The ISME Journal (2018) 12:2086–2089
https://doi.org/10.1038/s41396-017-0022-8

BRIEF COMMUNICATION

Methanobactin from *Methylosinus trichosporium* OB3b inhibits N_2O reduction in denitrifiers

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Received: 9 August 2017 / Revised: 9 November 2017 / Accepted: 17 November 2017 / Published online: 12 January 2018

Abstract

Methanotrophs synthesize methanobactin, a secondary metabolite that binds copper with an unprecedentedly high affinity. Such a strategy may provide methanotrophs a "copper monopoly" that can inhibit the activity of copper-containing enzymes of other microbes, e.g., copper-dependent N_2O reductases. Here, we show that methanobactin from *Methylosinus trichosporium* OB3b inhibited N_2O reduction in denitrifiers. When *Pseudomonas stutzeri* DCP-Ps1 was incubated in cocultures with *M. trichosporium* OB3b or with purified methanobactin from *M. trichosporium* OB3b, stoichiometric N_2O production was observed from NO_3^- reduction, whereas no significant N_2O accumulation was observed in cocultures with a mutant defective in methanobactin production. Copper uptake by *P. stutzeri* DCP-Ps1 was inhibited by the presence of purified methanobactin, leading to a significant downregulation of *nosZ* transcription. Similar findings were observed with three other denitrifier strains. These results suggest that in situ stimulation of methanotrophs can inadvertently increase N_2O emissions, with the potential for increasing net greenhouse gas emissions.

Climate change is driven by increased emissions of CO_2, CH_4, and N_2O. Unlike CO_2, both production and removal of CH_4 and N_2O are largely driven by microbial processes [1, 2]. CH_4 is produced in highly reduced anoxic environments by methanogenic microorganisms [3]. N_2O is produced in more oxidized environments, primarily as a byproduct of nitrification or denitrification [2]. Most CH_4 produced in subsurface anoxic environments is oxidized at the oxic–anoxic interface by the aerobic methanotrophs [4]. The sole biological sink of N_2O is via nitrous oxide reductase (NosZ) that converts N_2O to N_2, and significant correlations have been observed between N_2O emissions and the abundance and/or diversity of *nosZ* [2, 5, 6].

Aerobic methanotrophy is highly dependent on copper, with expression and activity of methane monooxygenases controlled by copper availability [7, 8]. Many of these microbes synthesize and utilize a copper binding compound termed methanobactin for copper acquisition [9]. Methanobactin binds copper ions with 1:1 stoichiometry and has an unprecedentedly high affinity for copper ions, with binding constants ranging from 10^{18} to 10^{58} M^{-1} (values vary depending on measurement technique used [10]). Interestingly, biological removal of N_2O is also strongly dependent on copper as NosZ has a high copper requirement [11]. Under copper stress induced with synthetic chelators, denitrification led to transient N_2O accumulation [12–14]. Given the importance of copper availability for both methanotrophy and N_2O reduction, we hypothesized that methanotrophs may be able to exert a "monopoly" over copper through production of methanobactin, leading to increased rates and amounts of N_2O production due to inactivation of NosZ.

N_2O production was first monitored upon incubations of the denitrifier *Pseudomonas stutzeri* DCP-Ps1 with NO_3^- as the terminal electron acceptor alone, in a coculture with *Methylosinus trichosporium* OB3b wildtype, or in a coculture with a mutant defective in methanobactin synthesis

Electronic supplementary material The online version of this article (https://doi.org/10.1038/s41396-017-0022-8) contains supplementary material, which is available to authorized users.

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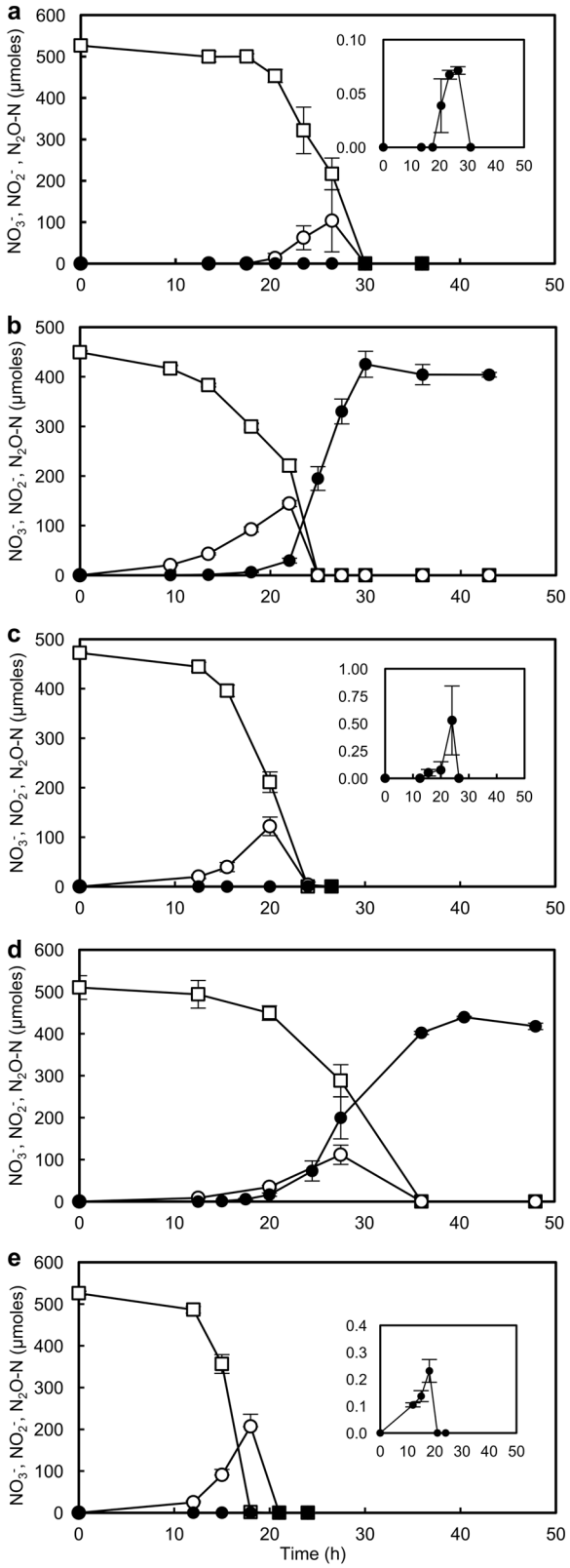

Fig. 1 N_2O production from denitrification of *P. stutzeri* DCP-Ps1 in **a** axenic cultures or **b** in cocultures with either *M. trichosporium* OB3b wildtype or **c** *M. trichosporium* OB3b *ΔmbnAN* mutant. The axenic cultures of *P. stutzeri* DCP-Ps1 with **d** 1 μM purified methanobactin and **e** 1 μM purified methanobactin pre-incubated with 1 μM Cu^{2+} were also examined. The initial measurement (t=0) was made immediately after inoculation of *P. stutzeri* DCP-Ps1. For each experiment, the amounts of NO_3^- (□), NO_2^- (○), and N_2O (●) in the culture vessel were monitored. The data points are the averages of triplicate experiments, with the error bars representing the standard deviations. The inserts are magnifications of N_2O measurements

(*ΔmbnAN* [15]). In the axenic cultures of *P. stutzeri*, transient N_2O production was observed (a maximum of 76 ± 8 nmol N_2O-N) but N_2O was eventually consumed (Fig. 1a). In the coculture with *M. trichosporium* OB3b wildtype, however, 430 ± 30 μmol N_2O-N permanently accumulated (Fig. 1b). In the presence of the *ΔmbnAN* strain, the N_2O production profile was indistinguishable from the axenic cultivation (Fig. 1c). Incubation of *P. stutzeri* with 1 μM purified methanobactin from *M. trichosporium* OB3b resulted in permanent production of N_2O (85.4% of the added NO_3^-; Fig. 1d), confirming that N_2O accumulation was due to the presence of methanobactin. When methanobactin was pre-loaded with the stoichiometric concentration of copper (1:1 molar ratio) and then added to cultures of *P. stutzeri*, no substantial accumulation of N_2O was observed, indicating that NosZ activity was not affected (Fig. 1e).

Copper uptake by *P. stutzeri* DCP-Ps1 in the absence and the presence of 1 μM methanobactin was then assayed by examining the partitioning of copper after incubation with 5 mM NO_3^- (Supplementary Table S2). In the absence of methanobactin, 0.14 ± 0.03, 0.28 ± 0.06, and 0.38 ± 0.04 μg of copper were recovered from the cell biomass, the wash solution, and the spent medium, respectively, out of 0.77 ± 0.10 μg (0.24 ± 0.03 μM) initially added to the medium. In the presence of methanobactin, the partitioning of copper changed significantly ($p < 0.01$), with 0.02 ± 0.01, 0.06 ± 0.00, and 0.61 ± 0.02 μg recovered from the cell biomass, the wash solution, and the spent medium, respectively, out of 0.67 ± 0.06 μg (0.21 ± 0.02 μM) initially added to the medium. The total mass of cellular copper was 0.69 ± 0.14 μg Cu•mg protein^{-1} upon incubation in the absence of methanobactin, while this value decreased significantly to 0.10 ± 0.03 μg Cu•mg protein^{-1} in the presence of 1 μM methanobactin ($p < 0.01$). The presence of methanobactin inhibited adsorption of copper onto cellular surfaces, as well, suggesting a lower affinity of methanobactin–Cu complex to the outer membrane than free Cu^{2+} or Cu-EDTA. Such inhibition of copper uptake reduced *nosZ* transcription. In the absence of methanobactin, *nosZ* was expressed at 4.42 ± 1.13 *nosZ* transcript/*recA* transcript, while in the presence of methanobactin,

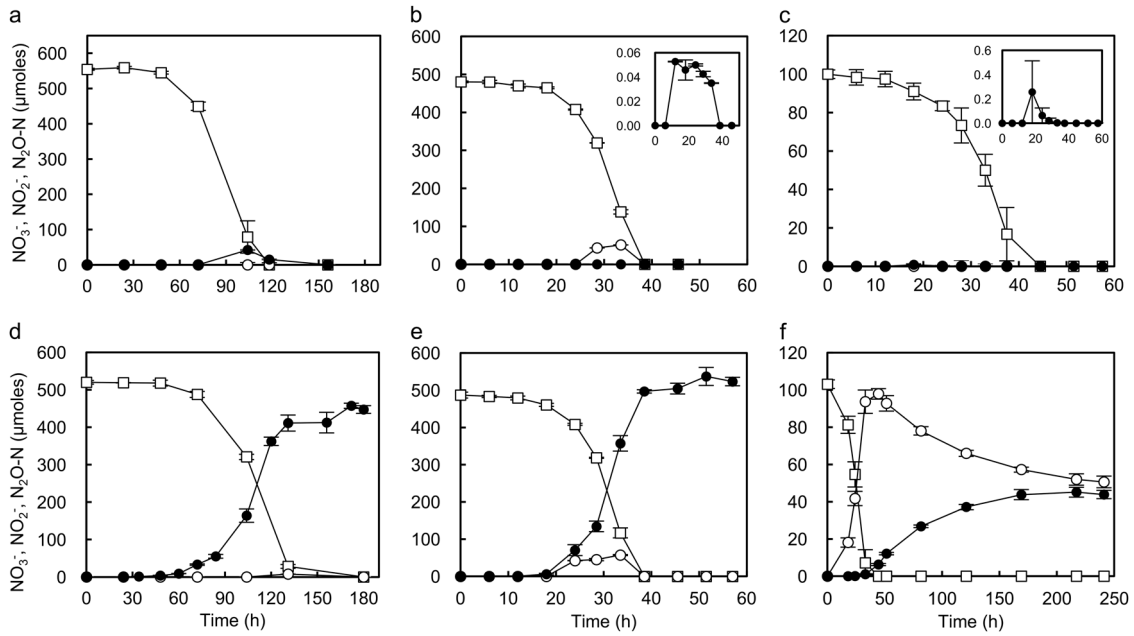

Fig. 2 N_2O production from denitrification in axenic cultures of *Dechloromonas aromatica* RCB (**a**, **d**), *Paracoccus denitirificans* ATCC17741 (**b**, **e**), and *Shewanella loihica* PV-4 (**c**, **f**) without (**a–c**) and with (**d-f**) 1 μM methanobactin. For each experiment, the amounts of NO_3^- (□), NO_2^- (○), and N_2O (●) in the culture vessel were monitored until the reaction was completed. The data points are the averages of triplicate experiments, with the error bars representing the standard deviations

nosZ transcription decreased significantly ($p < 0.05$) to 1.59 ± 0.55 *nosZ* transcript/*recA* transcript (a $\Delta\Delta C_t$ value of 1.669). Furthermore, the addition of methanobactin pre-loaded with copper (such that it could not bind any additional copper) had no significant effect on the expression of *nosZ* by *P. stutzeri* DCP-Ps1 (Supplementary Table S3). Thus, the effect of methanobactin on *nosZ* expression was in the reduction of bioavailable copper.

The effect of methanobactin was also examined with three other denitrifers: *Dechloromonas aromatica* RCB, a denitrifier with a clade II NosZ structurally and kinetically distinct from the clade I NosZ in *P. stutzeri* DCP-Ps1 [16, 17]; *Paracoccus denitrificans* ATCC17741, previously found to synthesize copropophryin III suggested to be used as a copper chelator [18]; and *Shewanella loihica* PV-4, a denitrifier utilizing NirK (copper-dependent) for NO_2^- reduction to NO [19]. In the absence of methanobactin, no significant accumulation of N_2O was observed in the three denitrifier cultures (Fig. 2a, b, c). When these denitrifiers were cultured with 1 μM methanobactin, near stoichiometric accumulations of N_2O were observed from denitrification (Fig. 2d, e, f). NirK-mediated NO_2^- reduction was also partially inhibited in *S. loihica* (Fig. 2f) ; nevertheless, eventual permanent production of 43.8 ± 2.3 μmol N_2O-N was observed before NO_2^- reduction came to a complete stop with 50.6 ± 3.1 μmol NO_2^- remaining. This suggests that NirK is less sensitive to copper deprivation than NosZ, possibly due to its lower copper requirement [20].

The threshold concentration of bioavailable copper for unhindered NosZ activity has been estimated to be on the order of 10^{-15}–10^{-24} M [12, 21]. Previously, such low copper concentrations could only be attained in water stripped of metals with help of artificially synthesized ligands, e.g., triethylenetriamine and tetrathiolmolybdate [12, 21]. Thus, enhanced release of N_2O due to the inhibition of N_2O reduction caused by the lack of bioavailable copper was previously deemed environmentally unlikely [14]. Contradicting these previous findings, we show here that a biogenic copper chelator produced by methanotrophs blocks N_2O reduction, eliciting N_2O emissions from incomplete denitrification. These findings have significant relevance for greenhouse gas emissions in situ as co-occurrence of methanotrophy and denitrification is not rare. As the main substrates for aerobic methanotrophs are CH_4 and O_2, which diffuse towards the oxic–anoxic interface from opposite ends of the oxygen gradient, the largest abundance of methanotrophs is often found at the oxic–anoxic interfaces in subsurface environments [22]. The oxic–anoxic interface is also often a "hotspot" for soil denitrification in agricultural soils, as the major source of the electron acceptors for denitrifiers is NO_3^-/NO_2^- leaching from the oxic upper layers of soil where nitrifiers oxidize NH_4^+-N [23]. In such ecological niches where methanotrophs and denitrifiers coexist, these findings suggest that hitherto unrecognized microbial competition for copper may be a significant mechanism controlling N_2O emissions.

An open question is how much methanobactin may be produced in situ. Levels of methanobactin have yet to be reported in environmental samples, but genomic analyses, as well as physiological and biochemical investigations, indicate that methanobactin synthesis is a widely distributed phenotype among methanotrophs at environmentally relevant copper concentrations [10]. Given that methanotrophs constitute a substantial portion of the active microbial community in soils, it seems likely that denitrifier activity can be affected in situ by production of methanobactin. Methanotroph–denitrifier interactions must be more fully explored to better understand how best to predict, and possibly minimize, net greenhouse gas emissions.

Acknowledgements This work was supported by the National Research Foundation of Korea (Grants 2017R1D1A1B03028161 and 2015M3D3A1A01064881) and the U.S. Department of Energy, Office of Science (Grant #DE-SC0006630). The authors were also financially supported by Korea Ministry of Land, Infrastructure and Transport (MOLIT) as U-City Master and Doctor Course Grant Program and the Brain Korea 21 Plus Project (Grant 21A20132000003).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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