken together, we propose the provisional taxonomic assignment, named *Candidatus* Methanogranum caenicola, for the enriched archaeon Kjm51a belonging to Group E2. We also propose to place the methanogenic lineage of the class *Thermoplasmata* in a novel order, *Methanomassiliicoccales* ord. nov.

**Key words:** Methanogranum caenicola, methanogen, *Thermoplasmata*, rice cluster III, anaerobic digested sludge

Until recently, the class *Thermoplasmata* had consisted of mainly acidophilic, aerobic, mesophilic to thermophilic, and sulfur-reducing archaea such as genera *Acidiplasma* (15), *Ferroplasma* (14), *Picrophilus* (47), *Thermoplasma* (4), *Thermogymnomonas* (28), and *Candidatus* Aciduliprofundum boonei (50). Archaeal members of those genera mainly inhabit extreme environments such as acidic and solfataric fields. On the other hand, culture-independent approaches have retrieved a diverse array of environmental clones belonging to the class *Thermoplasmata* from ordinary environments, and many of these clones form huge uncultured archaeal lineages at the order level, so-called Groups E2 and E3 (6, 33, 39). Groups E2 and E3 consist of sublineages such as Marine group II, deep-sea hydrothermal vent Euryarchaeotic group 1 and 2 (DHVE1 and DHVE2), and rice cluster III (RC-III), which is derived from the alimentary canal (12, 19, 54), anaerobic digester (13), contaminated aquifer (8), deep-sea hydrothermal vent (40, 55), marine plankton (5, 7), and rice field soil (3, 17, 30). More recently, a uniformly shaped pure culture B10$^T$, given the name *Methanomassiliicoccus luminyensis*, was isolated from human feces, and revealed to be a methanol-reducing, mesophilic, slightly alkaliphilic methanogen belonging to the class *Thermoplasmata* (9). These findings suggest that the class *Thermoplasmata* is a phenotypically versatile taxon; however, very little is known about the phylogenetic diversity and ecological distribution of methanogens in the class *Thermoplasmata*.

In our previous study, members of RC-III within Group E2 in the *Thermoplasmata* as well as *Methanoculleus*, *Methanosarcina*, and *Methanothermobacter* species have been detected from methanogenic bioreactors (1, 21, 43, 44, 46). To obtain cultures of those methanogens, we conducted

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enrichment cultures from methanogenic digester sludge and eventually succeeded in enriching a novel methanogen belonging to Group E2 in class *Thermoplasmata*. Thus, this paper deals with the phylogenetic characterization of the enriched methanogen in *Thermoplasmata* and the provisional characterization of the phenotypes.

## Materials and Methods

### *Sampling*

The anaerobic sludge was collected from a methanogenic packed-bed reactor at Kajima Technical Research Institute on 16th December 2004. The reactor, which was packed with carbon fiber textile as supporting media (43–45), had been properly operated at 55°C and was stably producing methane gas from garbage slurry as feedstock. The garbage slurry was prepared from kitchen waste from the company cafeteria. It was diluted with an equal amount of water after removing non-biodegradable materials and then pulverized using a homogenizer. The physicochemical properties of the slurry were as follows: pH 5.2; chemical oxygen demand (COD), approx. 203 g $L^{-1}$; and volatile suspended solids (VSS), approx. 104 g $L^{-1}$.

### *Enrichment from the sludge*

The basal medium was used with or without 0.01% (w/v) yeast extract (Becton Dickinson, Franklin Lakes, NJ, USA), designated YB and B media, respectively, in this study. Basal medium was composed of ($L^{-1}$): 0.54 g $NH_4Cl$, 0.14 g $KH_2PO_4$, 0.20 g $MgCl_2 \cdot 6H_2O$, 0.15 g $CaCl_2 \cdot 2H_2O$, 2.5 g $NaHCO_3$, and 1.0 mL trace element solution (58) containing 4.0 mg $Na_2WO_4 \cdot H_2O$ and eliminating NaCl. Prior to inoculation, the pH of the medium was adjusted to 7.0 with 6 N HCl, dissolved oxygen was removed by flushing with $N_2$:$CO_2$ (4:1, v/v), and 10 mL vitamin solution ($L^{-1}$) (60) and 10 mL sterile stock solution of $Na_2S$/cysteine-HCl solution (each 50.0 g $L^{-1}$) (26) were added. $H_2$:$CO_2$ (4:1, v/v; approx. 150 kPa), formate, acetate, or methanol (all at 10 mM) was added to the basal medium as the sole substrate. For enrichment, 0.5 mL anaerobic sludge was inoculated into 20 mL of each medium and incubated at 30°C for a week. A stable enrichment culture was obtained after three cultivations in MYB medium, YB medium supplied with methanol. The enrichment culture was maintained in MYB medium by consecutive transfer monthly.

### *Preparation of DNA, PCR amplification, and DNA sequencing*

The genomic DNA was extracted from the enrichment culture and purified as described previously (49). The archaeal and bacterial 16S rRNA genes were amplified by PCR using the following primers: A10F (5'-TCYGGTTGATCCYGCCRG-3') and A1400R (5'-ACGGGCGGTGTGTGCAAG-3') for the domain *Archaea*, U27F and U1492R (25) for the domain *Bacteria*. The PCR mixture (50 μL) contained 1×PCR buffer, 3.5 mM $MgCl_2$, 10 mM deoxynucleoside triphosphates (dNTPs), 1.25 U AmpliTaq Gold (each from Applied Biosystems, Foster City, CA, USA), and 0.4 μM of each forward and reverse primer. Approximately 100 ng genomic DNA was used as a template under the following cycling conditions: initial AmpliTaq Gold activation at 95°C for 9 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 30 s, extension at 72°C for 1.5 min, and a final extension step at 72°C for 5 min. The *mcrA* gene encoding the alpha-subunit of methyl-coenzyme M reductase was also partially amplified by PCR with primers MR1mod and ME2mod (35) under almost the same PCR conditions except for its cycle number (40 cycles) and time of extension step (1 min) in the cycle. The PCR product was purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany), and sequenced using the BigDye terminator v3.1 cycle sequencing kit with a 3130*xl* genetic analyzer (both from Applied Biosystems).

### *Clone library*

The purified archaeal 16S rRNA and *mcrA* genes were cloned with a pT7Blue T-vector kit (Novagen, Madison, WI, USA). The clonal DNAs were amplified from randomly selected recombinants by direct PCR with M13 primers, and then used as templates for sequencing. A universal primer 907r (56) and T7 promoter primers were used for sequencing the cloned 16S rRNA and *mcrA* genes, respectively. The obtained sequences of all the 16S rRNA gene clones (~690 bp) and the *mcrA* gene clones (~475 bp) were compared with those in the GenBank database using the BLAST program (NCBI-BLAST, www.ncbi.nlm.nih.gov/BLAST), and aligned using the CLUSTAL_X program. Sequence identity of 99% was used as the cut-off value for grouping the sequences into different operational taxonomic units (OTUs).

### *Phylogenetic analyses*

Almost full-length 16S rRNA gene and partial *mcrA* gene sequences were determined for phylogenetic analysis. The following primers were used for sequencing the PCR product of the archaeal 16S rRNA gene: A10F, Kjm700F (5'-TGGGGTAGGGGTAAA ATCCT-3'), Kjm1000F (5'-ACTCACCAGGGGAGACTGTT-3'), A500R (5'-GTGTTACCGCGGCKGCTGG-3'), Kjm700R (5'-GTG GTCCTTCTAGGATTACA-3'), and A1400R, that of the bacterial 16S rRNA gene: U520F (5'-GTGCCAGCAGCCGCGG-3') and U1492R, and that of *mcrA* gene: MR1mod and ME2mod. Sequences were compared using the BLAST program with those available in the DDBJ/EMBL/GenBank databases. Phylogenetic analyses were carried out using the 16S rRNA gene sequence and deduced amino acid sequence of the *mcrA* gene. The 16S rRNA gene sequences were aligned with an ARB data set using ARB software (32). According to the previously described method (27), thirty-five reference sequences of the phylogenetically related archaea and environmental clones were selected as authentic sequences located in the class *Thermoplasmata*. The data set of deduced McrA amino acid sequences was aligned using the CLUSTAL_X program. Phylogenetic trees were constructed by the neighbor-joining (NJ) method with the CLUSTAL_X program (42, 57) and the maximum-likelihood (ML) method with MORPHY software version 2.3b3 (10, 20). In addition, the posterior probabilities of branching points were estimated by Bayesian inference using MrBayes 3.1 (23, 41).

### *Fluorescence* in situ *hybridization*

The enriched archaeon Kjm51a grew on the aforementioned MYB medium for 8 d. The harvested cells were fixed in 4% paraformaldehyde at 4°C for 2 h and stored in 99% ethanol–phosphate-buffered saline (1:1). The fixed cells were incubated in hybridization buffer (0.9 M NaCl, 0.01% sodium dodecyl sulfate, 20 mM Tris-HCl, pH 7.2, and an appropriate amount of formamide) containing fluorescently labeled probes (0.5 pmol $\mu L^{-1}$). After incubation at 46°C for 10 h, the buffer was replaced with washing solution (378 mM NaCl, 0.01% sodium dodecyl sulfate, 20 mM Tris-HCl, pH 7.2, and 5 mM EDTA). The sample was incubated at 46°C for 20 min. and then stained with 1 μg $mL^{-1}$ of 4',6-diamidino-2-phenylindole (DAPI). The sample obtained was observed under a confocal laser scanning microscope (LSM710; Carl Zeiss Microscopy, Tokyo, Japan). A specific oligonucleotide probe targeting the 16S rRNA gene of the enriched archaeon Kjm51a (RC281r2, 5'-AAGGCCCATACCCGTCATC-3') was designed using the Probe Design tool of the ARB software package (32). The overall Gibbs free energy of this probe and target sequence calculated with the mathFISH web server was –9.2 kcal $mol^{-1}$ (62). The probes were labeled with fluorescent dye, Alexa Fluor 555 (Japan Bio Services, Saitama, Japan). Two domain-specific probes were also used: EUB338 labeled with Alexa Fluor 647 for detection of almost all bacteria (2), and ARC915 labeled with Alexa Fluor 488 for detection of almost all archaea (53). The stringency of hybridization was adjusted by adding formamide to the hybridization buffer (15% [v/v] for all the probes used in this study). More than 8,000 DAPI-stained cells were counted to determine the ratio of ARC915-hybridized cells to EUB338-hybridized cells.

*Physiological characteristics*

Growth conditions were determined using MYB medium. Aerobic and microaerobic conditions were prepared by the substitution of air and the addition of 2% (v/v) oxygen, respectively, with filtration through a 0.2 μm-pore membrane filter. Prior to inoculation, acetate, lactate, or pyruvate (all at 10 mM) were added as carbon sources instead of yeast extract. 2-Bromo ethanesulfonate (BES, final concentration 20 mM) was added as the inhibitor of methane production. Then, 0.2 mL of the preculture of the enrichment was inoculated into 20 mL fresh medium containing each substrate. The culture was incubated at 30°C for two weeks. After the transfer twice, hydrogen and methane concentrations in the headspaces of serum bottles were determined with a gas chromatograph (GC-14A; Shimadzu, Kyoto, Japan) equipped with a thermal conductivity detector and a porapack Type Q 80–100, mesh 80–100 (Waters, Tokyo, Japan). The analysis conditions were as follows; column temperature, 60°C; injector temperature, 80°C; and detector temperature, 100°C; current, 80 mA; carrier gas, $N_2$.

*Accession numbers*

The 16S rRNA gene and *mcrA* gene sequences of the enriched archaeon Kjm51a have been deposited in the DDBJ/EMBL/NCBI, and GenBank nucleotide sequence databases under accession numbers AB749767 and AB749768, respectively.

## Results

*Methanogenic enrichment cultures from anaerobic sludge*

A methanogenic enrichment culture was obtained from anaerobic digester using MYB medium containing methanol and yeast extract. The archaeal population in the culture was analyzed using archaeal 16S rRNA gene- and *mcrA* gene-specific primers. A total of 113 and 61 clones were obtained, respectively. A sole phylotype was obtained in both clone libraries, *i.e.*, all the cloned 16S rRNA gene and *mcrA* gene sequences in the two libraries were almost identical to the sequence identities of 99.4–100% and 99.5–100%, respectively.

An almost full-length 16S rRNA gene sequence (1,309 bp) was determined for a novel archaeon, designated phylotype Kjm51a, in the enrichment culture. In the phylogenetic trees of the 16S rRNA gene sequences constructed using NJ, ML, and Bayesian methods, the enriched archaeon Kjm51a was placed into an uncultured archaeal lineage, Group E2, in the class *Thermoplasmata* (6) (Fig. 1). The topologies of the trees generated by the three phylogenetic analysis methods were almost identical, and were supported by high bootstrap values (99–100%). Archaeon Kjm51a was a member of RC-III, a sublineage within Group E2, and showed the highest sequence similarities (91.3–96.2%) to the environmental clones derived from anaerobic digesters, bovine rumen, and landfill leachate (13, 22, 54, 61). The nearest cultivated neighbor of archaeon Kjm51a was *Methanomassiliicoccus luminyensis* B10$^T$ with 87.7% sequence identity. A partial *mcrA* gene sequence (1,109 bases) was also determined for the enriched archaeon Kjm51a. The NJ tree constructed using the McrA amino acid sequence deduced from the *mcrA* gene sequence demonstrated that archaeon Kjm51a formed a monophyletic cluster together with *M. luminyensis* B10$^T$ and environmental clone sequences derived from anaerobic bioreactor and bovine rumen (Fig. 2), and that the cluster was apparently distinct from the four known methanogenic lineages, the classes *Methanobacteria*, '*Methanomicrobia*', *Methanococci*, and *Methanopyri*. The McrA amino acid sequence of the enriched archaeon Kjm51a showed 76.0% identity with that of the closest species, *M. luminyensis* B10$^T$.

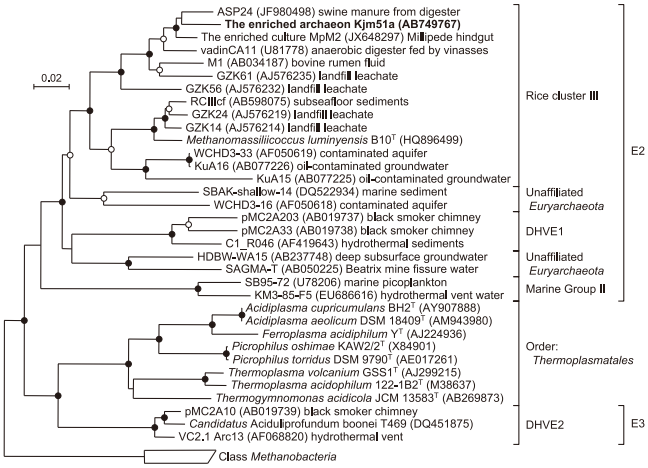


**Fig. 1.** Phylogenetic affiliation of the enriched archaeon Kjm51a within Group E2 in the class *Thermoplasmata* on the basis of the 16S rRNA gene sequences. The tree was constructed using the neighbor-joining method. Solid circles at branching nodes indicate supporting probabilities above 95% by all the phylogenetic analysis methods (NJ, ML, and Bayesian), and open circles indicate probabilities above 85% by two or more analyses. Bar, 0.02 substitutions per nucleotide position.

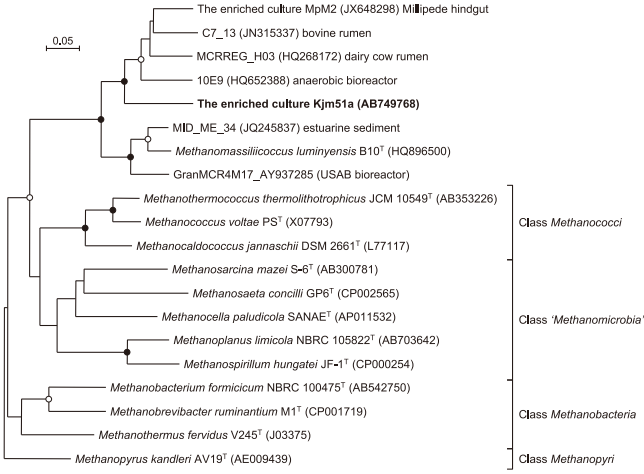


**Fig. 2.** Deduced McrA amino acid sequence-based phylogeny showing the phylogenetic relationships among the enriched archaeon Kjm51a, its related archaeon and environmental clones, and other known methanogens. Solid circles at branching nodes indicate supporting probabilities above 95%, and open circles indicate probabilities above 80%. Bar, 0.02 substitutions per nucleotide position.

Coccoid- and rod-shaped cells were observed under the microscope. The cocci and rods were identified as archaeal and bacterial cells, respectively, by fluorescence *in situ* hybridization with archaeal and bacterial probes (Fig. 3A and B). A ratio of archaeal cells to total cells was at least 3.5±1.4% in the enrichment culture. Cocci were hybridized with a Kjm51a-specific probe, but rods were not (Fig. 3C). Cells hybridized with the Kjm51a-specific probe completely corresponded to those with the archaeal probe (Fig. 3B and D). The bacterial rods in the enrichment culture were provisionally identified as *Clostridium celerecrescens* (sequence

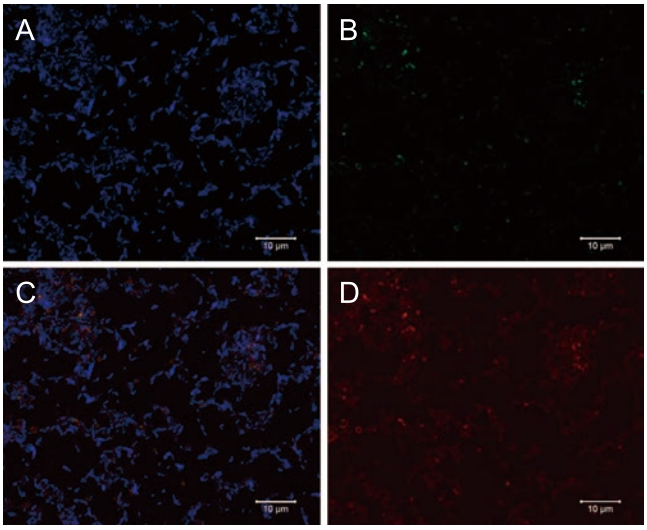


**Fig. 3.** Epifluorescence micrographs of *in situ* hybridization of the enrichment archaeon Kjm51a grown on MYB medium for a week. The same microscopic field is shown after hybridization with a Kjm51a-specific probe (red), an archaeal probe ARC915 (green), and a bacterial probe EUB338 (blue). A, blue color; B, green color; C, merge of red, green and blue colors; D, red color. Bars, 10 μm.

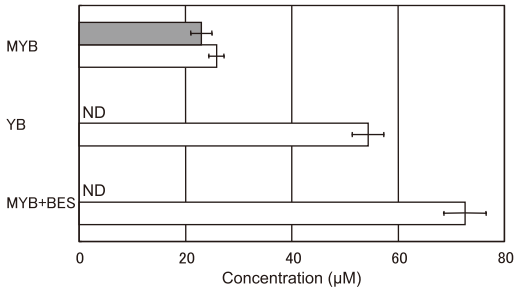


**Fig. 4.** Methane and hydrogen production in the enrichment culture obtained from anaerobic sludge. Filled bars, methane; open squares, hydrogen. Data points and bars are the means and standard deviations, respectively ($n$=3). Abbreviations: MYB, MYB medium supplied with methanol in YB medium; YB, YB medium; MYB+BES, MYB medium supplied with BES; ND, not detected.

identity: 99.9%, X71848) by bacterial 16S rRNA gene sequence analysis.

*Physiological property of the enriched archaeon Kjm51a*

The enriched archaeon Kjm51a was strictly anaerobic and was capable of growing in MYB medium under a $N_2/CO_2$ (4:1 [v/v]) atmosphere, but could not grow under microaerobic or aerobic conditions. Both methanol and yeast extract were required for the growth of archaeon Kjm51a. Acetate, lactate, and pyruvate were not utilized as carbon sources instead of yeast extract. Metabolic products of the enrichment culture with methanol and yeast extract were hydrogen and methane, and that with yeast extract and without methanol was hydrogen (Fig. 4). Growth of the enriched archaeon Kjm51a in the presence of methanol and its methane production were completely inhibited by the addition of BES. The amount of hydrogen in the presence of BES was approximately three times higher than that in the absence of BES.

## Discussion

The novel archaeon Kjm51a was successfully enriched from anaerobic sludge using MYB medium containing methanol and yeast extract. The archaeon was not yet purified in this study because *C. celerecrescens* was dominantly isolated in the presence of yeast extract, although we made a great effort to isolate the archaeon using agar plate culture and dilution-extinction culture. Clone library analysis demonstrated that cloned 16S rRNA and *mcrA* gene sequences obtained from the enrichment culture were almost identical in each. Furthermore, FISH analysis also showed that coccal cells hybridized with a Kjm51a-specific probe completely corresponded to those with an archaeal probe. These findings strongly support the archaeal purity of the enriched archaeon Kjm51a in MYB medium, although bacterial cells still coexisted.

The enriched archaeon Kjm51a was a strictly anaerobic and chemoheterotrophic cocci showing growth and methane production in the presence of methanol, the inhibition of methane production by BES, a well-known inhibitor of methanogenesis (18). Hydrogen production was also observed in the absence of methanol, which indicated that the coexisting bacterium, *C. celerecrescens*, produced hydrogen by its fermentation (38). Inhibition of methane production by BES resulted in increased hydrogen production. These physiological properties suggest that the enriched archaeon Kjm51a might be a methanol-reducing hydrogenotrophic methanogen.

As reported for *Methanosphaera stadtmanae* (34), *Methanomicrococcus blatticola* (51), and *Methanosarcina barkeri* strain Fusaro (36), the enriched archaeon Kjm51a is likely to produce methane by the hydrogen-dependent reduction of methanol through the following reaction: $H_2+CH_3OH \rightarrow CH_4+H_2O$ (11, 29, 52, 59). *Methanomassiliicoccus luminyensis*, a recently isolated methanogen from human feces, belonging to Group E2, also produced methane from methanol in the presence of hydrogen (9). The genome of *M. luminyensis* likely encodes only a partial methanogenesis pathway (16). Most recently, archaeon MpT1 in Group E2 was enriched from termite guts as a methanogen, which converted methanol to methane (39). Methanol may be a common substrate for methanogenesis in Group E2. Schink and Zeikus reported that heterotrophic microbes anaerobically produced methanol as a major end product from pectin, which is a component of plant tissue (48). Biodegradation of plants occurs in a wide variety of environments, such as the rumen, rice field soil and anaerobic digester treating garbage, and Group E2 methanogens may contribute to carbon flux.

The enriched archaeon Kjm51a is the first culture representative derived from an anaerobic methanogenic digester in Group E2. In the phylogenetic trees constructed using 16S rRNA gene sequences, the archaeon Kjm51a and *M. luminyensis* $B10^T$ were completely separated in Group E2 with their low sequence identity (87.7%), which was sufficiently low to classify them into different genera. The enriched archaeon Kjm51a and *M. luminyensis* $B10^T$ were affiliated with RC-III, one of the sublineages in Group E2. RC-III was clearly and completely separated from the validly described order *Thermoplasmatales*. Its monophyletic lineage

was strongly supported by the probability scores (>99%) calculated using all the phylogenetic analysis methods. The 16S rRNA gene sequence of the enriched archaeon Kjm51a and *M. luminyensis* B10$^T$ had similarities of only 77.1 to 80.3% with those of the known archaeal species in the order *Thermoplasmatales*. These similarities are lower than the 85% similarity that is generally used as a cut-off value for distinguishing lineages at the phylum, as suggested by Hugenholtz *et al.* (24). Therefore, RC-III composed of the enriched culture Kjm51a and *M. luminyensis* B10$^T$ is a distinct order level lineage in the class *Thermoplasmata*. Previously, Kemnitz *et al.* (30) reported that the RC-III archaea might heterotrophically grow using peptides, based on their enrichment culture experiment; however, taken together with the recent study (6), our findings clearly indicated that RC-III is a novel methanogenic lineage.

In conclusion, an archaeal representative enriched from the anaerobic methanogenic digester is a novel methanogen belonging to RC-III within Group E2 in the class *Thermoplasmata*. According to the recommendations of Murray and Stackebrandt (37), we propose the provisional taxonomic assignment of *Candidatus* Methanogranum caenicola for the enriched archaeon Kjm51a. Most recently, the order *Methanoplasmatales* was provisionally proposed for the deep-branching lineage accommodating *M. luminyensis* and the enriched archaea MpT1 and MpM2 (39); however, this lineage should be proposed as the *Methanomassiliicoccales* on the basis of Rule 47a of the Bacteriological Code to avoid bacteriological confusion (31). Consequently, we propose to rename the order '*Methanoplasmatales*' as *Methanomassiliicoccales* for the sublineage accommodating *M. luminyensis* B10$^T$ and the enriched archaeon Kjm51a as described below. The proposal of this novel order follows the description of the new family *Methanomassiliicoccaceae*. To purify the enriched archaeon Kjm51a and understand its ecological role in the methanogenic environment, further study via enrichment culture will be necessary.

### Description of *Candidatus* Methanogranum caenicola

Methanogranum caenicola (Me.tha.no.gra'num. cae.ni. co'la. N.L. n. methanum [from French n. méth(yle) and chemical suffix -ane], methane; N.L. pref. methano-, pertaining to methane; L. neut. n. granum, grain, kernel; N.L. neut. n. Methanogranum, a methane-producing grain: L. n. caenum, mud, sludge; L. suff. -cola [from L. n. incola], inhabitant, dweller; N.L. n. caenicola, an inhabitant of sludge).

Strictly anaerobic, chemoheterotrophic. Cells form cocci occurring as single cells. Produce methane dependent on hydrogen and methanol. Represent a distinct phylogenetic lineage in the class *Thermoplasmata* based on 16S rRNA gene sequence analysis. Enriched from an anaerobic sludge in a methanogenic digester.

### Description of *Methanomassiliicoccaceae* fam. nov.

*Methanomassiliicoccaceae* (Me.tha.no.mas.si.li.i.coc.ca'ce.ae. N.L. neut. n. *Methanomassiliicoccus* type genus of the family; *-aceae* ending to denote a family; N.L. fem. pl. n. *Methanomassiliicoccaceae* family of the genus *Methanomassiliicoccus*).

The family *Methanomassiliicoccaceae* is defined on the basis of a phylogenetic tree constructed by phylogenetic analysis of the 16S rRNA gene sequence of a single cultivated representative, of the enriched culture, and of environmental clone sequences derived mainly from the alimentary canal, anaerobic digester, landfill leachate, and rice field soil. The type genus is *Methanomassiliicoccus*.

### Description of *Methanomassiliicoccales* ord. nov.

*Methanomassiliicoccales* (Me.tha.no.mas.si.li.i.coc.cal'es. N.L. neut. n. *Methanomassiliicoccus* type genus of the order; *-ales* ending to denote an order; N.L. fem. pl. n. *Methanomassiliicoccales* order of the genus *Methanomassiliicoccus*).

The description is the same as that for the family *Methanomassiliicoccaceae*. The type genus is *Methanomassiliicoccus*.

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### References

1. Akuzawa, M., T. Hori, S. Haruta, Y. Ueno, M. Ishii, and Y. Igarashi. 2011. Distinctive responses of metabolically active microbiota to acidification in a thermophilic anaerobic digester. Microb. Ecol. 61:595–605.
2. Amann, R.I., B.J. Binder, R.J. Olson, S.W. Chisholm, R. Devereux, and D.A. Stahl. 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. 56:1919–1925.
3. Chin, K.-J., L. Thomas, and R. Conrad. 1999. Effect of temperature on structure and function of the methanogenic archaeal community in an anoxic rice field soil. Appl. Environ. Microbiol. 65:2341–2349.
4. Darland, G., T.D. Brock, W. Samsonoff, and S.F. Conti. 1970. A thermophilic, acidophilic mycoplasma isolated from a coal refuse pile. Science 170:1416–1418.
5. DeLong, E.F. 1992. Archaea in coastal marine environments. Proc. Natl. Acad. Sci. U.S.A. 89:5685–5689.
6. DeLong, E.F., and N.R. Pace. 2001. Environmental diversity of bacteria and archaea. Syst. Biol. 50:470–478.
7. DeLong, E.F., K.Y. Wu, B.B. Prezelin, and R.V. Jovine. 1994. High abundance of Archaea in Antarctic marine picoplankton. Nature 371:695–697.
8. Dojka, M.A., P. Hugenholtz, S.K. Haack, and N.R. Pace. 1998. Microbial diversity in a hydrocarbon- and chlorinated-solvent-contaminated aquifer undergoing intrinsic bioremediation. Appl. Environ. Microbiol. 64:3869–3877.
9. Dridi, B., M.-L. Fardeau, B. Ollivier, D. Raoult, and M. Drancourt. 2012. *Methanomassiliicoccus luminyensis* gen. nov., sp. nov., a methanogenic archaeon isolated from human faeces. Int. J. Syst. Evol. Microbiol. 62:1902–1907.
10. Felsenstein, J. 1981. Evolutionary trees from DNA sequences: a maximum likelihood approach. J. Mol. Evol. 17:368–376.
11. Fricke, W.F., H. Seedorf, A. Henne, M., Krüer, H. Liesegang, R. Hedderich, G. Gottschalk, and R.K. Thauer. 2006. The genome sequence of *Methanosphaera stadtmanae* reveals why this human intestinal archaeon is restricted to methanol and $H_2$ for methane gormation and ATP synthesis. J. Bacteriol. 188:642–658.
12. Friedrich, M.W., D. Schmitt-Wagner, T. Lueders, and A. Brune. 2001. Axial differences in community structure of *Crenarchaeota* and *Euryarchaeota* in the highly compartmentalized gut of the soil-feeding termite *Cubitermes orthognathus*. Appl. Environ. Microbiol. 67:4880–4890.

13. Godon, J.-J., E. Zumstein, P. Dabert, F. Habouzit, and R. Moletta. 1997. Molecular microbial diversity of an anaerobic digestor as determined by small-subunit rDNA sequence analysis. Appl. Environ. Microbiol. 63:2802–2813.
14. Golyshina, O.V., T.A. Pivovarova, G.I. Karavaiko, *et al.* 2000. *Ferroplasma acidiphilum* gen. nov., sp. nov., an acidophilic, autotrophic, ferrous-iron-oxidizing, cell-wall-lacking, mesophilic member of the *Ferroplasmaceae* fam. nov., comprising a distinct lineage of the *Archaea*. Int. J. Syst. Evol. Microbiol. 50:997–1006.
15. Golyshina, O.V., M.M. Yakimov, H. Lünsdorf, M. Ferrer, M. Nimtz, K.N. Timmis, V. Wray, B.J. Tindall, and P.N. Golyshin 2009. *Acidiplasma aeolicum* gen. nov., sp. nov., a euryarchaeon of the family *Ferroplasmaceae* isolated from a hydrothermal pool, and transfer of *Ferroplasma cupricumulans* to *Acidiplasma cupricumulans* comb. nov. Int. J. Syst. Evol. Microbiol. 59:2815–2823.
16. Gorlas, A., C. Robert, G. Gimenez, M. Drancourt, and D. Raoult. 2012. Complete genome sequence of *Methanomassiliicoccus luminyensis*, the largest genome of a human-associated Archaea species. J. Bacteriol. 194:4745.
17. Großkpof, R., S. Stubner, and W. Liesack. 1998. Novel euryarchaeotal lineages detected on rice roots and in the anoxic bulk soil of flooded rice microcosms. Appl. Environ. Microbiol. 63:4983–4989.
18. Gunsalus, R.P., J.A. Romesser, and R.S. Wolfe. 1978. Preparation of coenzyme M analogues and their activity in the methyl coenzyme M reductase system of *Methanobacterium thermoautotrophicum*. Biochemistry 17:2374–2377.
19. Hara, K., N. Shinzato, M. Seo, T. Oshima, and A. Yamagishi. 2002. Phylogenetic analysis of symbiotic archaea living in the gut of xylophagous cockroaches. Microb. Environ. 17:185–190.
20. Hasegawa, M., H. Kishino, and T.A. Yano. 1985. Dating of the human ape splitting by a molecular clock of mitochondrial-DNA. J. Mol. Evol. 22:160–174.
21. Hori, T., S. Haruta, Y. Ueno, M. Ishii, and Y. Igarashi. 2006. Dynamic transition of a methanogenic population in response to the concentration of volatile fatty acids in a thermophilic anaerobic digester. Appl. Environ. Microbiol. 72:1623–1630.
22. Huang, L.N., Y.Q. Chen, H. Zhou, S. Luo, C.Y. Lan, and L.H. Qu. 2003. Characterization of methanogenic Archaea in the leachate of a closed municipal solid waste landfill. FEMS Microbiol. Ecol. 46:171–177
23. Huelsenbeck, J.P., and F. Ronquist. 2001. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17:754–755.
24. Hugenholtz, P., C. Pitulle, K.L. Hershberger, and N.R. Pace. 1998. Novel division level bacterial diversity in a Yellowstone hot spring. J. Bacteriol. 180:366–376.
25. Iino, T., K. Mori, K. Tanaka, K. Suzuki, and S. Harayama. 2007. *Oscillibacter valericigenes* gen. nov., sp. nov., a valerate-producing anaerobic bacterium isolated from the alimentary canal of a Japanese corbicula clam. Int. J. Syst. Evol. Microbiol. 57:1840–1845.
26. Iino, T., K. Suzuki, and S. Harayama. 2009. *Lacticigenium naphtae* gen. nov., sp. nov., a novel halotolerant and motile lactic acid bacterium isolated from crude oil. Int. J. Syst. Evol. Microbiol. 59:775–780.
27. Iino, T., K. Mori, Y. Uchino, T. Nakagawa, S. Harayama, and K. Suzuki. 2010a. *Ignavibacterium album* gen. nov., sp. nov., a moderately thermophilic anaerobic bacterium isolated from microbial mats at a terrestrial hot spring and proposal of *Ignavibacteria* classis nov., for a novel lineage at the periphery of green sulfur bacteria. Int. J. Syst. Evol. Microbiol. 60:1376–1382.
28. Itoh, T., N. Yoshikawa, and T. Takashina. 2007. *Thermogymnomonas acidicola* gen. nov., sp. nov., a novel thermoacidophilic, cell wall-less archaeon in the order *Thermoplasmatales*, isolated from a solfataric soil in Hakone, Japan. Int. J. Syst. Evol. Microbiol. 57:2557–2561.
29. Keltjens, J.T., and C. van der Drift. 1986. Electron transfer reactions in methanogens. FEMS Microbiol. Rev. 39:259–303.
30. Kemnitz, D., S. Kolb, and R. Conrad. 2005. Phenotypic characterization of Rice Cluster III archaea without prior isolation by applying quantitative polymerase chain reaction to an enrichment culture. Environ. Microbiol. 7:553–565.
31. Lapage, S.P., P.H.A. Sneath, E.F. Lessel, V.B.D. Skerman, H.P.R. Seeliger, and W.A. Clark (ed.). P.H.A. Sneath. editor for 1992 edition (1992) International Code of Nomenclature of Bacteria, 1990 Revision, Published for the International Union of Microbiological Societies by the American Society for Microbiology. Washington, D.C.
32. Ludwig, W., O. Strunk, R. Westram, *et al.* 2004. ARB: a software environment for sequence data. Nucleic Acids Res. 32:1363–1371.
33. Mihajlovski, A., M. Alric, and J.F. Brugére. 2008. A putative new order of methanogenic Archaea inhabiting the human gut, as revealed by molecular analyses of the *mcrA* gene. Res. Microbiol. 159:516–521.
34. Miller, T.L., and M.J. Wollin. 1985. *Methanosphaera stadtmaniae* gen. nov., sp. nov.: a species that forms methane by reducing methanol with hydrogen. Arch. Microbiol. 141:116–122.
35. Mori, K., T. Iino, K. Suzuki, K. Yamaguchi, and Y. Kamagata. 2012. Aceticlastic and NaCl-requiring methanogen "*Methanosaeta pelagica*" sp. nov., isolated from marine tidal flat sediment. Appl. Environ. Microbiol. 78:3416–3423.
36. Müller, V., M. Blaut, and G. Gottschalk. 1986. Utilization of methanol plus hydrogen by *Methanosarcina barkeri* for methanogenesis and growth. Appl. Environ. Microbiol. 52:269–274.
37. Murray, R.G.E., and E. Stackebrandt. 1995. Taxonomic Note: Implementation of the provisional status *Candidatus* for incompletely described procaryotes. Int. J. Syst. Bacteriol. 45:186–187.
38. Palop, M.L.L., S. Valles, F. Piñaga, and A. Flors. 1989. Isolation and characterization of an anaerobic, cellulolytic bacterium, *Clostridium celerecrescens* sp. nov. Int. J. Syst. Bacteriol. 39:68–71.
39. Paul, K., J.O. Nonoh, L. Mikulski, and A. Brune. 2012. *"Methanoplasmatales*," *thermoplasmatales*-related archaea in termite guts and other environments, are the seventh order of methanogens. Appl. Environ. Microbiol. 78:8245–8253.
40. Reysenbach, A.L., K. Longnecker, and J. Kirshtein. 2000. Novel bacterial and archaeal lineages from an in situ growth chamber deployed at a Mid-Atlantic Ridge hydrothermal vent. Appl. Environ. Microbiol. 66:3798–3806.
41. Ronquist, F., and J.P. Huelsenbeck. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19:1572–1574.
42. Saitou, N., and M. Nei. 1987. A neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4:406–425.
43. Sasaki, K., S. Haruta, Y. Ueno, M. Ishii, and Y. Igarashi. 2006a. Archaeal population on supporting material in methanogenic packed-bed reactor. J. Biosci. Bioeng. 102:244–246.
44. Sasaki, K., S. Haruta, M. Tatara, A. Yamazawa, Y. Ueno, M. Ishii, and Y. Igarashi. 2006b. Microbial community in methanogenic packed-bed reactor successfully operating at short hydraulic retention time. J. Biosci. Bioeng. 101:271–273.
45. Sasaki, K., S. Haruta, Y. Ueno, M. Ishii, and Y. Igarashi. 2007. Microbial population in the biomass adhering to supporting material in a packed-bed reactor degrading organic solid waste. Appl. Microbiol. Biotechnol. 75:941–952.
46. Sasaki, D., T. Hori, S. Haruta, Y. Ueno, M. Ishii, and Y. Igarashi. 2011. Methanogenic pathway and community structure in a thermophilic anaerobic digestion process of organic solid waste. J. Biosci. Bioeng. 111:41–46.
47. Schleper, C., G. Pühler, H.P. Klenk, and W. Zillig. 1996. *Picrophilus oshimae* and *Picrophilus torridus* fam. nov., gen. nov., sp. nov., two species of hyperacidophilic, thermophilic, heterotrophic, aerobic archaea. Int. J. Syst. Bacteriol. 46:814–816.
48. Schink, B., and J.G. Zeikus. 1980. Microbial methanol formation: a major end product of pectin metabolism. Curr. Microbiol. 4:387–389.
49. Schmidt, T.M., E.F. DeLong, and N.R. Pace. 1991. Analysis of a marine picoplankton community by 16S rRNA gene cloning and sequencing. J. Bacteriol. 173:4371–4378.
50. Shouten, S., M. Baas, E.C. Hopmans, A.-L. Reysenbach, and J.S.S. Damsé. 2008. Tetraether membrane lipids of *Candidatus* "*Aciduliprofundum boonei*", a cultivated obligate thermoacidophilic euryarchaeote from deep-sea hydrothermal vents. Extremophiles 12:119–124.
51. Sprenger, W.W., M.C. van Belzen, J. Rosenberg, J.H.P. Hackstein, and J.T. Keltjens. 2000. *Methanomicrococcus blatticola* gen. nov., sp. nov., a methanol- and methylamine-reducing methanogen from the hindgut of the cockroach *Periplaneta Americana*. Int. J. Syst. Evol. Microbiol. 50:1989–1999.
52. Sprenger, W.W., J.H.P. Hackstein, and J.T. Keltjens. 2005. The energy metabolism of *Methanomicrococcus blatticola*: physiological and biochemical aspects. Antonie van Leeuwenhoek. 87:289–299.

53. Stahl, D.A., and R. Amann. 1991. Development and application of nucleic acid probes, p. 205–248. *In* E. Stackbrandt and M. Goodfellow (ed.), Nucleic Acid Techniques in Bacterial Systematics. John Wiley & Sons, New York.
54. Tajima, K., T. Nagamine, H. Matsui, M. Nakamura, and R.I. Aminov. 2001. Phylogenetic analysis of archaeal 16S rRNA libraries from the rumen suggests the existence of a novel group of archaea not associated with known methanogens. FEMS Microbiol. Lett. 200:67–72.
55. Takai, K., and K. Horikoshi. 1999. Genetic diversity of archaea in deep-sea hydrothermal vent environments. Genetics 152:1285–1297.
56. Tamaki, H., Y. Sekiguchi, S. Hanada, K. Nakamura, N. Nomura, M. Matsumura, and Y. Kamagata. 2005. Comparative analysis of bacterial diversity in freshwater sediment of a shallow eutrophic lake by molecular and improved cultivation-based techniques. Appl. Environ. Microbiol. 71:2162–2169.
57. Thompson, J.D., T.J. Gibson, F. Plewniak, F. Jeanmougin, and D.G. Higgins. 1997. The Clustal_X Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tool. Nucleic. Acids Res. 24:4876–4882.
58. Touzel, J.P., and G. Albagnac. 1983. Isolation and characterization of *Methanococcus mazei* strain $MC_3$. FEMS Microbiol. Lett. 16:241–245.
59. van de Wijngaard, W.M.H., J. Creemers, G.D. Vogels, and C. van der Drift. 1991. Methanogenic pathways in *Methanosphaera stadtmanae*. FEMS Microbiol. Lett. 80:207–212.
60. Wolin, E.A., M.J. Wolin, and R.S. Wolfe. 1963. Formation of methane by bacterial extracts. J. Biol. Chem. 238:2882–2886.
61. Xia, Y., D.I. Masse, T.A. McAllister, Y. Kong, R. Seviour, and C. Beaulieu. 2012. Identity and diversity of archaeal communities during anaerobic co-digestion of chicken feathers and other animal wastes. Bioresour. Technol. 110:111–119.
62. Yulmaz, J.S., and D.R. Noguera. 2004. Mechanistic approach to the problem of hybridization efficiency in fluorescent in situ hybridization. Appl. Environ. Microbiol. 70:7126–7139.

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**Special issue:** *Significance of culturing microbes in the omics era*

## *Minireview*

# Cultivation of Uncultured *Chloroflexi* Subphyla: Significance and Ecophysiology of Formerly Uncultured *Chloroflexi* 'Subphylum I' with Natural and Biotechnological Relevance

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Cultivation-independent molecular surveys have shown members of the bacterial phylum *Chloroflexi* to be ubiquitous in various natural and artificial ecosystems. Among the subphylum-level taxa of the *Chloroflexi* known to date, the formerly uncultured 'subphylum I' had well been recognized as a typical group that contains a number of environmental gene clones with no culture representatives. In order to reveal their ecophysiology, attempts were made over the past decade to domesticate them into laboratory cultures, and significant advances have been made in cultivating strains belonging to the group. The microorganisms characterized so far include seven species in six genera, i.e., *Anaerolinea*, *Levilinea*, *Leptolinea*, *Bellilinea*, *Longilinea*, and *Caldilinea*, and were proposed to represent two classes, *Anaerolineae* and *Caldilineae*, providing solid insights into the phenotypic and genetic properties common to the group. Another subphylum-level uncultured group of the *Chloroflexi*, *i.e.*, the class *Ktedonobacteria*, has also been represented recently by a cultured strain. In addition to the results from these tangible cultures, data obtained from functional analyses of uncultured *Chloroflexi* populations by assessing substrate uptake patterns are accumulating at an encouraging rate. In this review, recent findings on the ecological significance and possible ecophysiological roles of '*Chloroflexi* subphylum I' are discussed based on findings from both the characteristics of the cultured *Chloroflexi* and molecular-based analyses.

**Key words:** *Chloroflexi*, *Anaerolineae*, *Caldilineae*, uncultured microorganism

### *Introduction*

Cultivation-independent molecular methods have provided new tools to study the microbial world, enabling us to understand the actual microbial diversity that traditional cultivation-based methods have never unveiled (32). With the application of these techniques, it has become evident that the majority of microorganisms in the environment are uncultured, and that the ecophysiology of these organisms remains largely unknown. The finding of yet-to-be cultured microorganisms have driven renewed efforts in the cultivation and isolation of such microbes, because the domestication (cultivation) of microorganisms into laboratory cultures is still the best means to gain solid insights into metabolic ability and detailed genomic traits of individual microbes. In the past few years, new microorganisms have been successfully isolated that belong to uncultured taxa with environmental and biotechnological relevance, and the information of their physiology in conjugation with phylogeny has been updated (63). *Chloroflexi* subphyla are also examples where such microbial groups have recently been cultured and characterized.

The phylum *Chloroflexi*, formerly known as 'Green nonsulfur bacteria', has been recognized as a typical ubiquitous bacterial taxon containing a number of diverse environmental 16S rRNA gene clones with a limited number of cultured representatives (33, 63). Formerly, the phylum had been divided into four major subphylum (class)-level taxa on the basis of 16S rRNA/rRNA gene sequences, i.e. 'subphyla I, II, III, and IV (Fig. 1) (33), but the class *Thermomicrobia* has been reclassified into the phylum as an additional subphylum (34). The phylogenetic depth of the phylum is comparable with that of the phylum *Proteobacteria* (20). In addition to the major five subphyla, other uncultured lineages at the subphylum level were also identified (14, 63). Among the subphyla, 'subphylum III', known as the class *Chloroflexi*, has been best represented by cultured organisms belonging to the genera *Chloroflexus*, *Oscillochloris*, *Chloronema*, *Heliothrix*, *Herpetosiphon*, and *Roseiflexus*. These organisms mostly possess filamentous morphotypes, and show photoheterotrophic and/or chemolithoheterotrophic growth under mesophilic or moderately thermophilic conditions. The class *Thermomicrobia* also involves cultured organisms belonging

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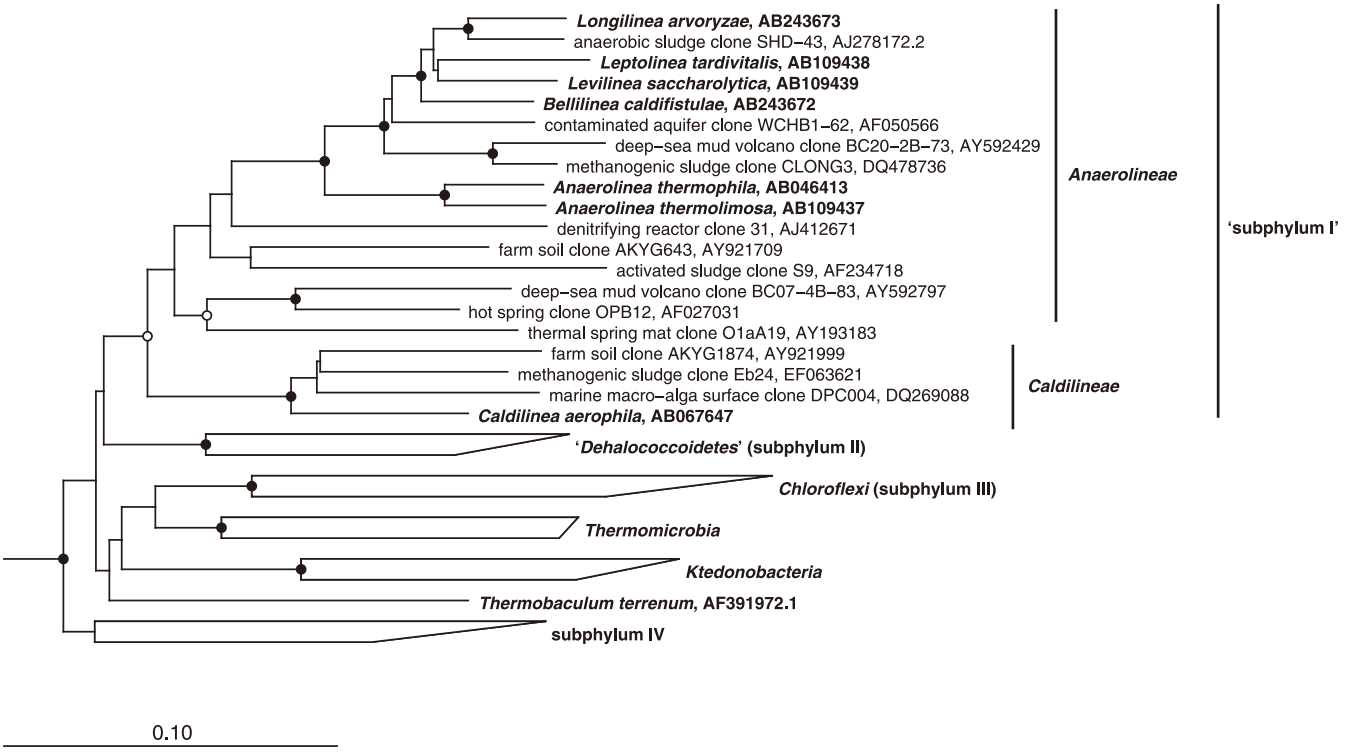


**Fig. 1.** Evolutionary distance dendrogram of the bacterial phylum *Chloroflexi* derived from a comparative analyses of 16S rRNA gene sequences, showing the phylogenetic relationship of the subphyla (class)-level taxa of the phylum. The sequences were aligned, and the phylogenetic tree was constructed by the neighbor-joining (NJ) method with the ARB software package (52). The fidelity of the topology of the NJ tree was also confirmed by bootstrap resampling (based on 1,000 replicates) with the neighbor-joining (PAUP* 4.0 program package) and maximum likelihood (TREEFINDER program package) methods. Nodes highly supported with bootstrap values higher than 85% by both analyses are marked as circles (filled circles, >95%; open circles, 95–85%). Nodes without symbols were not highly resolved (<85%) as specific groups in either analysis. The bar represents 10 nucleotide substitutions per 100 nucleotides.

to the genera *Thermomicrobium* and *Sphaerobacter* (34), which are rod-shaped, moderately thermophilic or hyperthermophilic, chemoheterotrophic aerobes. In addition, *Thermobaculum terrenum*, a moderately thermophilic chemolithoheterotrophic aerobe, represents a distinct lineage in the phylum, forming a new class-level taxon (9). The other three major subphyla (I, II, and IV) had been comprised solely of a variety of environmental clones except for purified, coccoid-shaped organisms, '*Dehalococcoides ethenogenes*' and related strains (e.g., (1, 53)) able to reductively dechlorinate chlorinated compounds, being classified into 'subphylum II' (class '*Dehalococcoidetes*') (31, 33). 'Subphylum I' contains the most diverse environmental clones among the four subphyla of the *Chloroflexi*; in the current 16S rRNA Ribosome Database Project (RDP) database (release 10.11), 'subphylum I' phylotypes are most frequently represented among the subphyla known to date and make up approximately >70% (>5,000 entries) of all the deposited sequences relative to the *Chloroflexi* phylum. Although the past two decades have seen a number of papers reporting the detection of '*Chloroflexi* subphylum I' in various ecosystems (see below), there had long been no description of cultivable microbes. However, in recent years, aerobic and anaerobic strains have successfully been cultivated and characterized that belong to 'subphylum I'. The microorganisms characterized so far are seven species in six genera in total, and were proposed to represent two distinct classes *Anaerolineae* and *Caldilineae*. In addition, an aerobic strain has recently been isolated and characterized that belongs to another uncultured lineage at the subphylum level in the *Chloroflexi* phylum, representing a new class, *Ktedonobacteria*.

In this review, recent findings on the ecological significance and possible ecophysiological roles of the formerly uncultured *Chloroflexi* subphyla are discussed based on findings from rRNA-based community analyses for the environment, as well as from the characteristics of recently cultured *Chloroflexi*. In addition, recent studies on the ecophysiology of these organisms in engineered systems through the evaluation of their substrate uptake pattern are described. Special emphasis is placed on the ecology and function of *Chloroflexi* 'subphylum I' members with natural and biotechnological relevance, particularly those found in waste/wastewater treatment systems.

## *Ecological significance of* Chloroflexi *subphyla*

In 1998, Hugenholtz *et al.* analyzed 16S rRNA gene sequences of 5,224 cultured bacteria and 2,918 environmental gene clones retrieved from a wide range of natural and artificial ecosystems, revealing ubiquitous bacterial groups to be those of the phyla *Proteobacteria*, *Actinobacteria*, *Firmicutes*, *Bacteroidetes*, *Planctomycetes*, *Acidobacteria*, *Verrucomicrobia*, and *Chloroflexi*, and the candidate phylum OP11 (33). In the past decade, a flood of papers reporting the molecular detection of *Chloroflexi* phylotypes in a variety of environments have been published, further supporting their

**Table 1.** Relative abundance of *Chloroflexi* phylotypes in natural and artificial ecosystems as determined by 16S rRNA gene-based clone library analyses

| Subphyla | Natural habitats | | | | | | | | | | | Artificial habitats | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Sediment | | | | Soil | | | Hot spring | Freshwater | Ocean | Hypersaline lake | Wastewater treatment | | Lagoon | Mine drainage | Microbial fuel cell system |
| | Deep seafloor | Sea | Lake | River | Agricultural | Geothermal | Meadow | | | | | Aerobic | Anaerobic | | | |
| Subphylum I (*Anaerolineae* and *Caldilineae*) | ○[a] | ○ | ● | ●-●● | ●●● | ○ | ○ | ○-● | ○ | — | ○-●●● | ● | ○-●●● | ○ | ● | ○-●● |
| Subphylum II ('*Dehalococcoidetes*') | ○ | — | — | ●● | — | — | — | — | — | — | — | — | — | — | — | — |
| Subphylum III (*Chloroflexi*) | — | — | — | — | — | — | — | ○-● | — | — | ○-●●● | ○ | — | — | ● | — |
| Subphylum IV | ○-●●● | — | — | — | — | — | — | — | ● | ○ | — | — | — | — | — | — |
| New subphyla | ○ | — | — | ○ | — | — | ● | — | — | — | — | — | — | — | — | — |
| References | 59 | 54 | 82 | 46 | 5 | 78 | 22 | 48 | 85, 99 | 29 | 50 | 42, 45, 56 | 3, 24, 58, 64, 90 | 97 | 79 | 39, 64 |

[a] Frequency of clones assigned to a subphylum of *Chloroflexi* as a percentage of the total number of bacterial sequencs analyzed: —, 0%; ○, 0.1–5%; ●, 5–15%, ●●, 15–25%, ●●●, >25%.

ubiquity in natural and engineered environments (Table 1). For example, *Chloroflexi* phylotypes were found as the most numerous bacterial group (nearly 80% of all bacterial gene clones analyzed) in an organic-rich deep subseafloor biosphere (38), where these phylotypes mainly fall into 'subphyla I, II and IV'. Similarly, *Chloroflexi* are often one of the most dominating bacterial phyla in various deep subseafloor sediments (*e.g.*, (7), see also a recent review by Fry *et al.* (27)), some of which were associated with methane hydrate-bearing sites (38, 59). Other natural environments where *Chloroflexi* phylotypes were detected in abundance are hot springs (*e.g.*, (4, 48)), hypersaline microbial mats (*e.g.*, (50)), soil (*e.g.*, agricultural soils (5, 75), geothermal soils (78), and low-temperature meadow soils (14)), sediment (*e.g.*, sea and lake (river) sediment (18, 35, 46, 54, 82, 89), and hydrothermally active sediment (79, 83)), chlorinated-solvent-contaminated aquifer sites (21, 26, 88)), oceans (*e.g.*, (6, 29, 63, 87)), and freshwater (*e.g.*, (85, 99)).

Recently, Lau *et al.* analyzed the bacterial communities of microbial mats from five hot springs (temperature: 60–65°C), indicating that 10–15% of the phylotypes detected were related with the *Chloroflexi* phylum (48). These *Chloroflexi* phylotypes fall into 'subphyla I and III'. Similarly, *Chloroflexi* phylotypes were found in a hypersaline (salinity: 8%) microbial mat as the majority of the mat constituents (21–39% of the bacterial rRNA clones analyzed were those of the *Chloroflexi* phylum) (50). These *Chloroflexi* phylotypes, again, fall into the 'subphyla I and III'. In this study, quantitative rRNA-targeted dot blot hybridization was conducted with an oligonucleotide probe specific for the phylum *Chloroflexi* (GNSB941 probe, Fig. 2, Table 2), estimating their abundance to be 22–41% of the total rRNA. These microbial mats were fueled by sunlight, and therefore photosynthetic bacteria of the class *Chloroflexi* ('subphylum III') were found in association with uncultured 'subphylum I' organisms. Costello *et al.* determined the bacterial community of a tundra wet meadow soil, where the annual soil temperature was 0.3°C and the temperature was stable throughout the year, revealing *Chloroflexi* phylotypes to make up 16% of the bacterial rRNA gene clones (14). In this case, the phylotypes found were classified into 'subphylum I' as well as other formerly and currently uncultured, previously unrecognized groups at the subphylum level, including the class *Ktedonobacteria* (see below). These findings suggest a wide range of temperature (0 to 65°C) and salinity for the habitats of *Chloroflexi*.

Soil is believed to be one of the most complex environments for microbial life (15). Concerning *Chloroflexi* phylotypes in soil environments, a recent review by Janssen showed the *Chloroflexi* to be one of the most dominant phyla in soils: 32 previously published clone libraries for different soil samples were re-evaluated and the mean contribution of the *Chloroflexi* phylum to soil bacterial communities was found to be 3% (range: 0–16%) (40). Similarly, pyrosequencing of rRNA gene clone libraries (*ca.* 150,000 clones in total) for different soils further supported the significance and genetic diversity of the *Chloroflexi* population (22, 65). Concerning the *Chloroflexi* phylum in oceanic and freshwater bacterioplanktons, environmental clones belonging to 'subphylum IV' (formerly known as cluster SAR202) were found in the Sargasso sea (4% of bacterial clones) (29), and similar phylotypes were detected in different ocean samples (6). Freshwater bacterioplankton also often contain the *Chloroflexi* phylum (average: 1%, range: 0–4%), most of them affiliated with 'subphyla I and IV' (85, 99).

*Chloroflexi* phylotypes have also been found in abundance in artificial and engineered ecosystems, such as lagoons (*e.g.*, (97)), mine drainage (*e.g.*, (73, 79)), anaerobic sludges for waste and wastewater treatment (*e.g.*, (2, 12, 17, 19, 25, 30, 43, 51, 57, 58, 64, 66, 71, 74, 76, 90, 94)), activated sludge systems (*e.g.*, (3, 8, 13, 25, 42, 45, 47, 55, 56, 60, 62, 77, 80, 98)), and microbial fuel cell systems (39, 61). Among these ecosystems, waste/wastewater treatment facilities are perhaps the best-recognized habitat where *Chloroflexi* phylotypes reside in abundance. For example, Rivière *et al.* evaluated bacterial and archaeal community structures of seven mesophilic (29–37°C), anaerobic (methanogenic) digesters decomposing municipal sewage sludge and found the *Chloroflexi* to be the most abundant bacterial phylum (average: 32% of all the bacterial clones analyzed, range: 15–45%)

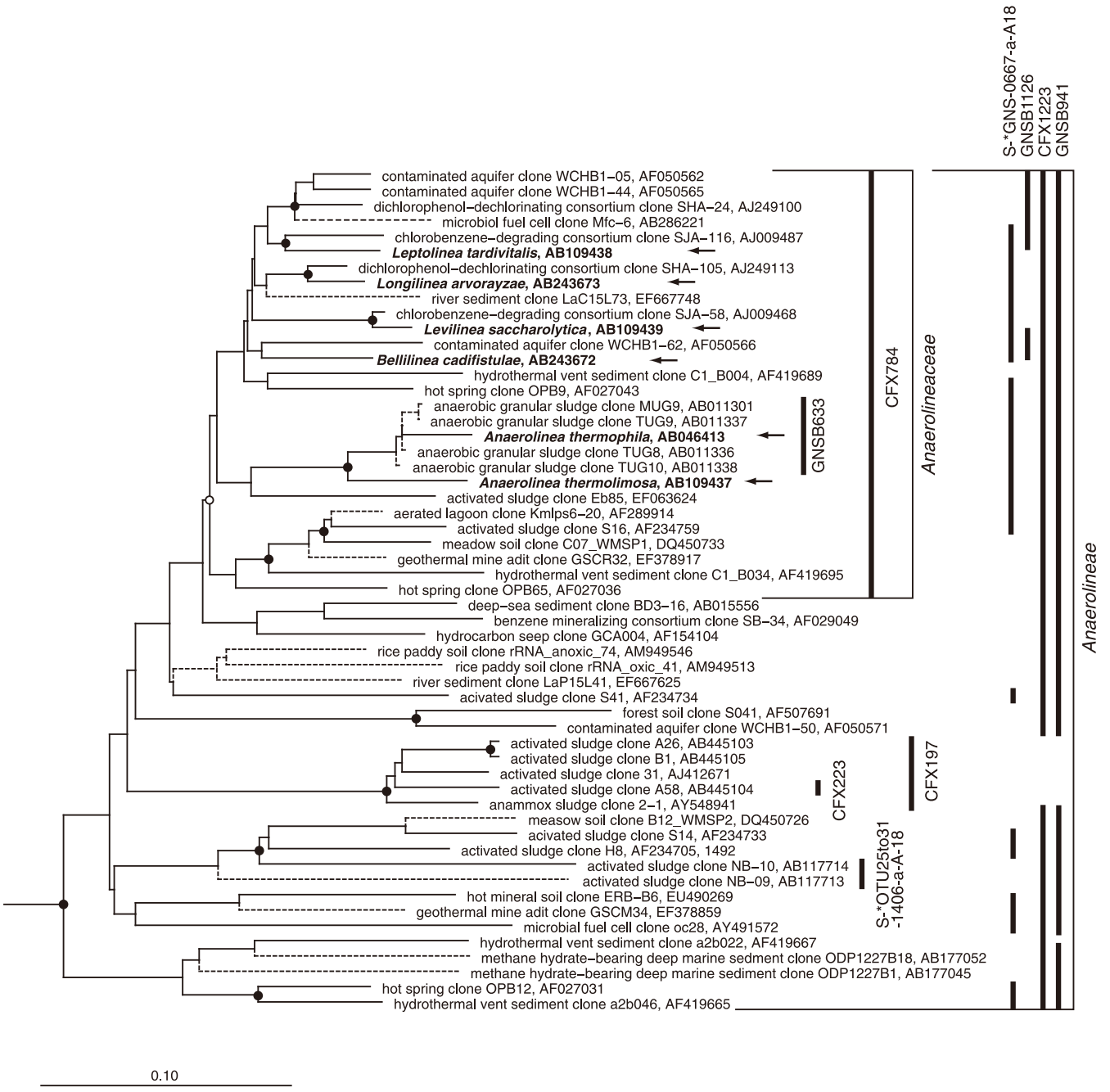


**Fig. 2.** Evolutionary distance dendrogram of the class *Anaerolineae* derived from a comparative analyses of 16S rRNA gene sequences, showing the phylogenetic positions of cultivated strains belonging to the class (indicated by arrow) and other related gene clones that were retrieved from a variety of environments. The sequences were aligned, and the phylogenetic tree was constructed by the NJ method with the ARB software package (52). The base tree was constructed with >1,200 nt sequences. Partial sequences of <1,200 nt (highlighted with dotted branches) were inserted into the base tree using the parsimony insertion tool of the ARB program. 16S rRNA gene sequences of members affiliated with the phylum *Thermotogae* were used as outgroups (not shown in the tree). The bar represents 10 nucleotide substitutions per 100 nucleotides. The fidelity of the topology of the NJ tree was also confirmed by bootstrap resampling (based on 1,000 replicates) with the neighbor-joining (PAUP* 4.0 program package) and maximum likelihood (TREEFINDER program package) methods. Nodes highly supported with bootstrap values higher than 85% by both analyses are marked as circles (filled circles, >95%; open circles, 95–85%). Nodes without symbols were not highly resolved (<85%) as specific groups in either analysis. Probe specificity is shown to the right of the figure with the probe name; the bars indicate a perfect match between the probe and target sequences.

(64). All the *Chloroflexi* phylotypes detected were affiliated with 'subphylum I'. Chouari *et al.* conducted quantitative rRNA-targeted dot blot hybridization with an oligonucleotide probe specific to the phylum *Chloroflexi* (GNSB1126 probe, Fig. 2, Table 2) for a mesophilic (33°C), anaerobic digester for sewage sludge, estimating their abundance to be 20% of the total rRNA (12). Similarly, Narihiro *et al.* estimated the microbial diversity of twelve different types of mesophilic (35–40°C), anaerobic (methanogenic) sludges treating organic wastewaters, detecting *Chloroflexi* phylotypes as a predominant phylum (average: 12% of the bacterial clones analyzed, range: 0–32%) (58). The abundance of

**Table 2.** 16S rRNA-tageting oligonucleotide probes used for in situ detection of the *Chloroflexi* phylum and 'subphylum I'

| Oligonucleotide | Target group | Probe sequences (5'-3') | Target site (*E. coli* position) | Length (nt) | References |
|---|---|---|---|---|---|
| GNSB941 | virtually all members of the phylum *Chloroflexi* | AAACCACACGCTCCGCT | 941–957 | 17 | 28 |
| CFX1223 | virtually all members of the phylum *Chloroflexi* | CCATTGTAGCGTGTGTGTMG | 1223–1242 | 20 | 8 |
| GNSB1126 | members of the class *Anaerolineae* | AACACACAGCGAGGG | 1112–1126 | 15 | 12 |
| CFX784 | members of the class *Anaerolineae* | ACCGGGGTCTCTAATCCC | 784–801 | 18 | 8 |
| GNSB633 | *Anaerolinea thermophila* | TAGCCCGCCAGTCTTGAACG | 633–652 | 18 | 68 |
| S-*OTU25to31-1406-a-A-18 | uncultured phylotypes of the class *Anaerolineae* | CCAGCTCCCATGACGTGA | 1406–1423 | 18 | 42 |
| S-*GNS-0667-a-A18 | uncultured phylotypes of the class *Anaerolineae* | CACCCSGAATTCCACRTT | 667–684 | 18 | 45 |
| CFX197 | uncultured phylotypes of the class *Anaerolineae* | TCCCGGAGCGCCTGAACT | 197–214 | 18 | 80 |
| CFX223 | uncultured phylotypes of the class *Anaerolineae* | GGTGCTGGCTCCTCCCAG | 223–240 | 18 | 80 |

Groups targeted by the probes are shown in Fig. 2.

these phylotypes varied depending on wastewater type, and all of them fell into 'subphylum I'. Similar phylotypes ('subphylum I') have been frequently found in anaerobic sludges that had treated wastewaters containing compounds recalcitrant to biodegradation, such as phenol (11% of bacterial clones) (24), phthalates (4–7%, (51, 90)), 4-methylbenzoate, (7%, (90)), 2,4-dinitroanisole and n-methyl-4-nitroaniline (36–42%, (3)).

*Chloroflexi* phylotypes are often present in activated sludge systems; phylogenetic analysis of activated sludge clones belonging to the *Chloroflexi* phylum indicated that they are affiliated with 'subphyla I and III' (8, 47). These clones were most abundant in submerged membrane bioreactors treating municipal wastewater (*Chloroflexi* phylum-specific probes, GNSB941 and CFX1223, were used for in situ detection and the probe-reactive cells accounted for 14–26% of the total, (56)). The phylotypes found in the systems again fall into 'subphyla I and III'. Nitrifying systems were also shown to contain these *Chloroflexi* in abundance; Kindaichi *et al.* found that phylotypes belonging to 'subphylum I' were dominant (13% of bacterial clones analyzed) in nitrifying biofilms formed in a submerged rotating disk reactor (45). *Chloroflexi* cells were found in abundance in nitrifying-denitrifying systems, in which uncultured 'subphylum I' cells (as detected using the probe S-*OTU25to31-1406-a-A-18, Fig. 2) accounted for 16% of the cells (42). Another example of these engineered ecosystems is the microbial fuel cell. The microbial fuel cell is a bio-electrochemical system that generates electric power from organic matter, in which *Chloroflexi* phylotypes are often found. For example, a phylotype affiliated with 'subphylum I' was one of the most dominant (17% of bacterial clones) in a microbial fuel cell system fed with cellulose (39).

These molecular inventories of *Chloroflexi* phylotypes in a wide range of natural and human-made ecosystems strongly suggest the ecological significance and physiological breadth of these organisms, playing indispensable roles in such habitats.

### *Cultivation of uncultured Chloroflexi at the subphylum level*

**The classes *Anaerolineae* and *Caldilineae* as '*Chloroflexi* subphylum I'.** Phylotypes affiliated with 'subphylum I' are detected in a wide range of environments, and the group now contains the most diverse rRNA gene sequences among known subphyla with strong natural and biotechnological relevance. To unveil their physiology, attempts were made to cultivate them, and the first pure culture representing the group, *i.e.*, *Anaerolinea thermophila*, was obtained in 2001 from a thermophilic (55°C) anaerobic sludge treating organic wastewater (71, 72). Simultaneously, the second tangible organism of the group, *Caldilinea aerophila*, was obtained from a hot spring (72). Later, a new thermophilic species of the genus *Anaerolinea* and four species of four genera, *i.e.*, *Levilinea*, *Leptolinea*, *Bellilinea*, and *Longilinea*, were successfully cultivated and characterized (92, 93). To our knowledge, these seven species are only the cultivated organisms of 'subphylum I'.

The morphology, physiology, and genetic properties of cultivated strains of the two classes are shown in Table 3. The strains are anaerobic or aerobic, mesophilic or moderately thermophilic, multicellular filamentous, chemolitho-organoheterotrophic organisms degrading carbohydrates and amino acids (or peptides). No growth was found in the dissimilatory reduction of nitrate and sulfate. It may not be appropriate to conclude that 'subphylum I' is comprised solely of such heterotrophs, but based on the unveiled physiological traits of these microorganisms, common features that make 'subphylum I' recalcitrant to isolation are likely to be (1) a relatively slow growth rate compared to commonly cultivable microbes and/or (2) the need to associate with other microbes (syntrophy) for efficient growth. In fact, the *Anaerolinea*-type anaerobes cultivated so far are all very slow growers (doubling time: 45–92 hrs), and hence are easily outcompeted by fast-growing heterotrophic anaerobes like *Clostridia*- and *Thermoanaerobacter*-type cells. We actually found that irrelevant fast-growing microbes immediately outcompeted *Anaerolinea*-type cells when we

**Table 3.** Characteristics of cultivated species belonging to classes *Anaerolineae* and *Caldilineae* in the phylum *Chloroflexi*

| Characteristic | Class *Anaerolineae* | | | | | | Class *Caldilineae* |
|---|---|---|---|---|---|---|---|
| | *Anaerolinea thermophila* | *Anaerolinea thermolimosa* | *Levilinea saccarolytica* | *Leptolinea tardivitalis* | *Bellilinea caldifistulae* | *Longilinea arvoryzae* | *Caldilinea aerophila* |
| Type strain | strain UNI-1$^T$ | strain IMO-1$^T$ | strain KIBI-1$^T$ | strain YMTK-2$^T$ | strain GOMI-1$^T$ | strain KOME-1$^T$ | strain STL-6-O1$^T$ |
| Cell diameter (μm) | 0.2–0.3 | 0.3–0.4 | 0.4–0.5 | 0.15–0.2 | 0.2–0.4 | 0.4–0.6 | 0.7–0.8 |
| Temperature range (°C) | 50–60 | 42–55 | 25–50 | 25–50 | 45–65 | 30–40 | 37–65 |
| Optimum growth temperature (°C) | 55 | 50 | 37–40 | 37 | 55 | 37 | 55 |
| pH range | 6.0–8.0 | 6.5–7.5 | 6.0–7.2 | 6.0–7.2 | 6.0–8.5 | 5.0–7.5 | 7.0–9.0 |
| Optimum growth pH | around 7.0 | around 7.0 | around 7.0 | around 7.0 | around 7.0 | around 7.0 | around 7.5–8.0 |
| Doubling time (h) | 72 (48)* | 48 (10)* | 56 (56)* | 50 (50)* | 45 (29)* | 92 (38)* | 5 (N.D)* |
| $O_2$ respiration | – | – | – | – | – | – | + |
| Major cellular fatty acids | $C_{16:0}$, $C_{15:0}$, $C_{14:0}$ | ai-$C_{17:0}$, i-$C_{15:0}$, $C_{16:0}$ | $C_{14:0}$, i-$C_{15:0}$, $C_{16:0}$ | Branched $C_{17:0}$, $C_{16:0}$, $C_{14:0}$ | $C_{16:0}$, $C_{14:0}$, i-$C_{15:0}$ | i-$C_{15:0}$, ai-$C_{15:0}$, $C_{14:0}$, | $C_{18:0}$, $C_{16:0}$, $C_{17:0}$ |
| Major quinone | – | – | – | – | – | – | MK-10 |
| DNA G+C content (mol%) | 54.5 | 53.3 | 59.5 | 48.2 | 54.7 | 54.5 | 59.0 |
| Utilization in the presence of yeast extract of: | | | | | | | |
| Tryptone | ± | + | + | + | ± | + | + |
| Betain | ND | – | ± | + | – | – | ND |
| Pyruvate | ± | + | + | ± | + | – | + |
| Glucose | + | + | + | + | + | – | + |
| Mannose | + | + | ± | + | + | – | – |
| Galactose | + | + | ± | ± | + | – | ND |
| Fructose | + | + | + | + | + | ± | – |
| Arabinose | ± | + | – | ± | + | – | – |
| Xylose | ± | + | + | + | ± | – | – |
| Ribose | ± | + | + | + | + | – | – |
| Pectin | ± | ± | ± | + | + | + | ND |
| Starch | + | ± | – | ± | – | – | + |
| Isolation source | Thermophilic UASB sludge | Thermophilic UASB sludge | Mesophilic UASB sludge | Mesophilic UASB sludge | Thermophilic anaerobic sludge | Rice paddy soil | Hot spring |
| Reference | 71, 72 | 93, 94 | 93, 94 | 93, 94 | 92 | 92 | 72 |

* Doubling time in parentheses indicates that for sytrophic growth with hydrogenotrophic methanogens.
–, Negative; ±, variable; +, positive; ND, not determined. Only differences found among the strains are listed. All strains showed the following characteristics: multicellular filamentous morphology; growth under anaerobic conditions (fermentation).

attempted to isolate them (70, 71). This is probably the primary reason why many attempts to isolate 'subphylum I' organisms have failed. Consequently, selecting appropriate inocula, in which 'subphylum I'-type cells are highly abundant, is one of the keys to success (71). In fact, the cultivation and isolation of *Anaerolinea thermophila* was possible only when we used spine-like structures of sludge granules as the inoculum, in which *Anaerolinea* cells were highly concentrated. In the isolation, highly enriched portions of *Anaerolinea* cells were found by fluorescence *in situ* hybridization (FISH) with the probe GNSB633 (Fig. 2, Table 2), and were carefully washed and serially diluted in liquid medium. In this case, the fast-growing anaerobes outgrew the *Anaerolinea* cells in lower dilutions, but the *Anaerolinea* grew slowly in the highest dilution, in which growth was also checked as determined by FISH with GNSB633. Such rRNA-directed cultivation may be also important for cultivating uncultured cells. In fact, four strains of the genera *Anaerolinea*, *Levilinea* and *Leptolinea* were successfully isolated by this approach, with rRNA-directed cultivation using inocula that contain 'subphylum I' cells in abundance (71, 94).

Another approach that eliminates irrelevant fast-growing heterotrophic microbes (i.e., 'subphylum I' cells) is to establish primary enrichment cultures that allow the growth of other slow growing bacteria, such as syntrophic propionate-oxidizers (37). When we constructed methanogenic, syntrophic propionate-degrading enrichment cultures, we found that they contained GNSB941 probe-positive filamentous cells as a concomitant population (92). Therefore, the enrichment cultures were transferred to anaerobic media that support the growth of *Anaerolinea*-type cells, resulting in the cultivation and isolation of two additional anaerobes of the genera *Bellilinea* and *Longilinea* (92). In primary enrichment cultures, they might survive on certain remnants from the propionate-oxidizing community. Similarly, *Caldilinea aerophila* was isolated from a primary aerobic enrichment culture to focus on the isolation of chemolithotrophic thermophiles (72). For the primary enrichment culture, thiosulfate was used as the sole energy source, and the cultured cells were subsequently transferred to an aerobic organic medium, resulting in the cultivation and isolation of *C. aerophila*. Similar to *Bellilinea* and *Longilinea*, *C. aerophila* survived on remnants from the community that formed during the

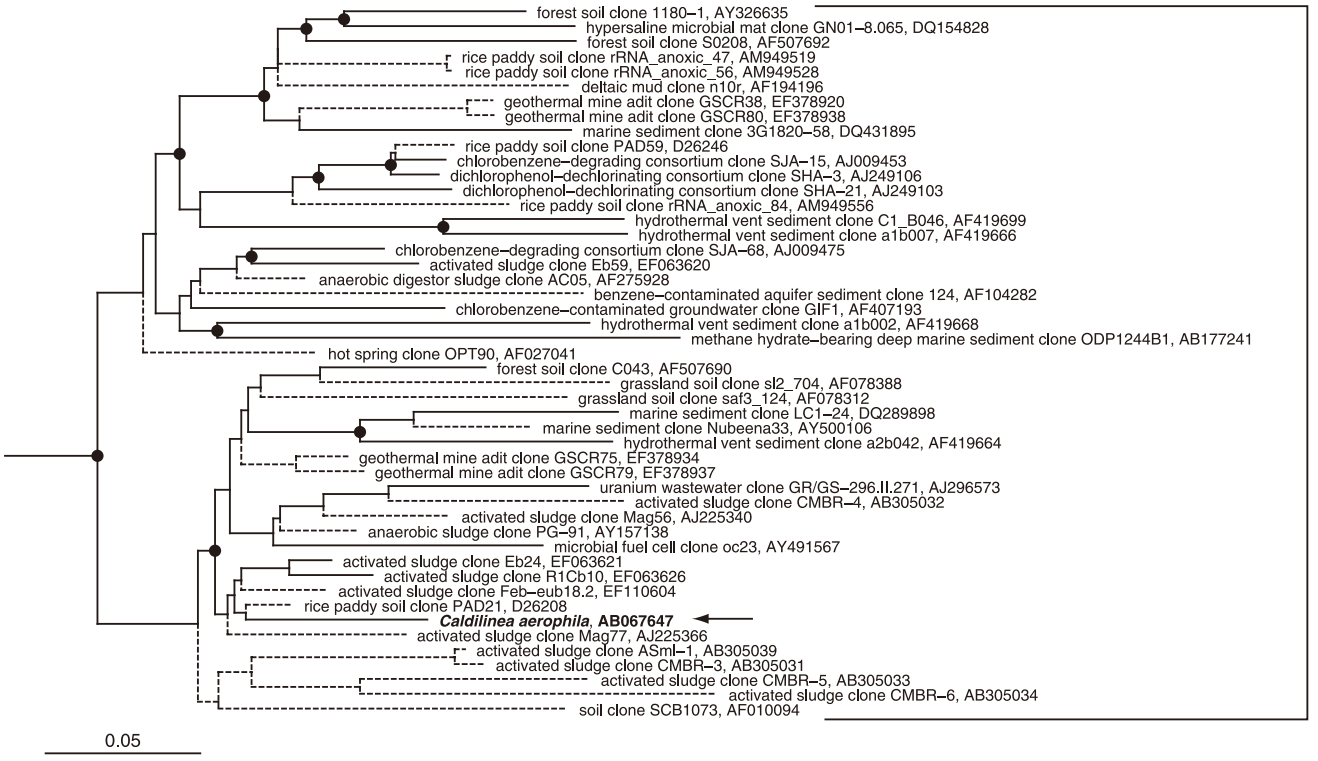


**Fig. 3.** Evolutionary distance dendrogram of the class *Caldilineae* derived from a comparative analysis of 16S rRNA gene sequences, showing the phylogenetic positions of *Caldilinea aerophila* (indicated by arrow) and other related gene clones that were retrieved from a variety of environments. The tree was constructed and marked as described in the legend of Fig. 2. The bar represents 5 nucleotide substitutions per 100 nucleotides.

primary enrichment, because *C. aerophila* cannot utilize thiosulfate as an energy source. In contrast, inoculating the original sample (hot spring microbial mat) directly into the same aerobic, organic medium resulted in the cultivation of typical fast growers like *Thermus*-type cells (72). These cases also demonstrated that the elimination of fast-growers from an inoculum is indispensable to the cultivation of 'subphylum I'.

Co-cultivation is an additional strategy. In the enrichment of *Anaerolinea*-type anaerobes, hydrogenotrophic methanogens were added beforehand, which stimulated the growth of 'subphylum I' anaerobes (94). Interestingly, some 'subphylum I' anaerobes produced hydrogen as an end product of fermentation, and grew more rapidly when co-cultivated with hydrogenotrophic methanogens (Table 3), indicating that they are "semi-syntrophic" bacteria requiring a hydrogen-scavenging partner for efficient growth (71, 72, 92, 93). Because microorganisms rarely live in pure cultures, this approach may be generally applicable to uncultured strains, whereby in situ conditions are appropriately mimicked, using co-cultivation for example.

These results indicated that it is still feasible to apply traditional cultivation techniques to the isolation of unseen microbes, if they are used thoughtfully in combination with molecular tools, and with carefully selected inocula, which contain sufficient amounts of targeted cells. By using a combination of these approaches, more novel microbes belonging to 'subphylum I' may be obtained.

Concerning the higher taxonomy of these organisms, the monophyly of 'subphylum I' was evaluated in detail based on the 16S rRNA gene sequences of these cultured organisms and environmental gene sequences, and the group was found not to be a monophyletic taxon (93), as suggested by Hugenholtz and Stackebrandt (34). In particular, the *Caldilinea* cluster did not often form a clade with other members of *Anaerolineae* (93). Thus, it was concluded that 'subphylum I' should be phylogenetically subdivided into at least two class-level taxa (Fig. 1), the class *Anaerolineae* representing the majority of lineages in 'subphylum I' (Fig. 2) and the class *Caldilineae* representing a deeply branched lineage relative to the *Anaerolineae* (Fig. 3). Physiologically, distinct differences, such as aerobic respiration, were found in the cultured members of the two classes (Table 3). Whole-genome analyses of *A. thermophila* and *C. aerophila* are now underway to unveil detailed genetic properties of these organisms (http://www.bio.nite.go.jp/ngac/e/project-e.html), which may also help to further assess the evolutional relationship among the *Chloroflexi* subphyla.

**Other cultured subphyla of the *Chloroflexi* phylum.** Recently, a novel strain was isolated from a soil and named *Ktedobacter racemifer* (11). The bacterium is a Gram-positive, aerobic, chemolithoorganoheterotrophic organism that produces branched vegetative mycelia, growing well under microaerobic conditions; this finding further expanded the morphological diversity of the phylum. Phylogenetically, the bacterium represents a new subphylum-level clade of the *Chloroflexi* phylum, to which the name *Ktedonobacteria* classis nov. has been given (11, 23). The subphylum (class)-level clade contains a variety of environmental gene clones (>100 sequences in public databases) retrieved mainly from

soil samples (these sequences can be browsed on the recent version of the greengene database (http://greengenes.lbl.gov/).

In addition to the class *Ktedonobacteria*, Davis *et al.* successfully isolated a strain belonging to a formerly uncultured *Chloroflexi* clade at the subphylum level, called the Ellin7237 lineage, from a soil sample using nontraditional aerobic media solidified with gellan (16). The lineage contains a relatively small number of environmental gene sequences mainly from soil environments (these sequences can also be seen on the greengene database (http://greengenes.lbl.gov/). Although detailed physiological properties of the strain have not yet been published, the bacterium will also provide new information on the genetic and phenotypic traits of the newly cultured subphylum. Gellan-based methods were found to be more effective than conventional agar-based techniques for culturing uncultured strains (10, 41, 44), resulting in the isolation of novel bacterial lineages, even at the phylum level, using media solidified with gellan (16, 81). This approach may also be useful for further isolating and characterizing uncultured *Chloroflexi* phylotypes.

### *Ecophysiology and function of the* Chloroflexi *'subphylum I'*

**Members of the class *Anaerolineae* in anaerobic sludge.** The cultured *Anaerolineae* (the cultured members of the genera *Anaerolinea*, *Levilinea*, *Leptolinea*, *Bellilinea*, and *Longilinea*) share common physiological and morphological traits, such as anaerobic (fermentative) growth on carbohydrates and/or peptides (amino acids) and a multicellular filamentous morphology. Considering that the class *Anaerolineae* contains a number of rRNA gene clone sequences mainly retrieved from anoxic environments, most of which were obtained from anaerobic (methanogenic) sludges (2, 12, 30, 51, 57, 58, 64, 66, 71, 74, 90, 94), the common physiological traits of the cultured *Anaerolineae* are likely to represent those of organisms in the *Anaerolineae*, at least those of the *Anaerolineaceae* lineage (Fig. 2). For example, a layered microbial structure of different trophic groups of anaerobes was often found within granular sludges in upflow anaerobic sludge blanket (UASB) systems treating organic wastewaters (e.g., (36, 70)), with the *Anaerolineae*-type populations often found in the outer most layer of such sludge granules (66, 70, 71, 94). This unique architecture of sludge granules is considered to be a result of substrate profiles formed within the granules (68). That is, because the methanogenic conversion of organic matter is driven by a food web of different trophic groups of anaerobes, i.e., fermentative heterotrophs, proton-reducing syntrophic bacteria, and methanogenic archaea (68), fermentative heterotrophs, that mainly utilize primary substances in wastewaters such as carbohydrates, mainly reside in the outer layer of granules. That *Anaerolineae* populations were found in the outer layers of sludge granules suggests them to be heterotrophic degraders, decomposing carbohydrates, for example. In addition, because yeast extract and peptides are good substrates for cultured *Anaerolineae* (Table 3), and some *Anaerolineaceae*-type filaments were also found inside of sludge granules (94), they may be able to act as degraders of cellular materials (like amino acids) that are present inside sludge granules.

Based on FISH using oligonucleotide probes for *Chloroflexi* members (Fig. 2 and Table 2), the *Anaerolineae* in anaerobic sludge were shown to be filamentous. For example, FISH using a probe (GNSB 633) specific for *Anaerolinea thermophila* showed that all of the probe-reactive cells in thermophilic sludges had a thin-filamentous morphotype (70, 71). FISH experiments using GNSB633 or a *Chloroflexi*-specific probe (GNSB941) for various types of anaerobic sludge also indicated that the probe-reactive cells were all filamentous with a wide range of thicknesses (66, 70, 71, 94). Considering that almost all the *Chloroflexi*-related 16S rRNA gene sequences in anaerobic sludges are classified into the class *Anaerolineae*, the filamentous morphology observed with the GNSB941 probe may be a common trait of the *Anaerolineae* lineage.

Interestingly, this filamentous morphotype was found to be important for biotechnological reasons: *Anaerolineae* members are considered important for the granulation of sludge in UASB reactors, as well as the formation of fluffy sludge (bulking) in similar treatment systems. The granulation of sludge (formation of sludge granules with good settleability) is the major premise for the start-up and stable operation of UASB reactors (67, 69). It was reported that the granulation of thermophilic sludges was difficult to achieve when volatile fatty-acid mixtures were used as the sole substrate, while the addition of sucrose or glucose to the influent wastewater resulted in the formation of a granular sludge with good settleability (84, 86). In thermophilic UASB reactors having well-settleable sludge granules, *Anaerolinea*-type filamentous microbes predominated on the surface of the granules (70, 71, 94). Considering these findings together with the physiological properties of the cultured *Anaerolineae*, *Anaerolinea*-type filaments are indispensable organisms to the granulation of thermophilic UASB sludges, forming a web-like coating on the surface of granules (70, 71, 94).

Besides their importance in thermophilic UASB reactors, *Anaerolinea*-type organisms microorganism are a potential causative agent for the bulking of granular sludges (71). Once these filaments outgrew in the system, the sludge became fluffy, resulting in flotation and washout of the sludge from the system (71, 95). Similar observations were made in mesophilic anaerobic systems (71, 91, 95, 96). Therefore it is important to control the growth of these filamentous *Anaerolineae* not only to enhance the formation of granules but also to prevent the bulking of sludge granules.

**Members of the class *Anaerolineae* in activated sludge systems.** Although *Anaerolineae* strains have not yet been cultivated and isolated from oxic environments, phylotypes of the class have been frequently found in oxic systems like activated sludges (Fig. 2). FISH for the detection of *Anaerolineae*-type cells in activated sludges revealed that all of the probe-reactive cells were filamentous (8, 45, 55, 56, 60, 80). For example, Björnsson *et al.* used a probe (CFX784) specific for part of the class *Anaerolineae* and a probe (CFX109) for the class *Chloroflexi* ('subphylum III') for *in situ* detection of these organisms in various activated sludge systems and found that *Anaerolineae* in sludge samples were generally thin (<1 μm), smooth filaments, although they were less abundant than filamentous *Chloroflexi* ('subphylum III')

cells as detected with CFX109 (8).

To reveal the ecophysiology of these *Anaerolineae*-type organisms in activated sludge systems, functional analyses were conducted using microautoradiography-fluorescent *in situ* hybridization (MAR-FISH) (45, 49). For example, using the MAR-FISH method, substrate uptake patterns of uncultured *Chloroflexi*, particularly those of the class *Anaerolineae*, were studied for autotrophic nitrifying biofilms and revealed that the microbes aerobically utilized N-acetyl glucosamine, a major structural component of bacterial cells, as well as amino acids, implying that they metabolize cellular materials in the biofilms (45, 60). Similarly, Miura *et al.* (55, 56) found using MAR-FISH that filamentous cells of the class *Anaerolineae* in membrane bioreactors (MBR) were metabolically versatile and preferentially utilized N-acetyl glucosamine and glucose under oxic and anoxic conditions. Zang *et al.* observed that *Anaerolineae*-related cells incorporated decayed tritium-labeled bacterial cells in activated sludges (98). Based on these observations, it may be concluded that *Anaerolineae*-type organisms in activated sludge systems are likely to utilize carbohydrates, as well as to scavenge cellular materials formed in the systems, similar to the cultured *Anaerolineae* in anaerobic sludges.

The *Chloroflexi* filaments in activated sludge may provide a stabilizing backbone for sludge flocs, explaining one important role of these organisms in the systems (8). Miura *et al.* indicated that *Anaerolineae*-type filaments were responsible for the degradation of soluble microbial products, including carbohydrates and cellular materials from cells, consequently reducing membrane fouling potential in membrane bioreactors (55, 56). *Anaerolineae*-type filaments were also shown to be a causative agent for the filamentous bulking in activated sludge systems as well. Recently, Speirs *et al.* reported that *Anaerolineae*-type filamentous cells, as detected with the probes CFX197 and CFX223 (Table 2 and Fig. 2), are causative agents for aerobic filamentous bulking, which had long been recognized as Eikelboom Type 0092 (80). These studies demonstrate the importance of *Anaerolinea* organisms in activated sludge systems, drawing an analogy between their functions in oxic and anoxic (methanogenic) biological waste/wastewater treatment systems.

**The class *Caldilineae* in natural and biotechnological systems.** No detailed in situ studies have been conducted for the class *Caldilinea*, *i.e.*, no specific oligonucleotide probes have been designed and no substrate-uptake properties have yet been elucidated. Although 16S rRNA gene sequences have been obtained from various ecosystems (Fig. 3), including hot springs (33), anaerobic sludges (76), aerobic sludges (13, 25, 56), geothermal soil (79), marine sediment (35), hydrothermal vents (83), rice paddy soils (75) and chlorinated-solvent-contaminated aquifers (88), the ecophysiology and functions of this class remain to be clarified.

### *Concluding remarks and perspectives*

In summary, recent cultivation and molecular-based studies suggest that microbes in the formerly uncultured *Chloroflexi* subphyla, *Anaerolineae* and *Caldilineae* in particular, may be filamentous, slowly growing, aerobic and anaerobic heterotrophs decomposing carbohydrates and amino acids, and often need to be associated with other microbes (syntrophy) for growth. They are ubiquitous in natural and artificial environments, and likely to play indispensable roles in ecosystems. They are often closely associated with the process performance of biological treatment systems, such as granule and floc formation and/or sludge bulking. Their ecophysiology and function have been well established based on information from cultured representatives, as well as from molecular-based ecological analyses, including an assessment of their substrate uptake patterns with the MAR-FISH technique. This synergism between traditional (or rRNA-directed) cultivation and molecular ecological analyses may be a promising strategy for further elucidating the function of these yet-to-be cultured lineages.

The cultivated *Anaerolineae* only make up part (the family *Anaerolineaceae* in Fig. 2) of the *Anaerolineae* lineage, and the class still contains surprisingly diverse, yet-to-be cultured environmental clades even at the subclass level (Fig. 2). The phylogenetic depth of the class is the highest among the *Chloroflexi* phylum (approximate rRNA gene sequence divergence of the class is 18%), possibly suggesting the presence of more genetically (and phenotypically) diverse *Anaerolineae* organisms than the cultured *Anaerolineae* strains. Similarly, the recently cultured classes, *Caldilineae* and *Ktedonobacteria*, contain only single cultured strains. Other uncultured subphyla of the *Chloroflexi* phylum remain to be characterized (63). To further unveil the function of these lineages that are less represented by cultured organisms, rRNA-directed cultivation and molecular ecological analyses may be useful, and should be applied to environments where targeted populations are abundant. To efficiently isolate and cultivate these organisms, it may be necessary to employ newly developed cultivation approaches for yet-to-be cultured microbes (10). More comprehensive studies of these *Chloroflexi* subphyla, involving cultivation, molecular ecological analyses, (meta-) genomics, and transcriptomics, will allow us to gain deeper insight into their functions, which may answer questions such as why these organisms are so abundant and ubiquitous in the environment.

### References

1. Adrian, L., U. Szewzyk, J. Wecke, and H. Görisch. 2000. Bacterial dehalorespiration with chlorinated benzenes. Nature 408:580–583.
2. Ariesyady, H.D., T. Ito, and S. Okabe. 2007. Functional bacterial and archaeal community structures of majar trophic groups in a full-scale anaerobic sludge digester. Water Res. 41:1554–1568.
3. Arnett, C.M., G. Rodriguez, and S.W. Maloney. 2009. Analysis of bacterial community diversity in anaerobic fluidized bed bioreactors treating 2,4-dinitroanisole (DNAN) and n-methyl-4-nitroaniline (MNA) using 16S rRNA gene clone libraries. Microbes Environ. 24:72–75.
4. Bachar, A., E. Omoregie, R.D. Wit, and H.M. Jonkers. 2007. Diversity and function *Chloroflexus*-like bacteria in a hypersaline microbial mat: Phylogenetic characterization and impact on aerobic respiration. Appl. Environ. Microbiol. 73:3975–3983.
5. Baertsch, C., T. Paez-Rubio, E. Viau, and J. Peccia. 2007. Source tracking aerosols released from land-applied class B biosolids during high-wind events. Appl. Environ. Microbiol. 73:4522–4531.
6. Bano, N., and J.T. Hollibaugh. 2002. Phylogenetic composition of bacterioplankton assemblages from the Arctic Ocean. Appl. Environ. Microbiol. 68:505–518.

7. Biddle, J.F., S. Fitz-Gibbon, S.C. Schuster, J.E. Brenchley, and C.H. House. 2008. Metagenomic signatures of the Peru Margin subseafloor biosphere show a genetically distinct environment. Proc. Natl. Acad. Sci. USA 105:10583–10588.
8. Björnsson, L., P. Hugenholtz, G.W. Tyson, and L.L. Blackall. 2002. Filamentous *Chloroflexi* (green non-sulfur bacteria) are abundant in wastewater treatment processes with biological nutrient removal. Microbiology 148:2309–2318.
9. Botero, L.M., K.B. Brown, S. Brumefield, M. Burr, R.W. Castenholz, M. Young, and T.R. McDermott. 2004. *Thermobaculum terrenum* gen. nov., sp nov.: A non-phototrophic gram-positive thermophile representing an environmental clone group related to the *Chloroflexi* (green non-sulfur bacteria) and *Thermomicrobia*. Arch. Microbiol. 181:269–277.
10. Cardenas, E., and J.M. Tiedje. 2008. New tools for discovering and characterizing microbial diversity. Curr. Opin. Biotechnol. 19:544–549.
11. Cavaletti, L., P. Monciardini, R. Bamonte, P. Schumann, M. Rohde, M. Sosio, and S. Donadio. 2006. New lineage of filamentous, spore-forming, gram-positive bacteria from soil. Appl. Environ. Microbiol. 72:4360–4369.
12. Chouari, R., D.L. Paslier, P. Daegelen, P. Ginestet, J. Weissenbach, and A. Sghir. 2005. Novel predominant archaeal and bacterial groups revealed by molecular analysis of an anaerobic sludge digester. Environ. Microbiol. 7:1104–1115.
13. Christensson, M., L.L. Blackall, and T. Welander. 1998. Metabolic transformations and characterization of the sludge community in an enhanced biological phosphorus removal system. Appl. Microbiol. Biotechnol. 49:226–234.
14. Costello, E.K., and S.K. Schmidt. 2006. Microbial diversity in alpine tundra wet meadow soil: Novel *Chloroflexi* from a cold, water-saturated environment. Environ. Microbiol. 8:1471–1486.
15. Curtis, T.P., W.T. Sloan, and J.W. Scannell. 2002. Estimating prokaryotic diversity and its limits. Proc. Natl. Acad. Sci. USA 99:10494–10499.
16. Davis, K.E.R., S.J. Joseph, and P.H. Janssen. 2005. Effects of growth medium, inoculum size, and incubation time on culturability and isolatiion of soil bacteria. Appl. Environ. Microbiol. 71:826–834.
17. Delbès, C., R. Moletta, and J.-J. Godon. 2008. Monitoring of activity dynamics of an anaerobic digester bacterial community using 16S rRNA polymerase chain reaction-single-strand conformation polymorphism analysis. Environ. Microbiol. 2:506–515.
18. Dhillon, A., A. Taske, J. Dillon, D.A. Stahl, and M.L. Sogin. 2003. Molecular characterization of sulfate-reducing bacteria in the Guaymas Basin. Appl. Environ. Microbiol. 69:2765–2772.
19. Diaz, E.E., A.J.A. Stams, R. Amils, and J.L. Sanz. 2006. Phenotypic properties and microbial diversity of methanogenic granules from a full-scale upflow anaerobic sludge bed reactor treating brewery wastewater. Appl. Environ. Microbiol. 72:4942–4949.
20. Dojka, M.A., J.K. Harris, and N.R. Pace. 2000. Expanding the known diversity and environmental distribution of an uncultured phylogenetic division of bacteria. Appl. Environ. Microbiol. 66:1617–1621.
21. Dojka, M.A., P.H. Hungenholtz, S.K. Haack, and N.R. Pace. 1998. Microbial diversity in a hydrocarbon- and chlorinated-solvent-contaminated aquifer undergoing intrinsic bioremediation. Appl. Environ. Microbiol. 64:3869–3877.
22. Elshahed, M.S., N.H. Youssef, A.M. Spain, and *et al.* 2008. Novelty and uniqueness patterns of rare members of the soil biosphere. Appl. Environ. Microbiol. 74:5422–5428.
23. Euzéby, J. 2007. List of new names and new combinations previously effectively, but not validly, published. Int. J. Syst. Evol. Microbiol. 57:433–434.
24. Fang, H.H.P., D.W. Liang, T. Zhang, and Y. Liu. 2006. Anaerobic treatment of phenol in wastewater under thermophilic condition. Water Res. 40:427–434.
25. Fernández, N., R.S.-Alvarez, J.A. Field, R. Amils, and J.L. Sanz. 2008. Microbial community dynamics in a chemolithotrophic dinitrification reactor inoculated with methanogenic granular sludge. Chemosphere 70:462–474.
26. Ficker, M., K. Krastel, S. Orlicky, and E. Edwards. 1999. Molecular characterization of a toluene-degrading mathanogenic consorium. Appl. Environ. Microbiol. 65:5576–5585.
27. Fry, J.C., R.J. Parkes, B.A. Cragg, A.J. Weightman, and G. Webster. 2008. Prokaryotic biodiversity and activity in the deep subseafloor biosphere. FEMS Microbiol. Ecol. 66:181–196.
28. Gich, F., J. Garcia-Gil, and J. Overmann. 2001. Previously unknown and phylogenetically diverse members of the green nonsulfur bacteria are indigeneous to freshwater lakes. Arch. Microbiol. 177:1–10.
29. Giovannoni, S.J., M.S. Rappe, K.L. Vergin, and N.L. Adair. 1996. 16S rRNA genes reveal stratified open ocean bacterioplankton populations related to the Green Non-Sulfur bacteria. Proc. Natl. Acad. Sci. USA 93:7979–7984.
30. Godon, J.-J., E. Zumsten, P. Dabert, F. Habouzit, and R. Moletta. 1997. Molecular microbiol diversity of an anaerobic digestor as determined by small-subunit rDNA sequence analysis. Appl. Environ. Microbiol. 63:2802–2813.
31. Hiraishi, A. 2008. Biodiversity of dehalorespiring bacteria with special emphasis on polychlorinated biphenyl/dioxin dechlorinators. Microbes Environ. 23:1–12.
32. Hugenholtz, P. 2002. Exploring prokaryotic diversity in the genomic era. Genome Biol. 3:reviews0003.1–reviews0003.8.
33. Hugenholtz, P., B.M. Goebel, and N.R. Pace. 1998. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. J. Bacteriol. 180:4765–4774.
34. Hugenholtz, P., and E. Stackebrandt. 2004. Reclassification of *Sphaerobacter thermophilus* from the subclass *Sphaerobacteridae* in the phylum *Actinobacteria* to the class *Thermomicrobia* (emended description) in the phylum *Chloroflexi* (emended description). Int. J. Syst. Evol. Microbiol. 54:2049–2051.
35. Hunter, E.M., H.J. Mills, and J.E. Kostka. 2006. Microbial community diversity associated with carbon and nitrogen cycling in permeable shelf sediments. Appl. Environ. Microbiol. 72:5689–5701.
36. Iguchi, A., T. Terada, T. Narihiro, T. Yamaguchi, Y. Kamagata, and Y. Sekiguchi. 2009. In situ detection and quantification of uncultured members of the phylum *Nitrospirae* abundant in methanogenic wastewater treatement systems. Microbes Environ. 24:97–104.
37. Imachi, H., Y. Sekiguchi, Y. Kamagata, A. Ohashi, and H. Harada. 2000. Cultivation and in situ detection of a thermophilic bacterium capable of oxidizing propionate in syntrophic association with hydrogenotrophic methanogens in a thermophilic methanogenic granular sludge. Appl. Environ. Microbiol. 66:3608–3615.
38. Inagaki, F., T. Nunoura, S. Nakagawa, and *et al*. 2006. Biogeographical distribution and diversity of microbes in methane hydrate-bearing deep marine sediments on the Pacific Ocean Margin. Proc. Natl. Acad. Sci. USA 103:2815–2820.
39. Ishii, S., T. Shimoyama, Y. Hotta, and K. Watanabe. 2008. Characterization of a filamentous biofilm community established in a cellulose-fed microbial fuel cell. BMC Microbiol. 8:6.
40. Janssen, P.H. 2006. Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes. Appl. Environ. Microbiol. 72:1719–1728.
41. Janssen, P.H., P.S. Yates, B.E. Grinton, P.M. Taylor, and M. Sait. 2002. Improved culturability of soil bacteria and isolation in pure culture of novel members of the divisions *Acidobacteria, Actinobacteria*, *Proteobacteria*, and *Verrucomicrobia*. Appl. Environ. Microbiol. 68:2391–2396.
42. Juretschko, S., A. Loy, A. Lehner, and M. Wagner. 2002. The microbiol community composition of a nitrifying-denitrifying activated sludge from an industrial sewage treatment plant analyzed by the full-cycle rRNA approach. Syst. Appl. Microbiol. 25:84–89.
43. Kaksonen, A.H., J.J. Plumb, P.D. Franzmann, and J.A. Puhakka. 2004. Simple organic electron donors support diverse sulfate-reducing communities in fludized-bed reactors treating acidic metal- and sufate-containning wastewater. FEMS Microbiol. Ecol. 47:279–289.
44. Kamagata, Y., and H. Tamaki. 2005. Cultivation of uncultured fastidious microbes. Microbes Environ. 20:85–91.
45. Kindaichi, T., T. Ito, and S. Okabe. 2004. Ecophysiological interaction between nitrifying bacteria and heterotrophic bacteria in autotrophic nitrifying biofilms as determined by microautoradiography-fluorescence in situ hybridization. Appl. Environ. Microbiol. 70:1641–1650.
46. Kittelmann, S., and M.W. Friedrich. 2008. Identification of novel perchloroethene-respiring microorganisms in anoxic river sediment by RNA-based stable isotope probing. Environ. Microbiol. 10:31–46.
47. Kragelund, C., C. Levantesi, A. Borger, and *et al.* 2007. Identity, abundance and ecophysiology of filamentous *Chloroflexi* species present in activated sludge treatment plants. FEMS Microbiol. Ecol.

59:671–682.
48. Lau, M.C.Y., J.C. Aitchison, and S.B. Pointing. 2009. Bacterial community composition in thermophilic microbial mats from five springs in central Tibet. Extremophiles 13:139–149.
49. Lee, N., P.H. Nielsen, K.H. Andreasen, S. Juretschko, J.L. Nielsen, K.-H. Schleifer, and M. Wagner. 1999. Combination of fluorescent in situ hybridization and microautoradiography—a new tool for structure-function analyses in microbial ecology. Appl. Environ. Microbiol. 65:1289–1297.
50. Ley, R.E., J.K. Harris, J. Wilcox, and *et al.* 2006. Unexpected diversity and complexity of the Guerrego Negro hypersaline microbial mat. Appl. Environ. Microbiol. 72:3685–3695.
51. Liang, D.-W., H.H.P. Fang, and T. Zhang. 2009. Microbial characterization and quantification of an anaerobic sludge degrading dimethyl phthalate. J. Appl. Microbiol. 106:296–305.
52. Ludwig, W., O. Strunk, R. Westram, and *et al.* 2004. ARB: A software environment for sequence data. Nucleic Acids Res. 32:1363–1371.
53. May, H.D., G.S. Miller, B.V. Kjellerup, and K.R. Sowers. 2008. Dehalorespiration with polychlorinated biphenyls by an anaerobic ultramicrobacterium. Appl. Environ. Microbiol. 74:2089–2294.
54. Mills, H.J., E. Hunter, M. Humphrys, L. Kerkhof, L. McGuinness, M. Huettel, and J. Kostka. 2008. Characterization of nitrifying, denitrifying, and overall bacterial communities in permeable marine sediments of the northeastern gulf of mexico. Appl. Environ. Microbiol. 74:4440–4453.
55. Miura, Y., and S. Okabe. 2008. Quantification of cell specific uptake activity of microbial products by unclutured *Chloroflexi* by microautoradiography combined with fluorescence in situ hybridization. Environ. Sci. Technol. 42:7380–7386.
56. Miura, Y., Y. Watanabe, and S. Okabe. 2007. Significance of *Chloroflexi* in peformance of submerged membrane bioreactors (MBR) treating municipal wastewater. Environ. Sci. Technol. 41:7787–7794.
57. Narihiro, T., and Y. Sekiguchi. 2007. Microbial communities in anaerobic digestion processes for waste and wastewater treatment: a microbiological update. Curr. Opin. Biotechnol. 18:273–278.
58. Narihiro, T., T. Terada, K. Kikuchi, A. Iguchi, M. Ikeda, T. Yamauchi, K. Shiraishi, Y. Kamagata, K. Nakamura, and Y. Sekiguchi. 2009. Comparative analysis of bacterial and archaeal communities in methanogenic sludge granules from upflow anaerobic sludge blanket reactors treating various food-processing, high-strength organic wastwaters. Microbes Environ. 24:88–96.
59. Nunoura, T., F. Inagaki, M.E. Delwiche, F.S. Colwell, and K. Takai. 2008. Subseafloor microbial communities in methane hydrate-bearing sediment at two distinct locations (ODP Leg204) in the Cascadia margin. Microbes Environ. 23:317–325.
60. Okabe, S., T. Kindaichi, and T. Ito. 2005. Fate of $^{14}$C-labeled microbial products derived from nitrifying bacteria in autotrophic nitrifying biofilms. Appl. Environ. Microbiol. 71:3987–3994.
61. Phung, N.T., J. Lee, K.H. Kang, I.S. Chang, G.M. Gadd, and B.H. Kim. 2004. Analysis of microbial diversity in oligotrophic microbial fuel cells using 16S rDNA sequences. FEMS Microbiol. Lett. 233:77–82.
62. Qin, Y.-Y., D.-T. Li, and H. Yang. 2007. Investigation of total bacterial and ammonia-oxidizing bacterial community compostion in a full-scale aerated submerged biofilm reactor for drinking water pretreatment in China. FEMS Microbiol. Lett. 268:126–134.
63. Rappé, M.S., and S.J. Giovannoni. 2003. The uncultured microbial majority. Annu. Rev. Microbiol. 57:369–394.
64. Rivière, D., V. Desvignes, E. Pelletier, S. Chaussonnerie, S. Guermazi, J. Weissenbach, T. Li, P. Camacho, and A. Sghir. 2009. Towards the definition of a core of microorganisms involved in anaerobic digestion of sludge. ISME J. 3:700–714.
65. Roesch, L.F., R.R. Fulthorpe, A. Riva, and *et al.* 2007. Pyrosequencing enumerates and contrasts soil microbial diversity. ISME J. 1:283–290.
66. Satoh, H., Y. Miura, I. Tsushima, and S. Okabe. 2007. Layered structure of bacterial and archaeal communities and their in situ activities in anaerobic granules. Appl. Environ. Microbiol. 73:7300–7307.
67. Schmidt, J.E., and B.K. Ahring. 1996. Granular sludge formation in upflow anaerobic sludge blanket (UASB) reactors. Biotechnol. Bioeng. 49:229–246.
68. Sekiguchi, Y. 2006. Yet-to-be cultured microorganisms relevant to methane fermentation processes. Microbes Environ. 21:1–15.
69. Sekiguchi, Y., and Y. Kamagata. 2004. Microbial community structure and functions in methane fermentation technology for wastewater treatment, p.361–384. *In* M.M. Nakano, and P. Zuber (ed.), Strict and Facultative Anaerobes: Medical and Environmental Aspects. Horizon Bioscience, Wymondham, UK.
70. Sekiguchi, Y., Y. Kamagata, K. Nakamura, A. Ohashi, and H. Harada. 1999. Fluorescence *in situ* hybridization using 16S rRNA-targeted oligonucleotides reveals localization of methanogens and selected uncultured bacteria in mesophilic and thermophilic sludge granules. Appl. Environ. Microbiol. 65:1280–1288.
71. Sekiguchi, Y., H. Takahashi, Y. Kamagata, A. Ohashi, and H. Harada. 2001. *In situ* detection, isolation, physiological properties of a thin filamentous microorganism abundant in methanogenic granular sludges: a novel isolate affiliated with a clone cluster, the green non-sulfur bacteria, subdivision I. Appl. Environ. Microbiol. 67:5740–5749.
72. Sekiguchi, Y., T. Yamada, S. Hanada, A. Ohashi, H. Harada, and Y. Kamagata. 2003. *Anaerolinea thermophila* gen. nov., sp. nov. and *Caldilinea aerophila* gen. nov., sp. nov., novel filamentous thermophiles that represent a previously uncultured lineage of the domain Bacteria at the subphylum level. Int. J. Syst. Evol. Microbiol. 53:1843–1851.
73. Senko, J.M., P. Wanjugi, M. Lucas, M.A. Bruns, and W.D. Burgos. 2008. Characterization of Fe(II) oxidizing bacterial activities and communities at two acidic Appalachian coalmine drainage-impacted sites. ISME J. 2:1134–1145.
74. Shigematsu, T., Y. Tang, H. Kawaguchi, K. Ninomiya, J. Kijima, T. Kobayashi, S. Morimura, and K. Kida. 2003. Effect of dilution rate on structure of a mesophilic acetate-degrading methanogenic community during continous cultivation. J. Biosci. Bioeng. 96:547–558.
75. Shrestha, P.M., M. Kube, R. Reinhardt, and W. Liesack. 2009. Transcriptional activity of paddy soil bacterial communities. Environ. Microbiol. 11:960–970.
76. Smith, N.R., Z. Yu, and W.W. Mohn. 2003. Stability of the bacterial community in a pulp mill effluent treatment system during normal operation and a system shutdown. Water Res. 37:4873–4884.
77. Snaider, J., R. Amann, I. Huber, W. Ludwig, and K.-H. Schleifer. 1997. Phylogenetic analysis and *in situ* identification of bacteria in activated sludge. Appl. Environ. Microbiol. 63:2884–2896.
78. Soo, R.M., S.A. Wood, J.J. Grzymski, I.R. McDonald, and S.C. Cary. 2009. Microbial biodiversity of thermophilic communities in hot mineral soils of Tramway Ridge, Mount Erebus, Antarctica. Environ. Microbiol. 11:715–728.
79. Spear, J.R., H.A. Barton, C.E. Robertson, C.A. Francis, and N.R. Pace. 2007. Microbial community biofabrics in a geothermal mine adit. Appl. Environ. Microbiol. 73:6172–6180.
80. Speirs, L., T. Nittami, S. Mcllroy, S. Schroeder, and R.J. Seviour. 2009. Filamentous bacterium Eikelboom Type 0092 in activated sludge plants in Australia is a member of the phylum *Chloroflexi*. Appl. Environ. Microbiol. 75:2446–2452.
81. Stott, M.B., M.A. Crowe, B.W. Mountain, A.V. Smirnova, S. Hou, M. Alam, and P.F. Dunfield. 2008. Isolation of novel bacteria, including a candidate division, from geothermal soils in New Zealand. Environ. Microbiol. 10:2030–2041.
82. Tamaki, H., Y. Sekiguchi, S. Hanada, K. Nakamura, N. Nomura, M. Matsumura, and Y. Kamagata. 2005. Comparative analysis of bacterial diversity in freshwater sediment of a shallow eutrophic lake by molecular and improved cultivation-based techniques. Appl. Environ. Microbiol. 71:2162–2169.
83. Teske, A., K.U. Hinrichs, V. Edgcomb, A. de Vera Gomez, D. Kysela, S.P. Sylva, M.L. Sogin, and H.W. Jannasch. 2002. Microbial diversity of hydrothermal sediments in the Guaymas Basin: Evidence for anaerobic methanotrophic communities. Appl. Environ. Microbiol. 68:1994–2007.
84. Uemura, S., and H. Harada. 1995. Inorganic composition and microbial characteristics of methanogenic granular sludge grown in a thermophilic upflow anaerobic sludge blanket reactor. Appl. Microbiol. Biotechnol 43:358–364.
85. Urbach, E., K.L. Vergin, L. Young, A. Morse, G.L. Larson, and S.J. Giovannoni. 2001. Unusual bacterioplankton community structure in ultra-oligotrophic Crater Lake. Limnol. Oceanogr. 46:557–572.
86. van Lier, J.B. 1996. Limitations of thermophilic anaerobic wastewater

treatment and the consequences for process design. Antonie van Leeuwenhoek 69:1–14.
87. Venter, J.C., K. Remington, J.F. Heidelberg, and *et al.* 2004. Environmental genome shotgun sequencing of the Sargasso Sea. Science 304:66–74.
88. vonWintzingerode, F., B. Selent, W. Hegemann, and U.B. Göbel. 1999. Phylogenetic analysis of an anaerobic, trichlorobenzen-transforming microbial consortium. Appl. Environ. Microbiol. 65:283–286.
89. Wise, M.G., J.V. Mcarthur, and L.J. Shimkets. 1997. Bacterial diversity of a carolina bay as determined by 16S rRNA gene analysis: Cofirmination of novel taxa. Appl. Environ. Microbiol. 63:1505–1514.
90. Wu, J.-H., W.-T. Liu, I.-C. Tseng, and S.-S. Cheng. 2001. Characterization of microbial consortia in a terephthalate-degrading anaerobic granular sludge system. Microbiology 147:373–382.
91. Wu, W.-M., J.H. Thiele, M.K. Jain, H.S. Pankratz, R.F. Hickey, and J.G. Zeikus. 1993. Comparison of rod-versus filament-type methanogenic granules: microbial population and reactor performance. Appl. Microbiol. Biotechnol. 39:795–803.
92. Yamada, T., H. Imachi, A. Ohashi, H. Harada, S. Hanada, Y. Kamagata, and Y. Sekiguchi. 2007. *Belllinea caldifistulae* gen. nov., sp. nov. and *Longilinea arvoryzae* gen. nov., sp. nov., strictly anaerobic, filamentous bacteria of the phylum *Chloroflexi* isolated from methanogenic propionate-degrading consortia. Int. J. Syst. Evol. Microbiol. 57:2299–2306.
93. Yamada, T., Y. Sekiguchi, S. Hanada, H. Imachi, A. Ohashi, H. Harada, and Y. Kamagata. 2006. *Anaerolinea thermolimosa* sp. nov., *Levilinea saccharolytica* gen. nov., sp. nov. and *Leptolinea tardivitalis* gen. nov., sp. nov., novel filamentous anaerobes, and description of the new classes *Anaerolineae* classis nov. and *Caldilineae* classis nov. in the bacterial phylum *Chloroflexi*. Int. J. Syst. Evol. Microbiol. 56:1331–1340.
94. Yamada, T., Y. Sekiguchi, H. Imachi, Y. Kamagata, A. Ohashi, and H. Harada. 2005. Diversity, localization, and physiological properties of filamentous microbes belonging to *Chloroflexi* subphylum I in mesophilic and thermophilic methanogenic sludge granules. Appl. Environ. Microbiol. 71:7493–7503.
95. Yamada, T., T. Yamauchi, K. Shiraishi, P. Hugenholtz, A. Ohashi, H. Harada, Y. Kamagata, K. Nakamura, and Y. Sekiguchi. 2007. Characterization of filamentous bacteria, belonging to candidate phylum KSB3, that are associated with bulking in methanogenic granular sludge. ISME J. 1:246–255.
96. Yoda, M., M. Kitagawa, and Y. Miyaji. 1989. Granular sludge formation in the anaerobic expanded microcarrier bed process. Water Sci. Technol. 21:165–173.
97. Yu, Z., and W.W. Mohn. 2001. Bacterial diversity and community structure in an aerated lagoon revealed by ribosomal intergenic spacer analysis and 16S ribosomal DNA sequencing. Appl. Environ. Microbiol. 67:1565–1574.
98. Zang, K., F. Kurisu, I. Kasuga, H. Furumai, and O. Yagi. 2008. Analysis of the phylogenetic diversity of estrone-degrading bacteria in activated sewage sludge using microautoradiography-fluorescence *in situ* hybridization. Syst. Appl. Microbiol. 31:206–214.
99. Zwart, G., B.C. Crump, M.P.K.V. Agterveld, F. Hagen, and S.K. Han. 2002. Typical freshwater bacteria: An analysis of available 16S rRNA gene sequences from plankton of lakes and rivers. Aquat. Microb. Ecol. 28:141–155.

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3. Sambrook, J., and D.W. Russell. 2001. Molecular Cloning: A Laboratory Manual, 3rd ed. Cold Spring Harbor Laboratory Press, New York.
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