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Exploring diversity of Nitrate reducing thermophiles in Nevada hot springs

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High rates of denitrification have been measured in Nevada geothermal hot springs, but little is known about the thermophiles that contribute to this activity. We hypothesize that heterotrophic bacteria in the genus *Thermus* are the most important denitrifiers in the springs. Alternatively, other microorganisms including chemolithotrophs may also be important. To test these hypotheses, several different strategies will be used to try to enrich and isolate nitrate-reducing microorganisms. Isolates will be identified by 16S rRNA gene PCR and sequencing. Subsequently, representative isolates will be chosen for nitrate reductase gene (*narG*) sequencing and for studies on the kinetics of nitrate reduction at high temperature. These data will provide information on how these microorganisms may behave *in situ* and how their activities may affect nitrogen cycling in the hot springs.

Exploring Diversity of Nitrate Reducing Thermophiles in Nevada Hot Springs

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Introduction

High rates of denitrification have been measured in Nevada geothermal springs, but little is known about the thermophiles that contribute to this activity [1]. Denitrification is a form of anaerobic respiration in which nitrate (NO_3^-) is converted to nitrogen gas (N_2) in a multi-step pathway, involving various intermediates (Fig. 1). It is necessary to cultivate and characterize nitrate reducing microorganisms in order to determine which thermophiles contribute to denitrification. We hypothesize that heterotrophic bacteria in the genus *Thermus* are the most important denitrifiers in the springs. Alternatively, other microorganisms including chemolithotrophs may also be important. To test these hypotheses, several different strategies were used to try to enrich and isolate nitrate-reducing microorganisms. Subsequently, microorganisms were identified and their nitrate reduction activities were characterized. By learning more about these microorganisms we may be able to obtain information on how they behave in situ and how their activities may affect nitrogen cycling in the hot springs.

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$$

Figure 1. The process of denitrification with all the nitrogenous intermediates.

Aims and Methods

1) Expand collection of heterotrophic nitrate reducing thermophiles from Great Boiling Spring (GBS) and Sandy's Spring West (SSW).

2) Identify isolates by using 16S rRNA gene PCR and sequencing.

3) Determine the stoichiometry of products of nitrate reduction using representatives of each species.

4) Attempt to cultivate chemolithotrophic and denitrifying thermophiles.

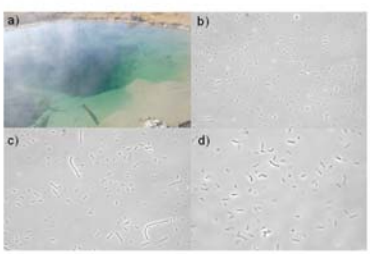

Figure 2. Geothermal spring and bacteria that inhabit it. a) Great Boiling Spring in Gerlach, NV. b) *Thermus oshimai* strain GBS-JL-2. c) *Thermus thermophilus* strain GBS-JL-6. d) *Anoxybacillus kualawohkensis* strain GBS-JL-25.

Results

Identification of isolates and qualitative analysis of nitrate reduction

Heterotrophs were enriched from sediment, water, and neuston samples with media containing different electron donors and incubated at 70°C. Pure cultures were isolated from positive enrichments. 16S rRNA gene PCR was performed on DNA from pure cultures. The 16S rRNA genes were sequenced using Sangar method. Isolates were identified using EzTaxon, Ribosomal Database Project, and NCBI.

- Three different species of *Thermus* were found.
- One species of *Anoxybacillus* was found.
- One species of *Geobacillus* was found.

Table 1. Identification of different bacterial species found in the hot springs. All isolates were isolated aerobically, then selected cultures were grown anaerobically, and tested for nitrite and nitrous oxide production.

16S rRNA group	Number of Isolates	In SSW	In GBS	Anaerobic Growth	NO_2^-	N_2O
Thermus thermophilus	37	9	28	21/29[a]	21/29	21/29
Thermus oshimai	4	3	1	3/4	3/4	3/4
Thermus aquaticus	7	3	4	0/2[b]	0/2	0/2
Anoxybacillus kualawohkensis	1	0	1	1/1	1/1	1/1
Geobacillus vulcani	2	0	2	1/1[c]	nd	nd

[a] Eight of these isolates have not been specified.
[b] Five of these isolates have not been specified.
[c] One of these isolates has not been specified.

Isolate sources, isolation strategy, and electron donors

Table 2. Isolates were obtained from different locations in the hot springs by direct plating or by plating after enrichment with different organic compounds.

16S rRNA group	*Thermus thermophilus*	*Thermus oshimai*	*Thermus aquaticus*	*Anoxybacillus kualawohkensis*	*Geobacillus vulcani*
Sediment	2	3	2	0	1
Sediment w/ YE and P	26	1	0	0	1
Spring Water	0	0	0	0	0
Spring Water w/ YE and P	3	0	3	0	0
Neuston w/ YE and P	0	0	0	1	0
Sediment w/ casamino acids	2	0	2	0	0
Sediment w/ FLAP	1	0	0	0	0
Sediment w/ BSA	1	0	0	0	0
Neuston w/ glycogen	1	0	0	0	0
Neuston w/ lactate	1	0	0	0	0
Total amount of strains	**37**	**4**	**5**	1	**2**

Quantitative analysis of denitrification during growth

Thermus thermophilus and *Thermus oshimai* were both grown anaerobically at 70°C with Castenholz medium D, which contains 9 mM nitrate. Nitrite, nitrous oxide and nitrogen gas concentrations were measured periodically throughout incubation. Nitrite concentrations were measured colorimetrically using the diazotization method with reagents from LaMotte. Nitrous oxide and nitrogen gas were measured using gas chromatography (GC-ECD and GC-TCD, respectively). Total gas concentrations were determined using Henry's Law.

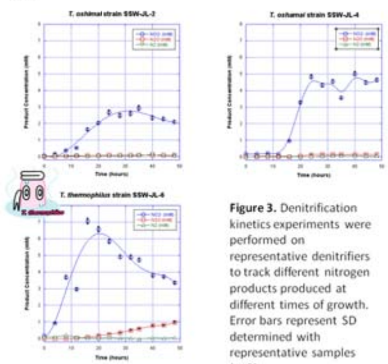

Figure 3. Denitrification kinetics experiments were performed on representative denitrifiers to track different nitrogen products produced at different times of growth. Error bars represent SD determined with representative samples (n=3).

Chemolithotrophic nitrate reduction

Electron donor stimulation experiments

Electron donor stimulation experiments were performed *in situ* to assess which electron donors are coupled to denitrification. Sediment slurries in anaerobic spring water were stimulated with 1 mM nitrate and several different possible electron donors and the final step in denitrification was blocked by addition of 10% acetylene. Slurries were incubated in the spring and sampled for nitrous oxide production. Nitrous oxide was quantified by GC-ECD and total nitrous oxide production was calculated using Henry's Law.

- Only yeast extract and peptone stimulated denitrification.

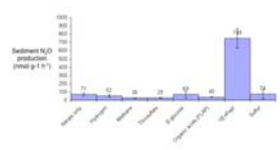

Figure 4. Results of acetylene block experiment. Most electron donors added to 1 mM. Yeast extract + peptone in excess.

Cultivation experiments

To further examine the possible existence of chemolithotrophic nitrate reducers, sediment was incubated in spring water or Castenholz Medium D at 70°C containing 9 mM nitrate and the inorganic electron donors hydrogen, sulfur, and thiosulfate. All cultures were examined microscopically and tested for nitrate and nitrite concentration.

- Growth in samples containing inorganic electron donors was very weak.
- Nitrate and nitrite assays were inconclusive.

Discussion

Very little is known about denitrification in hot springs, despite the fact that nitrate (NO_3^-) is an extremely favorable terminal electron acceptor for anaerobic respiration [3]. In this study, we isolated a large collection of thermophilic nitrate reducers from Great Basin hot springs, studied their nitrate reduction activities, and assessed the relative contributions of heterotrophs and chemolithotrophs to denitrification *in situ*.

Nitrate reducing thermophiles belonged to three species, *Thermus thermophilus, Thermus oshimai, and Anoxybacillus sp*. These two genera are known for their ability to respire nitrate. Four species of *Thermus* can reduce nitrate: *T. thermophilus, T. oshimai, T. scotodus, and T. brockianus*. *T. thermophilus* is capable of complete denitrification to nitrogen gas [2]. The biochemistry, genetics, and evolution of the nitrate reduction pathway is well-characterized. In contrast, although one reference to denitrification by *Anoxybacillus* strains exists, nitrate reduction activities are poorly described [4].

Nitrite is the major product by the three *Thermus* strains tested, suggesting a role in conversion of nitrate to nitrite *in situ*. In addition, *T. thermophilus* produces large amounts of nitrous oxide, consistent with high nitrous oxide fluxes measured at GBS.

An electron donor stimulation experiment suggested that denitrification is mainly coupled to heterotrophy, not chemolithotrophy, which is similar to aquatic and soil ecosystems.

Future directions

- Continue to expand collection of nitrate reducing thermophiles from Nevada hot springs.
- Continue trying to cultivate chemolithotrophic nitrate reducing thermophiles.
- Improve kintics experiments by adding cell growth data.
- Perform denitrification kinetics experiments with *Anoxybacillus kualawohkensis* to detemine the stoichiometry of nitrogen products from denitrification.
- Quantify heterotrophic and chemilothotrophic nitrate reducers in sediments using quantitative PCR for nitrate reductase genes.

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