DOI: 10.5846/stxb201307231936
周京勇, 刘冬秀, 何池全, 刘晓艳, 沈燕芬, 龙锡恩, 陈学萍.土壤中甲烷厌氧氧化菌多样性的分子检测.生态学报,2015,35(11):3491-3503.
Zhou J Y, Liu D X, He C Q, Liu X Y, Shen Y F, Long X E, Chen X P.Molecular detection of diversity of anaerobic methanotroph in soil.Acta Ecologica Sinica,2015,35(11):3491-3503.

土壤中甲烷厌氧氧化菌多样性的分子检测

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摘要:甲烷厌氧氧化作用是减少海洋底泥甲烷释放的重要生物地球化学过程, 然而在陆地生态系统中甲烷厌氧氧化作用及其功能菌群的生态功能仍然不确定。对甲烷厌氧氧化菌多样性的研究可为减少甲烷排放提供重要科学依据。与传统的分离培养方法比较,分子检测方法是一种更为快速和高效的研究手段,可直接和全面的反映参与甲烷厌氧氧化作用的功能微生物。以 DNA 分子标记物为研究对象,重点探讨三类主要的分子标记基因,即 16S rRNA, *mcr*A 和 *pmo*A,所采用的相关探针和引物信息,同时从定性和定量两个角度综述土壤甲烷厌氧氧化菌的多样性研究的主要进展,最后提出厌氧甲烷氧化菌多样性研究中存在的一些问题和相应的解决思路。

关键词:土壤; 甲烷厌氧氧化菌; 功能基因; 多样性

Molecular detection of diversity of anaerobic methanotroph in soil

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Abstract: Anaerobic oxidation of methane is the most important biogeochemical process to reduce methane released into the atmosphere from marine sediments, however, the anaerobic oxidation of methane and related functional microorganisms in soil still remain uncertain. Therefore, the studies on the diversity of anaerobic methanotrophs may be able to assist with reducing methane emissions from soil. Compared with traditional culture-dependent methods, molecular methods independent of culture techniques has vastly improved the knowledge on microbial diversity. This review mainly focused on the recent progress surrounding abundance and diversity of anaerobic methanotrophs in soils with emphasis on the molecular gene markers including 16S rRNA, *mcr*A and *pmo*A used for detecting anaerobic methanotrophs. Furthermore, the questions existing in the present research as well as the related resolution were also discussed. Methane oxidation in anoxic environments is microbially mediated and of global significance. In the last decade, the diversity of anaerobic methane oxidation populations has been studied intensively. Initially, most studies concerning environmental AOM were carried out in anaerobic marine waters and sediments where AOM was coupled to sulfate reduction. It is now known that there are also some microorganisms capable of coupling AOM to denitrification. Fluorescence in situ hybridization with target probes firstly showed that the sulfate dependent AOM archaea were in the absence of close physical association with sulfate reducing

基金项目:国家自然科学基金项目(41101230)
收稿日期:2013-07-23; **网络出版日期**:2014-06-12
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bacteria. With the development of probes, different types of AOM consortia were visualized. In addition, most investigations on the diversity of AOM archaea involved in the consortia were based on the 16S rRNA or *mcr*A gene phylogeny. Three lineages of the sulfate dependent AOM have been identified that are referred to as ANME-1, ANME-2, ANME-3. The first nitrate dependent methane oxidation cultures were initially enriched anaerobically, which contained a bacterium belonging to the candidate division NC10. "*Candidatus*, Methylomirabilis oxyfera," a member of the uncultured NC10 phylum, forms a novel taxonomic group of bacterial methanotrophs. Recently, special primers targeting methane monooxygenase (pMMO) for detection of anaerobic methanotrophs were developed. Based on these probes and primers, culture independent approaches were used to screen samples from several oxygen-limited habitats for the presence of both sulfate and nitrate dependent methane oxidation bacteria and archaea, e.g. quantitating the abundance of anaerobic methanotrophs by quantity PCR, detecting the community structure by clone library. Although methane oxidation occurs in a variety of different habitats and appears to be performed by different organisms, the distribution of AOM organisms in aquatic and terrestrial ecosystems remains to be fully revealed. Thus, several suggestions for future research on AOM processes and related microorganisms are put forward as follows: 1) to investigate more diverse terrestrial environments where AOM may occur or is known to occur based on genomic and biomarker -related methods. 2) to combine the enrichment culture with molecular method to better understand the mechanism of AOM and related microorganisms. The enrichment or isolation of these organisms will allow for a variety of novel physiological, biochemical, and genomic studies of AOM one or more key organisms. 3) to detect the environmental factors affecting the AOM process or organisms. Future biogeochemical studies also hold the potential to further our understanding of this process. 4) to explore new types of AOM microorganisms coupled with SO_4^{2-}, Mn^{4+}, Fe^{3+}, NO_3^- acting as the electron acceptors. Understanding AOM communities and the environmental conditions under which they consume methane may help to refine computational models for methane cycling on earth and should improve the accuracy of long-term climate change projections.

Key Words: soil; anaerobic methanotrophs; functional gene; diversity

甲烷(CH_4)是一种温室气体,其温室效应是二氧化碳的 26 倍,对全球变暖的"贡献率"达到 15%[1]。当今国际重大环境科学计划(例如 IGBP、WCRP、IHDP、GCTE、IPCC)中,陆地生态系统碳循环是其中的核心研究内容[2-4]。陆地湿地甲烷排放是温室气体甲烷的最大排放源[5],据估计天然湿地每年向大气中排放 110 Tg CH_4,占全球 CH_4排放总量的 15%—30%[6]。此外,IPCC 报告指出全球稻田 CH_4排放量每年高达 35—56 Tg,约占全球 CH_4排放总量的 1/5[4]。众所周知,甲烷厌氧氧化作用是海洋底泥中减少甲烷释放到大气中的重要生物地球化学过程,尽管在陆地生态系统中甲烷厌氧氧化作用广泛存在,但其过程及关键微生物尚未清楚[7]。例如,有人应用^{13}C 同位素估算垃圾填埋场渗滤液污染羽土壤,发现甲烷厌氧氧化作用消耗了 80%—90% CH_4[8]。吕镇梅等[9]同样也证实了水稻田土壤甲烷厌氧氧化过程的存在,但结果表明水田土壤中的甲烷厌氧氧化活性远低于甲烷好氧氧化活性,如以两者的氧化活性作为对甲烷氧化的贡献来计,则甲烷厌氧氧化作用的贡献率一般都在整个甲烷氧化的 10%以下。但在水田土壤淹没的情形下,由于土壤厌氧条件的形成和甲烷扩散受阻,甲烷厌氧氧化的速率明显超过好氧氧化的速率,甲烷厌氧氧化在整个甲烷氧化中的贡献率可到达 30%以上。甲烷在这些厌氧生境中由产甲烷菌形成以后,经土壤和水层,逸散至大气,在途经土壤和水层时可被栖息于其间的甲烷厌氧氧化菌(AOM)所氧化。因此探索土壤甲烷厌氧氧化菌的多样性有助于深入认知渍(淹)水土壤中甲烷厌氧消耗的微生物学机制,为减少甲烷排放通量提供科学基础,对减缓因温室效应而带来的气候变暖具有重要意义。

近几十年来,分子生态学方法已成为土壤甲烷厌氧氧化菌多样性研究的关键手段,并取得了丰硕的成果[10-12]。近几年,国内也开始广泛关注甲烷厌氧氧化作用及其功能菌,多个课题组已经对其多方面进行了综述[13-15]。本文重点对土壤甲烷厌氧氧化菌的功能基因(16S rRNA, *mcr*A, *pmo*A)所采用的相关探针和引物序

列进行了综述,并基于这些功能基因总结其在土壤甲烷厌氧氧化菌定性和定量分子检测方面的应用。

1　甲烷厌氧氧化菌的培养和分类

1.1　甲烷厌氧氧化菌的培养

由于土壤中微生物群落及环境因子极其复杂,能够被培养并分离出的微生物只是非常小的一部分,因此传统上依赖于培养的方法仅能反映不到1%的微生物种类多样性[16]。由于在富集培养条件下甲烷厌氧氧化菌生长速率慢,倍增时间长达数月,并且因其生物特性须在严格厌氧的条件下富集培养、工艺条件严格和影响因子复杂,客观上阻碍了对甲烷厌氧氧化菌功能和作用机理研究。许多科学家曾认为无法富集纯化依赖于硝酸根的甲烷厌氧氧化菌[17-20]。迄今,仅获得此类微生物的富集培养物。最初由 Ettwig 课题组从新西兰淡水底泥中富集得到硝酸盐甲烷厌氧氧化菌[21-23],此后陆续有来自其他淡水生境和人工污水处理系统中的富集培养的报道(混合培养活性污泥和淡水底泥富集[24-27])。Vecherskaya 等[28]从甲烷驯化的微氧反硝化生物反应器中筛选纯化到一株甲烷厌氧氧化菌,系统发育分析发现其属于 *Methylocystis parvus*。与硝酸盐甲烷厌氧氧化菌不同,硫酸盐甲烷厌氧氧化菌大多富集培养于海洋底泥,如 Eckernförde 海湾沉积物[29-31]、Aarhus 海湾沉积物[32]、Monterey 海湾沉积物[33]。国内闵航等[34]首次报道了 1 株从浙江象山市郊青紫泥水稻田土壤中分离到的能独立厌氧氧化甲烷的菌株。因此,到目前为止,仅有少数几个课题组能富集培养有限生境中甲烷厌氧氧化菌,不能充分描述甲烷厌氧氧化菌的多样性,而分子生态学方法的应用,能从分子水平上较为客观地揭示微生物的多样性,有效地克服了传统培养方法的不足,提高了分析检测的速度及结果的准确性和完整性。

1.2　甲烷厌氧氧化菌的分类

1.2.1　硫酸盐甲烷厌氧氧化菌(SAMO)

参与 SAMO 反应的甲烷厌氧氧化古菌(ANME)往往与硫酸盐还原细菌(SRB)形成共生体,因此甲烷氧化的同时伴随着硫酸盐的还原。根据系统发育分析,通常这类厌氧甲烷氧化菌分为三类: ANME-1(Anaerobic methanotrophic archaea)、ANME-2 、ANME-3,均属于广古菌门[35]。其中,ANME-1 与产甲烷微菌目(*Methanomicrobiales*)和产甲烷八叠球菌目(*Methanosarcinales*)有较近的亲缘关系,ANME-2 属于产甲烷八叠球菌目(*Methanosarcinales*),ANME-3 与甲烷拟球菌属(*Methanococcoides*)亲缘关系较近[35]。这三类古菌彼此间的进化距离较远,序列相似度仅为 75%—92%。即使在 ANME-2 中,分枝 ANME-2a、-2b 与-2c 相似度也较低。因此,虽然 ANME-1、ANME-2 、ANME-3 属于不同的目或科,但是都具有在各种生境厌氧氧化甲烷的能力。然而,与 ANME-2 的同源性较高的 ANME 的一个新的分枝 GoM Arc1,试验表明它并不具备氧化甲烷的能力,也不与硫酸盐还原菌(SRB)组成共生菌群[36-38]。因此,此类甲烷厌氧氧化菌在甲烷的生物地球化学循环过程中的作用还有待进一步研究。ANME-1 可以单细胞形式存在[39-40],也可以与硫酸盐还原菌以共生体的形式存在[39],在黑海中甚至以编绕式存在[40-41]。ANME-3 同样可以单细胞形式存在,或者与硫酸盐还原菌形成外壳型或者混合型的共生体。ANME-2 往往与硫酸盐还原菌以外壳型或者混合型共生体存在[40,42]。

1.2.2　硝酸盐甲烷厌氧氧化菌 (DAMO)

硝酸盐甲烷厌氧氧化菌能够在完全无氧的情况下和反硝化耦联将甲烷氧化,它的电子受体是 NO_2^- 和 NO_3^-。到目前为止,所有与反硝化过程耦合的甲烷厌氧氧化富集培养中都含有一定量(30%—80%)NC10 门细菌,而且无论生境的地理差异都与 *Methylomirabilis oxyfera* 有较高的同源性,可能是同一菌种的不同菌株(16S rRNA 同源性>97.5%)。而 NC10 门中其他种类的细菌是否具有甲烷厌氧氧化的能力不得而知。根据 16S rRNA 系统发育树,*M. oxyfera* 代表了新的甲烷营养型细菌分枝。*M. oxyfera* 和 *Verrucomicrobia* 是目前已知的唯一两类非变形菌门的甲烷营养型细菌。在过去的一个世纪里,已经分离鉴定出 100 多种甲烷氧化菌[43]。其分类及其亲缘关系的主要特点是 C1 同化途径和细胞质膜均含有大量 pMMO 酶,从而将甲烷氧化细菌主要分成Ⅰ、Ⅱ两类。Ⅰ型利用磷酸核酮糖途径(RuMP)或者 Calvin-Benson-Bassham(CBB)循环和 C1 同化途径丝氨酸循环过程的酶,属于 γ-变形菌门。II 型主要是丝氨酸同化途径,属于 α-变形菌门。根据系统发育分析,

M. oxyfera 是有别于Ⅰ、Ⅱ型的甲烷氧化菌。由于分离纯培养的困难,其生理生化特点还有待进一步研究[44]。

2 甲烷厌氧氧化菌多样性的主要分子标记物

鉴于自然环境中微生物群落的复杂性和传统的培养方法的局限性,分子生物学技术的应用越来越广泛,它能提高分子检测的速度和分析结果的准确性。检测甲烷氧化菌多样性的检测方法主要有温度梯度凝胶电泳(TGGE)、变性梯度凝胶电泳(DGGE)、荧光原位杂交(FISH)、末端限制性片段长度多态性分析(T-RFLP)、高通量测序等新兴分子生态学技术。这些分子生态学技术都需要建立在目标微生物群落的分子标记物的基础上,目前已有的甲烷厌氧氧化菌的分子标记物主要包括特异基因探针,16SrRNA,功能基因 *mcrA* 以及 *pmo*A 基因。

2.1 探针

基因探针(probe)又称“寡核苷酸探针”,简称“探针”,是一种核酸杂交应用。由于核酸分子杂交的高特异性及检测方法的高灵敏性,基因探针已经广泛用于环境微生物学中,检测土壤等生境中微生物多样性,鉴别功能基因,定性、定量分析环境微生物的存在、丰度、分布等。荧光原位杂交的是荧光标记特异核酸探针,然后与被检测的染色体或 DNA 片段变性-退火-复性进行杂交,通过荧光显微镜观察荧光信号,从而对所测目标进行定性、定量或相对定位分析。

表 1 总结了鉴定甲烷厌氧氧化菌及硫酸盐还原菌常用的一些特异性探针。这些探针中,ANME2-712 的信号比 ANME-1-538 弱,Eel-MS932 同时可以检测到 ANME-3,但是错配的几率较高,因此不推荐用此探针。引物 ANME3-1249 几乎可以覆盖 ANME-3 的所有序列,而且具有特异性。引物 AR468f 几乎可以覆盖 ANME-2c 的所有序列,但是对于 ANME-2c 没有严格的特异性。

基于上述探针,已经成功地将荧光原位杂交技术(FISH)技术应用于甲烷厌氧氧化菌种群的鉴定和种群密度的定量表达[35]。通过 FISH 试验, Boetius 等[42]首次从生物学角度证明甲烷氧化古菌与硫酸盐还原菌存在共生关系,并观察到外壳型的共生体。Raghoebarsing 等[21]同样应用 FISH 方法鉴定了甲烷氧化菌与反硝化菌的共生体:甲烷氧化菌成簇存在于细胞聚集体中央, 反硝化菌则聚集在周围。Wankel 等[52]利用 FISH 技术检测热液沉积物中中温和嗜热厌氧甲烷氧化菌,结果发现所有沉积层孔隙中存在的厌氧甲烷氧化菌为 ANME-1a,并且脱硫叠球菌属-脱硫球菌属这类厌氧甲烷菌只有在较低温度下才能观察到。Maignien 等[53]利用 FISH 技术发现 AOM 过程中总细胞的 79%为厌氧甲烷氧化菌 ANME-1 并且大部分 ANME-1 细胞形成单一反应链。随着研究深入,FISH 技术和离子质谱分析法相结合可以将 AOM 联合体中古菌的系统发育和功能结合进行研究。Orphan 等人[54]应用此技术直接证明了甲烷厌氧氧化偏好利用轻的碳同位素,与其他菌群的同化途径不同。Ettwig 等[22]将 FISH 和基质辅助激光解吸电离飞行时间质谱法相结合,发现甲烷氧化速率增加伴随古菌细胞数目下降,说明细菌可能在厌氧甲烷氧化过程中起主导作用。

2.2 16S rRNA

Woese 等[55]利用 16S 或 18S rRNA/rDNA 技术比较了二百多种原核生物和真核生物的序列图谱之后,定义并建立了古菌界,建立了真细菌(后更名为细菌)、古细菌(古菌)和真核生物三大主干。在众多生物类群中,核糖体序列保守,结构也保守,再加上 16S rRNA 相比于细菌核糖体的两外两种类型 5S rRNA 和 23S rRNA,遗传信息比较多,核苷酸数量适中,因此 16S rRNA 被认为是生物系统发育最为合适的指标,已成为应用最为广泛的标记基因[56]。

许多研究以 16S rRNA 基因作为标记基因,对不同生境中甲烷厌氧氧化菌的多样性进行了表征。Girguis 等[57]最先应用古菌通用引物对 Arch21F/Arch958R 建立克隆文库对富集培养物进行多样性分析,并设计专性引物对 AR468f/AR736r 对 ANME-2c 定量分析。Miyashita[58]设计了一系列特异性扩增甲烷厌氧氧化菌 16S rRNA 基因的一些引物对(ANME-1, ANME-2a, ANME-2b, ANME-2c and ANME-3)(表 2),并成功应用于硫酸盐浓度较低的厌氧生境中甲烷厌氧氧化菌多样性的检测,如产甲烷污泥,水稻土壤,莲底泥和天然气土壤

等。硝酸盐甲烷厌氧氧化菌是采用古菌的通用引物(如 8F/1492R) 进行 16S rRNA 基因的扩增,并通过系统发育分析进行甲烷厌氧氧化菌的分类鉴定。Ettwig 等[23]利用 FISH 探针设计成引物对,对富集培养物进行系统发育验证,并设计引物对 qP1F/qP1R, qP2F/qP2R 定量分析富集培养物的生物量。

表 1　靶标甲烷厌氧氧化菌及硫酸盐还原菌的一些特异性探针

Table 1　Oligonucleotide probes for ANME (Anaerobic Methanotroph) archaea, their sulfate-reducing partners and denitrifying methane-oxidizing bacteria

类群 Group	探针 Probe	序列 (5′-3′) Sequence (5′-3′)	靶标类型 Target type	参考文献 References
SAMO	ANME 1-350	AGTTTTCGCGCCTGATGC	ANME-1	[42]
	ANME1-862	GGCGGGCTTAACGGGCTTC	ANME-1	[45]
	ANME 2-538	GGCTACCACTCGGGCCGC	ANME-2, limnic AAA, Methanolobus tindarius, Methanococcus aeolicus	[46]
	ANME 2-712	TTCGCCACAGATGGTCCC	most ANME-2	[35]
	Eel-MS932	AGCTCCACCCGTTGTAGT	ANME-2, (ANME-3)	[42]
	ANME 2a-647	TCTTCCGGTCCCAAGCCT	ANME-2a	[40]
	ANME 2c-622	CCCTTGGCAGTCTGATTG	ANME-2c	[40]
	ANME 2c-760	CGCCCCCAGCTTTCGTCC	ANME-2c	[40]
	ANME 3-1249	TCGGAGTAGGGACCCATT	ANME-3	[47]
	ANME-3-1249H3	GTCCCAATCATTGTAGCCGGC	Helper probe for ANME-3-1249	[48]
	ANME-3-1249H5	TTATGAGATTACCATCTCCTT		[48]
	MBGB525	AGAGCTGGTTTTACCGCG	Marine benthic group B/ Deep-sea	[40]
	MBGB335	TGCGCCTCGTAAGGCCTG	archaeal group (MBGB, DSAG)	[40]
	MBGB380	GTAACCCCGTCACACTTT		[40]
	AAA-FW-641	GGT CCC AAG CCT ACC AGT	AAA	[49]
	AAA-FW-834	TGC GGT CGC ACC GCA CCT	AAA,	[49]
SRB	DSS 658	TCCACTTCCCTCTCCCAT	Desulfosarcina/Desulfococcus (including ANME-2 partners)	[50]
	DBB 660	GAATTCCACTTTCCCCTCTG	Desulfobulbus	[51]
	DBBA 655	CACTTTCCCCTCTAGTAC	ANME-3-partner (Desulfobulbus relatives)	[48]
DAMO	S-*-DBACT-0193-a-A-18	CGCTCGCCCCCTTTGGTC	Denitrifying methane oxidizing bacteria	[21]
	S-*-DBACT-0447-a-A-18	CGCCGCCAAGTCATTCGT		
	S-*-DBACT-1027-a-A-18	TCTCCACGCTCCCTTGCG		

SAMO: 硫酸盐甲烷厌氧氧化菌 Sulphate-dependent anaerobic methane oxidation;SRB:硫酸盐还原菌 Sulfate Reducing Bacteria;DAMO:硝酸盐甲烷厌氧氧化菌 Denitrification-dependent anaerobic methane oxidation

表 2　靶标甲烷厌氧氧化菌的一些 16S rRNA 基因引物

Table 2　16S rRNA gene primers targeting methanotrophs

类群 Group	引物 Primer	序列 (5′-3′) Sequence (5′-3′)	靶标类型 Targeted type	参考文献 References
SAMO	ANME-1 337f	AGGTCCTACGGGACGCAT	ANME-1	[57]
	ANME-1 724r	GGTCAGACGCCTTCGCT		
	ANME1-395F	AAC TCT GAG TGC CTC CTA AAC TCT GAG TGC CTC CAA AAC TCT GAG TGC CCC CTA	ANME-1	[58]
	ANME1-1417R	CCT CAC CTA AAC CCC ACT CCT CAC CTA AAT CCC ACT		[58]
	ANME1GBHS-183F	ATA CCT GGA ATG GGC GGA	GBHS clone group within the ANME-1	[58]

续表

类群 Group	引物 Primer	序列 (5′-3′) Sequence (5′-3′)	靶标类型 Targeted type	参考文献 References
	ANME1GBHS-841R	AAC ACC GGC ACC ACT CGT		[58]
	ANME2a-426F	TGT TGG CTG TCC GGA TGA TGT TGG CTG TCC AGA TGA TGT TGG CTG TCC AGA TGG	ANME-2a	[58]
	ANME2a-1242R	AGG TGC CCA TTG TCC CAA		[58]
	ANME2b-402F	AGT GCC AGT ACT AAG TGC	ANME-2b	[58]
	ANME2b-1251R	TTT CGA GGT AGG TAC CCA		[58]
	ANME2c-AR468f	CGC ACA AGA TAG CAA GGG CGC GCA AGA TAG CAA GGG AGC ACA AGA TAG CAA GGG	ANME-2c	[58]
	ANME2c-1411R	CCA AAC CTC ACT CAG ATG		[58]
	AR468f	CGCACAAGATAGCAAGGG	ANME-2c	[57]
	AR736r	CGTCAGACCCGTTCTGGTA		[57]
	ANME 111f	GGCTCAGTAACACGTGGA	ANME-1and ANME-2	[59]
	ARC915r	GTGCTCCCCCGCCAATTCCT		[60]
	ANME3-140F	GGA TTG GCA TAA CAC CGG	ANME-3	[58]
	ANME3-1249	TCG GAG TAG GGA CCC ATT		
	Arch20F/21F	TTCCGGTTGATCCYGCCGGA	Archaea/ANMEs	[57]
	Arch958R	YCCGGCGTTGAMTCCAATT		[57]
	ANMEF	GGCUCAGUAACACGUGGA	Archaea/ANMEs	[59]
	907R	CCGTCAATTCCTTTRAGTTT		[59]
	ARC-8f	TCCGGTTGATCCTGCC	Archaea/ANMEs	[61]
	ARC-1492r	GGCTACCTTGTTACGACTT		[61]
DAMO	202F	GACCAAAGGGGGCGAGCG	M. oxyfera	[23]
	1545R	CAKAAAGGAGGTGATCC	M. oxyfera	[62]
	8F	AGAGTTTGATYMTGGCTCAG	NC10 bacteria	[62]
	193F	GACCAAAGGGGGCGAGCG		[23]
	1043R	TCTCCACGCTCCCTTGCG		[23]
	qP1F	GGGCTTGACATCCCACGAACCTG	M. oxyfera	[23]
	qP1R	CGCCTTCCTCCAGCTTGACGC	M. oxyfera	[23]
	qP2F	GGG GAA CTG CCA GCG TCA AG	M. oxyfera	[23]
	qP2R	ŁCTC AGC GAC TTC GAG TAC AG	M. oxyfera	[23]

2.3 *mcr*A

逆甲烷生成途径是最早被提出，也是研究最多的关于甲烷厌氧氧化途径的假说。研究发现，产甲烷过程涉及的大部分酶所催化的反应都是可逆的，即在不同反应条件下，同一反应在酶的催化下可向不同方向进行，这为逆甲烷产生理论提供了理论支持。硫酸盐甲烷厌氧氧化菌在酶作用下将甲烷最终转化为 CO_2（反向产甲烷），该过程所释放的电子通过某种电子传递体转移到 SRB 中，从而使硫酸盐发生还原作用。已有研究发现 SAMO 过程中的确存在某种酶能够催化甲烷的氧化，这种酶非常类似产甲烷过程的关键酶-甲基辅酶 M 还原酶（Methyl-coenzyme Mreductase，MCR），该酶在产甲烷过程中能够催化甲烷的形成[63]。*mcr*A 基因编码甲基辅酶 M 还原酶（MCR）的 α 亚基，而 ANME-1 和 ANME-2 都有 *mcrA* 基因，在甲基辅酶 M 还原酶的作用下，甲烷首先被氧化为甲醇，再经过一系列脱氢酶的作用，最终转化为 CO_2。

表 3　靶标硫酸盐型甲烷厌氧氧化菌的一些 *mcrA* 基因引物

Table 3　Some *mcrA* gene primers targeting SAMO

引物 Primer	序列 (5′-3′) Sequence (5′-3′)	靶标类型 Targeted type	参考文献 References
MCRf	TAY GAY CAR ATH TGG YT	Methanogens/ANMEs	[64]
MCRr	ACR TTC ATN GCR TAR TT		[64]
ME1	GCM ATG CAR ATH GGW ATG TC	Methanogens/ANMEs	[65]
ME2	TCA TKG CRT AGT TDG GRT AGT	mcrA group a and c to e	[65]
AOM39_F	GCTGTGTAGCAGGAGAGTCA	Methanogens/ANMEs mcrA group b	[66]
AOM40_R	GATTATCAGGTCACGCTCAC		[66]
Forward primer	TGGTTCGGAACGTACATGTC	mcrA group a-b	[67]
Reverse primer	TCTYYTCCAGRATGTCCATG		[67]
Forward primer	GCTCTACGACCAGATMTGGCTTGG	mcrA group c-d	[67]
Reverse primer	CCGTAGTACGTGAAGTCATCCAGCA		[67]
Forward primer	CHCTGGAAGATCACTTCGGTGGTTC	mcrA group e	[67]
Reverse primer	RTATCCGAAGAARCCSAGTCKRCC		[67]
MCR-IRDf	TWY GAC CAR ATM TGG YT	Methanogens/ANMEs	[68]
MCR-IRDr	ACR TTC ATB GCR TAR TT		[68]
mcrA forward	GGTGGTGTMGGATTCACA CARTAYGCWACAGC	Methanogens/ANMEs	[69]
mcrA reverse	TTCATTGCRTAGTTWGGRTAGTT		[69]
ANME1-MCRf	GAC CAG TTG TGG TTC GGA AC	Methanogens/ANME1	[68]
ANME1-MCRAr	ATC TCG AAT GGC ATT CCC TC		[68]

2.4　pmoA

对于甲烷氧化菌的研究,应用较多的基因是编码甲烷单加氧酶(pMMO)的 *pmoA* 基因,是好氧甲烷氧化第一步($CH_4+2H^++O_2\rightarrow CH_3OH+H_2O$)的一个关键酶。*M. oxyfera* 是一个新的甲烷氧化菌的种属,能在缺氧条件下从亚硝酸盐氧化甲烷的反应中获取能量。*M. oxyfera* 厌氧微生物中 *pmoA* 基因的存在说明其特殊代谢过程:分子态的氧被从氮氧化物中还原出来,然后用生成的氧气通过由 pMMO 开始的完整好氧途径来氧化甲烷[70]。由于 *M. oxyfera* 的 *pmoA* 序列尤其是在反引物上有几个关键碱基的错配,所以用常用的好氧甲烷氧化菌 *pmoA* 基因引物对 A189/A682[71],Mb661[72]/A650[73]都不能扩增 *pmoA* 基因。Luesken 等[26]在前引物 A189 上把一个不稳定碱基替换,就变成了一个兼并引物 A189_b,它能够匹配大多数的甲烷氧化菌。同时又设计了针对亚硝酸盐为电子受体的厌氧甲烷氧化菌特异的 nest-PCR 引物,命名为 cmo182 和 cmo568。这些新引物对最早检测到 Ooijpolder 排水沟底泥中亚硝酸盐甲烷厌氧氧化菌的 DAMO 多样性[23],并且得到了特殊脂肪酸的验证[74]。到目前为止,这些引物对陆续检测了一些低浓度氧气生境的 DAMO,例如新西兰的废水处理(wastewater treatment plants (WWTP)[75-76],中国的高寒泥炭沼泽[77],德国的污染水体[78]。

表 4　靶标甲烷厌氧氧化菌的一些 *pmoA* 基因引物

Table 4　Primers targeting *pmoA* gene of methanotrophs

引物 Primer	序列 (5′-3′) Sequence (5′-3′)	特异性 Specificity	参考文献 References
A189_b	GGNGACTGGGACTTYTGG	*M. oxyfera*	[26]
cmo682	AAAYCCGGCRAAGAACGA	*M. oxyfera*	[26]
cmo182	TCACGTTGACGCCGATCC	*M. oxyfera*	[26]
cmo568	GCACATACTCCATCCCCATC	*M. oxyfera*	[26]
NA437Rdeg	RAATGTTCGRAGCGTVCCBC	NC10	[78]
NA555R	TCCCCATCCACACCCACCAG	NC10	[78]

3 甲烷厌氧氧化菌的分子多样性特征

3.1 硫酸盐甲烷厌氧氧化菌

16S rRNA 的系统发育分析发现,古菌域中至少有 3 个不同的组代表了甲烷营养型古菌:ANME-1(包括 a、b 两个分支)、ANME-2(包括 a、b、c、d 四个分支)、ANME-3。但是,根据 ANME-*mcrA* 基因的系统发育分析,甲烷厌氧氧化菌则归属于 6 个不同的发育型(a, b, c, d, e, f),其系统发育位置离产甲烷八叠球菌目等产甲烷菌较远。然而,从 16S rRNA 或 ANME-*mcrA* 建立的系统发育关系是一致的,比如基于 16S rRNA 的 ANME-1,-2c,-2a,-3 分别对应于 ANME-*mcrA* 的 a-b,c-d,e,f 分支。基于这些分子标记的系统发育分析发现,不同的发育类型即可以共同存在于一个海洋甲烷渗漏区[42],也可能以某一类型优势存在于一个生境中,比如在黑海生物垫中主要存在 ANME-1,而在水合物脊的渗漏底泥(seep-sediment from Hydrate Ridge)主要是 ANME-2[42]。此外,即使在同一生境中也呈现出不同的群落结构,比如黑海的微生物垫中同时存在 ANME-1 和 ANME-2,ANME-1 聚在内层,而 ANME-2 包围在外层,说明不同的甲烷厌氧氧化菌群落偏好不同的生态环境。ANME-1 和-2 两大类群在研究的众多生境中都是主要类群,ANME-3 仅在少数几个生境中报道过。

自然环境中甲烷的厌氧氧化最早在海底沉积物中发现。20 世纪 70 年代以来,开展了大量针对海底沉积物厌氧甲烷氧化古菌生理特性及其多样性的研究工作。一般认为海洋中 SAMO 与 SRB 形成共生体,但是陆地生态系统中硫酸盐浓度较低,认为其可能限制了 SRB 的生长,从而限制了共生的 SAMO 的生长。例如,Kadnikov 等[79]发现了贝加尔湖底泥表层(0—20 cm)硫酸根浓度最高仅约 0.17 mmol/L,而大于 20 cm 深度的底泥中均低于 0.04 mmol/L,并且建立的古菌克隆文库中没有发现 SAMO 和 SRB。直到 2006 年,Alain 等人[80]首次在陆地生态系统(喀尔巴阡山脉的泥火山)中发现大量沉积有机物转化为甲烷并释放到大气中,并且 ANME-2a 是主要的功能古菌。之后陆续有学者在垃圾填埋场[81]、厌氧水体[82]中检测到少量(<1%)ANME-1 和 ANME-2 古菌的存在。除此以外,还在众多土壤生境中发现了另一类名为 AAA 的甲烷厌氧氧化菌(表 5),此类甲烷厌氧氧化菌与 ANME-2 有最近的亲缘关系,但是与 ANME-2 的任何一个分支都不同源。除了 ANME-3,在陆地生态系统中发现了其他各类甲烷厌氧氧化菌,有着较高多样性。此外,从功能基因的定量分析的结果判断,土壤不同生境中存在着活跃的甲烷厌氧氧化菌。例如 Chang 等人[83]应用 ANME-2a 的特异性引物检测发现中国台湾泥火山 7 cm 和 29 cm 深度的土壤中厌氧甲烷菌最丰富,高达 1.4×10^7 和 2.15×10^7 copies/g 沉积物,而其他深度的土壤中约 10^4 copies/g 沉积物。Wrede 等人[84]建立了古菌的克隆文库发现 ANME-2a 占 14%,所有硝酸盐甲烷厌氧氧化菌则占古菌克隆文库的 22%。Takeuchi 等人[85]在日本的 Kanto 平原土壤中发现甲烷厌氧氧化菌的拷贝数也达到 10^4—10^6 copies/g 湿土。但是,一般海洋中甲烷厌氧氧化菌数量>10^{10}个/cm^3,在研究最多的黑海的 Hydrate Ridge 中优势菌 ANME-2 最高可达 10^8个/cm^3[35]。

3.2 硝酸盐甲烷厌氧氧化菌

目前硝酸盐/亚硝酸盐甲烷厌氧氧化菌均属于 NC10 门,经基因组测序、蛋白表达、生理研究确定此类细菌命名为 *Candidatus* Methylomirabilis oxygera。虽然 16S rRNA 基因与此类细菌同源的细菌分布在各种生境中[23],但是目前关于硝酸盐/亚硝酸盐甲烷厌氧氧化菌的富集培养只存在于两个生态系统中:淡水沉积物和污水处理污泥。然而,*Candidatus* Methylomirabilis oxygera 是否是唯一的硝酸盐/亚硝酸盐甲烷厌氧氧化菌还不得而知。根据 NC10 门设计的特异引物[23],将基因库中的序列比对之后发现,此类细菌可以细分为 4 个类群:a,b,c 及 d。然而,目前所富集的细菌均归属于 a 类群,说明 a 类群是硝酸盐甲烷厌氧氧化作用的主要功能群。

学者们在德国寡营养湖(Constance 湖[78])的深水底泥表层、日本淡水湖(Biwa 湖[92])的深水底泥表层、内陆浅水湖泊底泥表层[93]均能检测到 DAMO 菌,并且,用同样的引物定量分析发现,Biwa 湖和西湖中 DAMO 的数量分别为 10^5—10^6 copies/mL 沉积物及 10^5 copies/g 干土。此外,在其他生境中,也发现了一定数量的 DAMO。例如,引物的设计者 Ettwig 等[18]在新西兰的一个富营养化的沟渠中发现了 10^7—10^{10} copies/mg DNA

的 DAMO。Brunssummerheide 泥炭地中维管植物(具有根际泌氧能力)的根系最深达 60 cm,因此在 80—100 cm 深度发现了大量的 DAMO(3.2×10^7个/g 干土)。Wang 等检测了长期施氮肥的水稻土 0—100 cm 的 DAMO 的分布,结果发现表层(0—10 cm)中拷贝数最高($(1.0\pm0.1)\times10^5$—$(7.5\pm0.4)\times10^4$ copies/g 干土),40 cm 以下深度要比表层少一个数量级,70 cm 以下则低于检测限[94]。因此,在不同的土壤生境中存在丰富的 DAMO。

表 5　不同土壤生境中甲烷厌氧氧化菌的类型

Table 5　Overview of ANME habitats. Sequences released until December 2013 have been considered

研究生境 Habtat site	引物 Primer	甲烷厌氧氧化菌类型 Anaerobic methanotrophic archaea groups						参考文献 References
		ANME-1	ANME-2a	ANME-2b	ANME-2c	ANME-3	AAA	
美国北方泥炭地酸性草甸沼泽 Bear Meadows bog acidic, boreal peatland, USA	A21f/ Eury498				+			[86]
罗马尼亚喀尔巴阡山泥火山沉积物 Carpathian mountains mud volcano sediments, Rumania	A21f/A958r	+	+					[80]
美国佛罗里达沼泽 Florida Everglades soil, USA	23F/1492R						+	[87]
日本平原地下沉积物 Kanto Plain subsurface sediment,Japan	Arc109F/ Arc915R	+						[88]
美国密歇根草本泥炭沼泽 Michigan Hollow fen soil, USA	A1F/1492R						+	[89]
美国泥炭地 Peatland soil, USA	1AF/1100R						+	[90]
日本石油污染土壤 Petroleum-contaminated soil, Japan	A341f/ A1063r						+	[91]
中国西藏高原湿地土壤 Tibetan plateau wetland soil, China	915F/ 1492R						+	[79]
瑞士湖泊沉积物 Sediment of Lake Cadagno, Switzerland	A20f/A958r						+	[49]
德国泥火山 Nirano mud volcano field, Germany	A21f/A958r	+	+	+				[84]
中国台湾泥火山 Mud Volcano in eastern Taiwan, China	A8f/U1513	+	+				+	[83]

"+"表示对应生境中检测到的甲烷厌氧氧化菌类型

4　总结

分子检测方法的应用为揭示自然环境中甲烷厌氧氧化菌的多样性提供了科学准确的研究工具,特别是基于功能基因方面的分子检测极大地促进了甲烷厌氧氧化菌多样性及生态功能方面的研究。迄今为止,对于自然环境中甲烷厌氧氧化菌主要分为 SAMO 和 DAMO 两种类型,分别与硫酸盐还原过程和硝酸盐还原过程耦合。由于陆地生态系统中 SO_4^{2-} 含量较少,因此对 SAMO 的研究一般针对海底沉积物,但是某些含有高浓度硫酸盐的农田土壤如反酸田等 SAMO 的研究还很缺乏。而且,关于 DAMO 氧化菌的富集培养只存在于淡水沉积物和污水处理污泥两个生态系统中,缺乏对农田土壤等不同生态系统的大范围研究。虽然学者们针对不同厌氧甲烷氧化过程的功能基因设计出很多引物序列,但是这些引物的特异性在不同土壤生态系统里的应用还有待检验。另外对其他类型的甲烷厌氧氧化过程研究甚少,如 Fe^{3+}、MnO_4^-和 ClO_4^-等,虽然有研究报道存在以这些离子为电子受体的 AOM 过程,但是缺乏对不同生态系统此过程的深入研究。因此,建议今后应用分子检测方法在以下几个方面开展深入研究:(1)根据 SAMO 和 DAMO 的发生特点,对我国不同土壤生态系统进行深入研究,扩大研究范围,分析可能发生的甲烷氧化过程及其分子机理;(2)将分离培养和分子检测相结合,分析不同土壤生态系统厌氧甲烷氧化菌的多样性及生态功能;(3)不同土壤生态系统中甲烷厌氧氧化菌的多样性及与植物、根系和环境因子之间的关系;(4)目前已研究发现极端环境中好氧甲烷氧化菌的广泛存

在[95],在此环境中是否存在新型的由微生物介导的甲烷厌氧氧化作用与机制。

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