SHORT COMMUNICATION

Succession of methanotrophs in oxygen–methane counter-gradients of flooded rice paddies

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Little is known about population dynamics and contribution of specific taxa to methane oxidation in flooded rice paddies. In this article we investigate the succession of methanotrophs in oxygen–methane counter-gradients. We used a gradient microcosm system that simulates oxic–anoxic interfaces of a water-saturated paddy soils, and measured *pmoA*-based (gene encoding particulate methane monooxygenase) terminal restriction fragment length polymorphism (T-RFLP) profiles at both the transcription (mRNA) and the population (DNA) levels. The DNA T-RFLP profiles indicated that the methanotrophic community present clearly differed from the active methanotrophic community. We observed a succession of the methanotrophic community over time without any direct effect of pore water chemistry on the community structure. Both the total population and the active subpopulation changed with time, whereas methane oxidation rates remained nearly constant. Hence, we suggest that a diverse microbial seed bank of methanotrophs is important in maintaining the function in a dynamic ecosystem.

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Atmospheric methane (CH₄) is the second most important greenhouse gas after carbon dioxide (Denman *et al.*, 2007). The largest sources are natural wetlands and wetland rice fields, which account for one-third of the annual global methane emissions (Conrad, 2009). In wetland rice fields, aerobic methanotrophs have a key role in controlling methane emission into the atmosphere. In this ecosystem, methanotrophs can reduce the potential methane emissions by 80% (Conrad and Rothfuss, 1991).

Aerobic methanotrophs are a physiologically distinct group of bacteria that can use methane as sole carbon and energy source. Taxonomically they belong to the *Proteobacteria* and *Verrucomicrobia*; methanotrophy in the latter has only recently been discovered (Dunfield *et al.*, 2007; Pol *et al.*, 2007; Islam *et al.*, 2008). Methanotrophs within the *Proteobacteria* are classified into the families Methylocystaceae and Beijerinckiaceae (type II methanotrophs) and Methylococcaceae (type I methanotrophs) (Bowman, 2006). Type I methanotrophs are further subdivided into types Ia and Ib based on phylogeny (Bodrossy *et al.*, 2003). Type Ia includes many cultivated representatives such as

Methylobacter, *Methylomonas*, *Methylosarcina* and uncultivated environmental clones. Type Ib includes besides clusters of uncultivated methanotrophs the genera *Methylococcus* and *Methylocaldum*.

Methanotrophs oxidize methane to carbon dioxide. A key enzyme in this process is the particulate methane monooxygenase (pMMO), which catalyzes the first step. The pMMO is present in all known methanotrophs except the acidophilic *Methylocella* spp. (Theisen *et al.*, 2005). Hence, the *pmoA* gene, which encodes a pMMO subunit, is an excellent molecular marker to identify methanotrophs in environmental samples (McDonald and Murrell, 1997).

When rice paddies are flooded, the bulk soil quickly becomes anoxic, and methane oxidation is restricted to oxic microsites at the soil surface layer and in the rhizosphere (Bosse and Frenzel, 1997). At the soil surface, physical and chemical gradients develop with time. These gradients have a great impact on the activity and structure of the microbial community (Noll *et al.*, 2005). Both type I and type II methanotrophs are present in the soil surface layer (Eller and Frenzel, 2001; Conrad, 2007). However, detailed information about the population dynamics of methanotrophs and the contribution of specific taxa to methane oxidation in nature is lacking.

Here we studied the succession of both present (DNA) and active (mRNA) methanotrophic populations in a model system simulating the oxic–anoxic interface of wetland soils. To link environmental

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parameters that might determine the population structure, we analyzed environmental soil parameters and correlated them to the methanotrophic community.

A microcosm model system was used that simulates the oxic–anoxic boundary layer of wetland soils. The microcosm used here focuses specifically on the active layer, where methanotrophs thrive in the counter-gradients of methane and oxygen. Hence, the vast number of resting methanotrophs in the bulk soil (Eller and Frenzel, 2001), normally diluting the active community due to the sample size, did not mask actual population changes. A total of 16 microcosms were set up and incubated in the dark at 25 °C. At each time point (3, 6, 9, 13, 16, 19, 25 and 30 days), two microcosms were sacrificed. Soil was homogenized, and aliquots were immediately frozen in liquid nitrogen and stored at –80 °C for further analysis. To amplify the *pmoA* gene, we used the forward primer A189f and the reverse primers A682r (Holmes *et al.*, 1995) or mb661r (Costello and Lidstrom, 1999), hereafter referred to as primer set I and II, respectively. The differences between these primer systems for the Vercelli rice fields are summarized in Supplementary Table S1. We analyzed mRNA and DNA *pmoA*-gene-based terminal restriction fragment length polymorphism (T-RFLP) (full methods in Supplementary Information).

The T-RFLP analysis resulted in 14 terminal restriction fragments (TRFs) that could be affiliated to OTUs of methanotrophs and ammonium-oxidizing bacteria (Supplementary Table S1). One TRF of 58 bp corresponding *in silico* to sequences representing the RA21-related group was excluded from analysis (Supplementary Table S1). Cloning and sequencing showed that this particular PCR product was amplified from the *rRNA* gene of the genus *Myxococcus* having by chance the same length as *pmoA* PCR products. Even removal of 16S *rRNA* genes with the Epicentre mRNA-ONLY Isolation Kit (Madison, WI, USA) did not prevent the appearance of unspecific TRFs.

We first compared the total with the active methanotrophic community, combining both primer sets in one correspondence analysis. The analysis depicted a clear separation of DNA and mRNA T-RFLP profiles, and a separation according to primer sets I and II (Figure 1; Supplementary Figure S1).

In a second analysis, we considered the primer sets at the DNA and mRNA levels separately. A redundancy analysis showed that T-RFLP community profiles were significantly affected by time (Figure 2). On the basis of this, we identified two stages, showing distinct methanotrophic communities (Figure 2). The DNA T-RFLP profiles were characterized by type Ib and type II methanotrophs and ammonium-oxidizing bacteria in the initial stage. The latter stage was characterized by a high dominance of type II methanotrophs (Supplementary Figure S1). The mRNA T-RFLP profiles showed a high activity of type Ia and type Ib methanotrophs in

Figure 1 Correspondence analysis (CA) of T-RFLP data at the DNA and mRNA levels with primer sets I and II. The first two axes explained 26.7% and 18.9% of the total inertia, respectively. Numbers next to species scores depict the size of TRFs representing different phylogenetic units of methanotrophs. The phylogenetic affiliation of the TRFs is given in Supplementary Table S1.

the initial stage, and also the activity of type II methanotrophs at the latter stage of the succession (Supplementary Figure S1).

Finally, the effect of pore water chemistry on the methanotrophic communities was analyzed using principal component analysis and a correlation of environmental factors (Supplementary Table S2; Supplementary Figure S2). DNA T-RFLP profiles of primer set I were influenced by ammonium and sulfate. The decrease in the ammonium content was correlated with the successional stages (Supplementary Figure S2A). In contrast, DNA T-RFLP profiles of primer set II were not affected by these environmental parameters. mRNA T-RFLP profiles of primer set II were significantly influenced by sulfate (Supplementary Figure S2B), but profiles of primer set I were not. CH₄ oxidation rates remained relatively constant, except on days 3 and 25, when the rate was higher than average (Supplementary Table S2).

Using *pmoA*-based T-RFLP profiles and multivariate ordination techniques, we showed that the active methanotrophic community clearly differed from the total methanotrophic community (Figure 1; Supplementary Figure S1). Analyzing the methanotrophic community at the DNA level, we observed a high relative abundance of type II methanotrophs, which is consistent with earlier work (Eller and Frenzel, 2001). However, type II methanotrophs did not start to become active until after 25 days (Supplementary Figure S1). There is some indication that type Ia methanotrophs require not only methane and oxygen for growth, but also high levels of other nutrients (Bodelier *et al.*, 2000; Noll *et al.*, 2008), whereas type II methanotrophs are less

Figure 2 Redundancy analysis of DNA T-RFLP data (a) and mRNA T-RFLP data (b) using primer sets I and II, and with time as the only constraint. The significance of the model was tested by *post hoc* ANOVA (primer set I, $F = 0.015$, $P < 0.01$; primer set II, $F = 0.013$, $P < 0.01$; RNA: primer set I, $F = 0.16$; not significant; primer set II, $F = 0.072$; $P < 0.05$). Different stages of methanotrophic communities are indicated by filled and open triangles, respectively, and are encircled.

demanding and therefore have an advantage when nutrients are limiting (Graham *et al.*, 1993). Hence, the increase in relative abundance of type II methanotrophs toward the end of our experiment might be caused by nutrient limitation (Supplementary Table S2). Several OTUs of type I methanotrophs were detected (Supplementary Figure S1). Interestingly, type Ib methanotrophs showed both a remarkable high relative abundance and formed a major group within the active methanotrophic community. Type Ib methanotrophs have been detected as an abundant group in rice fields (Lüke *et al.*, 2009), but their significance, that is, activity, had not yet been shown. Here we showed that these organisms actively transcribed the *pmoA* gene, which suggests that they contribute greatly to CH_4 oxidation in rice fields.

The environmental interpretation of the observed succession of methanotrophs indicated that the recorded soil parameters were not responsible for these patterns (Supplementary Table S2; Supplementary Figure S2). We believe that the decreasing NH_4^+ concentration solely influenced ammonium-oxidizing bacteria, because NH_4^+ was only significant when ammonium-oxidizing bacteria were

present (Supplementary Figure S2). We assume that the high SO_4^{2-} concentration had only indirect effects on the methanotrophic community.

Although direct influences can be correlated easily, indirect influences are rather difficult to link to the community structure, for example, the effect of protistan grazing (Murae and Frenzel, 2007). In addition, there might be inter- and/or intraspecific competition, for example, when protistan grazing depletes competitors, thus making new niches accessible for other methanotrophs.

A succession of microbial communities is often explained by the general ecological concept of r- and K-type strategy that was adapted to microbial ecology by Andrews and Harris (1986). Our study data indicate that the r- and K-type strategy is not applicable to higher taxonomic levels, because both type I and II methanotrophs contain ecophysiologically very different species. However, the concept may be applicable to single representatives of the traditional type I and II groups as already suggested (Steenbergh *et al.*, 2009).

Methanotrophs can form drought-resistant cysts and exospores (Whittenbury *et al.*, 1970; Bowman *et al.*, 1993) and have been reported to survive

unfavorable conditions for up to 170 years (Rothfuss *et al.*, 1997). Hence, differences between the present and the active methanotrophic community might indicate that certain organisms become active during different periods of the season. The abundance of resting stages provides a seed bank from which methanotrophs can become active under optimal environmental conditions (Eller *et al.*, 2005). The succession of methanotrophs observed in our study supports this hypothesis. Primer set II, which best covers the known groups of type I and type II methanotrophs (Bourne *et al.*, 2001), clearly showed a succession of methanotrophs at both the DNA and the mRNA levels, specifically of type I methanotrophs (Figure 2; Supplementary Figure S1). Primer set II also revealed an increase of type II methanotrophs (Supplementary Figure S1). In contrast, primer set I revealed an apparent succession of methanotrophs at the DNA level, but not at the mRNA level. This apparent succession of methanotrophs at the DNA level was actually due to ammonium-oxidizing bacteria, which are partially covered by primer set I. In addition, type Ib methanotrophs, represented by a TRF of 79 bp, were amplified differently by primer sets I and II (Supplementary Figure S1). It is known that this TRF represents several clusters/genera of methanotrophs that were not all amplified by both primer sets (Supplementary Table S1). Hence, different clusters/genera might occur and become active at different stages resulting in different patterns. Conclusively, the patterns of methanotrophic diversity probably would not have been fully resolved if we had used only one primer set.

Finding a TRF derived from ribosomal RNA instead of *pmoA* transcripts emphasizes that phylogenetic assignments in T-RFLP analysis should always be verified by cloning and sequencing. The parallel analysis of mRNA and DNA showed that only a subset of the methanotrophic community present was active at different times. As the population structure changed, methane oxidation rates remained relatively stable. Hence, we suggest that a diverse microbial seed bank together with the functional complementarity of methanotrophs maintains functioning in dynamic ecosystems.

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