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DOI
10.1111/1758-2229.12630

Publication date
2018

Document Version
Final published version

Published in
Environmental Microbiology Reports

Citation (APA)

Important note
To cite this publication, please use the final published version (if applicable). Please check the document version above.

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Growth yield and selection of nosZ clade II types in a continuous enrichment culture of \( \text{N}_2\text{O} \) resiping bacteria

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Summary

Nitrous oxide (\( \text{N}_2\text{O} \)) reducing microorganisms may be key in the mitigation of \( \text{N}_2\text{O} \) emissions from managed ecosystems. However, there is still no clear understanding of the physiological and bioenergetic implications of microorganisms possessing either of the two \( \text{N}_2\text{O} \) reductase genes (\( \text{nosZ} \)), clade I and the more recently described clade II type \( \text{nosZ} \). It has been suggested that organisms with \( \text{nosZ clade II} \) have higher growth yields and a lower affinity constant (\( K_s \)) for \( \text{N}_2\text{O} \). We compared \( \text{N}_2\text{O} \) reducing communities with different \( \text{nosZI/nosZII} \) ratios selected in chemostat enrichment cultures, inoculated with activated sludge, fed with \( \text{N}_2\text{O} \) as a sole electron acceptor and growth limiting factor and acetate as electron donor. From the sequencing of the 16S rRNA gene, FISH and quantitative PCR of \( \text{nosZ} \) and \( \text{nir} \) genes, we concluded that betaproteobacterial denitrifying organisms dominated the enrichments with members within the family \( \text{Rhodocyclaceae} \) being highly abundant. When comparing cultures with different \( \text{nosZI/nosZII} \) ratios, we did not find support for (i) a more energy conserving \( \text{N}_2\text{O} \) respiration pathway in \( \text{nosZ} \) clade II systems, as reflected in the growth yield per mole of substrate, or (ii) a higher affinity for \( \text{N}_2\text{O} \), defined by \( \text{I}_{\text{max}}/K_s \), in organisms with \( \text{nosZ clade II} \).

Introduction

Nitrous oxide (\( \text{N}_2\text{O} \)) reducing microorganisms, both denitrifying and non-denitrifying, can contribute to the \( \text{N}_2\text{O} \) sink capacity of ecosystems and may be key in reducing emissions of this potent greenhouse gas (Hallin et al., 2018). The phylogeny of the nitrous oxide reductase (NosZ), encoded by the \( \text{nosZ} \) gene, has two major clades, clade I and II (Jones et al., 2013). A high abundance and diversity of \( \text{N}_2\text{O} \) reducing bacteria harboring \( \text{nosZ clade II} \), in particular, has been linked to an increased \( \text{N}_2\text{O} \) reduction potential in soils as well as lower \emph{in situ} \( \text{N}_2\text{O} \) emissions (Jones et al., 2014; Domeignoz-Horta et al., 2017), but a mechanistic explanation for this is lacking. \( \text{nosZ clade I and clade II} \) differ in (i) the co-occurrence with other denitrification genes, with \( \text{nosZ clade II} \) being more often associated to non-denitrifiers (Graf et al., 2014) and (ii) the accessory proteins associated to the \( \text{nos} \) operon. For example, \( \text{nosR} \) and \( \text{nosB} \) genes encode proteins likely to be involved in electron transport to the NosZ of clade I and clade II respectively (Sanford et al., 2012). It is not understood if these differences between the two types of NosZ, apparent on the genome level, result in a differentiation in the ecophysiology of \( \text{N}_2\text{O} \) reducers harboring either \( \text{nosZ} \) clade.

Physiological studies with clade II-type \( \text{N}_2\text{O} \) reducers are scarce, but Yoon and colleagues (2016) recently compared five \( \text{N}_2\text{O} \) reducing bacterial species and reported lower whole-cell half-saturation constants (\( K_s \)) for \( \text{N}_2\text{O} \) and up to 1.5 times higher biomass yields per mole of \( \text{N}_2\text{O} \) for the \( \text{nosZ clade II} \) \( \text{N}_2\text{O} \) reducers compared to those harboring \( \text{nosZ clade I} \). A lower \( K_s \) would confer \( \text{nosZ clade II} \) \( \text{N}_2\text{O} \) reducers a selective advantage during competition for limiting amounts of \( \text{N}_2\text{O} \), whereas a higher biomass yield implies a greater efficiency of energy conservation in the \( \text{nosZ clade II} \) associated electron transport chain (ETC). Extra charge separations during \( \text{N}_2\text{O} \) reduction could hypothetically be mediated by the predicted transmembrane protein encoded by \( \text{nosB} \) present in \( \text{nosZ clade II} \) organisms. It is an attractive hypothesis that \( \text{nosZI} \)-associated ETCs generate a greater proton motive force per electron accepted than the \( \text{nosZI} \) equivalent, which would explain niche...
differentiation between the two clades. To test the competition between nosZ clade I and clade II N₂O reducers, we recently analyzed the performance of an enrichment culture growing for a large number of generations with N₂O as the sole electron acceptor under different dilution rates and with either the electron donor (acetate) or N₂O as the limiting factor (Conthe et al., 2018). Continuous systems with enrichment cultures are optimal to study the potential dichotomy in N₂O reducer ecophysiology, as it allows competition experiments based on the affinity for a limiting substrate within a fairly complex community and provides prolonged steady state conditions to obtain reliable biomass yields. Nevertheless, irrespective of whether N₂O or acetate was the growth limiting substrate in the culture, nosZ clade I N₂O reducers dominated the enrichment. This led us to reject the hypothesis that nosZ clade II-harboring organisms have a higher overall affinity for N₂O than organisms with nosZ clade I, with affinity being determined by the ratio of $\mu_{\text{max}}$ over $K_s$. Since we did not enrich for a significant community of nosZ clade II N₂O reducers under the different operational conditions, we were unable to compare growth yields amongst N₂O reducers of both clades (Conthe et al., 2018). However, we did observe an increase in nosZ clade II when the dilution rate switched from high to low, which suggest that the $\mu_{\text{max}}$ was important in the selection of N₂O reducers.

The aim of the present study was to compare the results from the period with low dilution rate and N₂O limitation from our previous experiment with an independently enriched N₂O-fed chemostat culture subject to the same conditions. Even though a functional steady state had been achieved in the previous study, a steady state in terms of microbial community composition and nosZII/nosZI ratio had not. Additionally, the history of reactor operation likely affects the selection of community members, and in the present study, we directly started off with continuous operation under conditions of N₂O limitation and low dilution rate without a preceding period of higher dilution rate or acetate limiting conditions. With the new enrichment approach, the abundance of nosZ clade II bacteria was significantly increased, which allowed us to compare the thermodynamic efficiency of nosZ clade II- versus clade I-associated ETCs and to gain further insight into the role of the NosZ type in the microbial competition for N₂O. The abundance of N₂O reducers was determined using quantitative real-time PCR (qPCR) of nosZI and nosZII along with the nitrite reductase genes nirS and nirK characteristic of denitrifying organisms. Additionally, the 16S rRNA genes were sequenced to obtain the composition of the enriched community, and fluorescent in situ hybridization (FISH) with probes targeting Bacteria and

<table>
<thead>
<tr>
<th>Compound conversion rates (mmol h$^{-1}$)</th>
<th>C-bal (%)</th>
<th>e–-bal (%)</th>
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<tbody>
<tr>
<td>CH₃COO⁻</td>
<td>3.88 ± 0.81</td>
<td>4.34 ± 0.05</td>
</tr>
<tr>
<td>CH₄, O₂</td>
<td>1.14 ± 0.08</td>
<td>1.39 ± 0.04</td>
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<tr>
<td>CH₃OH</td>
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</tr>
<tr>
<td>N₂O</td>
<td>0.30 ± 0.01</td>
<td>0.30 ± 0.01</td>
</tr>
<tr>
<td>N₂H</td>
<td>2.27 ± 0.13</td>
<td>2.27 ± 0.13</td>
</tr>
<tr>
<td>C₂H₆NO</td>
<td>1.25 ± 0.13</td>
<td>1.25 ± 0.13</td>
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<tr>
<th>Dilution rate (h$^{-1}$)</th>
<th>0.026 ± 0.001</th>
<th>0.026 ± 0.001</th>
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<tr>
<td>This study</td>
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<td>During period IV of operation.</td>
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<td>After day 21.</td>
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<th>Conversion rates in the chemostats (negative numbers = consumption, positive = production) under N₂O limitation and carbon (C) and electron (e–) balances over the conversions (mean ± SD, n = 5).</th>
<th>This study</th>
<th>Conthe and colleagues (2018)</th>
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Beta- and Gammaproteobacteria was performed to independently quantify the relative abundance of these taxa.

Results and discussion

Prolonged heterotrophic growth sustained by N₂O respiration

Activated sludge from the wastewater treatment plant of Harnaschpolder (the Netherlands) was used as the inoculum to enrich a microbial community growing with N₂O as the sole electron acceptor and using acetate as an electron donor at pH 7 and 20°C. After an initial batch start-up phase of 48 h, the culture was operated in continuous mode under N₂O limiting conditions during 72 days at a dilution rate of 0.027 h⁻¹ (specifically at 0.028 ± 0.001 h⁻¹ days 0–20 and 0.026 ± 0.001 h⁻¹ days 21–72; Supporting Information Fig. S1). Nitrous oxide was supplied to the reactor at a constant rate (Supporting Information Fig. S1) and the reactor set-up, medium composition, operation and sampling are described in detail in Conthe and colleagues (2018). The microbial community was growing by N₂O reduction to N₂ at the expense of acetate oxidation, as confirmed by the elemental and electron balances (Table 1), with acetate present in excess throughout the operation (Supporting Information Fig. S1). The compound conversion rates were comparable to those obtained in our previous experiment, showing that the community functioning was similar in the two, independent enrichments (Table 1). To confirm that N₂O was growth limiting in the system, the N₂O sparging rate was increased, which resulted in an immediate increase in the biomass specific N₂O conversion rate (data not shown).
The N\textsubscript{2}O reducing community was dominated by betaproteobacterial denitrifiers

The composition of the enrichment culture, sampled on 10 different days during chemostat operation, and of the activated sludge used as inoculum was determined by Illumina sequencing of the 16S rRNA gene (Fig. 1A and Supporting Information Tables S1 and S2). Bacteria belonging to the family Rhodocyclaceae, despite representing only a small percentage of sequences in the activated sludge inoculum, made up a significant part of the enrichment with a single OTU (1) covering 40 to 60% of the reads after day 30. However, FISH performed on day 34 suggests that the relative abundance of the dominant OTU, as reflected in the abundance of bacteria hybridizing with the betaproteobacterial probe, was even much higher than estimated by sequencing (70–90% of the biovolume vs. 40% of sequences; Fig. 2). As far as we could see, the cells stained with the betaproteobacterial probe had the same morphology.

The initial decrease of Pseudomonas sp. and Comamonas sp. that dominated at the startup of the reactor operation was followed by an increase in Cloacibacterium sp., Chryseobacterium sp. and Dechloromonas sp. This shift in community composition coincided with a decrease in nosZ clade I abundance and an increase in nosZ clade II (Fig. 1B). In agreement, sequenced genomes of the genera Pseudomonas and Comamonas harbor clade I nosZ, whereas Dechloromonas sp. and N\textsubscript{2}O reducers within Flavobacteriaceae harbor nosZ clade II. After day 20, Rhodocyclaceae (Dechlorobacter sp.) dominated the enrichment. Different species within the Rhodocyclaceae have been shown to harbor either nosZ clade I or II (Jones et al., 2014). The only sequenced genome of Dechlorobacter so far has a nosZ sequence similar to the nosZ clade I from Rhodotherax ferriredens and Ralstonia pickettii (Conthe et al., 2018). However, while OTU 1 was assigned to Dechlorobacter when using the Silva taxonomy, it was assigned to the genus Azonexus when using the rdp classifier, and sequenced genomes of Azonexus harbor nosZ clade II rather than clade I. This makes it difficult to speculate about the type of nosZ associated to this OTU. Instead, the similar abundance of both nosZ types suggests that OTU 1 could be a mix of closely related species within the Rhodocyclaceae family. Interestingly, reads related to nosZ clade II from Azonexus dominated the nosZ clade II community in the previous experiment under the same conditions used in the present study, although the corresponding 16S rRNA gene sequences could only be assigned at the family level (Conthe et al., 2018). Bacteria of the genus Pseudomonas, Comamonas and Dechloromonas, as well as many Rhodocyclaceae also possess genetic potential for denitrification, although the corresponding 16S rRNA gene copy numbers were two to three orders of magnitude lower than that of the 16S rRNA genes and two orders lower than the abundance of nir genes, characteristic of denitrifying organisms, were highly abundant in the culture (Fig. 1B), indicating that the N\textsubscript{2}O reducers dominating the enrichment were likely denitrifiers rather than non-denitrifying N\textsubscript{2}O reducers. This shows that the availability of N\textsubscript{2}O, even under N\textsubscript{2}O limiting conditions, is not a selective driver for non-denitrifying N\textsubscript{2}O reducers and highlights the strong competitive advantage of proteobacterial nirS-type denitrifiers under these conditions.

The vast majority of the community members were presumed to harbor the nosZ gene required for sustained growth on N\textsubscript{2}O respiration, translated in similar abundances of nosZ and 16S rRNA genes. However, the total nosZ gene copy numbers were two to three orders of magnitude lower than that of the 16S rRNA genes and two orders lower than the abundance of nir genes.
genes after the community shift on day 21 (Fig. 1B and Table 2). This is potentially due to an underestimation of nosZ genes or the presence of a population incapable of N₂O reduction that was not captured when sequencing the 16S rRNA gene. We also detected a relatively high abundance of the phylum Gracilibacteria and unclassified bacteria (Fig. 1A). The only genomes of Gracilibacteria available so far were obtained from single-cell sequencing of cells from the vicinity of hydrothermal vents of the East Pacific rise. Both of the two retrieved genomes are closely related, have low G + C content and are characterized as fermentative bacteria (Rinke et al., 2013). They do not have any nos genes that would indicate capacity for N₂O reduction, although they have a nitric oxide reductase. They may have co-existed in the chemostat by living off products of cell lysis or cross-feeding with N₂O reducers. The Gracilibacteria were also present in the enrichment in Conthe and colleagues (2018).

nosZ clade type is not a selective driver in the competition for N₂O

The nosZII/nosZI abundance ratio in the present enrichment culture was higher compared to that reported by Conthe et al. despite similar operating conditions (Table 2). Differences in the bacterial community composition of the inoculum or in reactor operation history, as well as a certain degree of stochasticity to be expected during colonization of any ecosystem (Roeselers et al., 2006), could explain the difference in community composition between the two enrichment cultures. However, the small difference in dilution rate between the studies (0.026 ± 0.001 in this study vs. 0.027 ± 0.001 in Conthe et al., 2018) could be an explanation considering that the minor change in dilution rate on day 21 coincided with a dramatic shift in the composition of the bacterial community (Fig. 1). Changes in community composition, either due to minor operational differences or due to potential interactions among community members, suggest that the competitive differences between nosZ clade I and II are small during N₂O limiting conditions.

The fact that the relative abundance of the two clades differed substantially between the two independent enrichment cultures, while conversion rates and biomass yields were very similar (Tables 1 and 2), suggests that competition among community members was not driven by the type of NosZ and that the overall energy conservation was similar in nosZ clade I and nosZ clade II-associated ETCs present in our system. Our finding that N₂O reduction kinetics and stoichiometric yields do not distinguish bacteria harboring NosZ clade I from those with NosZ clade II contradicts the study reporting lower whole-cell K₂ rates values and 50–80% higher growth yields in nosZ clade II N₂O reducers compared to organisms with nosZ clade I during growth on N₂O as the sole electron acceptor (Yoon et al., 2016). The species that were studied might not be representative for the extant diversity known for the two clades of NosZ and furthermore, the difference in apparent K₂ among the clade II species was as large as the differences among the clade I species, suggesting that differences in affinity might be taxa dependent rather than between nosZ clade I and II organisms. We conclude that there is no simple answer explaining the divergence and ecological differences of the two clades of NosZ observed in several studies of soils, sediments and rhizosphere (e.g., Tsiknia et al., 2015; Wittorf et al., 2016; Graf et al., 2016; Dini-Andreote et al., 2016; Juhanson et al., 2017).

Acknowledgements

This work was funded by the European Union (Marie Curie ITN NORA, FP7–316472) and the Swedish Research Council (VR grant 2016–03551 to SH). The authors would like to thank Camiel Parchen and Gerben Stouten for their help with the lab work.

References

deciphering the ecophysiology of N₂O respiring bacterial communities in a continuous culture. ISME J.


Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

Fig. S1. Chemostat operation over 72 days showing (a) the liquid medium and gas flow rates (the total gas flow consisting of pure N₂O diluted in N₂ or Argon) going into the reactor, (b) the incoming and outgoing acetate and NH₄⁺ concentrations in the medium and effluent and (c) the biomass concentration and optical density of the culture. Day 0 corresponds to the start of continuous operation. Medium A contained 90.6 mmol acetate (NaCH₃COO·3H₂O) per liter, and medium B contained 26.6 mmol NH₄Cl, 14.8 mmol K₂HPO₄, 4.2 mmol MgSO₄·7H₂O, 1 mmol NaOH, 4 mg yeast extract and 5 ml trace element solution (Vishniac and Santer, 1957) per liter. Both media were fed to the chemostat by means of one peristaltic pump with two pump heads. Even though the biomass concentration increased after day 21, growth yields remained the same. This is because the HRT decreased after replacing the influent pump tubing feeding mediums A and B to the reactor while the growth limiting substrate – N₂O – was supplied to the reactor at a constant gas flow rate. Recirculation was implemented on day 47 with the intention of reducing the amount of Argon gas used and to increase the mass transfer of gaseous N₂O to the liquid phase. However, the resulting increase in N₂O availability in the liquid was too small to be detected in the biomass yield of the culture.

Table S1. Assigned taxonomy for the main 16S rRNA-based OTUs (those with > 10% sequences) of the activated sludge inoculum using the Silva database.

Table S2. Assigned taxonomy for the main 16S rRNA-based OTUs in the enrichment using the Silva database. The main OTUs were considered to be those with > 5% sequences on any given sampling date, also see Fig. 1.