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Incomplete Denitrification in *Thermus* Species

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INCOMPLETE DENITRIFICATION IN *THERMUS* SPECIES

By

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Bachelor of Sciences - Biology
Montclair State University
2013

A thesis submitted in partial fulfillment
of the requirements for the

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ABSTRACT

INCOMPLETE DENITRIFICATION IN *THERMUS* SPECIES

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Members of the bacterial genus *Thermus* have been shown to be incomplete denitrifiers, terminating with nitrite or nitrous oxide (N₂O). However, the ability to carry out denitrification and the evolution of nitrogen oxide reductase genes in *Thermus* remains poorly understood. This study tests the hypothesis that incomplete denitrification is common in *Thermus* and seeks to uncover patterns in the evolution of denitrification pathways in *Thermus*. Denitrification capacity was determined in a collection of 25 strains representing ten species of *Thermus* and phylogenetic analysis was performed to determine whether denitrification genes evolved horizontally in *Thermus*. No strains in this study reduced nitrate to dinitrogen (N₂). Terminal products were nitrite, nitric oxide (NO), or nitrous oxide (N₂O), with most strains ending with N₂O as a final product. In most cases, denitrification phenotypes were consistent with the presence of denitrification genes and strains of the same species typically had the same denitrification phenotypes. Phylogenetic analysis and the pattern of extant nitrogen oxide reductases showed evidence for horizontal gene transfer (HGT) and gene loss/gain within *Thermus*. These results show that incomplete denitrification is prominent in the genus *Thermus*, which suggests *Thermus* may play a role in consortial denitrification at high temperatures.

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Totus Tuus

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CHAPTER 1

INTRODUCTION

1.1 The nitrogen cycle

Microbial ecology concerns the relationship of microbes with one another and their environment. This includes the study of microbes and their roles in the cycling of nitrogen. The nitrogen cycle is the process by which nitrogen is converted between its different physical and chemical forms (Fig. 1) (Madigan *et al.* 2012). These transformations are often facilitated by microbial processes in an effort to accumulate nitrogen for growth or harvest energy. Assimilatory processes of the nitrogen cycle include dinitrogen (N_2) fixation, in which nitrogen is combined with hydrogen other elements to form ammonia to incorporate into proteins. Ammonification describes the pathway by which ammonia is released from the decomposition of organic nitrogen compounds. Dissimilatory processes of the nitrogen cycle include dissimilatory nitrate reduction to ammonia (DNRA), nitrification, anaerobic ammonia oxidation (anammox), and denitrification. DNRA is the direct reduction of nitrate to ammonia and can occur in anoxic environments when nitrate is limiting. Nitrification is the process by which microbes sequentially oxidize ammonia to nitrite then nitrate as a final product. In anammox, ammonia is oxidized with nitrite as an electron acceptor and forms N_2 as a final product. Denitrification is the process by which microorganisms sequentially reduce nitrate or nitrite to the gaseous products nitric oxide (NO), nitrous oxide (N_2O), or N_2 through anaerobic respiration (Madigan *et al.* 2012) (Fig. 2). Denitrification will be discussed in greater detail in Section 1.3.

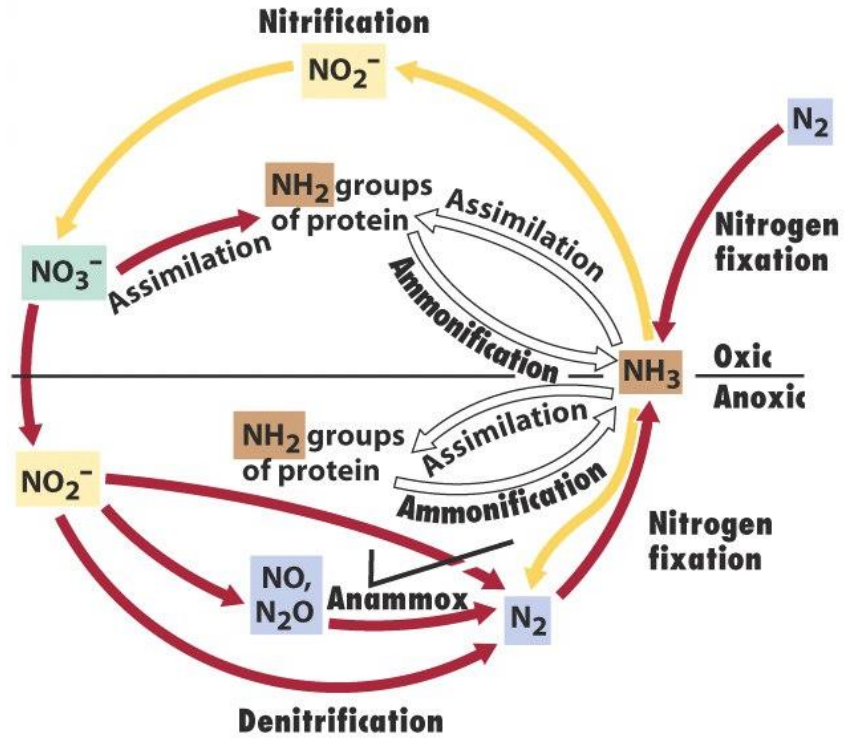


Figure 1. The nitrogen cycle (Madigan *et al.* 2012)

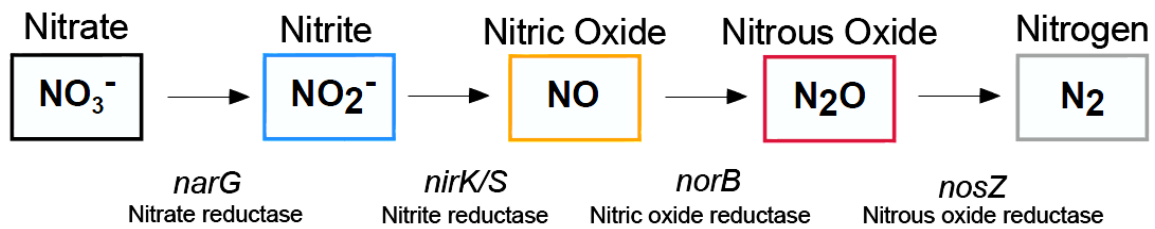


Figure 2. Complete denitrification pathway and corresponding reductase genes

1.2 High-temperature nitrogen cycling

Having an understanding of the biogeochemical cycles in high-temperature habitats is crucial to learning about the diversity of life at high temperatures. However, there is limited knowledge about the nitrogen cycle at high temperatures. Only recently has research begun to focus on the nitrogen cycle in various geothermal environments. For example, evidence N_2 fixation to ammonia in cultures of *Methanocaldococcus jannaschii* at 90°C has been shown using $^{15}N_2$ isotope tracer assays and expression experiments with *nifH* messenger RNA (Metha *et al.* 2006). Anammox has been detected in deep-sea hydrothermal vents using amplification of 16S rRNA gene sequences related to known anammox bacteria, ladderane lipids analysis, and $^{14}N^{15}N$ dinitrogen isotope-pairing experiments at 60°C and 85°C (Bryne *et al.* 2009). Other nitrogen cycling activities have been reported in terrestrial hot spring environments such as anammox, nitrite oxidation, N_2 fixation, and archaeal ammonia oxidation (Lebedeva *et al.* 2005; Hatzenpichler *et al.* 2008; de la Torre *et al.* 2008; Jaeschke *et al.* 2009; Hamilton *et al.* 2011; Edwards *et al.* 2013). Nitrite oxidation has been found in a pure culture of *Nitrolancetus hollandicus* cultivated on nitrite at ~63°C (Sorokin *et al.* 2012). DNRA has also been reported at 113°C in *Pyrolobus fumarii* (Blöchl *et al.* 1997). Interestingly, the collection of studies showing evidence for ammonia oxidizing archaea and nitrite oxidizing bacteria indicate that N_2 fixation and nitrification may decouple at high temperatures. These data show that both assimilatory and dissimilatory nitrogen cycling is active in geothermal environments. However, there does appear to be temperature limits for nitrogen cycle processes to occur due to changes in microbial diversity and energetics at high temperatures (Price and Sowers 2008; Cole *et al.* 2013a; Sharp *et al.* 2014), making geothermal system very different from all other habitat on Earth. Altogether, these studies

show the nitrogen cycle is an important process at extreme temperatures and should be carefully considered in the study of high temperature life.

1.3 Denitrification

Denitrification is an important part of the nitrogen cycle and is significant ecologically because it leads to the loss of nitrogen as a gaseous product from an ecosystem. Denitrification is the ecologically opposite pathway of N_2 fixation coupled with nitrification. The former consumes nitrate, while the latter produces nitrate. Thus, denitrification is a vital process, balancing the nitrogen budget of the biosphere by converting nitrate to N_2 . Though nitrate is a key plant nutrient and is not toxic in small amounts, it is a possible pollutant of drinking water and its reduction to nitrite can be toxic at high levels. Nitrite can reduce ferrous iron to ferric iron in hemoglobin, converting hemoglobin to methemoglobin. Methemoglobin has decreased ability to carry oxygen, leading to tissue hypoxia (Kim-Shapiro *et al.* 2004). Marine and freshwater ecosystems, livestock, and humans are all subject to nitrate toxicity. N_2O , as an intermediate of denitrification, is also of great concern. Atmospheric N_2O has increased over time from wastewater treatment plants (Otte *et al.* 1996) and fertilizer denitrification (Metz B *et al.* 2007), with microbes playing the large part in these changes. As a greenhouse gas, N_2O absorbs and emits radiation within the thermal infrared range, trapping heat within the surface-troposphere system that causes changes in the Earth's climate. Additionally, N_2O can be photochemically oxidized to NO, which then reacts with ozone in the upper atmosphere forming holes in the protective ozone layer. This increases the amount of UV radiation to the Earth's surface. Furthermore, the reaction between NO and ozone forms nitrite and returns to the Earth as nitric acid, or acid rain (Reddy and DeLaune 2008). In turn, this lowers the pH of soil leading to changes in microbial community structures and soil

fertility (Reddy and DeLaune 2008), and acidification of surface waters in poorly buffered areas (Lawrence 2002). These concerns increase efforts to understand denitrification and the role microbes have in this process.

1.4 Denitrification at high temperatures

When oxygen is plentiful, aerobic respiration can occur. However, oxygen solubility decreases as temperature increases. Thus, the need for organisms to carry out anaerobic respiration at high temperatures is vital for survival. In aerobic respiration, molecular oxygen serves as a terminal electron acceptor, accepting electron from electron carriers by way of an electron transport chain. Anaerobic respiration employs alternative terminal electron acceptors when oxygen is less abundant or absent. Anaerobic respiration, such as denitrification, is less energy efficient than aerobic respiration. Fewer protons are pumped across the membrane than when oxygen is the terminal electron acceptor; therefore, fewer ATP is produced from ATP synthase and the proton motive force generated. Alternative terminal electron acceptors, like nitrate, are less efficient electron acceptors than oxygen, but are still energetically favorable and makes respiration possible in environments where oxygen is absent. Thus, if nitrate is available, denitrification is a viable option to aerobic respiration at high temperatures.

High temperatures increase challenges for life to occur and influences microbial growth, maintenance, and survival. There is a greater energetic demand for life at high temperatures. The maintenance energy, or energy required for functions other than the production of new cell material (Pirt 1965), increases with temperature (Price and Sowers 2004) and could influence metabolic rates in microbes. Additionally, as temperature increases the rate at which proteins denature and molecules degrade increases. Together, this influences microbial diversity and composition in terrestrial geothermal environments. Previous work has shown a negative relationship between temperature

and sediment microbial community richness in geothermal sediment samples from Great Boiling Springs (Cole *et al.* 2013a). More recent work demonstrates temperature strongly impacts microbial community diversity and richness, and accounts for the variability in alpha diversity across several geothermal areas in Canada and New Zealand (Sharp *et al.* 2014). Moreover, temperature can play a selective pressure on denitrification, where some denitrifiers are observed in specific temperature ranges. In Great Boiling Spring, *T. thermophilus* was most abundant at 76°C and 79°C sites, but was replaced by other *Thermus* species, primarily *T. scotoductus*, at lower temperature. However, the low-temperature sites likely hosted other denitrifiers as well (Cole *et al.* 2013a). Additionally, high fluxes of N₂O in sites at or above 80°C in Great Boiling Spring are consistent with the presence of incomplete denitrifiers, such as *T. thermophilus* and *T. oshimai* (Hedlund *et al.* 2011). These studies indicate denitrification may be common in high temperature environments.

Although denitrification has been shown to take place at a range of temperatures in diverse groups of microorganisms (Zumft 1997), this work focuses on denitrification at high temperatures. Though there have only been a few studies that closely examine denitrification at high temperatures, previous work has shown denitrifiers to be present in terrestrial geothermal environments. Terrestrial geothermal environments host an array of thermophiles with differing nitrate reducing activities (Völkl *et al.* 1993; Cabello *et al.* 2004; Poli *et al.* 2009; Hedlund *et al.* 2015a; Hemp *et al.* 2015). Nitrate reduction is better known among thermophilic members of bacterial phyla, such as *Thermales*. *Thermales* species in the bacterial genus *Thermus* are widely distributed in geothermal systems and some species have been studied as models of thermophilic nitrate reduction and incomplete denitrification (Cava *et al.* 2009). Moreover, several genetic and genomic studies have shed light on nitrate reduction and denitrification pathways in a few *Thermus*

species (Ramirez-Arcos *et al.* 1998; Brüggemann *et al.* 2006; César *et al.* 2011 Gounder *et al.* 2011; Murugapiran *et al.* 2013a; Murugapiran *et al.* 2013b; Zhou *et al.* 2016; Mefferd *et al.* 2016). However, the capacity for denitrification in the genus as a whole has not been well characterized.

1.5 Incomplete denitrification in Thermus

Bacteria, archaea, and some microbial eukaryotes are capable of denitrification, most of which carry out complete denitrification by reducing nitrate to N₂ as the terminal product (Zumft and Kroneck 2007). However, microbes with truncated or incomplete denitrification pathways are also known to exist (Hart *et al.* 1965; Hemp and Gennis 2008; Sanford *et al.* 2012; Refojo *et al.* 2012 Hemp *et al.* 2015). It is possible that incomplete denitrification pathways may be an important factor in the nitrogen cycle in geothermal systems (Hedlund *et al.* 2011). Incomplete denitrification pathways may promote N-cycling within hot spring systems, slowing the rate at which nitrogen is removed from the system. Incomplete denitrification has been shown in some *Thermus* (Hedlund *et al.* 2011), but it is unknown whether this common across the entire genus.

The goal of this study was to characterize denitrification pathways in *Thermus* and determine the evolutionary forces driving denitrification potential across the genus. To determine the terminal denitrification products in *Thermus* species, a collection of 25 strains representing ten species of *Thermus* from hot springs in Yunnan Province, China, were grown under denitrifying conditions and nitrogenous products were measured to evaluate their denitrification capacity. Additionally, to determine the distribution of denitrification genes in the genus *Thermus*, denitrification genes were recovered from new and existing genomes and by PCR using *Thermus*-specific primers designed in this study. Finally, phylogenetic analyses and patterns of gene loss/gain used to untangle the impacts of vertical and horizontal evolution on denitrification genes

in *Thermus*. Results show that incomplete denitrification pathways are common in *Thermus* species; more specifically, nitrate reduction to either nitrite, NO, or N₂O as terminal products and the variable presence of nitrogen oxide reductase genes indicates varying denitrification capabilities within *Thermus*. Moreover, tree topologies from phylogenetic analyses of nitrogen oxide reductase genes show some evidence that HGT shaped the evolution and presence of denitrification genes in the genus *Thermus*.

CHAPTER 2

MATERIALS AND METHODS

Screen for nitrate reduction phenotype

The sources of *Thermus* strains are described in Table S1, Appendix. All strains were isolated from hot springs in Yunnan Province, with the exception of *T. arciformis* JCM15153, a type strain isolated from Guangxi Autonomous Region, China, which was included to increase taxonomic coverage. *Thermus* strains were revived from frozen stocks on Castenholtz Medium D (CMD) agar plates amended with 9 mM nitrate and supplemented with 0.1% yeast extract and 0.1% tryptone adjusted to a pH of 8.2 (Castenholtz 1969; Hedlund *et al.* 2011).

Thermus strains were grown and screened for nitrate reduction phenotype by testing for the ability to grow in anaerobic liquid CMD. Each strain was grown in anaerobic CMD amended with 4.5 mM nitrate. Nitrate, nitrite, and N₂O were then assayed after 96 hours using the methods described below. To detect N₂ gas production, *Thermus* cultures were screened for the ability to reduce nitrate to N₂ by testing their capability to produce N₂ gas in Durham vials in 25 mL glass Balch tubes with the same medium after 96 hours. All strains were capable of nitrate reduction and robust growth in the medium used for this work except strains *T. brockianus* YIM 77709, *T. caliditerrae* YIM77777, *T. scotoductus* YIM 77398, and *T. tengchongensis* YIM 77427, which were subsequently dropped from the study (Table S1, Appendix). All *Thermus* strains were tested in triplicate and data presented are from replicates with a final cell concentration of $\geq 1 \times 10^6$ cells/mL.

Cultivation of Thermus isolates for nitrate reduction experiments

For all nitrate reduction experiments, isolates were grown in liquid CMD supplemented with 4.5 mM nitrate, 0.1% yeast extract, and 0.1% tryptone and adjusted to a pH of 8.2. The medium was sparged with He for 45 min to remove oxygen and distributed into glass serum bottles, or Balch tubes with Durham vials, in a Coy Type B anaerobic chamber (Coy Laboratory Products Inc., Grass Lake, MI, USA) containing an atmosphere of N₂ (~90%), CO₂ (~5%), and H₂ (~5%). The culture bottles were sealed with butyl rubber stoppers and aluminum crimps and the headspace was exchanged prior to autoclaving by 5 cycles of evacuation (30 sec) and filling to 1 atm with 99.999% He.

A pure colony of each strain was suspended and grown in 10 mL of anaerobic medium in 25 mL glass serum bottles to serve as a starter culture. To dilute contaminating N₂ and ensure denitrification pathways were active, cells were grown to early stationary phase and passed using He-rinsed syringes with a 1:50 inoculum into pre-warmed medium twice before a final transfer into experimental bottles. 160 mL glass serum bottles containing 40 mL of liquid CMD described above, or Balch tubes with Durham vials with 10 mL of liquid CMD were used for the final transfer. Cultures in serum bottles were incubated in the dark at 60°C with rotary shaking at 100 rpm with serum bottles in a horizontal position to maximize gas equilibration. Cultures in Balch tubes were incubated in the dark at 60°C in a static incubator. Unless otherwise noted, cell density was measured using a Petroff-Hausser counting chamber on an Olympus BX-51 phase-contrast microscope with brightness and contrast optimized by using PictureFrame software (Optronics, Goleta, CA, USA).

Detection of terminal denitrification nitrogen products

To measure aqueous nitrate and nitrite concentrations, approximately 6 mL of liquid medium was sampled after a 96 hour endpoint, filtered through a 0.2 µm filters (28145-501 VWR, Radnor, PA, USA), stored at 4°C, and later analyzed by colorimetric methods or ion chromatography (IC) analysis. Nitrite concentrations were measured by diazotization with sulfanilamide, followed by coupling with *N*-(1-naphthyl)-ethylenediamine dihydrochloride (LaMotte, Chestertown, MD, USA). For nitrate measurements, powdered cadmium was used to reduce nitrate to nitrite prior to diazotization (LaMotte, Chestertown, MD, USA). To confirm colorimetric measurements, IC analysis was performed on samples from a subset of experiments as previously described (Hou *et al.* 2013) using a Dionex DX-500 Chromatograph with an AS22 anion exchange column with a 4.5 mM Na₂CO₃/0.8 mM NaHCO₃ eluent. To measure N₂O, headspace gas samples were collected from culture bottles at a 96 hour endpoint for gas chromatography (C2014 Shimadzu GC) analysis. Headspace N₂O concentration was measured by injecting a 2 mL headspace gas sample into a GC-2014 Nitrous Oxide Analyzer (Shimadzu, Moorpark, CA, USA) operated as described previously (Dodsworth *et al.* 2011a; Dodsworth *et al.* 2011b). To detect N₂ gas production, *Thermus* cultures were screened for the ability to grow and reduce nitrate to N₂ by testing their capability to produce N₂ gas in Durham vials in 25 mL glass Balch tubes after 96 hours. Statistical significance was calculated using a Student's T-test in R.

DNA extraction, PCR amplification of 16S rRNA genes, and DNA sequencing

DNA was extracted from *Thermus* cell pellets by using the FastDNA Spin Kit for Soil (MP Biomedicals) according to the manufacturer's protocol. 16S rRNA genes were amplified with PCR using primers specific for bacteria: 9bF (Eder *et al.* 1999) and 1512uR (Eder *et al.* 2001). The 25 μ L PCR mixture contained 1 μ L DNA extract, 200 nM of each primer, 200 μ M each dNTP (Promega, Madison, WI, USA), 1.5 mM MgCl₂, 0.625 U of GoTaq DNA polymerase (Promega), and 1x GoTaq buffer (Promega). Cycling conditions were as follows: denaturation at 95°C for 4 min followed by 30 cycles of denaturation (30 sec at 95°C), annealing (1 min at 55°C), and elongation (2 min at 72°C), with a final elongation step (7 min at 72°C). PCR products were sequenced using the Sanger method at Functional Biosciences, Madison, WI, using the forward and reverse PCR primer.

Design of PCR primers and amplification of nitrogen oxide reductase genes

Conserved regions for *narG*, *nirK*, *nirS*, and *norB* in *Thermus* species were chosen to design the primers used in this study. Existing primers for nitrogen oxide reductases (Braker *et al.* 1998; Phillippot *et al.* 2002; Throbäck *et al.* 2004) were shown or predicted to be ineffective with *Thermus* genes. *Thermus* denitrification gene sequences were harvested from genomes (Gounder *et al.* 2011; Murugapiran *et al.* 2013b; Zhou *et al.* 2016; Mefferd *et al.* 2016) available at the Joint Genome Institute's Integrated Microbial Genomes (IMG) website (Markowitz *et al.* 2013) and RAST (Aziz *et al.* 2008; Overbeek *et al.* 2014) and used for alignment. For each gene, the available sequences were aligned by using default parameters using MUSCLE (Edgar *et al.* 2004; Dereeper *et al.* 2008). Conserved regions used for primer design were chosen manually and were predicted to give PCR products of ~1000 bp and include conserved functional domains.

To optimize annealing temperatures for each primer set, the annealing temperature for gradient PCR amplification ranged from $\pm 5^{\circ}\text{C}$ from the mean of the calculated melting temperature for each primer set. Primer combinations and optimal annealing temperatures can be found in Table S2, Appendix. The sequences and locations of the binding sites of the primers are shown in Table 1.

Table. 1 Primer sequences and positions to amplify fragments from nitrogen oxide reductases

Target Gene	Primer ^a	Position ^b	Primer Sequence (5'-3')
<i>narG</i>	narGn2F_CC (F)	1013249-1013269	ACCACCCACGGGGTGAAGTGC
	narGn6R_CC (R)	1012013-1012030	CTGGGCCATGAGGAGGTC
	narGn7R_CC (R)	1012025-1012042	GAGGTCAAAGACGGTGGC
<i>nirK</i>	nirKn3F (F)	1025543-1025559	ATGTACCACTGCGCCCC
	nirKn3R (R)	1024599-1024615	GGGTGGATGCTGCAGATG
<i>nirS</i>	nirSn1F (F)	1023891-1023910	GCCACCCACATCCTGCGCT
	nirSn4R (R)	1022835-1022857	GCGGTGTTGTACACGTTGAAGT
	nirSn1FB (F)	1023891-1023910	GCCACCCACATCTTGCGCT
	nirSn925RB (R)	1023408-1023425	GCGTTGGCCGCCACAATG
<i>norB</i>	norBF1F_CC (F)	1028097-1028080	GCCCTYTGGTACTTCTGG
	norBn9R (R)	1026906-1026927	GCTCCACCATGGTYTGGGTGAA
	norBn925 (F)	1027686-1027703	CGGTGATGGTTATCTTCC

^a Forward and reverse primers are indicated by the letters F and R, respectively

^b Nucleotide positions in the *Thermus oshimai* JL-2 genome

Hot start PCR for amplification of *narG*, *nirK*, *nirS*, and *norB* was performed with DNA from each *Thermus* strain as template. The 25 μL PCR mixture contained 10-125 ng DNA, 200 nM of each primer, 1.5 mM MgCl_2 , 200 μM each dNTP, 0.625 U of GoTaq DNA Polymerase, and 1x Green GoTaq Reaction Buffer. Cycling conditions were as follows: denaturation at 95°C for 4 min followed by 35 cycles of denaturation (2.5 min at 95°C), annealing (1 min, see Table S2 for temperatures, Appendix), and elongation (2.5 min at 72°C), with a final elongation step at 72°C for 7 mins. PCR products were sequenced using the Sanger method at Functional Biosciences, Madison, WI, using the forward and reverse PCR primer.

Determining the evolutionary relationship in denitrification pathways in Thermus

To determine the evolutionary relationships among denitrification genes in *Thermus*, phylogenetic trees were constructed for each denitrification gene and compared to a reference tree with appropriate taxa constructed based on the 16S rRNA genes. For each denitrification gene, multiple sequence alignments were obtained using CLUSTAL_X (Thompson *et al.* 1997) taking into account the corresponding amino acid alignment. For 16S rRNA genes, multiple sequence alignments were obtained using mothur (Schloss *et al.* 2009) and bacterial and archaeal reference alignments from SILVA (Quast *et al.* 2013). Alignments also included the application of a SILVA-compatible lane mask sequence filter and manual editing to visually identify positions with uncertain alignment to be corrected for analysis. Maximum-likelihood analyses and tree construction was performed using the DNA model with the lowest Bayesian Information Criterion (BIC) score obtained for each gene using MEGA 5.02 (Tamura *et al.* 2011). Bootstrap analysis was performed using 1000 replications.

The Shimodaira-Hasegawa Test (SH-test) was performed to infer events of HGT and to detect phylogenetic incongruence in the topology of the gene trees (trees based on nitrogen oxide reductases) compared to the reference tree (tree based on 16S rRNA gene sequences). The SH-test finds the best topology between the gene tree and reference tree, compares the log-likelihood values of each candidate to the tree with the best topology, and determines p-values associated with the differences in those trees. In this study the SH-test was performed using pruned trees based on appropriate 16S rRNA and nitrogen oxide reductase gene sequences; if a given nitrogen oxide reductase gene was not found in a given taxon, then that taxon was removed from the reference tree for the SH-test. Additionally, to improve resolution of the trees, input for this analysis included taxa that would best represent visual signs of discordance. Only one strain was

used to represent a given *Thermus* species, unless there were visual signs of HGT. If visual evidence of HGT existed, the taxa suspected to have undergone HGT and another *Thermus* of the same species was included in the analysis.

CHAPTER 3

MANUSCRIPT FOR *FEMS MICROBIOLOGY ECOLOGY*

Incomplete Denitrification in *Thermus* Species from Tengchong, China

Chapter prepared for publication in *FEMS Microbiology Ecology*

Chrisabelle C. Mefferd^a, Enmin Zhou^{a,b,c}, Noel A. Bernardo^a, Shreya Srivasta^e, Amanda J. Williams, Wen-Jun Li^{b,c}, Brian P. Hedlund^{a,f}

Graduate Student's Involvement and General Background

This chapter represents a draft manuscript to be submitted for publication in a peer-reviewed scientific journal within the next few months. The research detailed in this chapter was conducted by me, other Hedlund lab members, and collaborators for my graduate thesis work during my tenure as a Master's student at the University of Nevada, Las Vegas. Specifically, the work conducted to determine the terminal nitrogen product of denitrification in the *Thermus* strains used in this research, detect denitrification genes by mining *Thermus* genomes and using PCR, and perform phylogenetic analysis was done by myself. Experiments were designed by myself, Williams, and Hedlund. Experiments were performed by myself, Bernardo, Srivasta, Williams, and Hedlund. The data was analyzed by myself and Bernardo. Reagents, materials, analysis tools, and isolates were provided by Zhou, Srivasta, Li, and Hedlund. The manuscript was written by myself. This manuscript highlights the denitrification capacity and phylogenetic analysis of nitrogen oxide reductase genes in a collection of 25 strains and ten species of *Thermus* from hot springs in Yunnan Province, China on several discrete sampling trips conducted in 2010 and 2011.

ABSTRACT

Members the bacterial genus *Thermus* have been shown to be incomplete denitrifiers, terminating with nitrite or nitrous oxide (N₂O). However, the ability to carry out denitrification and the evolution of nitrogen oxide reductase genes in *Thermus* remains poorly understood. This study tests the hypothesis that incomplete denitrification is common in *Thermus* and seeks to uncover patterns in the evolution of denitrification pathways in *Thermus*. Denitrification capacity was determined in a collection of 25 strains and ten species of *Thermus* and phylogenetic analysis was performed to determine whether denitrification genes evolved horizontally. Terminal products were nitrite, nitric oxide (NO), or nitrous oxide (N₂O), with most stains ending with N₂O as a final product. In most cases, denitrification phenotypes were consistent with the presence of denitrification genes and strains of the same species typically had the same denitrification phenotypes. Phylogenetic analysis and the pattern of extant nitrogen oxide reductases showed evidence for horizontal gene transfer (HGT) and gene loss/gain within *Thermus*. These results show that incomplete denitrification is prominent in the genus *Thermus*, which suggests *Thermus* may play a role in consortial denitrification at high temperatures.

INTRODUCTION

Denitrification is the process by which microorganisms sequentially reduce nitrate or nitrite to the gaseous products nitric oxide (NO), nitrous oxide (N₂O), or dinitrogen (N₂) through anaerobic respiration. Bacteria, archaea, and some eukaryotes are capable of denitrification, most of which carry out complete denitrification by reducing nitrate to N₂ as the terminal product (Zumft and Kroneck 2007). However, microbes with truncated or incomplete denitrification pathways are also known to exist. Some microbes have missing *nar* and *nir* genes, which code for nitrate and

nitrite reductases, but contain atypical N₂O reductase (*nosZ*) genes for N₂O reductases (Sanford *et al.* 2012). Some do not appear to have a cytochrome bc₁ complex needed from N₂O reduction to N₂, but have the alternative complex III (ACIII), which performs the same function as the bc complex by transferring electrons from the quinol pool to N₂O (Refojo *et al.* 2012). Other microbes contain novel, putative NO reductase genes, *sNOR*, *eNOR*, and *gNOR*, instead of *norB*, which likely encode the enzymes that reduce NO to N₂O (Hemp and Gennis 2008; Hemp *et al.* 2015). Others carry out incomplete denitrification ending with N₂O (Hart *et al.* 1965). This can occur for several reasons such as mutations in the *nosZ* gene (Zumft and Kroneck 2007), or absence of *nos* genes (Murugapiran *et al.* 2013a).

Terrestrial geothermal environments host an array of thermophiles with differing nitrate reducing abilities. Nitrate and nitrite reductase have been found in sequenced genomes of members of archaeal phyla such as Crenarchaeota (*Aeropyrum*, *Sulfolobus*, and *Pyrobaculum*) and Euryarchaeota (*Archaeoglobus*) (Cabello *et al.* 2004). However, nitrate reduction is better known in thermophilic members of several bacterial phyla, such as the *Aquificae* (Hedlund *et al.* 2015a), *Chloroflexi* (Hemp *et al.* 2015), *Firmicutes* (Poli *et al.* 2009), and *Thermales*. In particular, *Thermales* species of the bacterial genus *Thermus* are widely distributed in geothermal systems and have been studied as models of thermophilic nitrate reduction and denitrification (Cava *et al.* 2009). Some strains of *Thermus thermophilus* reduce nitrate to nitrite (Cava *et al.* 2008b), while some work shows that other isolates of *T. thermophilus* and *T. oshimai* are incomplete denitrifiers that terminate with N₂O as a final nitrogen product (Hedlund *et al.* 2011). Some species of *Thermus*, such as *T. Brockianus*, *T. antranikianii*, and *T. scotoductus*, are known nitrate reducers (da Costa *et al.* 2006), but their capacity for denitrification has not been well characterized.

Several genetic and genomic studies have shed light on nitrate reduction and denitrification pathways in *Thermus*. Genes for nitrate reduction are found within the *nar* gene cluster and neighboring genes code for nitrogen oxide reductases responsible for denitrification (Gounder *et al.* 2011, Murugapiran *et al.* 2013a; Murugapiran *et al.* 2013b, Zhou *et al.* 2016, Mefferd *et al.* 2016). *Thermus* denitrification genes are sometimes present on a plasmid (Ramirez-Arcos *et al.* 1998; Brüggemann *et al.* 2006; Murugapiran *et al.* 2013b). For example, genes encoding the ability to reduce nitrate to nitrite in *T. thermophilus* HB8 and NAR1 comprise three adjacent gene clusters, *nar*, *nrc*, and *dnr*, which are located on a megaplasmid, termed the nitrate conjugative element (NCE). The megaplasmid carrying the NCE can be transferred among *T. thermophilus* strains by conjugation (Ramirez-Arcos *et al.* 1998), implicating horizontal gene transfer (HGT) of the NCE is possible in *Thermus*. Other nitrogen oxide reductases can also be found on megaplasmids. Such is the case with *T. oshimai* JL-2 and *T. thermophilus* JL-18 whose megaplasmids, approximately 0.27 Mb and 0.26 Mb respectively, contain a gene cluster for the reduction of nitrate to N₂O (Murugapiran *et al.* 2013b). Additionally, in some *Thermus*, the megaplasmid has much lower genetic stability than the genome and appears to be evolving faster than the chromosome (Brüggemann *et al.* 2006; Murugapiran *et al.* 2013a). The possibility of HGT of denitrification genes is further supported by research done using *Thermus* species as models for thermophilic HGT. For instance, whole-genome studies of *T. scotoductus* SA-01 (Gounder *et al.* 2011) and *T. thermophilus* HB8 and HB27 (Kumwenda *et al.* 2014) have uncovered evidence for large-scale genetic loss, acquisition, and rearrangement. *T. thermophilus* HB27 is naturally competent, containing many proteins associated with competence and conjugation (Averhoff 2009), and can take up DNA at any stage during growth (Hidaka *et al.* 1994; César *et al.* 2011).

These studies have shed some light on denitrification pathways and nitrogen oxide reductase gene evolution in *Thermus*. However, these studies only encompass a few species of *Thermus* and do not provide a comprehensive characterization of the distribution and evolution of denitrification genes in the genus as a whole. The goal of this study was to characterize denitrification pathways in *Thermus* to gain insight into their potential roles in the nitrogen cycle in high-temperature environments and to determine the evolutionary forces driving denitrification potential across the genus *Thermus*. To determine the terminal denitrification products in *Thermus* species, a collection of 25 strains representing ten species of *Thermus* from hot springs in Yunnan Province, China, were grown under denitrifying conditions and nitrogenous products were measured to evaluate their denitrification capacity. Additionally, to determine the distribution of denitrification genes in the genus *Thermus*, denitrification genes were recovered from new and existing genomes and by PCR using *Thermus*-specific primers designed in this study. Finally, phylogenetic analyses and gene loss/gain studies were performed in an effort to untangle the impacts of vertical and horizontal evolution of denitrification genes in *Thermus*. Results show that incomplete denitrification pathways are common in *Thermus* species. These pathways terminate with nitrite, NO, or N₂O as terminal products of nitrate reduction, as evidenced by both growth experiments and the variable presence of nitrogen oxide reductase genes in *Thermus* genomes. Moreover, the resulting tree topologies and patterns of gene presence/absence show some evidence that HGT and loss/gain shaped the evolution of denitrification genes in the genus *Thermus*.

RESULTS AND DISCUSSION

Determination of nitrate reduction intermediates and terminal products

For this study, 29 *Thermus* strains were tested initially. *T. caliditerrae* YIM77777 did not reduce nitrate. *T. brockianus* YIM 77709, *T. scotoductus* YIM 77398, and *T. tengchongensis* YIM 77427 grew poorly on the medium used in this study. These four strains were removed from the remaining work. The remaining 25 *Thermus* included in this study reduced nitrate and failed to produce N₂ in Balch tubes with Durham vials within 96 hours of cultivation with 4.5 mM nitrate as the sole terminal electron acceptor. Activity of detected nitrogen oxide reductases varied among the isolates (Table 2), though generally strains of the same species of *Thermus* showed the same activity. For example, all *T. arciformis*, *T. antranikianii*, and *T. tengchongensis* strains appeared to be incomplete denitrifiers terminating with N₂O. By comparison, all *T. caliditerrae*, *T. igniterrae*, and *T. scotoductus* strains reduced nitrate to nitrite as a final product. This trend was not seen, however, in *T. amyloliquefaciens*, *T. brockianus*, *T. oshimai* and *T. thermophilus*, where different strains terminated with different intermediates of denitrification (Table 2). To confirm the nitrate reduction phenotype in *Thermus* in more detail, *T. arciformis* JCM15153 was selected for further analysis based on its consistent growth phenotype in several replicates (Fig. 3). In this strain, there was a long lag phase until exponential growth was detected after 36 hours. The results of this experiment were consistent with those found in the end point experiments, where N₂O accumulated in cultures over time as a final product of denitrification. Results also showed a near-stoichiometric conversion of nitrate to N₂O with transient production of nitrite and no production of N₂.

NO was not assayed with the methods used in this work, however, NO production can be inferred to be a terminal product in several cases. Nitrite reduction to NO is the probable terminal product for *T. brockianus* YIM 77420.2, *T. brockianus* YIM 77904, *T. brockianus* YIM 77927, *T. oshimai* YIM 77923.2, and *T. thermophilus* YIM 77318. In these cultures, nitrate was removed

during anaerobic growth with nitrate as the sole terminal electron acceptor, yet neither nitrite, N₂O, nor N₂ were detected. This phenotype, in combination with the presence of *narG* and *nirK/S*, and apparent absence of *nor* and *nos* (Table 2) point to NO as the most likely denitrification product in these strains.

Together, these results strongly suggest that incomplete denitrification pathways are common in *Thermus* species. Consistent with the incomplete denitrification phenotype found in other *Thermus* strains (Hedlund *et al.* 2011), nitrate reduction to either nitrite, NO, or N₂O as terminal products indicates varying denitrification capabilities within *Thermus*, with most strains ending with N₂O as a final product. These results point to important ecological implications to consider and test further. These data support the idea that *Thermus* in geothermal areas may serve as a source of N₂O, a strong greenhouse gas and stratospheric reactant, as has been measured in geothermal springs in the U.S. Great Basin (Hedlund *et al.* 2011). Incomplete denitrification pathways to N₂O may promote N-cycling within hot spring systems, slowing the rate at which N is removed from the system. However, single-cell genomic analysis of 'Aigarchaeota' in GBS show this microbial dark matter group is a predicted N₂O reducers (Rinke *et al.* 2013; Hedlund *et al.* 2015b) and is a possible metabolic partner of *Thermus*. More research is required to test this idea.

Table. 2 Measured reductase activity and gene amplification

Strain	Measured Activity				Amplified Genes
	Nar	Nir	Nor	Nos	
<i>T. amyloliquefaciens</i> YIM 77409	+	(+)	+	-	<i>narG, nirS, norB</i>
<i>T. amyloliquefaciens</i> YIM 77735.1	+	-	-	-	<i>narG, norB</i>
<i>T. antranikianii</i> YIM 77311.1	+	(+)	+	-	<i>narG</i>
<i>T. antranikianii</i> YIM 77430.1	{+}	(+)	+	-	<i>narG, nirK</i>
<i>T. antranikianii</i> YIM 77730	+	(+)	+	-	<i>narG, nirK</i>
<i>T. arciformis</i> JCM15153	+	(+)	+	-	<i>narG, nirS, norB</i>
<i>T. brockianus</i> YIM 77420.2	+	(+)	-	-	<i>narG, nirS</i>
<i>T. brockianus</i> YIM 77904	+	(+)	-	-	<i>narG, nirK, nirS</i>
<i>T. brockianus</i> YIM77927	+	(+)	-	-	<i>narG, nirK, nirS</i>
<i>T. brockianus</i> YIM 779134	+	(+)	+	-	<i>narG, nirK, nirS</i>
<i>T. caliditerrae</i> YIM77925	+	-	-	-	<i>narG, nirS, norB</i>
<i>T. igniterrae</i> YIM 77777.1	+	-	-	-	<i>narG</i>
<i>T. oshimai</i> YIM 77359	+	(+)	+	-	<i>narG, nirK, nirS, norB</i>
<i>T. oshimai</i> YIM 77838.1	+	(+)	-	-	<i>narG, nirK, nirS, norB</i>
<i>T. oshimai</i> YIM 77923.2	+	(+)	-	-	<i>narG, nirK, nirS</i>
<i>T. scotoductus</i> YIM 77445.2	+	-	-	-	<i>narG</i>
<i>T. tengchongensis</i> YIM 77357	+	(+)	+	-	<i>narG, nirS, norB</i>
<i>T. tengchongensis</i> YIM 77392	+	(+)	+	-	<i>narG, nirS, norB</i>
<i>T. tengchongensis</i> YIM 77392.1	+	(+)	+	-	<i>narG, norB</i>
<i>T. tengchongensis</i> YIM 77401	+	(+)	[+]	-	<i>narG, nirS, norB</i>
<i>T. tengchongensis</i> YIM 77410	+	(+)	+	-	<i>narG, nirS, norB</i>
<i>T. tengchongensis</i> YIM 77727	+	(+)	+	-	<i>narG, norB</i>
<i>T. tengchongensis</i> YIM 77924	+	(+)	+	-	<i>narG, nirS, norB</i>
<i>T. thermophilus</i> YIM 77318	+	(+)	-	-	<i>narG, nirK</i>
<i>T. thermophilus</i> YIM 77430.2	+	-	-	-	<i>norB</i>

Statistical significance was calculated using a Student's T-test in R.

+, Statistically significant decrease in a starting substrate (nitrate) and increase in product compared to uninoculated negative controls ($p \leq 0.1$).

(+), Inferred Nir activity due to a significant decrease in nitrite and significant increase in N_2O compared to negative controls, or presence of *nir* genes ($p \leq 0.1$).

[+], Incomplete denitrifier that produces ≥ 1 mM of N_2O in at least two replicates in multiple assays.

{+}, Decrease in nitrate and increase in nitrite compared to an uninoculated negative control ($p \leq 0.102$).

-, No reductase activity detected.

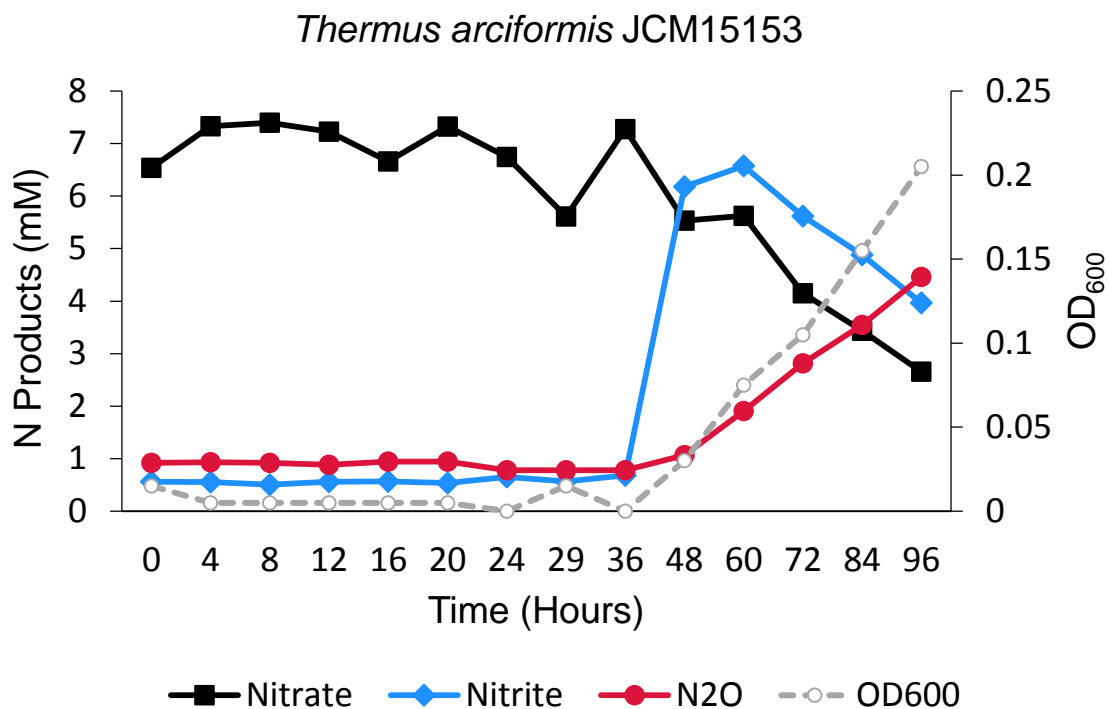


Figure 3. Near-stoichiometric conversion of nitrate to N₂O during growth. *Thermus arciformis* JCM15153 cells were grown with 9 mM NO₃⁻-amended CMD and sampled periodically for quantification of cell density using a Genesys 10 Series Spectrophotometer (Thermo, Madison, WI, USA) and possible denitrification intermediates (i.e. nitrite and N₂O). Reduction of nitrate to N₂ was never detected in Balch tubes with Durham vials. Data are representative of triplicate experiments; data from replicate experiments can be found in Figure S1, Appendix.

Presence and arrangement of denitrification gene clusters

Denitrification genes were recovered from new genomes of *T. arciformis* JCM15153, *T. brockianus* YIM 77927, *T. caliditerrae* YIM 77925, and *T. tengchongensis* YIM 77924, and previously available genomes of *T. amyloliquefaciens* YIM 77409 (Zhou *et al.* 2016) and *T. tengchongensis* YIM 77401 (Mefferd *et al.* 2016) (Fig. 4). The genome of *T. caliditerrae* YIM77777 did not contain nitrogen oxide reductase genes, consistent with the absence of growth in nitrate reduction experiments (Mefferd *et al.* 2016). The other six strains contained a complete nitrate reductase operon (*narGHJK*) and two nitrate/nitrite transporters (*narK1* and *narK2*). Genes

encoding nitrite reductase (*nirS*) and nitric oxide reductase (*norB* and *norC*) were also found in proximity to the nitrate reductase operon, with the exception of strain *T. brockianus* YIM 77927, which contained two nitrite reductases (*nirK* and *nirS*) and was missing *nor* genes. As noted previously (Murugapiran *et al.* 2013a, Zhou *et al.* 2016; Mefferd *et al.* 2016), nitrous oxide reductase (*nos*) genes were absent in all six *Thermus* strains examined. Nitrogen oxide reductase genes are co-localized and appear to have shared synteny, consistent with other *Thermus* genomes (Gounder *et al.* 2011; Murugapiran *et al.* 2013a; Murugapiran *et al.* 2013b). This suggests nitrogen oxide reductase genes in different species of *Thermus* have descended from the same ancestor and recombination events did not separate these loci.

It should be noted that the genome of *T. caliditerrae* YIM 77925 appears to contain C-terminal truncations in NirS and NorB (Fig. 4). *T. caliditerrae* YIM 77925 is also missing *norC*, an essential periplasmic *nor* subunit that shuttles electrons to the catalytic subunit *norB* (Thorndycroft *et al.* 2007; Hino *et al.* 2010). Together, this may explain why there was no detected activity of *nirS* and *norB* in *T. caliditerrae* YIM 77925 cultures (Table 2). Interestingly, there is no indication that truncations in *nirS* and *norB* include binding sites or amino acid residues of known functional importance (Figure S2 and Figure S3, Appendix; Rees *et al.* 1997; Watmough *et al.* 1999; Hemp and Gennis *et al.* 2008).

All 16S rRNA gene PCR amplicons were most closely related to known *Thermus* species, with DNA identities of 98-99% (Fig. 5). These data confirm the identity of the strains used in this work as *Thermus* species. All nitrogen oxide reductase PCR amplicons were most closely related to those from other *Thermus* species, with DNA identities of 82-99% (Fig. 6A-6D). Putative *narG* fragments were amplified from DNA extracts of all *Thermus* strains tested, except *T. thermophilus* YIM 77430.2. The presence of *nir* and *nor* gene fragments varied among the *Thermus* strains used

in this study. However, strains of the same species of *Thermus* showed similar genetic capacity for denitrification and some general patterns can be described (Table 2). For *T. antranikianii*, *narG* and *nirK* were detected in most strains. *narG*, *nirK*, and *nirS* were detected in most *T. Brockianus* strains. In most *T. oshimai* strains, *narG*, *nirK*, *nirS*, and *norB* were detected. *narG*, *nirS*, and *norB* were amplified in most *T. tengchongensis* strains. The presence/absence of nitrogen oxide reductase genes in these strains is generally consistent with phenotypes observed in these strains; however, there are instances where the detected reductase activity and the presence of a given denitrification gene did not agree (Table 2). *nar* genes were not found in *T. thermophilus* YIM 77430.2 using primers for this study, but nitrate reductase activity was detected. *nir* genes were not detected for *T. antranikianii* YIM 77311.1 and *T. tengchongensis* YIM 77392.1, though there was measured nitrite reductase activity. Finally, *nor* genes were not detected in all *T. antranikianii* strains and *T. Brockianus* YIM 7779134, though NO reduction to N₂O was detected. In these cases, inconsistencies can be attributed to the limited sensitivity of the PCR primers used in this work. Future work will be needed to design primers with better coverage within the *Thermales*, perhaps using the sequences similar to those in this work that include degenerate positions, or by incorporating alignments with denitrification gene sequences from other published genomes.

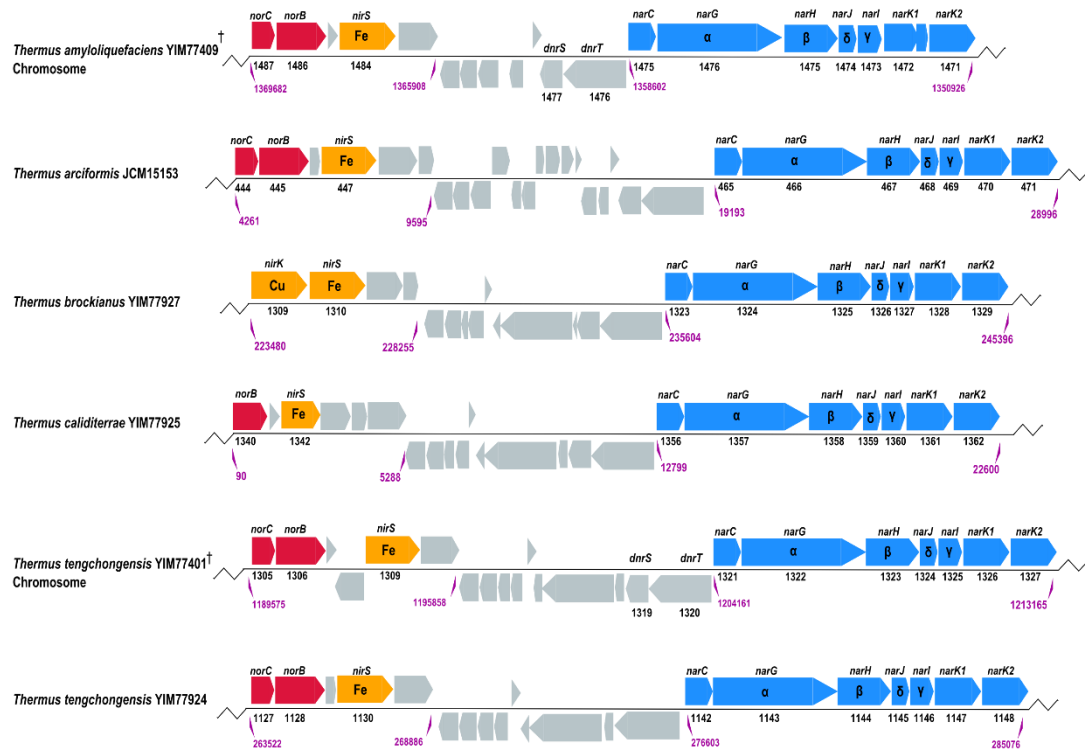


Figure 4. Map showing *nar* operon and neighboring genes involved in denitrification located on the chromosome of *T. amyloliquefaciens* YIM 77409, *T. arciformis* JCM15153 and *T. tengchongensis* YIM 77924. Numbers below genes indicate provisional ORF numbers found in RAST and IMG (indicated by †) for *T. tengchongensis* YIM 77401 (BS84DRAFT_1309) and *T. terrae* YIM 77409 (BS74DRAFT_1484). The locations in the chromosome are indicated below in purple text. *nar*: nitrate reductase; *nir*: nitrite reductase; *nor*: nitric oxide reductase; *dnr*: denitrification regulator. Figure modified from Murugapiran *et al.* 2013a.

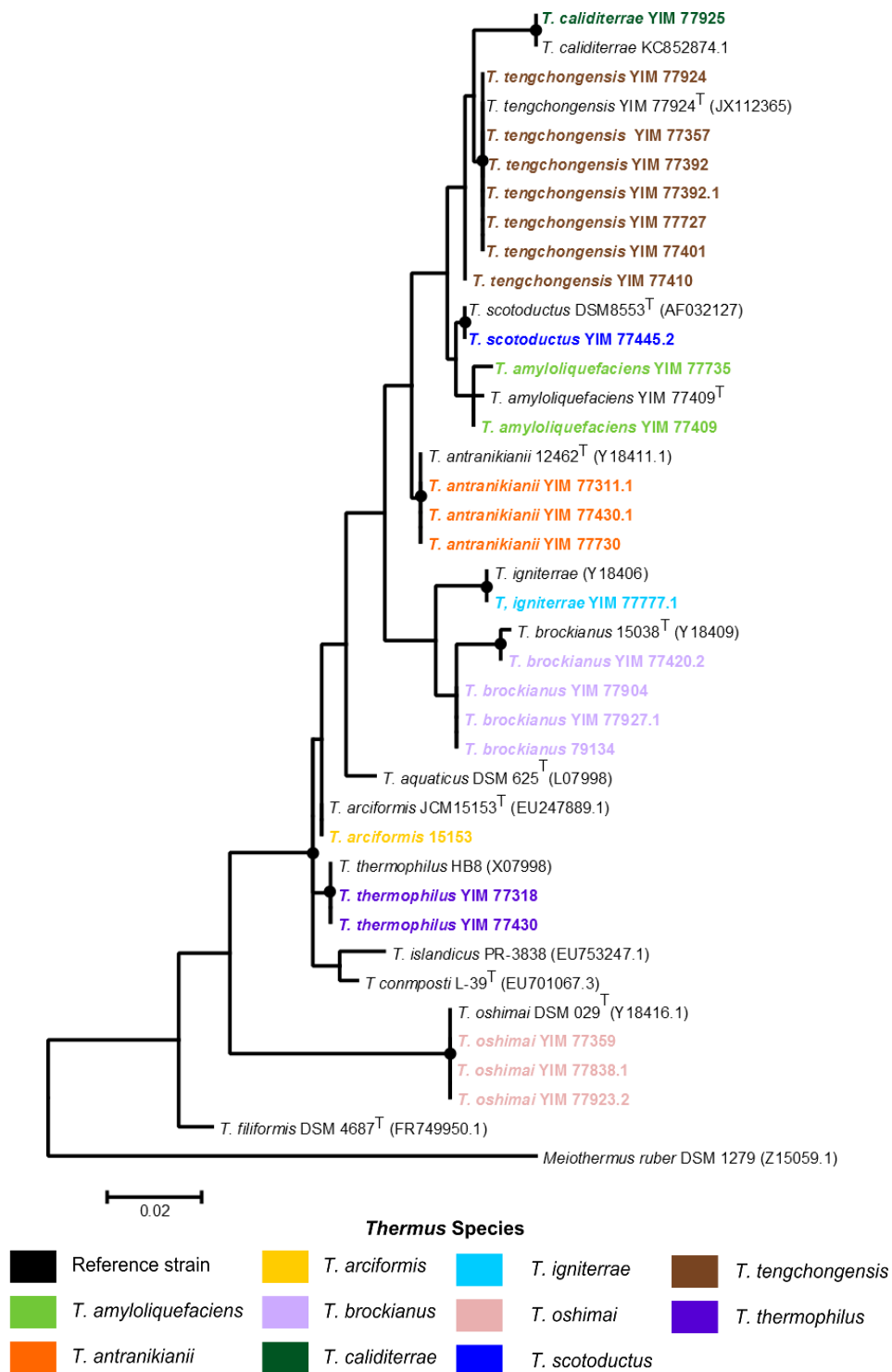


Figure 5. Maximum-likelihood phylogenetic tree based on 16S rRNA gene sequences of *Thermus* strains isolated from the Chinese geothermal areas. Bar indicates 0.02 substitutions per nucleotide position. Bootstrap values ≥ 80 are represented by filled circles. Constructed using the Tamura-Nei DNA substitution model (4031.6 BIC score). Only *Thermus* strains that have nitrogen oxide reductase genes were included.

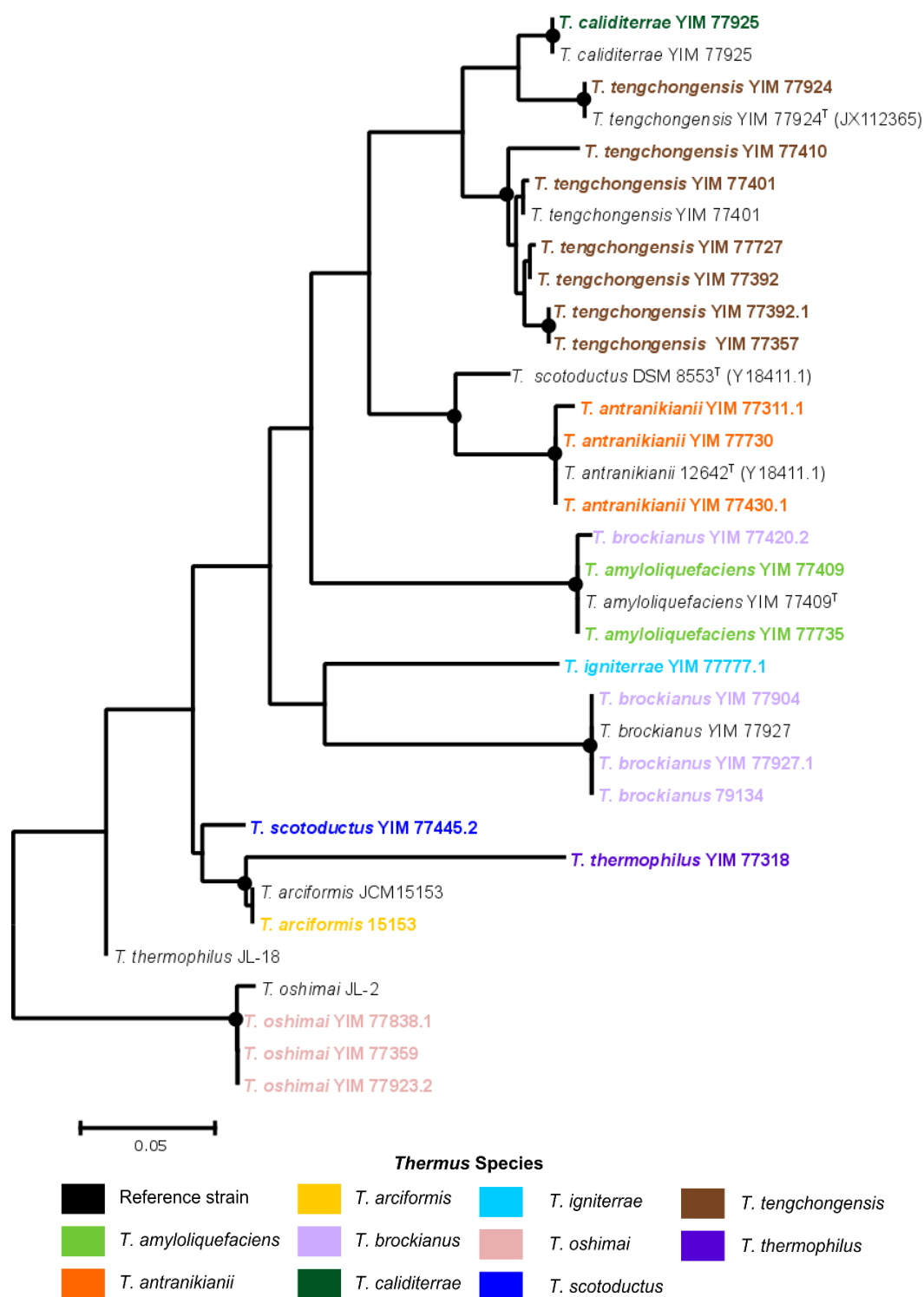


Figure 6A. Maximum-likelihood phylogenetic tree showing the relationships of the *narG* nucleotide sequences from *Thermus* strains. Bar indicates 0.05 substitutions per nucleotide position. Bootstrap values ≥ 80 are represented by filled circles. Constructed using the Tamura 3-parameter DNA substitution model with 5 gamma distributions (7124.6 BIC score).

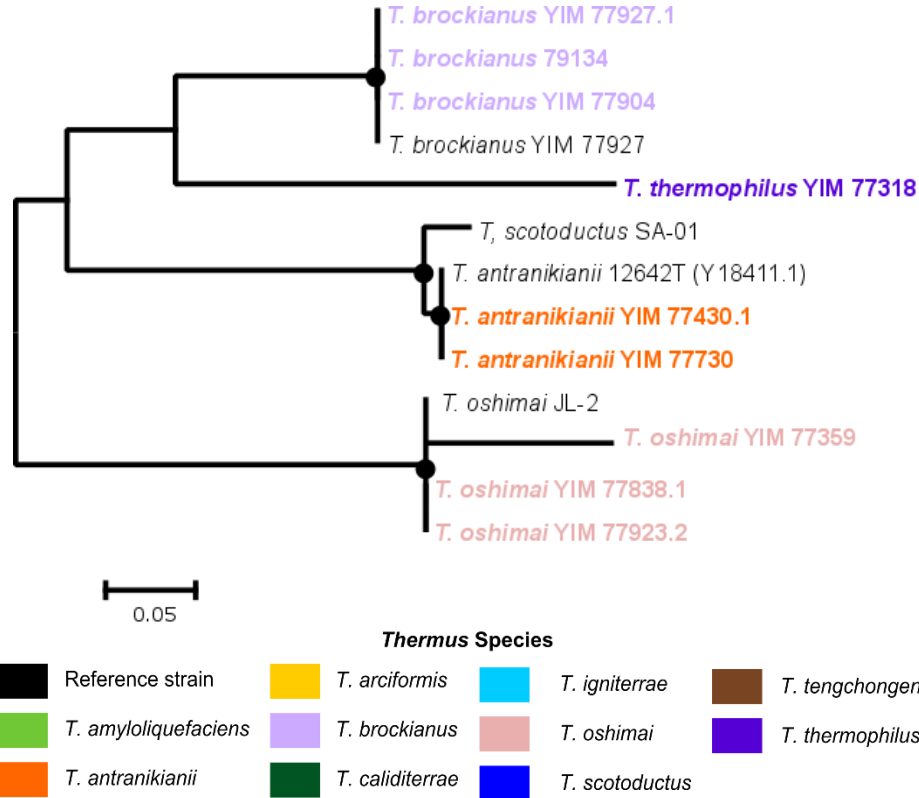


Figure 6B. Preliminary maximum-likelihood phylogenetic tree showing the relationships of the *nirK* nucleotide sequences from *Thermus* strains. Bar indicates 0.02 substitutions per nucleotide position. Bootstrap values ≥ 80 are represented by filled circles. Constructed using the Tamura 3-parameter DNA substitution model (3595.1 BIC score).

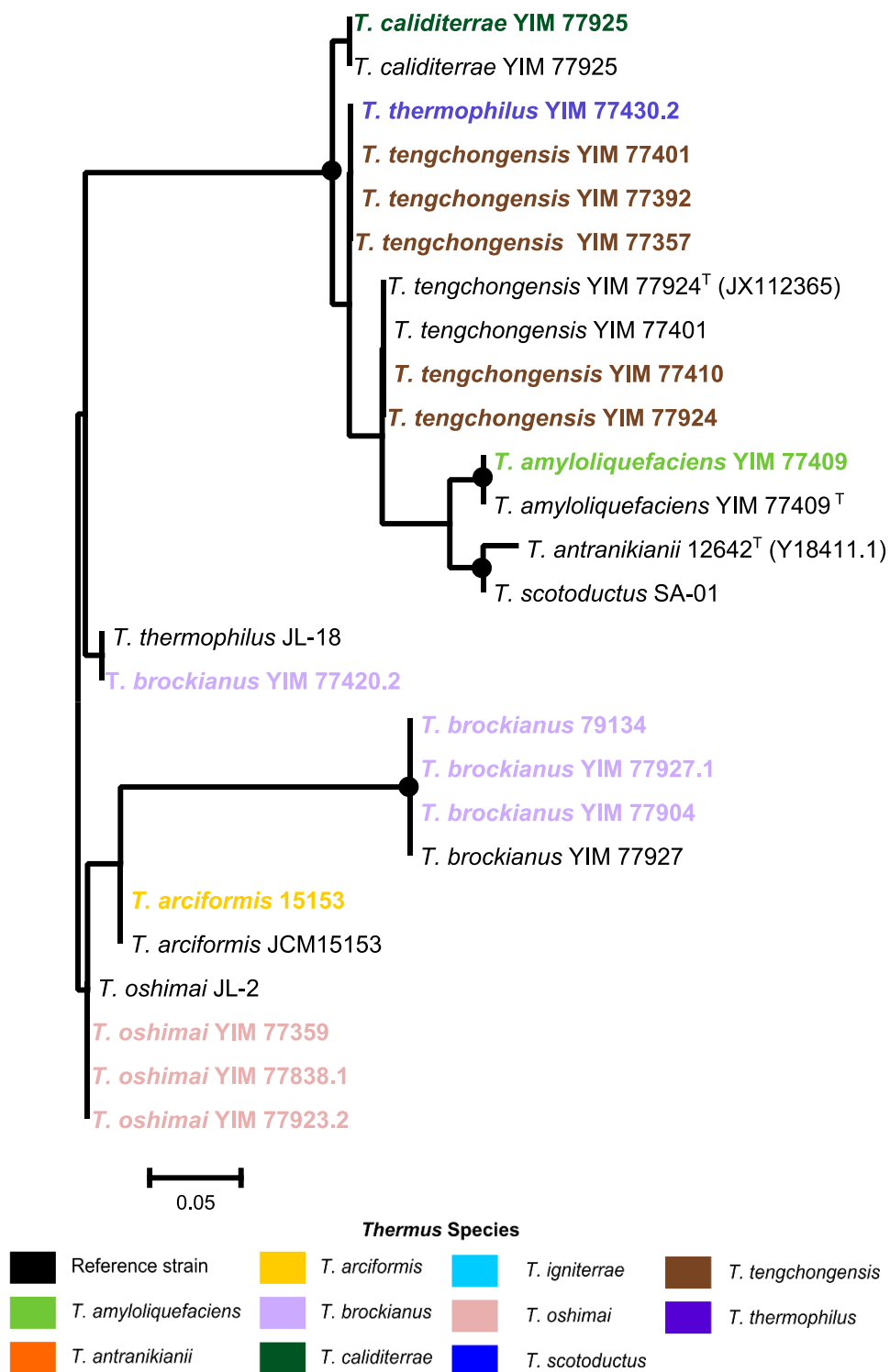


Figure 6C. Maximum-likelihood phylogenetic tree showing the relationships of the *nirS* nucleotide sequences from *Thermus* strains. Bar indicates 0.05 substitutions per nucleotide position. Bootstrap values ≥ 80 are represented by filled circles. Constructed using the Jukes-Cantor DNA substitution model (740.3 BIC score).

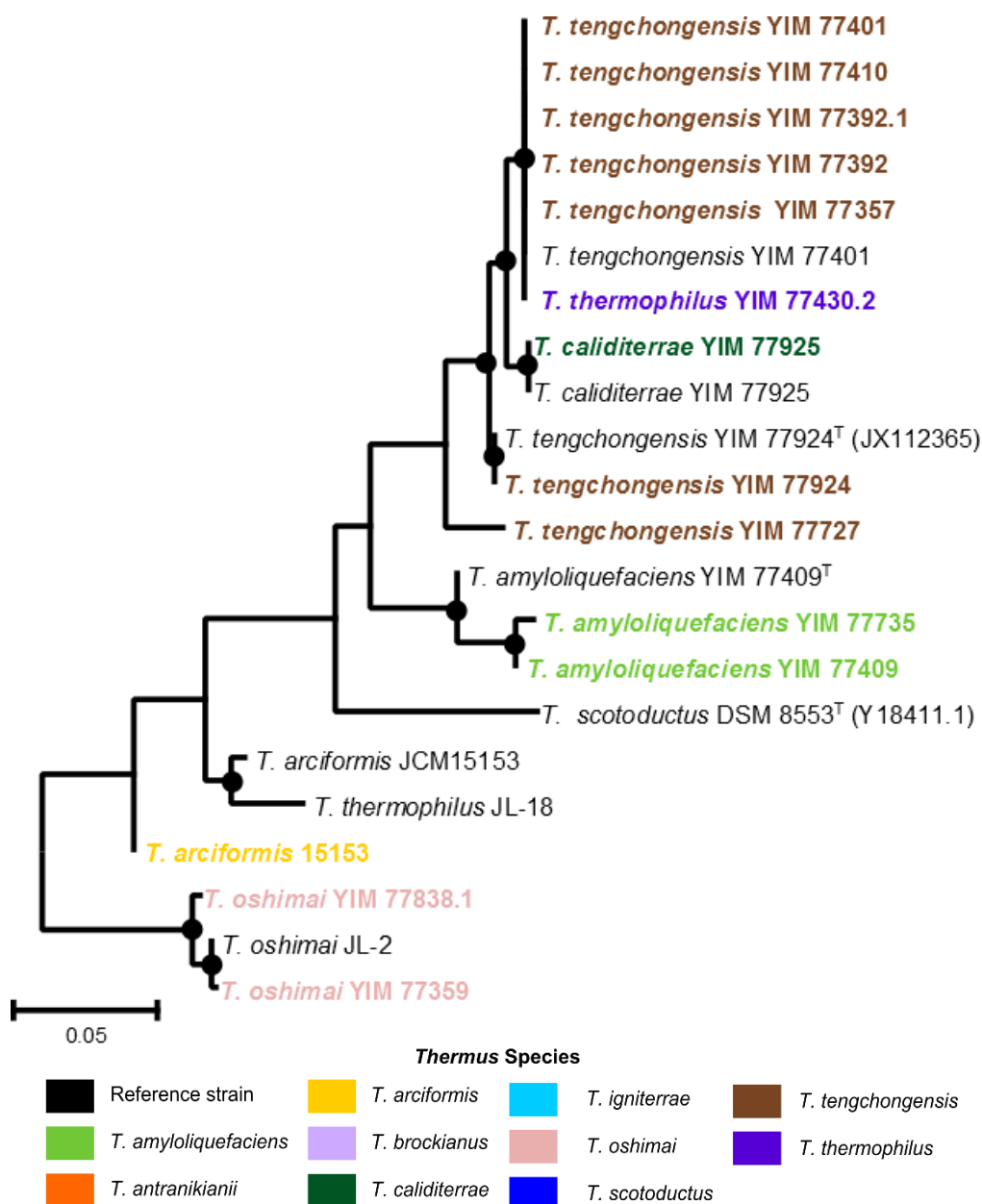


Figure 6D. Maximum-likelihood phylogenetic tree showing the relationships of the *norB* nucleotide sequences from *Thermus* strains. Bar indicates 0.02 substitutions per nucleotide position. Bootstrap values ≥ 80 are represented by filled circles. Constructed using the Hasegawa-Kishino-Yano DNA substitution model (4841.1 BIC score).

Phylogenetic analysis and inferring horizontal gene transfer

HGT can be inferred through visual observation of phylogenetic trees showing the relationship between nitrogen oxide reductase genes (Fig. 6A-6D). To illustrate, examples exist where the topology of the 16S rRNA reference tree (Fig. 5) is discordant with the trees based on nitrogen oxide reductase genes. From the topology of the *narG* genes, it appears that the *T. Brockianus* YIM 77420.2 nitrate reductase is more closely related to that in *T. Amyloliqefaciens* YIM77409. Likewise, the *T. scotoductus* YIM 77445.2 nitrate reductase is more closely related to that of *T. thermophilus* strains. Furthermore, the *T. Brockianus* YIM 77420.2 *nirS* is more closely related to the *T. thermophilus* JL-18. From the topology of the *norB* genes, *T. thermophilus* YIM 77430.2 NO reductase is more closely related to that from *T. tengchongensis* strains. Discordance appeared more frequently in some species than in others, specifically, in *T. Brockianus*, *T. thermophilus*, and *T. scotoductus* strains. On the other hand, some species showed no evidence of HGT, such as *T. oshimai*, where all nitrogen oxide reductase genes branched together in every tree.

Another method to assess HGT is to perform similarity searches using BLAST to find relatives of a given gene among a selection of available genomes. Phyletic patterns, differences in gene absence/presence in organisms and relatives, can be detected in this way. The most similar sequences found in genomes in the database can then be used to infer HGT (Zhaxybayeva 2009). A blastx search was performed to search for genes closely related to *T. oshimai* JL-2 *nirK* in sequenced genomes found in GenBank. With the exception of other *Thermus* species, *T. oshimai* JL-2 *nirK* relatives were not found in other members of the phylum *Deinococcus-Thermus*. However, genes related to the *T. oshimai* JL-2 *nirK* were present in diverse members the bacterial phylum *Fimicutes* (e.g., *Geobacillus*, *Paenibacillus*, and *Caldalkalibacillus* species), with related genes also in the genus *Thioalkalivibrio* in the *Proteobacteria* and "*Candidatus* Caldiarchaeum

subterraneum" in the candidate archaeal phylum "Aigarchaeota". Together, these data suggest *Thermus*, *Thioalkalivibrio*, and "*Candidatus* Caldiarchaeum subterraneum" may have inherited *nirK* from *Firmicutes*; for example, *Thermus* and "*Candidatus* Caldiarchaeum subterraneum" may have received *nirK* genes from *Geobacillus*, which cohabitates in terrestrial geothermal systems (Cole *et al.* 2013a; Cole *et al.* 2013b). However, this approach of using phyletic patterns and top-scoring BLAST hits to infer HGT is not the most reliable and relies on how broadly certain groups of organisms are represented in the database. Future analysis is required to more meaningfully map the evolutionary history of *nirK* in *Thermus*.

SH-test was performed to infer events of HGT. A statistically significant difference ($p < 0.0001$) was observed between the topology of the reference tree (tree based on 16S gene sequences) and gene trees based on *nirK*, *nirS*, and *norB*. Possible explanations for the differences in tree topology indicated by the SH-test include gene duplication, gene loss, or HGT (Dávalos *et al.* 2012; Ravenhall *et al.* 2015). When there are multiple copies of a gene, paralogs could result in significantly incongruent trees. In this case, a gene copy in one taxon is not orthologous to those of others and could have a different gene history. This can cause significant differences in the topology of the candidate trees. However, gene duplication in *Thermus* denitrification genes has not been reported and can therefore be eliminated as a possible source of discordance. Gene loss would result in missing branches of a gene tree, thus showing a significantly different topology from the reference tree. However, in this study the SH-test was performed using pruned trees. Thus, the discordance is not likely explained by gene loss, suggesting HGT represents the most likely cause of the differences in the topology trees based on the 16S rRNA and nitrogen oxide reductase gene sequences. Unfortunately, identifying all instances of HGT is quite difficult using the SH-test alone. It is difficult to use the SH-test to infer HGT between closely neighboring taxa,

as this type of transfer event cannot be easily detected with this method (Zhaxybayeva 2009). It is also possible a weak phylogenetic signal, or tree with insufficient bootstrap support in some branches, cannot be resolved sufficiently by this test. This may have been the case with the tree based on *Thermus narG* sequences, as the SH-test for this data set never came to completion. However, future work using methods such as ancestral state reconstruction (Carmel *et al.* 2010) and identification of differences nucleotide word usage patterns (Monier *et al.* 2007) can improve the work done in this study.

From the construction of phylogenetic trees (Fig. 6A-6D) and the physiological data (Table 2), some conclusions can be made on about gene loss and gain, especially in the case of nitrite reductase genes. *nirK* genes were detected in deeper lineages of *Thermus*, specifically in *T. antranikianii*. *nirS* genes were detected in more recent lineages of *Thermus*, specifically in *T. amyloliquefaciens* and *T. tengchongensis*. Interestingly, both *nirK* and *nirS* were detected in *T. brockianus* and *T. oshimai* strains, which appear to have evolved in the middle of old and recent lineages of *Thermus*. These observations can indicate that NirK is a protein found in *Thermus* ancestors and is lost in more recent lineages, while *nirS* is gained later in the evolutionary history of *Thermus*. The question regarding these observations of gene loss/gain of denitrification genes in *Thermus* remains open, and further phylogenetic analyses to model these changes will focus on this vital question.

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CHAPTER 4

MANUSCRIPT FOR *GENOME ANNOUNCEMENTS*

High Quality Draft Genomes from *Thermus caliditerrae* YIM 77777 and *T. tengchongensis* YIM 77401 Isolates from Tengchong, China

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Graduate Student's Involvement and General Background

This chapter represents a manuscript published in *Genome Announcements*. The research detailed in this chapter was conducted by me, Hedlund lab members, JGI representatives and researchers, and collaborators for my graduate thesis work during my tenure as a Master's student at the University of Nevada, Las Vegas. Experiments were designed by Zhou and Hedlund. Genomic prep was performed by Williams. Experiments and genome sequencing were performed by the team at JGI. The data was analyzed by myself, Zhou, Murugapiran, and the team at JGI. Reagents, materials, analysis tools, and isolates were provided by JGI, Zhou, Li, and Hedlund. The manuscript was written and prepared by myself. Work highlighting the denitrification capacity and nitrogen oxide reductase genes in the genomes of *T. tengchongensis* YIM 77401 and *Thermus caliditerrae* YIM 77777 was conducted by myself.

ABSTRACT: The draft genomes of *T. tengchongensis* (*Tt*) YIM 77401 and *Thermus caliditerrae* YIM 77777 are 2,562,314 and 2,218,114 bp and encode 2,726 and 2,305 predicted genes, respectively. Gene content and growth experiments demonstrate broad metabolic capacity, including starch hydrolysis, thiosulfate oxidation, arsenite oxidation (*Tt*), incomplete denitrification (*Tt*), and polysulfide reduction.

Bacterial strains YIM 77401 and YIM 77777, members of the order *Thermales*, class *Deinococci*, were isolated from Frog Mouth Spring (Hamazui), Rehai National Park, Tengchong County, Yunnan Province, China (Ming *et al.* 2014). The draft genomes of the two strains were generated at the DOE Joint Genome Institute (JGI), Walnut Creek, CA, USA using Pacific Biosciences (PacBio) technology. A PacBio SMRTbell™ library was created and sequenced using the PacBio RS platform, which generated 191,522 filtered subreads totaling 522 Mbp for strain YIM 77401 and 280,439 filtered subreads totaling 626 Mbp for strain YIM 77777. HGAP (version: 2.0.0) (Chin *et al.* 2013) was used to assemble raw reads. Genome annotation was performed using the JGI Prokaryotic Automatic Annotation Pipeline (Huntemann *et al.* 2015) with manual curation using GenePRIMP (Pati *et al.* 2010) and additional manual review using the Integrated Microbial Genomes - Expert Review (IMG-ER) platform (Markowitz *et al.* 2009). JGI's library construction and sequencing protocols can be found at <http://www.jgi.doe.gov>.

The strain YIM 77401 genome encoded 2,726 predicted genes in 5 contigs, including 47 tRNA-encoding genes, and 3 rRNA operons, and the strain YIM 77777 genome encoded 2,305 predicted genes in 4 contigs, including 50 tRNA-encoding genes, and 3 rRNA operons. Both genomes included at least one megaplasmid (>100 kb), based on the presence of plasmid replicon domains

(Jorgensen *et al.* 2014). Analysis of carbohydrate-active enzymes (CAZymes) found in strains YIM 77401 and YIM 77777 genomes revealed 39 and 32 CAZymes, respectively. Among these were 11 and 9 glycoside hydrolases (GHs) in strains YIM 77401 and YIM 77777, respectively, including GHs predicted for starch hydrolysis (GH13 and GH57) in both strains. This is consistent with amylase activity observed in both isolates. The genome of YIM 77401 featured genes involved in arsenite oxidation (*aioAB*), consistent with arsenite oxidation activity observed in this isolate. Both genomes contained a *sox* gene cluster comprised of 10 genes (*soxABCDVWXYZ*), predicted for thiosulfate oxidation (Friedrich *et al.* 2005), similar to other *Thermus* species (Gounder *et al.* 2011; Murugapiran *et al.* 2013a; Skirnisdottir *et al.* 2001); however, thiosulfate oxidation activity was only detected in YIM 77777.

Strain YIM 77401 contained a chromosomally encoded nitrate reductase gene cluster (*narGHJK*) and two nitrate/nitrite transporters (*narK1* and *narK2*), similar to other *Thermus* species (Murugapiran *et al.* 2013a). Genes encoding the catalytic subunit of a *cd*-cytochrome nitrite reductase (*nirS*) and nitric oxide reductase (*norBC*) were also found in this genome. However, nitrous oxide reductase (*nos*) genes, which catalyze the reduction of nitrous oxide to dinitrogen, were absent, consistent with the incomplete denitrification phenotype found in several *Thermus* species (Murugapiran *et al.* 2013a; Hedlund *et al.* 2011) and the production of N₂O as the terminal denitrification product by YIM 77401. Additionally, YIM 77401 and YIM 77777 contained genes for polysulfide reduction (*psrABC*), which is similar to other *Thermus* genomes (Murugapiran *et al.* 2013a) and consistent with polysulfide reductase activity in both isolates.

NUCLEOTIDE SEQUENCE AND ACCESSION NUMBERS: These Whole Genome Shotgun projects have been deposited in Genbank under accession nos. JQNC01000001-JQNC01000004 (YIM77777) and JQLK01000001-JQLK01000005 (YIM77401). The genome sequence is available from Genbank (NZ_JQNC000000000; GI: 740207912) for *Thermus caliditerrae* YIM77777, and from Genbank (NZ_JQLK000000000; GI:740202250) for *T. tengchongensis* YIM77401. The data are also available at the Joint Genome Institute (JGI) Integrated Microbial Genomes (IMG) system (Markowitz *et al.* 2013).

FUNDING INFORMATION: The work conducted by the U.S. Department of Energy Joint Genome Institute is supported by the Office of Science of the U.S. Department of Energy under Contract No. DE-AC02-05CH11231. Additional support was supported by NSF Grant Number OISE-0968421 to Brian P. Hedlund, and Key Project of International Cooperation of Ministry of Science & Technology (MOST) (No. 2013DFA31980) and Natural Science Foundation of China (No. 31470139) to Wen-Jun Li.

CHAPTER 5

CONCLUSION

The genus *Thermus* is characterized by incomplete denitrification to nitrite, NO, or N₂O, with no strains producing N₂ as a terminal product and most strains ending with N₂O. These data suggest a model where denitrification in *Thermus* terminating with N₂O is common at high temperatures. This is consistent with the demand for energy at high temperatures (Price and Sowers 2004). Denitrification to N₂O allows for the production of protons without the energetic cost of the expression of Nos. Varying terminal denitrification products and the varying presence of detected genes for nitrogen oxide reductases indicate differing denitrification capabilities within *Thermus*. Some patterns could be observed, where denitrification phenotypes were consistent with the presence of denitrification genes in *Thermus* and strains of the same species appeared to have the same denitrification phenotypes. This work suggests both nitrate reducers and incomplete denitrifiers may be present in high temperature sites.

The data reported here suggest denitrification may be decoupled at high temperatures. Denitrification has an optimal temperature ranging from 20-60°C and declines rapidly above this temperature (Dawson and Murphy 1972; Knowles *et al.* 1982). Thermophilic organisms, like *Thermus*, are responsible for activity above this temperature. Unfavorable kinetic conditions at high temperatures could be responsible for the decoupling of denitrification in *Thermus*, with the reduction of N₂O to N₂ carried out by predicted N₂O reducers such as 'Aigarchaeota' (Rinke *et al.* 2013; Hedlund *et al.* 2015b), *Thermoflexus* (unpublished data), and *Fervidibacteria* (Rinke *et al.* 2013). High temperature life has a high demand for maintenance energy. This could result in a greater proportion of organic carbon being mineralized, resulting in lower amounts of organic

carbon left in the system. Both a supply of nitrate and an organic carbon source is necessary for denitrification to occur (Dincer and Kargi 2000). Having a metabolic partner to split the need for organic carbon sources decreases this demand. A partner organism reducing only N_2O to N_2 ($\Delta G^\circ = -339.5 \text{ kJ/mol}$) still gains an energetically comparable amount of free energy compared to the reduction of NO to N_2O ($\Delta G^\circ = -306.3 \text{ kJ/mol}$) (Zumft 1997). For these reasons, it is possible incomplete denitrification at high temperature may be more common than complete denitrification to N_2 .

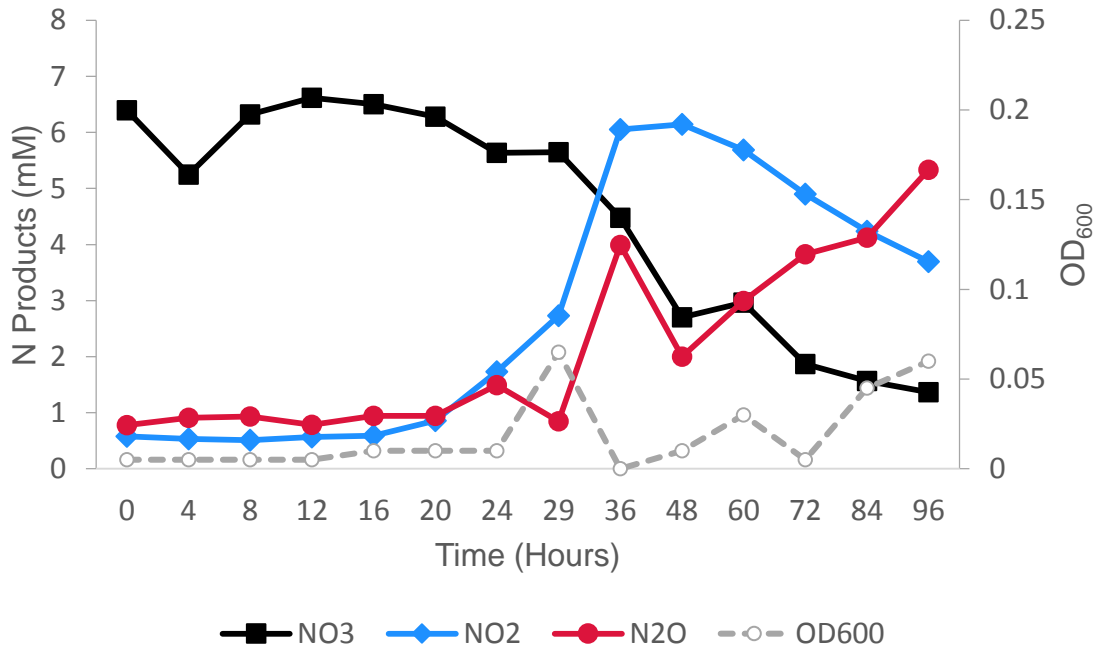
Moreover, observations made from constructed phylogenetic trees showed evidence for HGT, especially in *T. brockianus* and *T. scotoductus* strains, and gene loss/gain of nitrite reductase genes in *Thermus*. This is evident in the resulting topologies of the phylogenetic trees based on the DNA sequences of the PCR amplicons from this study. Rearrangements and loss of nitrogen oxide reductase genes has been shown in *Thermus* found in high-temperature environments (Gounder *et al.* 2011, Murugapiran *et al.* 2013a) as a means to lose/acquire genes to sustain life in variable conditions. Consistent with this, the work done here demonstrates a possible energy-conserving and survival strategy in *Thermus*. Loss of genes (i.e. *nir* and *nos*) prevents the cost of making proteins that are not necessary and genes that provide respiratory options when oxygen is low and conditions are stressful, such as those in geothermal regions in Yunnan, are kept.

Despite a few inconsistencies between detected reductase activity and amplified genes, this study provides a broad description of denitrification capabilities and the evolution of denitrification pathways in the genus *Thermus* as a whole. Incomplete denitrification to N_2O by *Thermus* may be a source of N_2O , which may be further reduced to N_2 by other organisms or be released to the atmosphere. Additionally, phylogenetic observations made from this study point to the importance of gene loss/gain and HGT as a means of bacterial adaption at high temperatures.

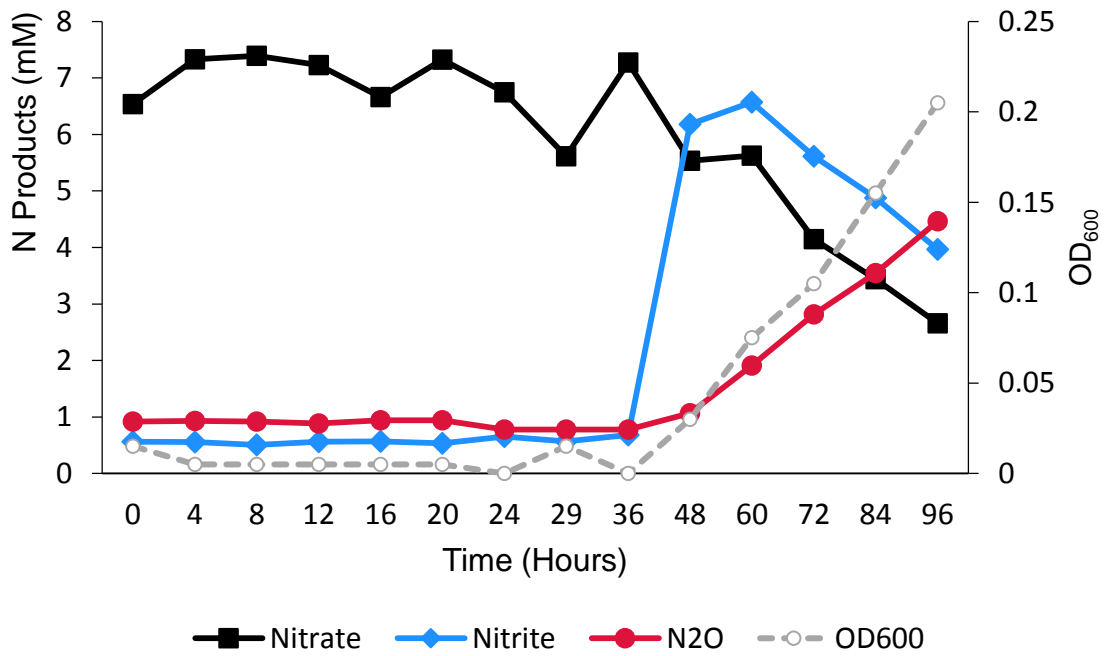
Overall, the results of this work provide greater insight in thermophilic denitrification and nitrogen cycling in geothermal environments.

APPENDIX

Thermus arciformis JCM15153 (Replicate A)



Thermus arciformis JCM15153 (Replicate B)*



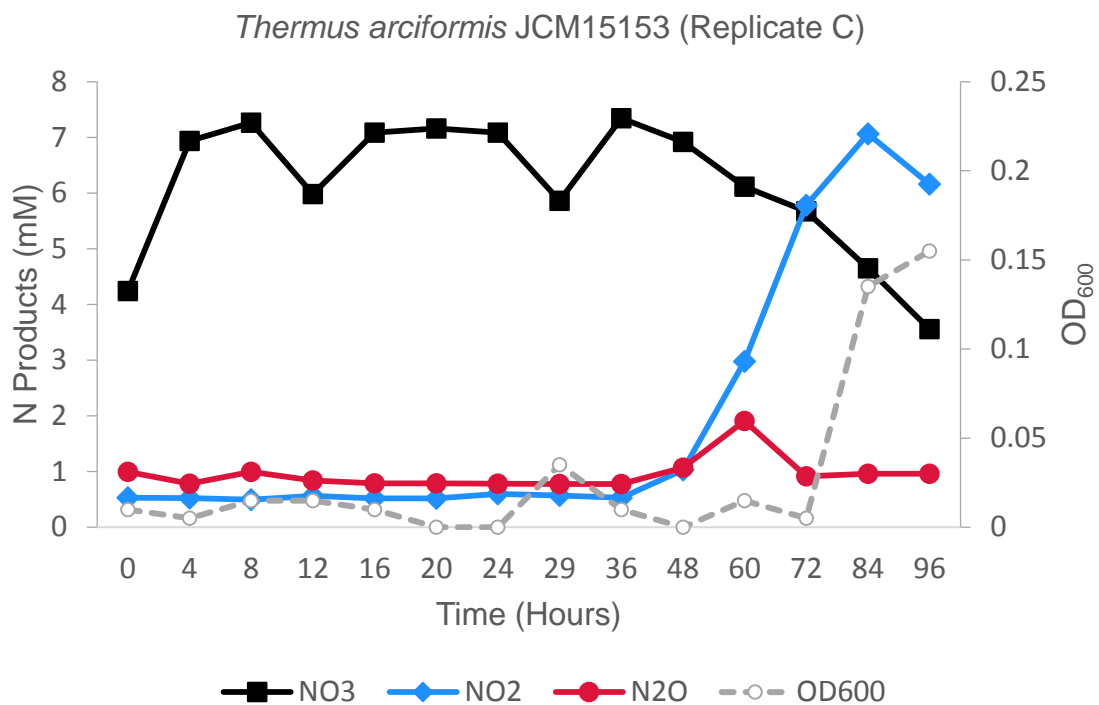


Figure S1. Near-stoichiometric conversion of nitrate to N₂O during growth. *Thermus arciformis* JCM15153 cells were grown with 9 mM NO₃⁻ amended CMD and sampled periodically for quantification of cell density using a Genesys 10 Series Spectrophotometer (Thermo, Madison, WI, USA) and possible denitrification intermediates (i.e. nitrite and N₂O). Reduction of nitrate to N₂ was never detected in Balch tubes with Durham vials. *Replicate B was determined to be most representative of the data.

PAO1	60	EFNEAKQIYFQRCAGCHGVL	RKGATGKPLTPDITQQR-----	GQQYLEALIT
JM300	35	EKEAAKKIYFERCAGCHGVL	RKGATGKNLEPHWEKTE-DGKKIEGGTLKLG	TKRLENI IA
Ps632	35	EKEASKQIYFERCAGCHGVL	RKGATGKNLEPHWSKTEADGKKTEGGTLNLG	TKRLENI IA
409	29	EREEAAKIYFDRCAGCHGVL	RKGATGPALDPKKMAEK-----	GLEYLKAVIF
153	18	EKERASQIYFDRCAGCHGVL	RKGATGPALDPKKMAER-----	GVEYLKAVIF
927	29	ERERASQIYFDRCAGCHGVL	RKGATGPALDPRRMAER-----	GLEYLKAVIF
925	29	EKEEAAKIYFDRCAGCHGVL	RKGATGPALDPKKMAEK-----	GVEYLKAAIF
401	14	EKEEAAKIYFDRCAGCHGVL	RKGATGPALDPKKMAEK-----	GVEYLKAVIF
924	29	EKEEAAKIYFDRCAGCHGVL	RKGATGPALDPKKMAEK-----	GVEYLKAVIF
PAO1	107	YGTPLGMPN	WGSSGELSKEQITLMAKYIQHTPPQPPEWGMPEMRESWKVLVKPEDRPPKKQ	
JM300	94	FGTEGGMVNYDD--ILT	AEEINLMARYIQHTPDIPPEFSLQDMKDSWNLIVPVAERP	PKKQ
Ps632	95	YGTEGGMVNYDD--ILT	KEEINMMARYIQHTPDIPPEFSLQDMKDSWNLIVPVEKRVT	KQ
409	76	GGLPGGMPDWGRQ	GILSEKDTELMARFLLEPPAPPVLT	FEDIRKTWKVHVPP
153	65	GGLPGGMPDWGRQ	GILSEKDTELMARFLLEPPPTPIPSYEEIRKTWKVYVP	PEKRPPKP
927	76	GGLPGGMPDWGRQ	GILSEKDTELVARFLLEPPAPPPIPSFEEVKKTWKVYVP	PEKRPNRP
925	76	GGLPGGMPDWGRQ	GILKEKDIELVARFLLEQPPAPPVPTFEEIKKTWKVQVP	PEKRPTKP
401	61	GGLPGGMPDWGRQ	GILKEKDIELVARFLLEQPPAPPVPTFEEIKKTWKVQVP	PEKRPTKP
924	76	GGLPGGMPDWGRQ	GILKEKDIELVARFLLEQPPAPPVPTFEEIKKTWKVQVP	PEKRPTKP

Figure S2. Amino acid sequences of NirS from *Pseudomonas aeruginosa* PAO1 (PAO1), *P. stutzeri* JM300 (JM300), *P. stutzeri* ZoBell 632 (Ps632), *T. amyloliquefaciens* YIM 77409 (409), *T. arciformis* JCM15153 (15153), *T. brockianus* YIM 77927 (927), *T. caliditerrae* YIM 77925 (925), *T. tengchongensis* YIM 77401 (401), and *T. tengchongensis* YIM 77924 (924) were aligned using ClustalW (Thompson *et al.* 1994). Methionine residues in green and residues in yellow indicate putative the heme *c*-binding sites.

Psd	202	WWWVVHLWVEGVWELIMGSMLAFVLIKITGVDREVVEKWLYVIIAMA--LITGIIGTGHH
409	202	WWWVVHLWVEATWEVLVGSIMAMALMHLLGTPRRIVETWLYLEVALV--FGTGILGLGHH
15153	202	WWWVVHLWVEATWEVLVGSIMAMALMHLLGTPRRIVETWLYLEVALV--FGTGILGLGHH
925	241	FQQFFWFYSHPTVYVMLLPYLGILAEVASTFSRKPLFGYKQMVWAQMGIVVLGTMVWAHH
401	202	WWWVVHLWVEATWEVLVGSIMAMALMHLLGTPRRIVETWLYLEVALV--FGTGILGLGHH
924	241	FQQFFWFYSHPTVYVMLLPYLGILAEVASTFARKPLFGYKQMVWAQMGIVVLGTMVWAHH

Figure S3. Amino acid sequences of NorB from *Pseudomonas denitrification* ATCC 13867 (Psd), *T. amyloliquefaciens* YIM 77409 (409), *T. arciformis* JCM15153 (15153), *T. caliditerrae* YIM 77925 (925), *T. tengchongensis* YIM 77401 (401), and *T. tengchongensis* YIM 77924 (924) were aligned using ClustalW (Thompson *et al.* 1994). Residues in pink indicate putative Fe_B ligands.

Table S1. *Thermus* master list and isolation source.

Strain	Hot Spring - Isolation source	GPS Coordinates
<i>T. amyloliquefaciens</i> YIM77409	Nuijia ancient hot spring	N 15.02333 E 99.98944
<i>T. amyloliquefaciens</i> YIM 77735.1	soil sample, near Shuirebaoza	N 24.95002 E 98.43742
<i>T. antranikianii</i> YIM 77311.1	soil sample near Shuirebaoza	N 24.95002 E 98.43742
<i>T. antranikianii</i> YIM 77430.1	soil sample near Shuirebaoza	N 24.95002 E 98.43742
<i>T. antranikianii</i> YIM 77730	soil sample near Shuirebaoza	N 24.95002 E 98.43742
<i>T. arciformis</i> JCM15153	Type strain	N 23.97111 E 109.75472
<i>T. brockianus</i> YIM 77420.2	Nuijia ancient hot spring	N 15.02333 E 99.98944
<i>T. brockianus</i> YIM 77709 ^a	soil sample near Shuirebaoza	N 24.95002 E 98.43742
<i>T. brockianus</i> YIM77904	Gongxiaoshe	N 25.44012 E 98.44081
<i>T. brockianus</i> YIM77927	Hehua	N 23.65489 E 97.87011
<i>T. brockianus</i> YIM79134	Shuirebaoza	N 24.95014 E 98.43743
<i>T. caliditerrae</i> YIM77925	Shuirebaozha	N 24.95014 E 98.43743
<i>T. caliditerrae</i> YIM 77777 ^a	Hamazui 2	N 24.95006 E 98.4380
<i>T. igniterrae</i> YIM 77777.1	Gongxiaoshe	N 25.44012 E 98.44081
<i>T. oshimai</i> YIM 77359	Hamazui 4	N 24.95006 E 98.4380
<i>T. oshimai</i> YIM 77838.1	Xianrendong	N 25.46721 E 98.49097
<i>T. oshimai</i> YIM 77923.2	Hehua	N 23.65489 E 97.87011
<i>T. scotoductus</i> YIM 77445.2	Hamazui 4	N 24.95006 E 98.43805
<i>T. scotoductus</i> YIM 77398 ^a	Nuijia ancient hot spring	N 15.02333 E 99.98944
<i>T. tengchongensis</i> YIM 77357	Shuirebaoza	N 24.95002 E 98.43742
<i>T. tengchongensis</i> YIM 77392	Shuirebaoza	N 24.95002 E 98.43742
<i>T. tengchongensis</i> YIM 77392.1	Shuirebaoza	N 24.95002 E 98.43742
<i>T. tengchongensis</i> YIM 77401	Hamazui 2	N 24.95006 E 98.4380
<i>T. tengchongensis</i> YIM 77410	Hamazui 2	N 24.95006 E 98.4380
<i>T. tengchongensis</i> YIM 77427 ^a	Hamazui 2	N 24.95006 E 98.4380
<i>T. tengchongensis</i> YIM 77727	Hamazui 2	N 24.95006 E 98.4380
<i>T. tengchongensis</i> YIM77924	soil sample near Shuirebaoza	N 24.95002 E 98.43742
<i>T. thermophilus</i> YIM 77318	Shuirebaoza	N 24.95014 E 98.43743
<i>T. thermophilus</i> YIM 77430.2	soil sample near Shuirebaoza	N 24.95002 E 98.43742

^aremoved from this study

Table S1. *Thermus* master list and isolation source (con't)

Strain	Spring Temp./pH	Date of sample collection
<i>T. amyloliquefaciens</i> YIM77409	84 °C / 7.4	03/2010
<i>T. amyloliquefaciens</i> YIM 77735.1	73 °C / 7.0	03/2010
<i>T. antranikianii</i> YIM 77311.1	73 °C / 7.0	03/2010
<i>T. antranikianii</i> YIM 77430.1	73 °C / 7.0	03/2010
<i>T. antranikianii</i> YIM 77730	73 °C / 7.0	03/2010
<i>T. arciformis</i> JCM15153	N/A	N/A
<i>T. brockianus</i> YIM 77420.2	84 °C / 7.4	03/2010
<i>T. brockianus</i> YIM 77709 ^a	73 °C / 7.0	03/2010
<i>T. brockianus</i> YIM77904	73.8 °C / 7.29	06/2011
<i>T. brockianus</i> YIM77927	73 °C / 7.0	06/2011
<i>T. brockianus</i> YIM79134	79.8 °C / 7.5	06/2011
<i>T. caliditerrae</i> YIM77925	79.8 °C / 7.5	06/2011
<i>T. caliditerrae</i> YIM 77777 ^a	85 °C / 8.0	03/2010
<i>T. igniterrae</i> YIM 77777.1	73.8 °C / 7.29	01/2011
<i>T. oshimai</i> YIM 77359	64 °C / 8.0	03/2010
<i>T. oshimai</i> YIM 77838.1	73 °C / 8.0	06/2011
<i>T. oshimai</i> YIM 77923.2	73 °C / 7.0	06/2011
<i>T. scotoductus</i> YIM 77445.2	64 °C / 8.0	03/2010
<i>T. scotoductus</i> YIM 77398 ^a	84 °C / 7.4	03/2010
<i>T. tengchongensis</i> YIM 77357	70 °C / 8.0	03/2010
<i>T. tengchongensis</i> YIM 77392	70 °C / 8.0	03/2010
<i>T. tengchongensis</i> YIM 77392.1	70 °C / 8.0	03/2010
<i>T. tengchongensis</i> YIM 77401	85 °C / 8.0	03/2010
<i>T. tengchongensis</i> YIM 77410	85 °C / 8.0	03/2010
<i>T. tengchongensis</i> YIM 77427 ^a	86 °C / 8.0	03/2011
<i>T. tengchongensis</i> YIM 77727	85 °C / 8.0	03/2010
<i>T. tengchongensis</i> YIM77924	82 °C / 7.5	06/2011
<i>T. thermophilus</i> YIM 77318	70 °C / 8.0	03/2010
<i>T. thermophilus</i> YIM 77430.2	73 °C / 7.0	03/2010

^a Removed from this study

Table S1. *Thermus* master list and isolation source (con't)

Strain	Enrichment - Lab or field?	Enrichment
<i>T. amyloliquefaciens</i> YIM77409	Lab-serial dilution technique	aerobic
<i>T. amyloliquefaciens</i> YIM 77735.1	Lab-serial dilution technique	aerobic
<i>T. antranikianii</i> YIM 77311.1	Lab-serial dilution technique	aerobic
<i>T. antranikianii</i> YIM 77430.1	Lab-serial dilution technique	aerobic
<i>T. antranikianii</i> YIM 77730	Lab-serial dilution technique	aerobic
<i>T. arciformis</i> JCM15153	N/A	aerobic
<i>T. brockianus</i> YIM 77420.2	Lab-serial dilution technique	aerobic
<i>T. brockianus</i> YIM 77709 ^a	Lab-serial dilution technique	aerobic
<i>T. brockianus</i> YIM77904	Lab-serial dilution technique	aerobic
<i>T. brockianus</i> YIM77927	Lab-serial dilution technique	aerobic
<i>T. brockianus</i> YIM79134	Field-Enrichment	aerobic
<i>T. caliditerrae</i> YIM77925	Field-Enrichment	aerobic
<i>T. caliditerrae</i> YIM 77777 ^a	Lab-serial dilution technique	aerobic
<i>T. igniterrae</i> YIM 77777.1	Lab-serial dilution technique	aerobic
<i>T. oshimai</i> YIM 77359	Lab-serial dilution technique	aerobic
<i>T. oshimai</i> YIM 77838.1	Lab-serial dilution technique	aerobic
<i>T. oshimai</i> YIM 77923.2	Lab-serial dilution technique	aerobic
<i>T. scotoductus</i> YIM 77445.2	Lab-serial dilution technique	aerobic
<i>T. scotoductus</i> YIM 77398 ^a	Lab-serial dilution technique	aerobic
<i>T. tengchongensis</i> YIM 77357	Lab-serial dilution technique	aerobic
<i>T. tengchongensis</i> YIM 77392	Lab-serial dilution technique	aerobic
<i>T. tengchongensis</i> YIM 77392.1	Lab-serial dilution technique	aerobic
<i>T. tengchongensis</i> YIM 77401	Lab-serial dilution technique	aerobic
<i>T. tengchongensis</i> YIM 77410	Lab-serial dilution technique	aerobic
<i>T. tengchongensis</i> YIM 77427 ^a	Lab-serial dilution technique	aerobic
<i>T. tengchongensis</i> YIM 77727	Lab-serial dilution technique	aerobic
<i>T. tengchongensis</i> YIM77924	Lab-serial dilution technique	aerobic
<i>T. thermophilus</i> YIM 77318	Lab-serial dilution technique	aerobic
<i>T. thermophilus</i> YIM 77430.2	Lab-serial dilution technique	aerobic

^a Removed from this study

Table S1. *Thermus* master list and isolation source (con't)

Strain	Enrichment nutrient source?	Reference
<i>T. amyloliquefaciens</i> YIM77409	T5	Yu 2015,Song 2014
<i>T. amyloliquefaciens</i> YIM 77735.1	T5	This study
<i>T. antranikianii</i> YIM 77311.1	T5	This study
<i>T. antranikianii</i> YIM 77430.1	T5	This study
<i>T. antranikianii</i> YIM 77730	T5	This study
<i>T. arciformis</i> JCM15153	autoclaved spring water, tryptone, and yeast extract	Zhang 2010
<i>T. brockianus</i> YIM 77420.2	T5	Yu 2015,Song 2014
<i>T. brockianus</i> YIM 77709 ^a	T5	This study
<i>T. brockianus</i> YIM77904	R2A	This study
<i>T. brockianus</i> YIM77927	R2A	This study
<i>T. brockianus</i> YIM79134	T5	Ming, 2014
<i>T. caliditerrae</i> YIM77925	T5	Ming, 2014
<i>T. caliditerrae</i> YIM 77777 ^a	T5	This study
<i>T. igniterrae</i> YIM 77777.1	R2A	This study
<i>T. oshimai</i> YIM 77359	R2A	This study
<i>T. oshimai</i> YIM 77838.1	R2A	This study
<i>T. oshimai</i> YIM 77923.2	R2A	This study
<i>T. scotoductus</i> YIM 77445.2	R2A	This study
<i>T. scotoductus</i> YIM 77398 ^a	T5	Yu 2015,Song 2014
<i>T. tengchongensis</i> YIM 77357	T5	This study
<i>T. tengchongensis</i> YIM 77392	T5	This study
<i>T. tengchongensis</i> YIM 77392.1	T5	This study
<i>T. tengchongensis</i> YIM 77401	T5	This study
<i>T. tengchongensis</i> YIM 77410	T5	This study
<i>T. tengchongensis</i> YIM 77427 ^a	T6	This study
<i>T. tengchongensis</i> YIM 77727	T5	This study
<i>T. tengchongensis</i> YIM77924	R2A	Yu, 2012
<i>T. thermophilus</i> YIM 77318	T5	This study
<i>T. thermophilus</i> YIM 77430.2	T5	This study

^a Removed from this study

Table S2. Primers for PCR amplification and annealing temperatures

Primer (forward/reverse)	Target Gene	Annealing Temperature
narGn2F_CC/narGn6R_CC	<i>narG</i>	61.3°C
narGn2F_CC/narGn7R_CC	<i>narG</i> (strain 318)	59.6°C
nirKn3F/nirKn3R	<i>nirK</i>	57.2°C
nirSn1F/nirSn4R	<i>nirS</i>	60.9°C
nirSn1FB/nirSn925RB	<i>nirS</i> (strain 925)	64.1°C
norBnF1F_CC/norBn9R	<i>norB</i>	54.9°C
norBn925/norBn9R	<i>norB</i> (strain 925)	55.4°C

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Genome Announcements

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Academic Background

University of Nevada, Las Vegas, NV (UNLV)
M.S. Biology, August 2013 – May 2016
Under the advisement of Dr. Brian P. Hedlund
Montclair State University, NJ
B.S., Biology, Completed May 2013
Magna cum laude
Cumulative GPA 3.746

Honors and Awards

UNLV Graduate College Recruitment Scholarship
Las Vegas, Nevada
Fall 2016, Spring 2017
Nevada NASA Space Grant Consortium RiSE and HoP Scholarship
Las Vegas, Nevada
November 2015
UNLV, Outstanding Graduate Student Teaching Award 2015-2016, Nominee
Las Vegas, NV
October 2015
Dean's List – Montclair State University
Montclair, NJ
Spring 2013, Fall 2012, Spring 2012, Spring 2011, Fall 2010, Spring 2010, Fall 2009

Research Experience

Graduate Assistant, under the advisement of Dr. Brian P. Hedlund
University of Nevada, Las Vegas
August 2013-Present
Undergraduate Research Assistant, under the advisement of Dr. Sandra Adams
Montclair State University, Montclair
September 2012 – May 2013

Relevant Skills

- Molecular Microbiology: DNA extraction, Gel electrophoresis, PCR, Sanger sequencing
- Instruments: Gas Chromatography-ECD, Gas Chromatography-TCD
- Cultivation of Microbes: Growth of anaerobic cultures
- Phylogenetic Analysis: R, ClustalX, MEGA 5.0, MOTHUR, MUSCLE, RAxML,
- Design: Inkscape

Professional Membership

The American Association for the Advancement of Science
August 2015 - Present
American Society of Microbiology, UNLV Student Chapter
January 2014 – Present

Manuscripts Accepted for Publication

Mefferd, Chrisabelle C., En-Min Zhou, Tian-Tian Yu, Hong Ming, Senthil K. Murugapiran, Marcel Huntemann, Alicia Clum, Manoj Pillay, Krishnaveni Palaniappan, Neha Varghese, Natalia Mikhailova, Dimitrios Stamatis, T.B.K. Reddy, Chew Yee Ngan, Chris Daum, Kecia Duffy, Nicole Shapiro, Victor Markowitz, Natalia Ivanova, Nikos Kyrpides, Amanda J. Williams, Tanja Woyke, Wen-Jun Li, Brian P. Hedlund. (2016). High quality draft genomes from *Thermus caliditerrae* YIM 77777 and *T. tengchongensis* YIM 77401 isolates from Tengchong, China. *Genome Announcements*. DOI 10.1128/genomeA.00312-16.

Zhou, En-Min, Senthil K. Murugapiran, **Chrisabelle C. Mefferd**, Lan Liu, Wen-Dong Xian, Yi-Rui Yin, Hong Ming, Marcel Huntemann, James Han, Amy Chen, Nikos Kyrpides, Konstantinos Mavrommatis, Victor Markowitz, Krishna Palaniappan, Natalia Ivanova, Andrew Schaumberg, Amrita Pati, Konstantinos Liolios, Henrik P. Nordberg, Michael N. Cantor, Susan Xinyu Huad, Tanja Woyke, Wen-Jun Li, and Brian P. Hedlund. (2016). High-quality draft genome sequence of the *Thermus amyloliquefaciens* type strain YIM 77409^T with an incomplete denitrification pathway. *Standards in Genomics Sciences*. DOI 10.1186/s40793-016-0140-3.

Presentations and Posters

Cempron C., Zhou E., Bernardo N., Li W., Hedlund B., (April 2016) Incomplete Denitrification Pathways in *Thermus* species from Yunnan Province, China. Poster presentation at 2016 Nevada NASA Statewide Meeting, Reno, Nevada

Malwane M., **Cempron C.**, Williams A., Hedlund B., (July 2014) Incomplete Denitrification Pathways in *Thermus* Species from Tengchong, China. Poster presentation at UNLV School of Life Sciences' Research Experience for Undergraduates Symposium, Las Vegas, Nevada

Cempron C., Adams S., (April 2013) Classification of Mycobacterium Phage Using Polymerase Chain Reaction. Poster presentation at annual Montclair State University Student Research Symposium, Montclair, New Jersey.

Cempron C., Adams S., (April 2013) Classification of Mycobacterium Phage Using Polymerase Chain Reaction. Poster presentation at annual meeting of New Jersey Academy of Science at Kane University, Union, New Jersey.

2nd Place Undergraduate Poster Presentation

Conferences

2016 Nevada NASA Statewide Meeting
Grand Sierra Resort, Reno, Nevada
April 29, 2016

NSF Partnership in International Research and Education (PIRE) Tengchong Summit
Northern Arizona University, Flagstaff, AZ
May 19-22, 2015

NASA Astrobiology Workshop
Desert Research Institute, Las Vegas, NV
March 21, 2014
Hot Life in the Desert Conference
Arizona State University, Phoenix, AZ
March 5-7, 2014

Teaching Experience

BIOL 251 General Microbiology, Lab Instructor
September 2014-December 2015
BIOL 196 Principles of Modern Biology, Lab Instructor
September 2013-May 2014

Research Mentoring

Jacob Villarama, UNLV UROP Undergraduate Researcher
January 2016-Present
Noel Adrian Bernardo, UNLV UROP Undergraduate Researcher
September 2014-March 2016
Michelle Malwane, NSF REU Environmental Microbiology Fellow
June 2014-August 2014

Work Experience

Care Giver, Employer: Grace Sullivan
Bloomfield, NJ
September 2010 – June 2013

Volunteer and Outreach Activities

Volunteer Youth/Young Adult Minister, Saint Joseph Husband of Mary Catholic Church
Las Vegas, NV
July 2013 – Present
UNLV ASM Student Chapter Science Workshops Coordinator
Las Vegas, NV
November 2015 – Present
UNLV ASM Student Chapter Outreach Committee
Las Vegas, NV
August 2015 – Present
UNLV Multicultural Program for STEM and Health Science, STEM Forum Speaker
Las Vegas, NV
November 19, 2015
UNLV ASM Student Chapter and Nevada Y.O.U. Science Fair Challenge
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October 2014 – March 2015